

Liposomes:

Systematic Screening and Characterisation of Lipid-Membrane Stability

Dissertation
zur Erlangung des Grades
"Doktor der Naturwissenschaften"
im Promotionsfach Pharmazie

am Fachbereich Chemie, Pharmazie, Geographie und Geowissenschaften
der Johannes Gutenberg-Universität Mainz

Lukas Gleue



Mainz, März 2025



JOHANNES GUTENBERG
UNIVERSITÄT MAINZ

Dekanin:

Univ.-Prof. Dr. Eva Rentschler

1. Berichterstatter:

Univ.-Prof. Dr. Mark Helm

2. Berichterstatterin:

Prof. Dr. Andrea Tüttenberg

Datum der mündlichen Prüfung:

D77 (Dissertation Mainz)

Die vorliegende Arbeit wurde am Institut für Pharmazeutische und Biomedizinische Wissenschaften der Johannes Gutenberg-Universität Mainz zur Erlangung des Grades „Doktor der Naturwissenschaften“ unter Betreuen von Univ.-Prof. Dr. Mark Helm zwischen dem 01.08.2020 und 18.03.2025 angefertigt.

1. Berichterstatter:

Univ.-Prof. Dr. Mark Helm
Fachbereich Chemie, Pharmazie, Geographie und Geowissenschaften
Institut für Pharmazeutische und Biomedizinische Wissenschaften
Johannes Gutenberg-Universität Mainz

2. Berichterstatterin:

Prof. Dr. Andrea Tüttenberg
Universitätsmedizin
Hautklinik and Poliklinik
Johannes Gutenberg-Universität Mainz

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Abstract

Liposomes are versatile nanoparticles for drug delivery, facilitated by surface modifications and controlled cargo release, making them a valuable tool in cancer treatment. However, despite their potential, the number of approved liposomal therapies is limited due to challenges in tumour targeting and drug release. To address this limitations, a liposome preparation technique published by Massing *et al.* in 2008, which uses dual centrifugation, was utilised. ¹ This method is particularly suitable for research due to its ability to produce multiple samples in parallel, its high encapsulation efficiency and the minimal amount of lipid required. ²⁻⁵ The first project investigated how the chain length of dialkyl lipids affects their membrane anchoring and migration rate into other membranes. This is particularly important for surface-functionalised liposomes, as the loss of modified lipids can significantly reduce therapeutic efficacy. By covalently linking lipids with 16, 18 and 20 carbon atoms in their dialkyl chains with fluorescent dyes, a reduced anchoring stability of C16 lipids in liposome membranes compared to longer chain lipids was observed. In addition, a correlation between the anchoring stability and the proportion of phospholipids in the lipid composition, as well as the type of phospholipids used, was investigated. Overall, this study provided new insights into the development of stably modified liposomes. ⁶ In the second project, dual centrifugation was utilised for parallel sample preparation to perform a screening aimed at identifying new pH-sensitive liposomes according to rational criteria. These liposomes were designed to release their cargo under acidic conditions, such as those found in the mature endosome. Initially, binary lipid compositions were evaluated for membrane stability, physicochemical properties and release characteristics. The selected and optimised candidates demonstrated improved intracellular release of the encapsulated cytostatic agent *in cellulo* compared to a liposomal control, while maintaining similar biodistribution over 24 h in a mouse model. Overall, this rational screening approach successfully identified three pH-

sensitive liposomes, demonstrating the practical applicability of the method.³ In the third study, a novel method was developed to assess the biological activity of FTAD, a potential methyltransferase inhibitor, which was previously not possible due to its lack of membrane permeability. This was achieved by using the fusogenic liposome LFT1 as a transfection system for small molecules.⁷ Physicochemical characterisation revealed minimal effect of encapsulated cargos on the particle properties of LFT1. The successful intracellular release was demonstrated using a fluorescent phosphatase substrate as a model cargo. Finally, the activity of FTAD via liposomal transfection was evaluated *in cellulo* by analysing the methylation levels of total tRNA and comparing it with the effects of the methyltransferase inhibitor sinefungin. FTAD showed no inhibitory activity, whereas sinefungin effectively reduced six total tRNA methylation sites under comparable conditions. This work highlights the utility of LFT1 as a tool for early assessment of the biological activity of new small molecules in drug development.

Zusammenfassung

Liposome sind vielseitige Nanopartikel zur Arzneimittelabgabe, die durch Oberflächenmodifikationen und kontrollierte Freisetzung der Ladung ein wertvolles Werkzeug in der Krebstherapie darstellen. Trotzdem ist die Anzahl zugelassener liposomaler Therapien aufgrund von Herausforderungen bei der gezielten Tumoradressierung und der Arzneimittelabgabe gering. Zur Überwindung dieser Limitierungen wurde eine von Massing *et al.* im Jahr 2008 veröffentlichte Herstellungsmethode für Liposome genutzt, basierend auf dualer Zentrifugation.¹ Diese Methode zeichnet sich durch die parallele Herstellung mehrerer Proben, hohe Einschlusseffizienzen und geringen benötigten Lipidmengen aus.²⁻⁵ Im ersten Projekt wurde der Einfluss der Kettenlänge von Dialkyllipiden auf die Membranverankerung und die Wanderungsrate in andere Membranen untersucht, mit dem Ziel einer Optimierung der Halbwertszeit von Oberflächenfunktionalisierungen. Durch die kovalente Konjugation von Lipiden mit 16, 18 und 20 Kohlenstoffatomen in ihren Dialkylketten mit fluoreszierenden Farbstoffen wurde eine reduzierte Ankerstabilität von C16-Lipiden in Liposomen im Vergleich zu länger-kettigen Lipiden festgestellt. Zusätzlich wurde eine Korrelation zwischen der Ankerstabilität und dem Phospholipidanteil in der Lipidzusammensetzung sowie der Art der verwendeten Phospholipide beobachtet. Insgesamt lieferte diese Studie neue Erkenntnisse zum Design stabil-modifizierter Liposomen.⁶ Im zweiten Projekt wurde die duale Zentrifugation für die parallele Liposomenherstellung zur Durchführung eines Screenings neuer pH-sensitiver Liposomen anhand rationaler Kriterien eingesetzt. Diese Liposomen wurden so konzipiert, dass sie ihre Ladung unter sauren Bedingungen, wie sie im späten Endosom vorliegen, freisetzen. Zwei-Lipidkompositionen wurden zunächst hinsichtlich ihrer Membranstabilität, ihrer physikochemischen Eigenschaften und ihrer Freisetzungseigenschaften charakterisiert. *In cellulo* demonstrierten die finalen, optimierten Kandidaten eine verbesserte intrazelluläre Freisetzung des verkapselten Zytostatikums im Vergleich zu

einem liposomalen Kontrollpräparat, während sie über 24 h in einem Mausmodell eine ähnliche Biodistribution aufwiesen. Zusammenfassend führte dieser rationale Screeningansatz erfolgreich zur Identifizierung von drei neuen pH-sensitiven Liposomen, was die praktische Anwendbarkeit der Methode demonstriert. ³ In der dritten Studie wurde eine Methode zur Messung der biologischen Aktivität von FTAD, einem potenziellen Methyltransferase-Inhibitor, dessen Aktivitätsbestimmung zuvor aufgrund fehlender Membranpermeabilität scheiterte, entwickelt. Dies wurde unter Verwendung des fusogenen Liposoms LFT1 als Transfektionsmittel erreicht. Physikochemische Charakterisierungen zeigten einen geringen Einfluss der eingeschlossenen Moleküle auf die Partikeleigenschaften von LFT1. Die erfolgreiche intrazelluläre Freisetzung wurde unter Verwendung eines fluoreszierenden Phosphatasesubstrates als Modellfracht demonstriert. Schließlich gelang die Bestimmung der Aktivität von FTAD durch liposomale Transfektion *in cellulo*, indem die Häufigkeit verschiedener Methylierungen in totaler tRNA analysiert und mit den Effekten des Methyltransferase-Inhibitors Sinefungin verglichen wurden. FTAD zeigte keine inhibierende Aktivität *in cellulo*, während Sinefungin unter vergleichbaren Bedingungen effektiv sechs Modifikationen reduzierte. Diese Arbeit unterstreicht die Anwendung von LFT1 als Werkzeug zur frühzeitigen Bewertung der biologischen Aktivität neuer Wirkstoffkandidaten in der Arzneimittelentwicklung.

Abbreviations

BPF	bilayer phospholipid fragment
Cas9	CRISPR-associated 9
cDNA	complementary DNA
CHEMS	cholesteryl-hemisuccinate
CHOL	cholesterol
COVID-19	coronavirus disease 2019
CRISPR	clustered regularly interspaced short palindromic repeats
DC	dual centrifugation
DiD	1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate salt
DiR	1,1'-dioctadecyl-3,3',3'-tetramethylindotricarbocyanineiodide
DLS	dynamic light scattering
DMEM	dulbeccos modified eagle medium
DNA	deoxyribonucleic acid
DNMT2	DNA methyltransferase 2
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
DODAP	1,2-dioleoyl-3-dimethylammonium-propane
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phospho-ethanolamine

DOPG	1,2-dioleoyl- <i>sn</i> -glycero-3-phospho-rac-(1-glycerol)
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium
DPPC	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
DPPG	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phospho-rac-(1-glycerol)
EDTA	ethylenediaminetetraacetic acid
EE	encapsulation efficiency
EPC3	egg phosphatidyl choline 3
EPR	enhanced permeability and retention
Fab	antigen binding fragment
Fc	crystallisable fragment
5-FAM	5-carboxyfluorescein
FDA	U.S. Food and Drug Administration
FDP	fluorescein diphosphate
FTAD	5-FAM-triazolyl-adenosyl-diaminobutyric
GSH	glutathione
GUV	giant unilamellar vesicle
HEK293	human epithelial kidney 293
HIFU	high-intensity focused ultrasound
HPLC	high-performance liquid chromatography
IC ₅₀	half maximal inhibitory concentration
K _D	dissociation constant
LC-MS	liquid chromatography–mass spectrometry
LNA	locked nucleic acids
LNP	lipid-based nanoparticle
LUV	large unilamellar vesicle
m ⁵ C	5-methylcytosine

MLV	multilamellar vesicle
MPS	mononuclear phagocyte system
mRNA	messenger RNA
MTases	methyltransferases
MV	multivesicular vesicle
NIR	near-infrared light
NSUN2	NOP2/Sun RNA methyltransferase 2
PA	phosphatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDI	polydispersity index
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PEG2000	2000 g/mol PEG
PET	positron emission tomography
PG	phosphatidylglycerol
pH	power of hydrogen
PhD	philosophiae doctor
pK _a	acid dissociation constant
PI	phosphatidylinositol
ROS	reactive oxygen species
PS	phosphatidylserine
rfu	relative fluorescence unite
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
SAH	<i>S</i> -adenosyl- <i>L</i> -homocysteine

SAM	<i>S</i> -adenosyl- <i>L</i> -methionine
SEC	size exclusion chromatography
SFG	sinefungin
siRNA	small interfering RNA
SPECT	single photon emission computed tomography
SRb	sulforhodamine B
SUV	small unilamellar vesicle
TME	tumour microenvironment
tRNA	transfer ribonucleic acid
UV	ultraviolet
VEGFR	vascular endothelial growth factor receptor

1 Introduction

1.1 Liposome diversity: Structural variation and lipid influence

In the field of pharmaceutical science and biomedical research, the search for effective drug delivery systems is a dynamic area of continuous innovation. In particular, liposomes stand out for their versatility and adaptability. These microscopic vesicles consist of one or more lipid bilayers about 5 nm thick surrounding an aqueous core (**Figure 1.1**).^{8–10} The lipid particles exhibit a diverse size distribution, encompassing several morphological subtypes. At the lower end of the spectrum are small unilamellar vesicles (SUVs), with diameters approximating 50 nm, which can be utilised to passively target tumour tissue and are preferred for the delivery of therapeutic cargo.^{11–13} The upper range includes large unilamellar vesicles (LUVs) with diameters <1000 nm, and giant unilamellar vesicles (GUVs) that can reach millimetre-scale dimensions and are typically used as a membrane model and to generate artificial cells.¹⁴ Multilamellar vesicles (MLVs) are characterised by multiple concentric lipid bilayers and typically have a diameter exceeding 500 nm. In contrast, multivesicular vesicles (MVVs), which consist of several smaller vesicles enclosed within a single outer bilayer, generally measure over 1000 nm in diameter.^{15,16}

1.1.1 Influence of lipid composition

The main components of the lipid bilayers are diacyl phospholipids. These lipids have an amphiphilic nature, which causes them to form a double membrane on contact with water. This effect is characterised by the hydrophobic diacyl chains orienting inwards into the membrane where they form van der Waals interactions. In contrast, the

hydrophilic phosphate groups are aligned towards the aqueous phases on both sides of the membrane, enabling the formation of hydrogen bonds. This arrangement allows the lipid bilayer to effectively separate the internal and external aqueous environments.¹⁷

Many phospholipids used in the manufacture of liposomes are derived from natural sources, particularly soyabeans, cottonseed and egg yolk.^{18,19} Among these, phosphatidylcholines (PC), also known as lecithin, and phosphatidylethanolamines (PE) are the prevalent lipids found in both plants and animals, making them the most frequently used excipients in liposomal formulations due to their widespread presence in biological systems. In addition, other naturally occurring phospholipids such as phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidic acid (PA) are also used, although less frequently than PC and PE.^{19–22}

The physicochemical properties of liposomal membranes are strongly influenced by the molecular characteristics of the lipids, particularly the charge of the polar head group and the nature of the esterified fatty acid chains. The origin of phospholipids influences the saturation of their fatty acids. Phospholipids from plant sources typically contain a higher percentage of unsaturated fatty acids. In contrast, phospholipids from animal sources predominantly consist of saturated and monounsaturated lipids with fatty acid chains of different lengths.²³ The degree of saturation determines the phase transition temperature of the lipids and thus affects the fluidity and permeability of the liposome membrane.²⁴ The phase transition temperature is defined as the temperature range at which the tightly packed phospholipid layer of the ordered gel phase begins to loosen by interrupting hydrogen bonds and van der Waals interactions, thereby increasing membrane fluidity. This allows water molecules to migrate between the lipids until a disordered liquid crystalline phase is reached. If the temperature drops below the phase transition temperature, the lipids revert to an ordered crystalline phase in which water molecules can remain in the membrane.^{25–28} This makes the lipid phase a significant parameter for influencing cargo release, mechanical membrane stability, cellular uptake and particle size.^{29–32}

1.1.2 Modulation of the phase transition temperature

One strategy for modulating the phase transition temperature is based on the addition of cholesterol to the lipid composition for the formation of lipid rafts. These are formed by the rearrangement of phospholipids by cholesterol, resulting in local areas with a

liquid ordered phase in the otherwise liquid disordered system. Thereby, the membrane permeability decreases, and the average particle diameter increases.^{33–39}

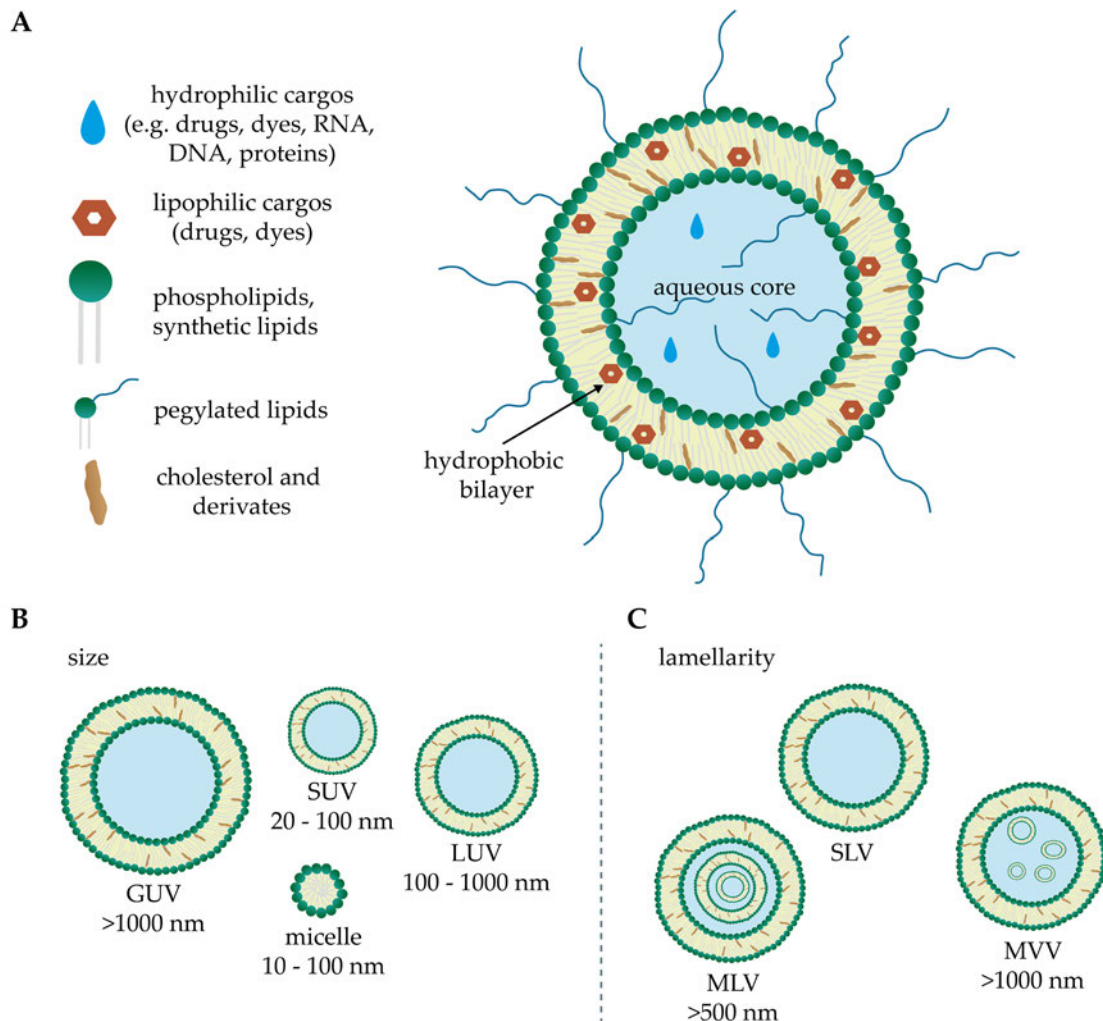


Figure 1.1: Structural overview and classification of liposomes. **A:** Schematic representation of liposomes based on the most common types of lipids and cholesterol for use in liposomal formulations. The figure shows compartments and potential options for encapsulating hydrophilic and hydrophobic cargo. **B:** Classification of liposomes according to size into small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and giant unilamellar vesicles (GUV). The structure of the micelles is also shown. **C:** Liposomes can exhibit varying numbers and configurations of bilayers. Those composed of a single phospholipid bilayer are classified as single lamellar vesicles (SLVs). Multilamellar lipid nanoparticles are categorized as multilamellar vesicles (MLVs) when they contain multiple bilayers, or as multivesicular vesicles (MVs) when several vesicles are enclosed within a single outer bilayer.^{16,40}

The selection of phospholipids with saturated or unsaturated fatty acids is another strategy for modulating membrane fluidity, as the phase transition temperature decreases with the increase in unsaturated double bonds in *cis*-conformation, whereas

trans-double bonds typically have the opposite effect. This is due to the fact that *cis*-double bonds lead to reduced order within the membrane, whereas *trans*-bonds increase it.^{41,42} In addition, bulky head groups such as cyclopropane rings and short carbon-hydrogen chains of fatty acids can also lower the phase transition temperature.^{43,44}

1.1.3 Characteristics of lipid particle with encapsulated nucleic acids

Besides the membrane fluidity, lipids also influence the surface charge of the nanoparticles. Anionic phospholipids such as PG, PS and PA shift the membrane charge in a negative potential, while neutral or zwitterionic lipids such as PC and PE have little effect on the overall membrane charge.^{45–47} The charge of liposomes plays a crucial role in determining their behaviour and functionality. Weakly charged and neutral liposomes tend to aggregate due to reduced electrostatic repulsion, affecting their stability. In contrast, neutral and anionic nanoparticles tend to circulate longer in the bloodstream and are less likely to be phagocytosed by macrophages than their cationic counterparts. In addition, anionic liposomes are often known to release their cargo more rapidly than neutral liposomes.^{48–53} Cationic lipids are primarily used for the complexation and transfection of RNA (ribonucleic acid) and DNA (deoxyribonucleic acid) due to their ability to interact electrostatically with nucleic acids. The electrostatic interaction of the cationic particles with the negatively charged cell membrane can facilitate rapid uptake into the cells. However, this property can also result in increased cell toxicity compared to anionic and neutral liposomes.^{54–56}

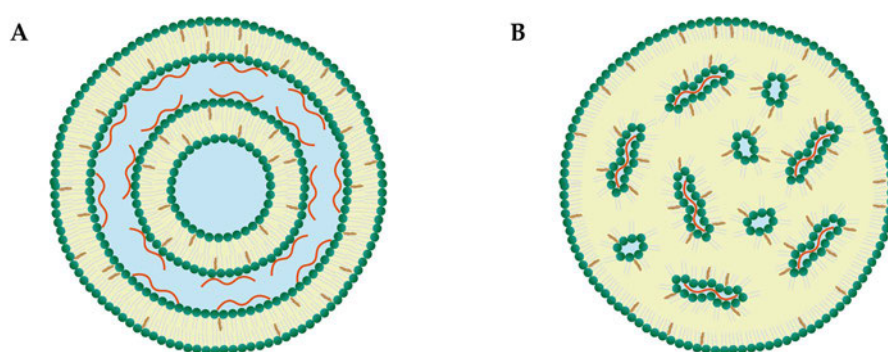


Figure 1.2: The exact structure of lipid nanoparticles containing nucleic acids remains unclear. Two possible arrangements have been considered: **A:** The cargo is intercalated between phospholipid layers or **B:** it is trapped in inverse micelles within the vesicle. (Figure inspired by Tenchov *et al.*)⁵⁷

It is important to note that nucleic acids encapsulated in lipid particles containing cationic lipids do not typically conform to the structure of liposomes, which usually have an aqueous core surrounded by a lipid bilayer. Various structures are discussed, such as the encapsulation of nucleic acids in inverted lipid micelles within the nanoparticle or their integration between lipid bilayers, commonly referred to as lipoplexes (**Figure 1.2**).⁵⁷ Lipid-based nanoparticles (LNPs) are an advanced form in which some of the cationic lipids are replaced by ionisable lipids, leading to reduced cytotoxicity.⁵⁸ Ionisable lipids, such as 1,2-dioleoyl-3-dimethylaminopropane (DODAP), are characterised by one or more tertiary nitrogen atoms. DODAP in its protonated state has an acid dissociation constant (pKa) of approximately 6.5, which means that it remains non-charged at physiological pH, but becomes positively charged in an acidic environment such as mature endosomes due to protonation of the tertiary nitrogen atom.⁵⁹ Despite the use of techniques like small-angle X-ray scattering, small-angle neutron scattering and electron microscopy to study these nanoparticles, their complex structure remains unclear. However, a deeper understanding of their architecture can be crucial for accurately predicting and determining the pharmacological properties of these nanoparticles.⁶⁰

A wide range of synthetic and semi-synthetic lipids are also available to further optimise liposome properties and to overcome the limitations of natural-based lipids (see **Sections 1.3** and **1.4**).

1.2 Liposome preparation techniques

1.2.1 Lipid particle preparation by thin film hydration

Since the first production of artificial liposomes in the 1970s, various techniques have been developed to prepare liposomes for specific applications and to overcome limitations. One of the first was the thin film hydration method, which was also developed by Bangham *et al.* and is still widely used today for the preparation of liposomes and related lipid particles in combination with extrusion. This technique can be used for the preparation of liposomes and lipid nanoparticles loaded with RNA, DNA and small molecules (**Figure 1.3 A**).^{61–69}

In this method, a thin lipid film is first formed on the inner side of a flask by evaporating the solvent of the dissolved lipids under reduced pressure. The lipid film is then hydrated, usually with an aqueous solution containing the dissolved cargo to be encapsulated. A typical protocol involves vigorous shaking of the water-lipid mixture to obtain a suspension, followed by storage at 4 °C overnight to ensure optimal hydration. To achieve uniformity and convert MLVs to SUVs, the emulsion is often first sonicated to break up aggregates and then successively extruded through membranes of decreasing pore sizes to obtain a homogeneous liposome suspension.⁷⁰

Various iterations of this protocol have been established over time, each optimised to specific lipid compositions, cargo types and production efficiencies. As an example, an alternative approach carries out the hydration at elevated temperatures (60-70 °C), reducing the hydration time to 2 h and improving the overall time efficiency of the process. Furthermore, by raising the temperature above the phase transition temperature of the lipids, this process can increase encapsulation efficiency through improved hydration.^{63,70–73}

This highlights the adaptability and broad applicability of the thin film hydration method to a wide range of lipid compositions. However, these advantages are offset by several limitations. The technique is often limited in its ability to encapsulate hydrophilic molecules, primarily due to the large volume required during the hydration phase. In addition, the production process is particularly time-consuming, does not allow parallel production of different samples, is limited in scalability and can require post-production sterilisation.^{63,74–76}

1.2.2 Liposome preparation by dual centrifugation

Closely related to the thin film hydration method is the approach based on dual centrifugation published in 2008 by Massing *et al.* Here, the sample is rotated around two axes simultaneously and at different angles, in contrast to conventional centrifugation, resulting in homogenisation of the sample (**Figure 1.3 B and C**).^{1,77} Dual centrifugation has emerged as a promising technique in liposome research, offering several advantages by allowing the simultaneous production of multiple liposome samples while typically achieving high encapsulation efficiencies (EE) of up to 50% of the introduced cargo. It also allows dynamic batch size adjustment and preparation under sterile conditions, increasing its versatility and applicability in various experimental settings.^{2–5,78}

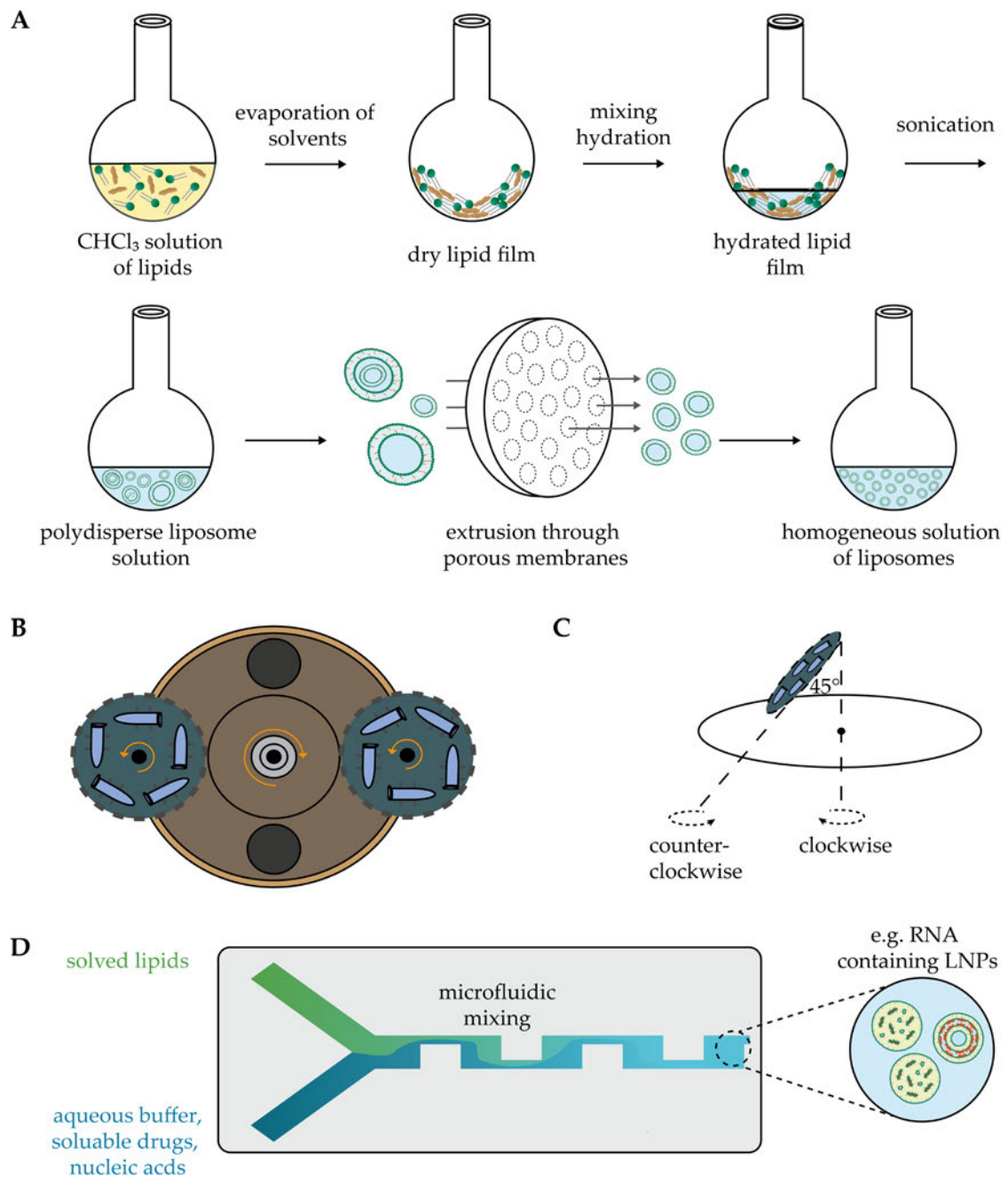


Figure 1.3: Overview of common liposome and lipid particle preparation techniques. **A:** Widely used process for the production of liposomes using thin film hydration technology followed by membrane extrusion to obtain homogeneous samples. **B:** Schematic representation of liposome preparation by dual centrifugation, whereby the two highlighted axes rotate simultaneously to achieve the mixing effect. **C:** Cross-sectional view of dual centrifugation showing the angle of the samples during homogenisation and the rotating axes. **D:** Illustration of a flow cell with stepwise mixing of the lipid stream and the aqueous phase for the production of LNPs and lipoplexes using baffle mixer microfluidics. (Part A inspired by Cazzolla *et al.*; part C inspired by Massing *et al.*; part D inspired by Osouli-Bostanabad *et al.*)^{1,71,79,80}

In contrast to other production methods, further extrusion of the liposome dispersion is often not necessary to reduce the diameter and polydispersity index (PdI), which describes the size distribution of the particles in a sample. Several articles report examples where the physicochemical properties of the prepared liposomes were between 100 nm and 200 nm in diameter and did not exceed a PdI of 0.3 a.u. (adjustable unit).^{2,4-6,81}

1.2.3 Preparation by ethanol injection

An alternative approach to liposome production is the ethanol injection method introduced by Batzri *et al.* in 1973.⁸² In this technique, an ethanolic lipid solution is injected into an aqueous medium. The rapid dilution of the ethanol leads to the formation of bilayer phospholipid fragments (BPFs), which then rapidly fuse to form vesicles as the open ends of the lipid bilayers, minimising contact with water.^{75,83,84} The ethanol injection technique is particularly suitable to produce small diameter vesicles due to the rapid fusion of the BPF ends. Additionally, it can provide precise control over particle size and distribution through several adjustable parameters, including choice of injected solvent (e.g. ethanol, isopropanol, acetone), injection speed, lipid concentration and optimisation of solvent diffusion.⁸⁵ However, a significant limitation of ethanol injection is the inability to encapsulate certain enzymes and macromolecules, as they become inactive in the presence of ethanol.⁶³

1.2.4 Microfluidic nucleic acid-lipid nanoparticle production

One of the most advanced techniques for lipid nanoparticle production is microfluidics, which involves controlled mixing of an organic phase with an aqueous phase on a specialised chip (**Figure 1.3 D**). The process starts with the laminar flow of both phases through the microfluidic chip. Turbulence is created by modulating the volume of the mixing path and the contact area to break the liquid into small units, forming particles during the mixing process.⁷⁹ Microfluidic technology is typically characterised by precise control of lipid particle size and high reproducibility, coupled with exceptional encapsulation efficiencies in the >90% range. However, an additional purification process is often required to remove solvents. Given these advantages, it is not surprising that a wide range of microfluidic techniques have been developed featuring various flow chamber designs. These innovations enable production from small batches for academia to industrial volumes.^{86,87} Due to its versatility and effectiveness, microfluidic technology has found widespread application in the

production of LNPs for various therapeutic purposes. It has been successfully employed for developing LNPs for CRISPR/Cas9-based (clustered regularly interspaced short palindromic repeats / CRISPR-associated 9) genome editing in cancer therapies, creating siRNA (small interfering RNA) and mRNA (messenger RNA) delivery systems, and manufacturing the COVID-19 (coronavirus disease 2019) vaccine produced by BioNTech and Pfizer.^{79,88–93}

1.3 Surface functionalisation for passive and active targeting

Conventional liposomes, composed of phospholipids and cholesterol, mimic the body's cell membranes, which can result in reduced immunogenicity, enhanced biocompatibility, lower drug degradation and improved delivery efficiency compared to free drugs.^{94,95} However, when administered, liposomes encounter various proteins and biomolecules in the biological environment, which typically attach to the liposome surface, forming a protein corona. The newly formed layer envelops the entire nanoparticle and plays a critical role in determining several key aspects of its *in vivo* and *in cellulo* behaviour.⁹⁶ The protein corona has the potential to influence the cellular uptake of liposomes, their distribution in the body, their clearance from the system and their interaction with macrophages. The challenge of protein corona formation and the low tissue specificity of conventional liposomes restrict the wide medical application of liposomes.⁹⁷ Consequently, innovative strategies to overcome these drawbacks and to extend the clinical utility of liposomes, such as the modification of the liposomal membrane, have become an important area of research.

1.3.1 Surface modification strategies

Membranes can be modified either before or after production. The pre-production lipid modification offers precise control and analysis of the modification level, making it particularly suitable for available molecules. However, this method has a 50% chance of incorporating the functionalised lipid into the inner membrane layer by the

liposome preparation, requiring a higher input of modified lipid. Moreover, it is often not suitable for biologically active macromolecules, which can lose their activity in organic solvents during liposome preparation.^{97–99}

For these molecules, as well as those with limited availability, post-production binding to liposomes is an avenue to be explored. This approach is typically based on chemical reactions between the ligands and reactive lipids, often containing alkyne, azide, cyclooctyne or maleimide groups. These reactive groups are frequently attached to the lipid by a PEG (polyethylene glycol) linker (**Figure 1.4 B**). Due to the different reaction conditions of the functional groups, double functionalisation are also possible.^{6,100–104} An alternative approach exploits the strong interaction between biotin and streptavidin, where streptavidin acts as an adapter protein with four biotin binding sites. In this method, both the liposomes and the ligand are biotinylated, allowing them to be linked via streptavidin.^{105,106} This strong affinity between the binding partners is based on the formation of multiple hydrogen bonds and van der Waals interactions, combined with conformational changes that trap biotin within streptavidin.¹⁰⁷ A third approach to liposome surface modification involves the fusion of two different lipid particles, resulting in the mixing of their membrane lipids and proteins. The fusion can occur between two synthetically produced lipid-based nanoparticles or between the liposome and the natural exosome. Literature reports the fusion of exosomes secreted by macrophages with liposomes, resulting in hybrid particles that mimic immune cells. These hybrid structures offer the potential for targeted delivery to specific tumour cells.^{108–110}

The choice of lipid for modification, whether by covalent or non-covalent binding, must consider its stability within the lipid bilayer. Previous research by Fritz *et al.* demonstrated significantly faster exchange rates of cholesterol linkers with other membrane systems, reducing the half-life of functionalisation considerably compared to dialkyl linkers. These finding highlights the importance of lipid choice in maintaining the lifetime and stability of liposome modifications.¹⁰²

1.3.2 Shielding of the liposome surface

PEGylation is a widely used surface modification technique for liposomes and related lipid particles, addressing the limited circulation time and stability of conventional particles in the bloodstream (**Figure 1.5**). This polymer-based approach creates a flexible, protective layer around the liposome surface, which can minimise the interactions with proteins and immune cells. As a result, PEGylated liposomes typically show reduced uptake by the mononuclear phagocyte system (MPS), earning

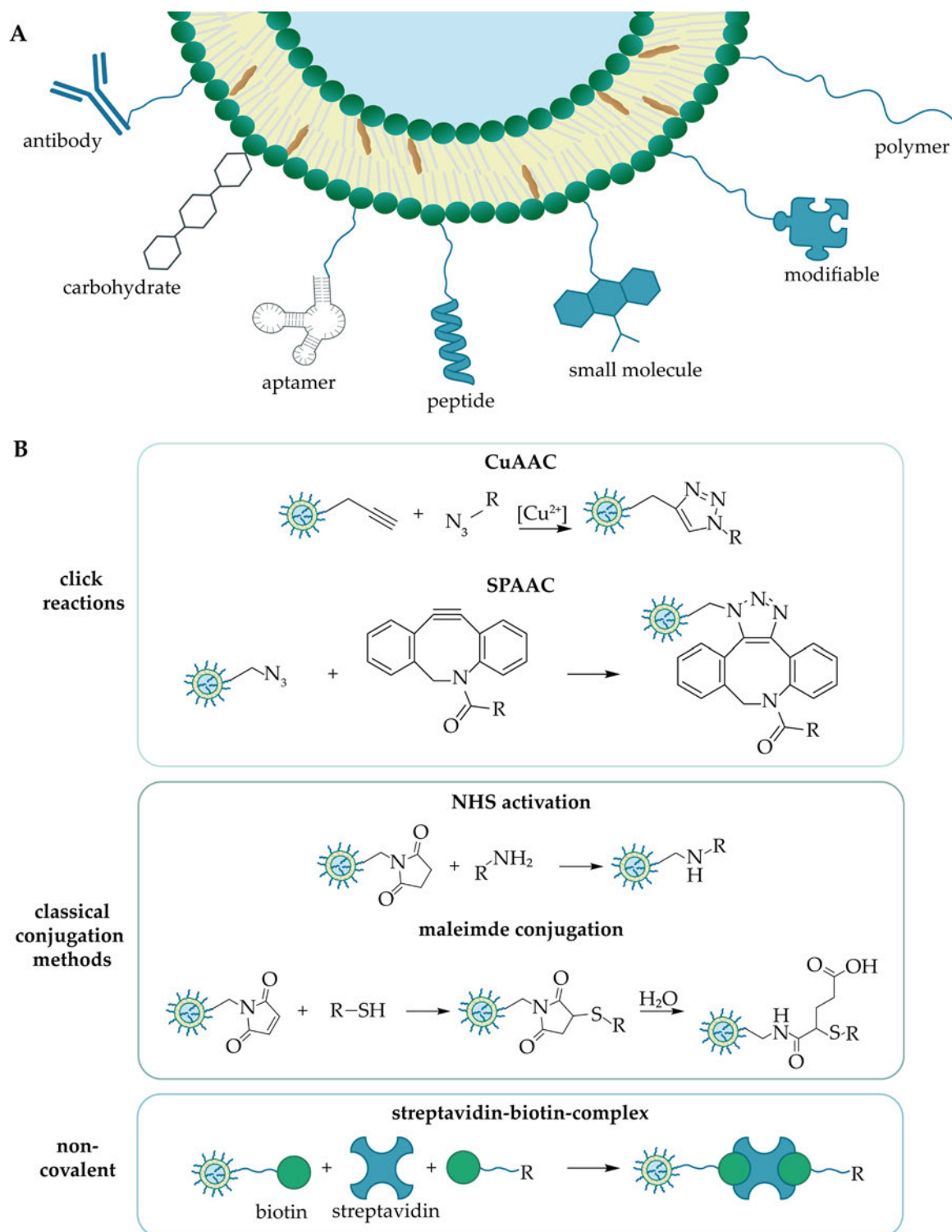


Figure 1.4: Liposome surface modification. **A:** Common surface modifications to enhance liposome specificity and performance. Their membrane distribution depends on functionalization timing. Pre-preparation results in both inner and outer surface presence, while post-preparation localizes them exclusively on the outer surface. **B:** Overview of commonly used covalent and non-covalent methods for attaching ligands to liposomes by using modified lipids. (Part A inspired by Khan *et al.*)^{111–115}

them the name "stealth liposomes" due to their ability to evade immune recognition and prolong their circulation within the bloodstream.^{116,117}

In liposome formulations, the most commonly used PEG chain length consists of 2000 g/mol PEG (PEG2000) units, with 5 mol-% typically used in the lipid composition.¹¹⁸ The PEGylation process can be implemented either through pre-production insertion or post-production modification.^{119,120} PEG2000 is incorporated into all five FDA-approved PEGylated liposomes and LNPs, including the BioNTech/Pfizer and Moderna COVID-19 vaccines and Doxil™, the first liposomal drug approved by the U.S. Food and Drug Administration (FDA) for the treatment of ovarian cancer.^{121–123}

As polyethylene glycol is widely used as an additive in pharmaceutical and cosmetic products, immune reactions leading to the formation of PEG antibodies are more common in certain patients when PEG is combined with nanocarriers.¹²⁴ In a study from 2023, elevated PEG antibody levels were found in 4 out of 5 samples (83%) of the german study participants, showing the widespread distribution of the antibodies. In particular, the highest antibody levels were found in 20-year-old subjects, with a gradual decline observed in older age groups.¹²⁵ However, the generalisability of these data to other regions of the world is difficult. When PEGylated therapeutics are administered again to a patient, they become targets for pre-existing anti-PEG antibodies, which rapidly recognise and bind to the PEGylated drug molecules, forming distinct immunological complexes. These newly formed complexes are subsequently recognised and taken up by Kupffer cells in the liver, which typically reduces the therapeutic value of the drug by shortening its circulation time in the bloodstream. In addition, this immunological process can potentially trigger allergic complications, further compromising the therapeutic efficacy.^{124,126–128} Several alternative polymers have been developed to address the issue of immune responses, including those derived from polysarcosine, heparin, hyperbranched polyglycerol, hyaluronic acid and polyvinylpyrrolidone, to name some examples. The polymers have been successfully used to decorate liposomes as well as LNPs such as for RNA delivery. Furthermore, some of these alternative polymer candidates have been shown to be less immunogenic than PEG.^{129–134}

1.3.3 Passive tumour targeting

Stealth liposomes can exploit the enhanced permeability and retention (EPR) effect, a characteristic of certain solid tumours which is caused by the increased permeability of tumour blood vessels (**Figure 1.5**). Since stealth liposomes often have a longer

circulation time than conventional liposomes, they can gradually accumulate in the tumour tissue.¹³⁵ This is mainly due to rapid and irregular angiogenesis, which is driven by the tumour's high nutrient demand and elevated levels of inflammatory hormones. In addition, the lymphatic system of the tumour is often unable to remove macromolecules effectively. Together, these factors promote the accumulation of nanoparticles in tumour tissue.¹³⁶ Numerous studies in animal models have demonstrated positive therapeutic outcomes using the EPR effect. However, in most studies, these results have not been effectively translated into clinical practise. This lack of translation may be due to several factors, including the remarkably low accumulation rate in tumour tissue of only 1-5% of the administered dose, as well as the inherent variability between different tumour types.¹³⁷

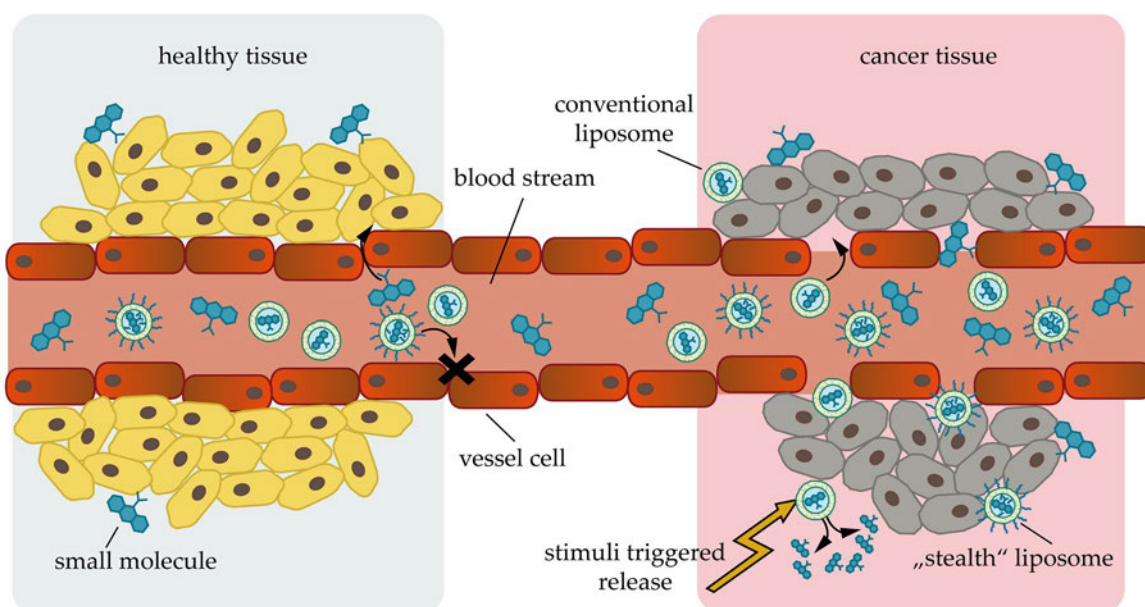


Figure 1.5: Passive tumour targeting via enhanced permeability and retention (EPR) effect. Solid tumours can have blood vessels that are more permeable to macromolecules than healthy tissue, allowing targeted penetration and accumulation of liposomes. This targeted tumour treatment has the potential to be further improved using stimuli-responsive particles. (Figure inspired by Franco *et al.*)¹⁰

1.3.4 Active targeting of tissue by antibodies

Besides extending the circulation time of liposomes, surface functionalisation is used for targeted delivery to tissue. The earliest method of tissue-specific targeting exploited the natural binding affinity of antibodies for their respective antigens. This technique was first applied to liposomes in 1980 by Leserman *et al.*, who successfully attached antibodies to liposomes.^{138–140} In this approach to tumour therapy, antibodies

are often used to target tumour-specific or over-expressed surface proteins and antigens.¹⁴¹ As antibodies tend to lose their biological activity in organic solvents, post-formulation conjugation is a widely used strategy for attaching them to liposomes.¹⁴² A particularly promising target is the overexpressed vascular endothelial growth factor receptor (VEGFR), which promotes angiogenesis and thus tumour growth.^{143–147} Besides VEGFR, immunoliposomes have demonstrated the ability to target a wide range of molecular markers, demonstrating their versatility in targeted drug delivery.^{148–151}

Two comparative studies in tumour mouse models evaluated the effect of antibody conjugation to conventional liposomes. In these studies, immunoliposomes achieved several times higher intracellular cargo accumulation in the tumour after one day compared to conventional liposomes. Importantly, there was no noticeable difference in total tumour tissue accumulation between the two groups. This finding indicates the enhanced cargo accumulation is likely due to receptor-mediated endocytosis, which facilitates more efficient cellular uptake.^{141,152,153} In addition to optimising liposomal lipid composition, antibody bioengineering is an active area of research aimed at improving pharmacokinetic properties and reducing immunogenicity. One approach involves generating antibody fragments through the removal of non-essential regions, such as the crystallisable fragment (Fc), yielding constructs containing only the antigen binding fragments (Fabs).¹⁵⁴

1.3.5 Surface decoration with peptides

Peptides offer a promising alternative strategy for the targeted delivery of particles. They typically have several advantages over antibodies, including smaller size, higher stability, easier production and a more favourable immunogenicity profile.¹⁵⁵ Nevertheless, it is important to note that antibodies usually have a higher binding affinity than peptides.¹⁵⁶ An essential peptide for targeted delivery is arginine-glycine-aspartic acid (RGD), which specifically targets $\alpha_v\beta_3$ integrins overexpressed on endothelial cells undergoing active angiogenesis, which is upregulated on several solid tumours.^{157–160} Beneficially, integrins have been shown to facilitate cellular internalisation of RGD-lipid particles.¹⁶¹ The applicability of the conjugates was demonstrated by the successful delivery of both cytostatic drugs and mRNA via RGD-LNPs and liposomes to tumour tissue, liver and spleen in a mouse model. In addition, conjugated liposomes demonstrated the ability to reduce blood clot aggregation by blocking glycoprotein IIb/IIIa integrin, offering an exciting approach to the treatment of thrombosis and atherosclerosis.^{157,158,160–162} The wide range of potential peptides

offers versatile targeting options for different types of cancer, providing a broad spectrum of therapeutic approaches.¹⁶³

1.3.6 Use of nucleic acids for tissue targeting

Aptamers, which can have similar properties to peptides, are short, synthetically produced DNA or RNA sequences designed to bind to specific targets.¹⁶⁴ According to the literature, DNA aptamers generally have higher overall stability, whereas RNA-based aptamers often offer greater structural flexibility at the expense of reduced stability.¹⁶⁵ The term 'aptamer' is derived from a combination of two Latin words: *aptus* 'to fit' and *meros* 'part'.¹⁶⁶ Chemical modifications of aptamers provide a way to tailor the critical properties of aptamers and improve the pharmacokinetic profile. These modifications can be used to alter immunogenicity, improve serum stability, and prolong half-life in the bloodstream. One approach is to replace the 2'-hydroxy group of ribose by either a fluoro or methoxy group. Another strategy uses locked nucleic acids (LNA), in which the 2'-O and 4'-O of the ribose are linked by a methylene bridge. These structural modifications are typically used to increase the overall stability of the aptamer.¹⁶⁷ Improving the stability of aptamers is particularly important to prevent rapid degradation after administration.¹⁶⁸ In addition to the advantages mentioned above, the strong negative charge of the aptamers' phosphate backbone can induce repulsive interactions with anionic membranes and charge-mediated release of liposomes from endosomes following endocytosis. The charge can also interact with cationic or ionised lipids, which may have a negative effect on particle properties.¹⁶⁹

Aptamers may have reduced specificity compared to antibodies, resulting in increased cross-reactivity with similar targets. This characteristic requires extensive validation and careful design to ensure optimal performance.¹⁷⁰ Unlike antibody conjugation, aptamer liposomes can often be prepared in the pre-production phase by incorporating lipid-bound aptamers into the lipid mixture, thereby retaining their activity. Additionally, post-production conjugation methods are available, including chemical conjugation techniques or the use of micelles to attach aptamers to the liposomal membrane.^{171,172} Studies have shown that aptamer-conjugated lipid particles can achieve targeted delivery to tumours, bone, muscle and various organs. However, a major challenge with this approach is usually the rapid degradation of the aptamer ligands.^{170,173,174}

1.3.7 Small molecule ligands

Certain tumour tissues show an overexpression of vitamin receptors on their surface compared to normal cells. These receptors, which include those for vitamin A, biotin or nicotinamide, provide an opportunity for targeted delivery using vitamin-liposome conjugates.¹¹⁵ One notable example is the folate receptor, which is overexpressed in several tumour tissues but lacking in normal tissues. When targeting this receptor, it is critical to conjugate folic acid to liposomes using a PEG linker, as direct attachment to the particle surface can prevent effective binding to the receptor.¹⁷⁵ This is a promising approach for the targeted delivery of cytostatic drugs using folate-conjugated liposomes.^{176,177} By exploiting the upregulated folate receptor on activated macrophages, this strategy could potentially enable lipid particle-based therapies for inflammatory diseases such as rheumatoid arthritis and atherosclerosis. However, despite these promising *in cellulo* results with folic acid conjugates, attempts to replicate these findings *in vivo* have often been unsuccessful.^{178,179}

In addition to targeting, small molecule ligands facilitate *in vivo* and *in cellulo* visualisation and tracking. Liposomes can be conjugated with fluorescent dyes, enabling *in vivo* distribution studies in living animal models. Furthermore, liposomes conjugated with chelators are used in imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) for tumour diagnosis and therapy in combination with radioactive isotopes.^{180–182}

1.3.8 Carbohydrate-decorated lipid nanoparticles

Carbohydrates and polysaccharides can exhibit high chemical stability, are often readily available, biocompatible, and have excellent water solubility, making them attractive ligands for targeting cellular sugar receptors, such as the C-type lectin receptors, which are found on many cell membranes.^{183,184} Experimental studies revealed that PEGylation of the lipid particle surface can reduce the binding efficiency of carbohydrate-conjugated liposomes to receptors. Consequently, direct conjugation of carbohydrates to the surface is recommended for optimal receptor targeting.¹⁸⁴ Using mannose- respectively trimannose-decorated liposomes, specific targeting of dendritic cells and macrophages in mice was achieved, as these cells express the mannose receptor, a subtype of the C-type lectin family, on their surface.^{185,186} This provides an opportunity to shift the polarisation of anti-inflammatory M2 macrophages to pro-inflammatory M1 subtypes, which have an anti-tumour effect.^{187,188} Moreover, targeted delivery of therapeutic agents to liver cells, pancreatic cancer and mucosal tissues through functionalisation mediated by various saccharides

is being investigated.¹⁸⁴ Apart from cell targeting, polysarcosine offers several advantages as an alternative polymer for the design of stealth liposomes. These advantages typically include low protein adsorption, excellent water solubility, high biocompatibility and minimal immunogenicity, making it particularly suitable for this application.^{184,189}

1.3.9 Exploiting exosomes for the design of liposomes

All the strategies discussed so far involve engineered particles designed to target specific tissues or to optimise particle properties. Furthermore, cells naturally secrete extracellular vesicles called exosomes, which are loaded with DNA, RNA or proteins for intracellular communication.¹⁹⁰ The exosomes typically are between 30 and 200 nm in diameter and, like liposomes, have a lipid bilayer membrane consisting of phospholipids, cholesterol and sphingolipids. However, their membranes also contain various transmembrane proteins, such as glycoproteins, adhesion proteins and antigen-presenting molecules, which facilitate cellular targeting, uptake and potential immune stimulation.¹⁹¹ Exosomes, as endogenous vesicles, may have unique biocompatibility, low immunogenicity and reduced toxicity, making them an attractive option for drug delivery systems. However, their clinical use is typically limited by several challenges, including difficulties in isolation and purification, low entrapment efficiency and short circulation time.¹⁹² One strategy for utilising natural vesicles is to create hybrid particles by fusing exosomes with liposomes, which can be loaded with lipophilic and lipophobic cargo. In addition, PEGylated hybrid particles showed a twofold increase in cellular uptake compared to their non-PEGylated counterparts. The authors suggest that PEGylation may reduce electrostatic repulsion with the cell membrane, thereby enhancing particle uptake.^{109,193} Lu *et al.*, adopted an alternative approach by designing lipid nanoparticles with fusogenic membrane properties for siRNA transfection based on the membrane composition of extracellular vesicles. These particles showed substantially improved cellular uptake compared to traditional lipid particles.⁷ Despite major progress in the use of exosomes as drug delivery systems and their advantageous properties as native vesicles, significant challenges remain before clinical application.¹⁹⁴

1.4 Stimulus responsive liposomes

An alternative, promising approach to tumour therapy uses stimuli-responsive liposomes, which have the potential to selective treatment of cancerous tissue while minimising damage to healthy cells. This approach uses generally external or internal stimuli, such as temperature changes, exposure to light, magnetic fields or pH changes, to precisely control the release of encapsulated therapeutic compounds (**Figure 1.6**).

1.4.1 Design of heat-sensitive liposomes

A complementary therapeutic strategy to classical chemotherapy is the application of mild hyperthermia to tumour tissue, which increases tissue perfusion and cellular permeability. This approach can be synergistically combined with thermosensitive liposomes to achieve highly targeted drug delivery.¹⁹⁵ Thermosensitivity can be achieved by using lipids with a phase transition temperature above body temperature. When the temperature exceeds this range, the membrane changes from the solid gel phase to the liquid crystalline phase, resulting in increased membrane permeability and cargo release. A typical lipid used for this purpose is DPPC, which has a phase transition temperature of 41.4 °C and is combined with other lipids to precisely modulate membrane properties.¹⁹⁶ In this process, conventional thermosensitive liposomes normally release their encapsulated cargo within a temperature range of 42-45 °C after 30 min.¹⁹⁷

1.4.2 Ultrasonic-induced particle release

In order to increase sensitivity and expand therapeutic applications, the concept of thermosensitive liposomes has been refined to implement advanced release mechanisms. One approach uses high-intensity focused ultrasound (HIFU) for targeted delivery, where tissue is irradiated with precisely focused ultrasound at frequencies above 20 kHz and provides deep tissue penetration. The sound waves heat both the tissue and the liposome membrane, which can cause a localised increase in temperature and subsequent release of the cargo. In addition, the process creates vapour bubbles in the membrane which rapidly collapse (cavitation), further compromising membrane integrity.¹⁹⁸ This method can be integrated with direct tissue ablation using HIFU, which exploits the ability to focus sound waves at a specific point. At the focal point, tissue temperatures can exceed 80 °C, resulting in rapid cell

death. Several studies are currently investigating the combined use of HIFU and thermosensitive liposomes to improve therapeutic efficacy.^{199,200}

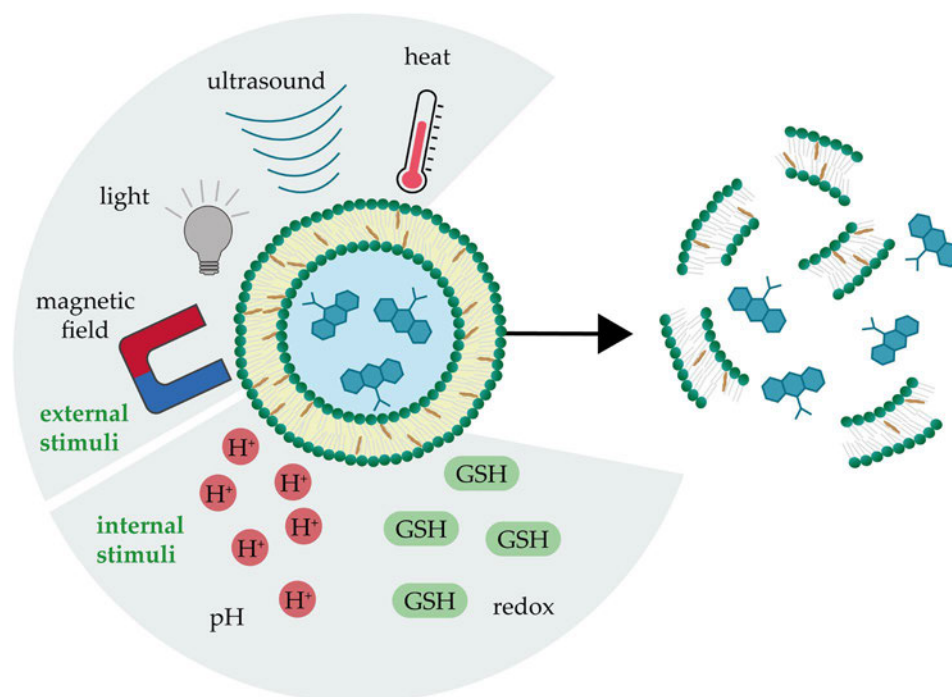


Figure 1.6: Summary of external and internal stimuli inducing the release of cargo from liposomes for the selective treatment of tumour tissue. (Figure inspired by Ashrafizadeh *et al.*)^{10,201}

1.4.3 Application of near-infrared light for release control

Near-infrared light (NIR, 750-2000 nm) is often used to trigger release from photosensitive liposomes, mirroring approaches used with other wavelengths. This longer wavelength light penetrates tissue more effectively than shorter wavelengths due to reduced absorption and scattering, despite carrying less energy per photon. Unlike ultraviolet (UV) radiation (100-400 nm), NIR light generally does not cause harmful changes to RNA, DNA or proteins, making it safer for biological applications.^{202,203} Based on this, design approaches were developed for photochemical-, photothermal- and photoisomerisation-induced release of the cargo from the liposomes.²⁰³ The photoisomerisation approach exploits UV-induced conformational changes of double bonds within the liposomal membrane, converting them from *trans* to *cis* configurations. This transformation increases the steric space requirement in the membrane, thereby increasing its permeability.²⁰⁴ For this purpose,

these liposomes contain lipids with an azo moiety, such as azobenzene, in which the conformational change is induced.²⁰⁵

Photochemically triggered liposomes can be created by incorporating photosensitisers into the lipid composition. When exposed to visible or NIR light, these photosensitisers generate reactive oxygen species (ROS) which subsequently damage the liposome membrane, facilitating the release of the encapsulated cargo.²⁰⁶ Generated ROS can in turn damage nearby tumour tissue, enhance tumour immunogenicity and antigen presentation while reducing immunosuppression in the tumour microenvironment (TME), leading to synergistic therapeutic effects.²⁰⁷ Several FDA-approved photosensitisers are available for therapeutic applications, most of which share an optimised porphyrin-oligomer core structure as the photoactive moiety with excitation wavelengths between 570 and 762 nm.²⁰⁸ Current research and approved formulations primarily use photosensitisers in liposomes to enhance pharmacokinetic properties by incorporating these molecules into the phospholipid bilayer, rather than for triggered cargo release. Visudyne, an approved formulation, is used in the treatment of age-related macular degeneration and scar tissue.^{209,210} Liposomal temoporfin has been approved for the palliative treatment of squamous cell carcinoma of the head and neck. The clinical approach of temoporfin is based on the accumulation of tissue followed by irradiation to activate the photosensitiser.²⁰⁶

Photothermal liposomes typically use a therapeutic strategy that combines heat-sensitive liposomes with incorporated photothermal agents capable of converting light energy into heat.²¹¹ One study demonstrated the efficacy of this approach by combining a water-soluble photothermal compound with a mild chemotherapeutic agent, resulting in a significantly enhanced anti-tumour effect. In addition, *in vivo* photothermal imaging, enabled by thermal imaging cameras, allows the visualisation of the conversion of radiation energy into thermal energy and the resulting heating of tissues as an indicator of liposome accumulation.²¹² Numerous studies have demonstrated the successful use of light-triggered thermal activation of liposomes in the treatment of various tumour types.²¹¹

1.4.4 Release of cargo by external magnetic fields

Unlike other liposome systems discussed, magneto-liposomes can be confined to the treated area by an applied magnetic field, preventing diffusion. This often sets liposomes apart from other nanoparticle systems due to their stability combined with a high loading of magnetic compounds. Additionally, the magnetic moieties in these liposomes allow visualisation by magnetic resonance imaging.^{201,213} In the preparation

of liposomes, magnetic substances such as iron oxides or magnetic nanoparticles can be incorporated into the phospholipid bilayer and the aqueous core.^{214,215} The precise performance of the liposomes is influenced by the frequency of the applied magnetic field. When magnetic liposomes interact with a high frequency magnetic field, it typically induces a temperature rise in the phospholipid bilayer. Above the phase transition temperature, the membrane permeability increases, which is consistent with the behaviour of thermosensitive liposomes. Low frequency magnetic fields do not cause an increase in temperature, but instead induce oscillation of the magnetic particles, which may also trigger the release of cargo, although the exact mechanism remains unclear. Benefits of using lower frequency ranges are generally their relative safety to the human body, the localised effect for a specific therapy, a higher cargo release and no hyperthermia of healthy tissue.^{216,217} Although the approach has been validated in several studies, it remains an active area of research requiring further refinement and optimisation.²¹⁷

1.4.5 Use of pH for cargo release

The concept of pH-sensitive liposomes exploits the pH difference between healthy tissue and the tumour microenvironment. While healthy tissue maintains a pH of 7.3-7.4, the TME has a more acidic pH of 5.5-6.8. This acidic state results from increased metabolic activity and an excess of CO₂, which contributes to tumour growth, resistance to therapy and a higher risk of metastasis.^{218,219} One approach induces liposome fusion with cell membranes by combining permanently charged cationic lipids with ionisable anionic lipids. The liposome surface charge is neutralised at a specific acidic pH, which favours fusion with the cell membrane. By adjusting the lipid ratio, the pH range at which membrane fusion occurs can be precisely controlled.²²⁰ Another strategy exploits pH-induced changes in lipid properties to disrupt the membrane and trigger cargo release. This method often uses dioleoylphosphatidyl ethanolamine (DOPE), which tends to form non-bilayer structures due to its conical geometry. However, when combined with a weakly acidic amphiphile such as cholesteryl hemisuccinate (CHEMS), stable liposomes can be formed. In an acidic environment, the anionic CHEMS is protonated and loses its ability to stabilise the bilayer membrane by electrostatic repulsion. This change triggers a phase transition in the DOPE, moving it to a hexagonal phase and consequently increasing membrane permeability.²²¹ In addition to the use of the phosphatidylethanolamine-based lipid DOPE, several other lipids have been investigated for the design of pH-responsive liposomes. These include the phosphoglycerol lipid 1,2-dipalmitoylsuccinylglycerol, oleic acid, polysaccharides and several polymers.^{222,223} Combined with active targeting

strategies, pH-sensitive liposomes offer a potentially powerful approach to enhance tissue specificity and accumulation, making them a promising strategy for tumour therapy.²²⁴

1.4.6 Exploitation of biochemical anomalies in tumours

Redox-sensitive liposomes can also be designed to exploit the biochemical differences between healthy and tumour tissue. This approach utilises the significantly elevated levels of glutathione (GSH) in tumour tissue, which can be up to 1000 times higher than in healthy cells. GSH plays a crucial role as an intracellular antioxidant and maintains cellular redox balance.²²⁵ An alternative approach focuses on the elevated levels of H₂O₂ in cancer cells, which are typically up to 100 times higher in comparison to healthy tissue.²²⁶ Using GSH as an example, stealth liposomes can be designed in which PEG chains are conjugated to liposomes via disulfide bonds. In the presence of high levels of GSH, the disulfide bonds are reduced, leading to cleavage of the PEG units and facilitating increased interaction between liposomes and cells.²²⁷ In a second approach, the lipid composition was supplemented with disilene conjugated to carboxylic acids. The cleavage of this linker was triggered by oxidation of disilene by reactive oxygen species, which subsequently initiated the release of the encapsulated cytostatic drug.²²⁸

1.5 Liposome delivery of polar methyltransferase inhibitors

The cellular phospholipid membrane typically allows uncharged and weakly polar small molecules to pass through. However, more polar molecules are often unable to cross this non-polar barrier due to their charge and hydrophilic nature.²²⁹ Liposomes offer a promising way to overcome this limitation by mediating the cellular uptake of polar molecules, such as inhibitors of RNA methyltransferases (MTases), which are inspired by the charged methyl donor *S*-adenosyl-*L*-methionine (SAM).^{230–232} Understanding and controlling RNA modifications is a major focus of current scientific research, as RNA methylation plays a critical role in many regulatory processes.²³³ The

importance of this field was further underlined in 2023 when Katalin Karikó and Drew Weissman were awarded the Nobel Prize, highlighting the critical importance of understanding and manipulating RNA modifications.²³⁴

1.5.1 The role of DNMT2

One area of RNA modification research has focused on the inhibition of DNA methyltransferase 2 (DNMT2), which catalyses the methylation of cytosine at position 38 to form 5-methylcytosine (m^5C) in human tRNA^{Asp(GUC)}, tRNA^{Gly(GCC)} and tRNA^{Val(AAC)} (Figure 1.7).^{230–232,235–237} The modification of cytosine, near the wobble position, typically has a substantial effect on tRNA stability and consequently on protein translation. This was demonstrated in mammalian cells by generating a double knock-out of DNMT2 and NOP2/Sun RNA methyltransferase 2 (NSUN2), both methyltransferases that catalyse the formation of m^5C . A clear reduction in the levels of unmethylated tRNAs was observed in the cells, leading to reduced protein translation.^{238,239} In addition, DNMT2 is thought to play a critical role in maintaining protein integrity by reducing the likelihood of misidentification between the methylated C38 in tRNA^{ASP(GUC)} and its related but unmethylated C38 in tRNA^{GLU(UUC)} during the process of protein translation.²³⁶

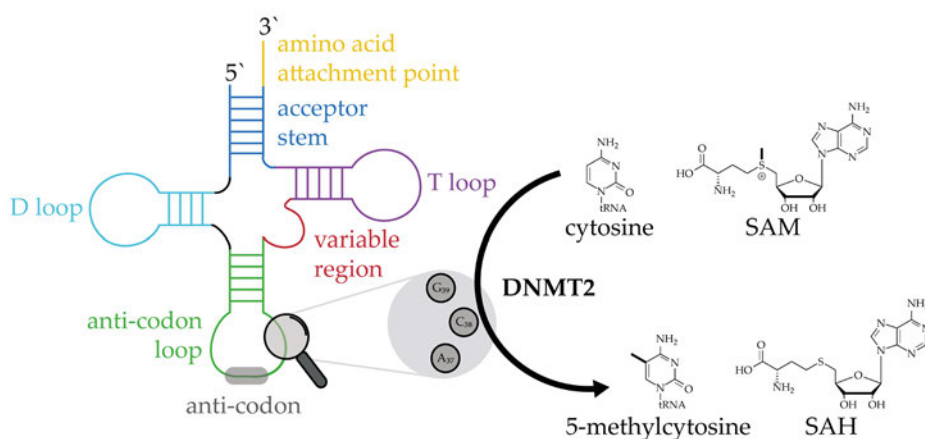


Figure 1.7: Schematic representation of a tRNA, highlighting its labelled loops and functional sections, as well as the methylation of cytosine to 5-methylcytosine catalysed by DNMT2 using SAM as methyl donor. (Figure inspired by Fischer *et al.*)^{240–242}

The role of DNMT2 in oncology is not well understood. In one study, increased enzymatic activity was observed in some somatic cancer mutations of DNMT2,

suggesting a possible role in tumour development.²⁴³ This is supported by the finding of a correlation between DNMT2 enzymatic activity, tumour proliferation, and metastasis in a study of liver cancer patients. However, further data are still very limited.²⁴⁴

1.5.2 Methyltransferase inhibitors

Besides the novel inhibitors inspired by natural SAM, several small molecules with inhibitory activity against various MTases have been reported in the literature.^{230–232} Methyltransferase inhibition can be achieved by leveraging *S*-adenosyl-*L*-homocysteine (SAH), the demethylated counterpart of SAM, which mediates non-specific feedback inhibition when its hydrolysis is blocked. This mechanism underlies the effect of the pan-inhibitors adenosine aldehyde and methylthioadenosine.^{235,245,246} In addition, the non-proteinogenic amino acid cycloleucine acts at the substrate level by inhibiting SAM synthesis via the methionine salvage pathway.^{247,248} Sinefungin, a small molecule with antifungal activity originally isolated from *Streptomyces griseolus*, was also reported to inhibit several methyltransferases due to its structural similarity to SAM. However, its inhibitory activity has only been studied in unicellular eukaryotes.^{230,242} Overall, these approaches result in a very limited range of available methyltransferase inhibitors.

2 Motivation and Objectives

The global burden of cancer remains a critical challenge, with statistics showing 20 million new diagnoses and 9.7 million deaths in 2022. These alarming numbers are further underscored by WHO projections of a potential 77% increase in cancer cases by 2050.^{249,250} The current standard of therapy, which often involves a combination of surgical removal of the tumour and chemotherapy, is facing increasing limitations. In particular, the significant damage caused to healthy tissue due to a lack of drug selectivity is hampering their effectiveness.²⁵¹ The development of novel, targeted therapeutic approaches is therefore urgently needed. Nanotechnology offers a promising avenue in this regard due to its inherent adaptability and potential for precise tissue targeting. Within the field of nanotechnology, liposomes have demonstrated considerable clinical potential, as evidenced by eight approvals in Europe and the USA. Despite this success, the development and approval of novel liposomal formulations remains a slow and challenging process. To address these challenges, the dual centrifugation-based production method for liposomes published by Massing *et al.* in 2008 offers a potent preparation technique. This technique provides unique advantages, including the ability to produce multiple samples simultaneously with minimal lipid consumption and high encapsulation efficiency for small molecules, making it a powerful tool for accelerating liposome research.

The first project of this thesis investigated the exchange kinetics of modified lipids between liposomes and cell membranes, a critical factor in maintaining surface modifications for targeted drug delivery. Building on the previous work of Fritz *et al.*, who observed a significantly higher transfer rate of modified cholesterol derivatives compared to dialkyl lipids from liposomes to cell membranes, this study aimed to elucidate the membrane anchoring stability of various modified dialkyl lipids.¹⁰² This

task was addressed through a combined approach involving highly efficient liposome preparation by dual centrifugation, covalent fluorescent labelling of lipids, and quantitative and qualitative fluorescence detection techniques. The integrated application of these methods to liposomes with labelled dialkyl lipids was intended to validate the lipid migration rate into other membrane systems *in cellulo*. In addition to a better understanding of the anchoring stability of dialkyl lipids, the influence of the total lipid composition of liposomes on lipid migration kinetics was also an area of interest.⁶

A major aim of this work was to develop novel pH-sensitive liposomes. Due to the large variety of available lipids, standard methods are not sufficient to produce and validate the properties of all potential lipid compositions. Therefore, an efficient workflow for liposome production and characterisation was required. To address these research objectives, we proposed an integrated methodological approach combining dual centrifugation for parallel liposome production with a suite of analytical techniques. The approach included physicochemical characterization methods, chromatographic analysis, and fluorescence-based liposome evaluation. By applying this comprehensive analytical framework to a diverse range of liposomal candidates, we aimed to elucidate the release properties and validate their potential as pH-responsive drug delivery systems. This multi-faceted strategy was expected to streamline the identification process of novel pH-sensitive liposomes.³

The third project focuses on the application of liposomes to transfect membrane-impermeable drug candidates for their evaluation of the *in cellulo* activity. For this purpose, a fusogenic liposome inspired by the lipid composition of natural exosomes and developed by Lu *et al.* was a promising avenue.⁷ To validate the liposomal system, dual centrifugation was used to produce samples with high entrapment efficiency together with quantitative and qualitative analysis techniques of cellular fluorescence as well as model substrates. This was complemented by chromatographic analysis methods. In the combination of the techniques, we aimed to standardize sample quality in order to assess the suitability of the approach *in cellulo* for the transfection of membrane impermeable drug candidates.

In addition to the main projects, emphasis was placed on cooperation in various projects. This involved the cultivation of various eukaryotic cell lines, the application of cell lysis methods, RNA extraction and the preparation of liposomes by dual centrifugation. The analytical evaluation of the samples was carried out using LC-MS. By integrating these techniques, the aim was to address questions related to liposomal drug delivery of an immunomodulator in a mouse model and to generate cell samples for tRNA composition analysis (data not shown in part).

3 List of Publications

Publications included in this thesis

1. Stability of Alkyl Chain-Mediated Lipid Anchoring in Liposomal Membranes

Gleue, L.; Schupp, J.; Zimmer, N.; Becker, E.; Frey, H.; Tuettenberg, A.; Helm, M. *Cells* 2020 Vol 9 Page 2213 2020, 9 (10), 2213. <https://doi.org/10.3390/CELLS9102213>.

Author contributions: L. Gleue prepared and purified the liposomes, including the subsequent chemical conjugation with fluorescent dyes. He also performed the flow cytometric experiments and sample preparation for confocal microscopy under the supervision of J. Schupp and N. Zimmer to investigate the influence of the chain length of fatty acids on the exchange rate between liposomal membranes and cell membranes. E. Becker synthesised the propargylated lipids with different chain lengths of the lipid anchors. The manuscript was written and illustrated by M. Helm and L. Gleue. M. Helm, A. Tuettenberg and H. Frey drafted the initial concept. ⁶

2. Dual Centrifugation-based Screening for pH-responsive Liposomes

Gleue, L.*; Gräfen, B.*; Voigt, M.*; Schupp, J.*; Schneider, D.; Fichter, M.; Kuske, M.; Mailänder, V.; Tüttenberg, A.; Helm, M. *ChemMedChem* 2024, e202400648. <https://doi.org/10.1002/cmdc.202400648>.

* These authors contributed equally to the publication.

Author contributions:

M. Voigt prepared the first liposomes and characterised the physicochemical particle properties and release rates under storage conditions and at acidic pH. J. Schupp developed an *in vivo*-based screening approach to test the delivery properties of the nanoparticles using flow cytometry and confocal microscopy. L. Gleue investigated the impact of serum albumins on liposomal stability and cargo release rate. He implemented a lipid quality control based on thin-layer chromatography to ensure the integrity of samples during the liposome preparation process. Together with B. Gräfen and supported by M. Kuske and M. Fichter, he investigated the *in vivo* circulation and organ distribution in albino black 6 mice. The concept was originally developed by M. Helm, D. Schneider, M. Mailänder and A. Tuettenberg. L. Gleue, M. Voigt, J. Schupp, B. Gräfen, A. Tuettenberg and M. Helm wrote and illustrated the manuscript.³

3. DORQ-seq: high-throughput quantification of femtomol tRNA pools by combination of cDNA hybridization and Deep sequencing

Kristen, M.; Lander, M.; Kilz, L.-M.; Gleue, L.; Jörg, M.; Bregeon, D.; Hamdane, D.; Marchand, V.; Motorin, Y.; Friedland, K.; Helm, M. *Nucleic Acids Res.* **2024**, 52 (18), e89. <https://doi.org/10.1093/nar/gkae765>.

Contribution as co-author:

L. Gleue cultivated several human cell lines as model samples for the publication.

4. In-Depth Immune-Oncology Studies of the Tumor Microenvironment in a Humanized Melanoma Mouse Model

Schupp, J.; Christians, A.; Zimmer, N.; Gleue, L.; Jonuleit, H.; Helm, M.; Tuettenberg, A. *Int. J. Mol. Sci.* **2021**, 22 (3), 1011. <https://doi.org/10.3390/ijms22031011>.

Contribution as co-author:

L. Gleue formulated liposomes loaded with tasquinimod, a checkpoint inhibitor, and established an LC-MS-based quantification method for the drug content in the lipid nanoparticles, which preceded this manuscript.

Publications not included

5. The P(III)-Amidite Based Synthesis of Stable Isotope Labeled mRNA-Cap-Structures Enables Their Sensitive Quantitation from Brain Tissue

Ripp, A.*; Krämer, M.*; Barth, V.; Moser, P.; Haas, T. M.; Singh, J.; Huck, T.; Gleue, L.; Friedland, K.; Helm, M.; Jessen, H. J. *Angew. Chem. Int. Ed.* **2024**, e202414537. <https://doi.org/10.1002/anie.202414537>.

* These authors contributed equally to the publication.

Contribution as co-author:

L. Gleue cultivated human cell lines as model samples for the extraction of messenger RNA in the process of method optimisation by M. Krämer.

4 Results and Discussion

4.1 Membrane anchoring of lipids in liposomal and cellular membranes

During his time as a PhD student in Mark Helm's group, Thomas Fritz worked on the surface modification of liposomes by adding synthetic lipids to the lipid composition, which subsequently enabled chemical conjugation with ligands. However, the conjugated liposomes, which in some experiments contained ligands to induce receptor-mediated particle uptake by cells, had no advantage over control groups. In follow-up experiments to investigate this observation, he replaced the ligand by fluorescent dyes and observed a rapid migration of cholesterol derivatives containing a fluorescent dye from the liposome membrane into the membrane of the incubated cells, while dye-conjugated dialkyl lipids remained stable in the liposome membrane. This finding was published 2016 in *Chemistry – A European Journal* in cooperation with scientists from the Institute of Organic Chemistry and the Institute of Nuclear Chemistry in Mainz ¹⁰².

Due to the importance of surface functionalised systems for the targeting of tissues and the modulating of particle properties, we decided to extend the study by Fritz *et al.* ^{252,253} We therefore decided to further improve our knowledge of suitable lipid linkers by studying the migration rate of diacyl lipids as a function of the number of carbon atoms in the hydrophobic tail region with a focus on dialkyl derivatives in a range of 16, 18 and 20 carbon atoms. We prepared liposomes containing small amounts of propargylated alkyl lipids of the appropriate chain lengths, which had previously been synthesised by Eyleen Becker from the Department of Chemistry. A fluorescent dye was then coupled to the corresponding propargylated lipid via a Huisgen cycloaddition. Jonathan Schupp from the Department of Dermatology and I successfully incubated melanoma cells with liposomes to quantify the incorporation

of labelled lipids in the cell membrane after 4 h and 24 h using flow cytometry. We discovered that the migration rate of dialkyl lipids with 16 carbon atoms per chain was significantly higher compared to the derivative with 18 and 20 carbon atoms. This was validated by confocal fluorescence imaging. In our final experiment, we investigated the effect of lipid composition on anchor stability. We did this by varying the ratio of the dialkyl lipid and by testing the effect of different dialkyl chain lengths. Our research showed that the ratio of dialkyl lipids in the lipid composition did not influence the exchange rate. However, the use of phospholipids with chains of 18 or 20 carbon atoms proved beneficial in reducing migration compared to inhomogeneous EPC3. ⁶

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Article

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Special Issue

Nanoparticles in Cancer Immunotherapy

Edited by

Prof. Dr. Stephan Grabbe



<https://doi.org/10.3390/cells9102213>



Article

Stability of Alkyl Chain-Mediated Lipid Anchoring in Liposomal Membranes

Lukas Gleue ¹, Jonathan Schupp ², Niklas Zimmer ², Eyleen Becker ³, Holger Frey ³ ,
Andrea Tuettenberg ^{2,*} and Mark Helm ^{1,*}

¹ Institute of Pharmaceutical and Biomedical Science, Johannes Gutenberg-University Mainz, 55128 Mainz, Germany; lugleue@uni-mainz.de

² Department of Dermatology, University Medical Center, Johannes Gutenberg-University Mainz, 55131 Mainz, Germany; jonschup@uni-mainz.de (J.S.); Niklas.Zimmer@unimedizin-mainz.de (N.Z.)

³ Department of Chemistry, Johannes Gutenberg-University Mainz, 55128 Mainz, Germany; ebecker@uni-mainz.de (E.B.); hfrey@uni-mainz.de (H.F.)

* Correspondence: antuette@uni-mainz.de (A.T.); mhelm@uni-mainz.de (M.H.)

Received: 30 July 2020; Accepted: 24 September 2020; Published: 29 September 2020



Abstract: Lipid exchange among biological membranes, lipoprotein particles, micelles, and liposomes is an important yet underrated phenomenon with repercussions throughout the life sciences. The premature loss of lipid molecules from liposomal formulations severely impacts therapeutic applications of the latter and thus limits the type of lipids and lipid conjugates available for fine-tuning liposomal properties. While cholesterol derivatives, with their irregular lipophilic surface shape, are known to readily undergo lipid exchange and interconvert, e.g., with serum, the situation is unclear for lipids with regular, linear-shaped alkyl chains. This study compares the propensity of fluorescence-labeled lipid conjugates of systematically varied lengths to migrate from liposomal particles consisting mainly of egg phosphatidyl choline 3 (EPC3) and cholesterol into biomembranes. We show that dialkyl glyceryl lipids with chains of 18–20 methylene units are inherently stable in liposomal membranes. In contrast, C16 lipids show some lipid exchange, albeit significantly less than comparable cholesterol conjugates. Remarkably, the C18 chain length, which confers noticeable anchor stability, corresponds to the typical chain length in biological membranes.

Keywords: liposomes; click chemistry; polyglycerol; bioconjugates; drug delivery

1. Introduction

Liposomes are spherical nanovesicles consisting of lipids, which were first produced in the 1960s by Alex Bangham et al. [1]. Due to the amphiphilic properties of phosphatidyl choline lipids in combination with cholesterol-based lipids, liposomes possess an aqueous core and one or more hydrophobic phospholipid bilayers. This enables the incorporation of hydrophobic molecules into the membranes as well as the transport of water-soluble molecules in the core compartment. Because of these properties, liposomes have found application in cancer treatment, gene therapy, cell biology and as a powerful drug delivery system [2–10].

Phospholipids, glycerophospholipids and derivatives of cholesterol can be used for the production of liposomes, and this variety of lipids allows the formulation of liposomes with different properties, especially adapted to the respective application.

For example, by adding cholesterol up to 50 mol-% to a liposome formulation, membrane stability and tightness can be increased or particle uptake by macrophages can be reduced by using lipids with linked polyethylene glycol (PEG) chains [11]. These commonly called “PEGylated” liposomes show peculiar behavior, known as the “stealth effect”, i.e., significantly increased blood circulation

half-life [12,13]. This effect results from shielding by the long polyethylene glycol chains, which reduces the binding of blood plasma components and interaction with macrophages, leading to increase half-life in blood circulation [14,15]. In addition to the classic example of PEGylation, the conjugation of liposomes to antibodies, folic acid, biotin or peptides can be used for specific tumor targeting [16–20].

We have previously established hyperbranched polyglycerol (*hbpG*)-derived stealth-like polyether lipid structures, possessing either a dialkyl or cholesterol anchor [21]. In human blood serum, liposomes containing hyperbranched polyglycerols showed lower aggregation than comparable PEGylated liposomes by comparable biodistribution [22,23]. Furthermore, *hbpG*-functionalization leads to increased interaction with macrophages, probably triggered by the lower amount of adsorbed proteins on the surface compared to classical PEGylated particles. This effect might possibly result from the larger number of free hydroxy groups in *hbpG*-functionalized liposomes, although experimental verification of this hypothesis is not yet available [5].

Cholesterol and many of its metabolic derivatives (such as steroid hormones) are distributed in the bloodstream by lipid–protein particles of variegated density. In addition to receptor-mediated uptake, steroid structures are thought to insert into, or pass through, cell membranes in the frame of a dynamic equilibrium [24,25]. Hence, lipid extraction from liposomes in a similar equilibrium is quite relevant for the sustained properties of liposomal formulations. Previous research of our group established the monitoring of lipid escape from the liposomal membrane and insertion into other membranes. This behavior was particularly evident for cholesterol lipids, which, presumably due to their irregular scaffold structure, are less stably anchored in membranes than typical biomembrane lipids whose two long hydrocarbon chains are known to form a dense network of van-der-Waals interactions in a symmetric lipophilic environment such as a cell membrane [26]. In order to demonstrate this effect, we prepared liposomes based on functionalizable cholesterol and dialkyl lipid derivatives, to which fluorescent dyes were covalently attached by click chemistry. After 4 h incubation of cells with the fluorescently labelled liposomes, confocal laser scanning microscopy images showed a significant fluorescence of labeled cholesterol derivatives in the cell membrane, in contrast to labeled dialkyl lipids which rarely inserted into the cell membrane [25].

The choice of appropriate lipid anchors can therefore influence a number of important parameters of liposomal formulations, e.g., the sustained transport (or release) of a model cargo during extended circulation, or the durability of molecular structures conjugated to the liposomal surface, which are important for stealth shielding or for targeting to specific receptors involving molecular recognition of bioconjugates [27,28].

In the present study, we identify a lower limit for dialkyl chain length in lipids, which leads to the exchange between the liposomal membrane and the cell membrane.

2. Materials and Methods

2.1. Synthesis of Dialkyl-Based Anchors

Three different hydrophobic anchor structures were synthesized for use as initiators for the anionic ring-opening polymerization (AROP) of epoxides. The initiators were prepared in a straightforward two-step procedure based on a procedure of Stauch et al., performing a Williamson etherification and using 1-*O*-benzyl glycerol and hexadecyl bromide, octadecyl bromide as well as icosane bromide, followed by hydrogenation to remove the benzyl protecting group [29]. In order to investigate the influence of the chain lengths, the anchors 1,2-bis-*n*-icosanyl glyceryl ether (BisID), 1,2-bis-*n*-hexadecyl glyceryl ether (BisHD) and 1,2-bis-*n*-octadecyl glyceryl ether (BisOD) were synthesized. The alkyl chain lengths of the anchor structures were 20 (BisID), 18 (BisHD) and 16 (BisOD) carbon atoms.

2.2. Synthesis of Amphiphilic Polyethers

The anionic ring-opening polymerization (AROP) of ethylene oxide was applied to synthesize well-defined PEG chains with tailored molecular weights [30]. The 1,2 bis-*n*-alkyl glyceryl ethers were

used as initiators for the polymerization to obtain amphiphilic polyether-based lipids (see supplement scheme S1). The combination of a hydrophobic initiator and a hydrophilic polyether results in polymers with amphiphilic behavior, which are suitable for the integration into liposomes to obtain sterically stabilized liposomal nanocarriers.

The AROP for the polymerization of ethylene oxide (EO) was carried out in dry tetrahydrofuran (THF), and potassium naphthalenide was used as a base to deprotonate the initiator. The deprotonation process was followed by color change of the initiator solution. The polymerization was carried out at 60 °C for 24 h to obtain full conversion. Molecular weights are controlled via the ratio of employed initiator and epoxide monomer.

2.3. Functionalization of Amphiphilic Polyethers with Propargyl Bromide

To attach alkyne-moieties, the polyether-based lipids were functionalized with propargyl bromide at the polyether end group. An alkyne-group enables the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) with functional groups, e.g., dyes like atto 488 azide or other azide-bearing molecules [25]. This reaction is known from literature and was adapted for the synthesized amphiphilic polyethers [31,32]. For this purpose, the terminal hydroxyl group of PEG was deprotonated using sodium hydride (NaH) (see supplement scheme S1). The terminal propargyl group was then introduced by a substitution reaction with propargyl bromide (structures: Figure 1B).

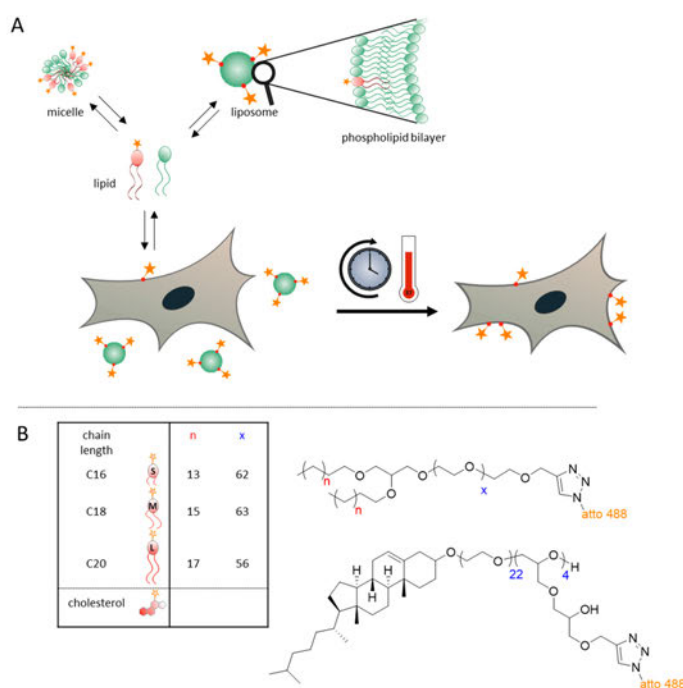


Figure 1. (A) Conceptual sketch of the approach. Lipid compounds are distributed among a variety of lipid-containing bodies in a complex equilibrium with ill-defined dynamics. The star symbolizes the attached fluorescence dye atto 488. (B) Synthetic experimental polyether lipids employed in the investigations: n describes the number of carbon atoms in alkyl chains, x the number of polyethylene glycol (PEG)-units.

2.4. Synthesis of Cholesterol-Based Linear-Hyperbranched Polyethers

As a drawback, despite its combination of favorable properties, such as excellent aqueous solubility, low toxicity and biocompatibility, PEG exhibits the disadvantage of low functionality, i.e., only the terminal hydroxyl groups [30,33]. Hyper-branched structures based on polyglycerol (*hbPG*) are therefore a promising alternative, due to the multitude of hydroxyl groups available for post-polymerization modification. Furthermore, *hbPG* is highly water-soluble and also exhibits excellent biocompatibility [34]. Hofmann et al. introduced amphiphilic *hbPG*s using different hydrophobic initiators (cholesterol, 1,2-bis-*n*-tetradecyl glyceryl ether (BisTD), 1,2-bis-*n*-hexadecyl glyceryl ether (BisHD) and 1,2-bis-*n*-octadecyl glyceryl ether (BisOD)) [21,35].

In the first reaction step a cholesterol-PEG-PEEGE precursor polymer was synthesized via AROP with EO and ethoxyethyl glycidyl ether (EEGE) (see supplement scheme S2). The polymerization of EEGE leads to linear structures (PEEGE) with acetal-protected hydroxyl groups, which can be released upon acidic treatment. The resulting linear polyglycerol (*linPG*) structure exhibits numerous hydroxyl groups and can therefore be used as a macroinitiator to prepare a hyperbranched polyglycerol (*hbPG*) block. Consequently, after the deprotection under acidic conditions, the cholesterol-PEG-*linPG* macroinitiator was used for “hypergrafting” of glycidol via the slow monomer addition (SMA) technique to obtain the cholesterol-PEG-*hbPG* polyether lipids (Figure 1B).

2.5. Functionalization of Cholesterol-PEG-*hbPG* with Propargyl Bromide

Polymer derivatization for subsequent functionalization by click chemistry was carried out using propargyl bromide. The hydroxyl groups of *hbPG* were deprotonated using sodium hydride (NaH). In the case of the functionalization of *hbPG*, the degree of functionalization was controlled via the amount of propargyl bromide employed. The average degree of functionalization was determined via ¹H NMR spectroscopy [29].

2.5.1. Liposome Preparation

In the first step, for the preparation of 5 mM liposomes, lipids dissolved in chloroform (see Table 1) were added to a PCR tube (#G001 F, Kisker Biotech, Steinfurt, Germany) depending on their percentage of total lipid amount and the solvent was removed by vacuum centrifugation (#5305, Eppendorf, Hamburg, Germany).

Table 1. Lipid compositions of liposomal formulations in mol-%.

Lipid 1	Percentage	Lipid 2	Percentage	Lipid 3	Percentage
EPC3	50 mol-%	Cholesterol	45 mol-%	C16 or C18 or C20 or	5 mol-%
EPC3	60 mol-%	Cholesterol	35 mol-%	Cholesterol alkyne lipid	
EPC3	70 mol-%	Cholesterol	35 mol-%		
DPPC	50 mol-%	Cholesterol	45 mol-%	C16 or C18 alkyne lipid	5 mol-%
DSPC	50 mol-%	Cholesterol	45 mol-%		
20:0 PC	50 mol-%	Cholesterol	45 mol-%		

EPC3: egg phosphatidyl choline (#527600, lipid, Ludwigshafen, Germany); DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (#850355C-200MG, Sigma-Aldrich, St. Louis, MO, USA); DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine (#850365C-200MG, Sigma-Aldrich); 20:0 PC: 1,2-diarachidoyl-sn-glycero-3-phosphocholine (#850368C-25MG, Sigma-Aldrich).

For complete removal of solvents, lipids were then freeze-dried at −80 °C (Alpha 2–4 LD, Martin Christ Gefriertrocknungsanlagen, Osterode/Harz, Germany) overnight and stored in a freezer until use at −20 °C.

To prepare liposomes, dried lipids were incubated together with 325 mg SiLibeads (#96035, Typ ZY-S 0.3–0.4 mm, Sigmund Lindner, Warmensteinach, Germany) and 9.3 µL DPBS (Dulbecco’s phosphate-buffered saline (#14190-094, Thermo Fisher Scientific, Waltham, MA, USA)) for 10 min at room temperature and then mixed for 20 min at 4 °C by dual centrifugation (#3200 + #3205,

Andreas Hettich, Tübingen, Germany) to form a phospholipid gel. To finally form liposomes, the gel was homogenized again together with 77.2 μ L DPBS twice for 2 min at 4 $^{\circ}$ C.

2.5.2. Liposome Modification and Purification

To label alkyne liposomes with fluorescent dye atto 488-azide, 30 μ L liposome stock was dissolved in 100 mM phosphate buffer pH 8 (PB) (94.7 mM disodium hydrogen phosphate (#P030.1, Carl Roth, Karlsruhe, Germany), 5.3 mM sodium dihydrogen phosphate dihydrate (#T879.1, Carl Roth)), 0.5 mM Tris (hydroxypropyltriethylammonium) amine (THPTA, Helm Group, Johannes Gutenberg University Mainz, Germany), 0.1 mM copper sulfate pentahydrate (#8175.1, Carl Roth), 2.5 mM sodium ascorbate (#3149.1, Carl Roth), 0.1 mM atto 488-azide (#AD 488-101, Atto-Tec, Siegen, Germany) in a PCR tube (#G001 F, Kisker Biotech) and filled up with MiliQ-Water (#ZRQSVR5WW, Merck Millipore, Darmstadt, Germany) to a final volume of 120 μ L.

The reaction mixture was agitated at room temperature for two hours and after completion of the transformation, 20 mM EDTA (ethylenediaminetetraacetic acid (#8040.1, Carl Roth, solved in water)) was added to stop the reaction.

2.5.3. Cell Culture and Cell Lines Employed

The human melanoma cell line UKRV-Mel-15a was cultured in RPMI 1640 medium (#31870, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (fetal bovine serum (#10500064, Thermo Fisher Scientific)), 1% GlutaMAXTM (#35050038, Thermo Fisher Scientific) and 0.1% primocin (#ant-pm-2, InvivoGen, San Diego, CA, USA). Cells were detached via incubation Trypsin-EDTA (#T3924, Merck, Darmstadt, Germany) for 5 min at 37 $^{\circ}$ C every 3 to 4 days.

2.5.4. Flow Cytometry

For analysis of lipid exchange via flow cytometry UKRV-Mel-15a cells were seeded in 24 well tissue culture plates (#3524, Corning, Corning, NY, USA) at a density of 100,000 in 1 mL medium and incubated for 4 or 24 h with liposomes at a concentration of 1% or 5% (v/v). For Section 3.5, adherent cells were treated additionally with 10 μ M cytochalasin D (#C2618, Merck) 30 min prior to liposome addition [36–38]. Cells were detached via incubation with Trypsin-EDTA (#T3924, Merck) for 5 min at 37 $^{\circ}$ C. Single cell suspension was stained with 200fold diluted Fixable Viability Dye eFluorTM 780 (#65-0865-18, Thermo Fisher Scientific) in DPBS (#14190-094, Thermo Fisher Scientific) for 20 min at 4 $^{\circ}$ C for dead cell exclusion, fixed with 4% PFA (paraformaldehyde (#0335.1, Carl Roth)) in DPBS for 20 min at 4 $^{\circ}$ C and measured in FACS buffer containing 0.5% HSA (#10530a/96, CSL Behring, Marburg, Germany), 1 mM EDTA (#A3553, AppliChem, Darmstadt, Germany), 10 μ g/mL human IgG (#EU/1/08/446/001, CSL Behring GmbH, Marburg, Germany) in DPBS.

Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and samples were measured by 488 nm (FITC-channel 505LP, 530/30) and by 633 nm (APC-channel 660/20; FVD780 735LP, 780/60). Data were analyzed by using Cytobank [39].

2.5.5. Microscopy

For the confocal imaging, the Leica SP8 with HyD Detector (Wetzlar, Germany) was used with lasers for 405 nm, 488 nm and 638 nm. Melanoma cell line UKRV-Mel-15a was cultured for 24 h in ibidi μ -slides 8 well (#80826, ibidi, Gräfelfing, Germany), 30,000 cells/well each, in supplemented RPMI 1640 medium (see cell culture cell line). After treatment for 4 and 24 h with the same endpoint, cells were checked for adherence and then fixed with 4% PFA (#0335.1, Carl Roth, Karlsruhe, Germany) in DPBS for 20 min at 4 $^{\circ}$ C. Additionally DNA was stained by Hoechst 33342 (#PK-CA707-40046, PromoCell, Heidelberg, Germany) and the membrane by DiD (1,1-Dioctadecyl-3,3,3-tetramethylindodicarbocyanine (#PK-CA707-30021, PromoCell, Heidelberg, Germany)) for 30 min at RT each. For image analysis, Fiji was used [40].

2.5.6. Dynamic Light Scattering

For liposome characterization, 20 μ L purified liposome suspension was diluted in 1 mL MilliQ-water and polydispersity (PDI), size and zeta-potential (ζ -potential) were measured in a disposable folded capillary cell (#dts1070, Malvern, Worcestershire, United Kingdom) at 25 °C by using a Malvern Zetasizer Nano series (Malvern). The refractive index was set to 1.33 (1.59 for liposomes) with a water viscosity of 0.8872 and the scattering angle was configured to 173.

3. Results

3.1. Concept and Approach

The strategy for monitoring the fate of different dye-conjugated lipids is shown in Figure 1A. The upper part depicts in a general manner the behavior of single lipid molecules in equilibrium between micelles, free lipids and liposomal structures. In *in vitro* cell culture, the latter also includes the cell membranes, which participate in an exchange equilibrium with liposomes.

To monitor the exchange equilibrium, we labelled the polyether model lipids with a fluorescent dye and traced their location both by microscopy and by flow cytometry. Suspension of liposomes containing fluorescence-labelled lipids were added to cell cultures, and after a certain incubation period, the localization and intensity of fluorescence in the cell membranes were analyzed. Of note, we are aware that we cannot directly distinguish between lipid exchange taking place at the cell membrane with liposomes in suspension or phagocytosis of liposomes and their subsequent degradation and incorporation into the cell membrane system. However, time-lapse imaging with this approach has previously enabled us to efficiently monitor the fast exchange of cholesterol derivatives between liposomes and cell membranes [26]. We observed a rapid exchange rate in the single-minutes digit for cholesterol derivatives, with fluorescent lipids first appearing in the cell membrane and only later being incorporated into intracellular vesicles [26]. In this work, we observed a significantly slower exchange for alkyl chains up to a time of 4 h, leading us to conduct the current observation at 4 h and 24 h. The latter time point was chosen to obtain a measure of the duration of the lipid exchange. To guard against the potential adverse effects of endocytosis, we also performed experiments in the presence of cytochalasin D, an endocytosis inhibitor.

3.2. Experimental Lipids and Liposome Preparation

Figure 1B depicts the different compounds employed in this setting, which were synthesized based on our prior discovery that revealed high dynamics of a cholesterol derivative in transitions from liposomal preparations to cell membranes [26]. The model compounds employed are conceptually similar to biological membrane lipids containing two long alkyl chains as well as a hydrophilic headgroup, which in the current work is a polyethylene glycol chain (or alternatively a *hbPG* structure) that additionally equips the liposomes with a stealth effect. The synthesis of these compounds was conducted along the lines described previously by Frey et al. [21,23,35]. Briefly, the synthesis of PEGylated lipids was performed by anionic ring-opening polymerization (ROP) starting from dialkyl-substituted glycerol. Subsequent to the polymerization of EO, the resulting PEGylated ether lipids were reacted with propargyl bromides to functionalize the chain ends with a propargyl-ether.

The terminal alkyne moieties allowed for subsequent derivatization by click chemistry either before (pre) or after (post) liposomal formulation. Our current investigation relies on post-formulation CuAAC of liposomes with fluorescent dyes, which were then purified by gel filtration chromatography. The advantages of this formulation–derivatization sequence include mild reaction conditions and fast product isolation by routine semi-automated methods [41].

The production of liposomes was conducted by dual centrifugation, a technique based on the simultaneous rotation of two sample plates around two axes. This results in the mixing of the samples instead of sedimentation as in classical centrifugation. The advantages of using dual centrifugation to produce liposomal formulations are the choice of flexible preparation sizes with simultaneous sterile

and endotoxin-free conditions. In addition, this cost-effective method allows for the simultaneous preparation of several samples [26,42].

The liposome suspensions prepared by dual centrifugation were then fully automatically purified by using semi-automated gel filtration on an HPLC (high-performance liquid chromatography) setup to remove free lipids. Routine characterization included size determination by dual light scattering and measurement of surface potential. For this work, we used liposomes with 162.60 ± 29.51 nm diameter, 0.26 ± 0.07 a.u. polydispersity and -13.87 ± 0.07 mV zeta-potential.

3.3. Analysis of Lipid Exchange Via Flow Cytometry

To compare the transfer of fluorescently labelled experimental lipids from liposomes to cells in tissue culture, cells were cultivated for 4 h and 24 h with liposomal formulations containing the fluorescence-labeled lipids in two different concentrations. In comparison to previously investigated RBE4 cells [26], we found the human melanoma cell line UKRV-Mel-15a more amenable to rapid and reproducible analyses, presumably because of their relatively large size. After incubation with liposomal preparations, the medium was removed, cells were fixed and then analyzed by flow cytometry.

The results after gating signals for the atto 488 are presented in Figure 2A,B. Figure 2A, reflecting the fluorescence intensity obtained after an incubation of 4h, clearly reproduces the behavior of cholesterol-anchored lipids as previously published, showing strong signals corresponding to a transfer of cholesterol derived lipids to the cell membranes [26]. For the dialkyl lipids, most signals remained near a baseline of 5.0×10^3 with the visually apparent, but not significant, exception of the liposomal preparation containing 5 mol-% C16 compounds, i.e., the shortest alkyl chain used in this study. Additionally, cells incubated with C18 and C20 liposomes for 4 h showed moderately, but not significantly, increased fluorescence.

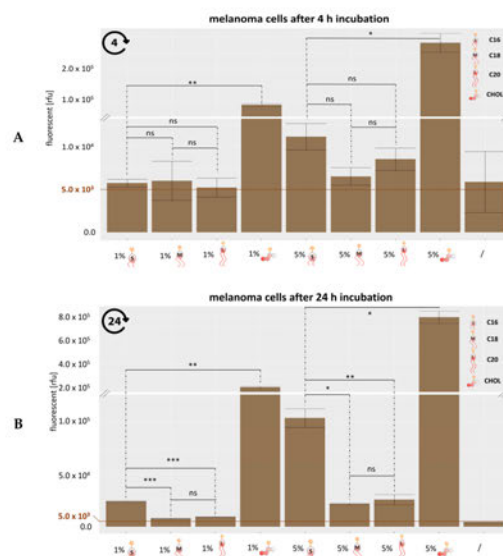


Figure 2. Quantification of lipid exchange after 4 h (A) and 24 h (B) by flow cytometry. 1.0×10^5 UKRV-Mel-15a cells were incubated with 1 or 5 vol-% atto 488 linked liposomes at 37°C and fixed with a 4% formaldehyde solution before flow cytometric analysis. The significance between two values is illustrated by * $p = 0.05$ – 0.01 , ** $p < 0.001$, *** $p < 0.0001$ and ns = no significance. The statistically significant was calculated using the Welch and Brown–Forsythe version of the one-way ANOVA test.

Data obtained after 24 h (Figure 2B) demonstrate the progression of lipid exchange for all preparations containing 5 mol-% polyether lipids, albeit only to a very limited extent for the C18 and C20 anchor chains. Meanwhile, in further confirmation of our previous observations, the signal resulting from the cholesterol derivative outdistanced all others by far, more subtle differences were observable among the polyether lipids.

Thus, the C16 preparations both at 1 mol-% and 5 mol-% now gave rise to significantly higher signals than those of the corresponding longer C18 and C20 alkyl chains. These observations suggest that for the anchoring of stealth structures with alkyl chains, a minimum alkyl chain length of C18 is very efficient, whereas a length of 16 carbon atoms delineates a border, below which anchoring might be insufficient. Future studies including, e.g., C14 and C12 polyether lipids, must be undertaken to understand if chain length is the only determinant of membrane anchorage.

3.4. Tracking Labeled Lipids by Fluorescence Microscopy

For a more spatially resolved, detailed investigation, cells exposed to liposomes as described above for the flow cytometry analyses were imaged by confocal fluorescence microscopy. The results for 4 h and 24 h incubation at 37 °C are shown side-by-side in Figure 3.

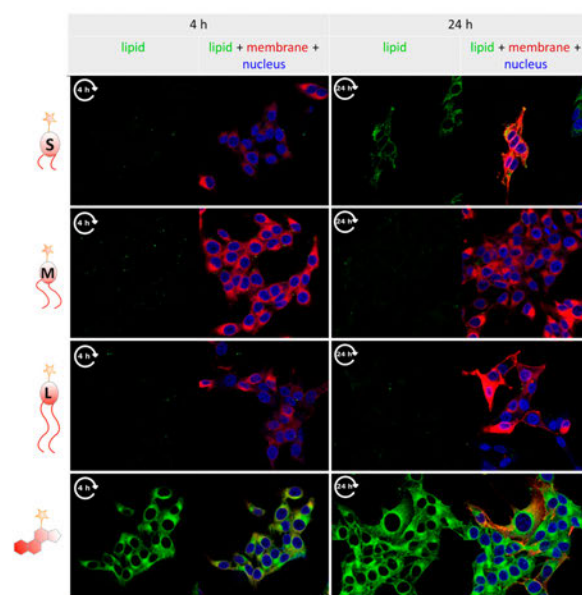


Figure 3. Confocal fluorescence imaging of UKRV-Mel-15a cells, observed at 4 h and 24 h after incubation with liposomes containing membrane label (DiD, red) and click-labeled experimental lipids with varied chain length (atto 488, green). Cell nuclei were stained with Hoechst (blue). Together with the cell membrane shown in red, the insertion of lipids becomes visible as an orange signal in the merge view.

As a positive control for lipid exchange, the liposomes contained a DiD label, which is observed to stain the cells' biomembranes in the red fluorescence channel clearly. In comparison to this efficient exchange from liposomes to cell membranes, C18 and C20 lipids hardly show signals of relocation, which is coherent with the observations in flow cytometry stated above. In contrast, relocation was easily visible for cholesterol-conjugated dye, which rapidly stained cell membranes, indicated by

green fluorescence from the atto 488 dye, or by an overlay of red and green fluorescence visualized in orange in Figure 3, this observation, too, is well in line with the flow cytometry data. To a lesser extent, but clearly visible, the relocation of green fluorescence was observed for the labeled C16 lipid after 24 h (upper right panel in Figure 3), signaling a certain extent of lipid exchange from liposomes to the cell membrane. In summary, these images thus confirm the tendency of C16 alkyl lipid to enter into a dynamic equilibrium as depicted in Figure 1A in a time frame comprising a few hours to one day.

3.5. Analysis of Lipid Exchange Depending on Liposome Composition

To further characterize the differential behavior of C16 versus longer alkyl lipids in some more depth, we investigated the influence of liposome composition on the lipid exchange. Liposomes containing 5 mol-% C16 versus C18 alkyl lipids were prepared with different EPC3: Chol ratios, namely 50/45, 60/35 and 70/25. Lipid exchange after 24 h incubation was analyzed by flow cytometry as described above. To suppress the potential influence of endocytotic uptake, cell culture experiments were carried out in the presence of 10 μ M endocytosis inhibitor cytochalasin D.

The results shown in Figure 4A show slightly decreased exchange and thus more stable anchoring for C18 lipids at higher EPC3 content, while the exchange of the C16 lipid peaks at medium EPC3 content. The principle insight obtained here, though, is that the difference between C16 and C18 is highly significant at all ratios, and that C16 maintains similarly high lipid exchange rates even at increased EPC3 content.

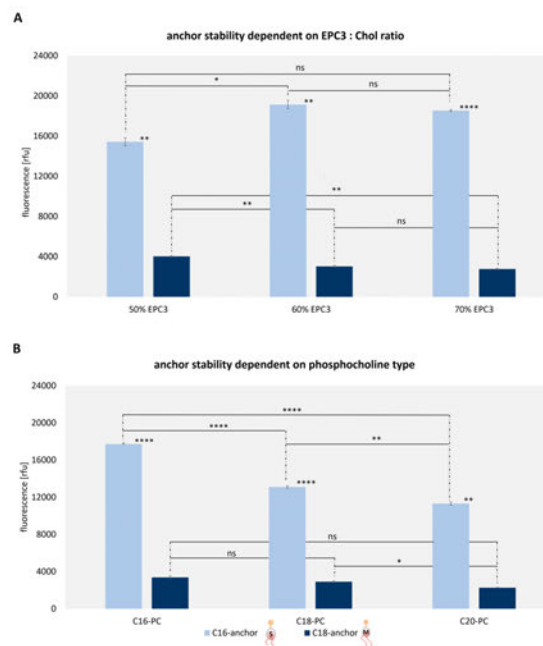


Figure 4. Quantification of lipid exchange depending on (A) the EPC3: Chol (cholesterol) ratio (B) and on the carbon atom number of phosphocholines used for liposome preparation. 1.0×10^5 UKRV Mel-15a cells were incubated with 5 vol-% atto 488 linked liposomes and cytochalasin D at 37 °C and fixed with a 4% formaldehyde solution before flow cytometric analysis. The significance between two values is illustrated by * $p = 0.05$ – 0.01 , ** $p < 0.001$, *** $p > 0.0001$ and ns = no significance. The statistically significant was calculated using the Welch and Brown–Forsythe version of the one-way ANOVA test.

In a similar experiment, we investigated the effect of phosphocholines with different dialkyl chain lengths. Correspondingly liposomes were prepared, which contained phosphocholine with chain lengths of C16, C18, or C20 instead of EPC3. Each formulation contained 5 mol-% C16 or C18 alkyne lipid. The results after 24 h incubation shown in Figure 4B suggest a slightly increased anchoring of our C18-hbPG-lipid in membranes containing long-chain phosphocholine, but this visual impression is not statistically significant. Similarly, our C16-hbPG-lipid displays reduced lipid exchange in longer EPC chains, and this effect is highly significant. Throughout, the mobility of the C16 compound is clearly higher than that of the C18 compound.

4. Discussion

In the present study, we showed results on the mobility of amphiphilic polyether lipids in liposomal membranes with respect to their propensity to insert into cell membranes in a time frame of up to one day, which is a relevant time span for testing liposomal formulations in either in vitro or in vivo.

We used fast exchanging cholesterol derivatives as a positive control in the comparative analysis of a mini-series of homologous lipids containing dialkyl chains of increasing length [26]. The data firmly established that dialkyl anchors of any tested length are much more stably anchored in the liposomal membrane than cholesterol derivatives, extending previous observations to lipids as short as C16 [26]. This is plausible, given the irregular shape of cholesterol, yet interesting with respect to the behavior of biological lipids with chain lengths shorter than the classical C18. Of interest, the lipids forming the liposomal double-membrane consisted of nearly equal parts cholesterol, and phosphatidylcholine, the latter being structurally more similar to the dialkyl lipids. Although the lipid-dye conjugate used here to trace the lipid exchange contained a rather hydrophilic polyglycerol chain, which is likely to enhance its extraction from a membrane, our results support a view of “aging” liposomes, which may dramatically change their composition when exposed to a “lipid extraction equilibrium” as depicted in Figure 1B. This may give rise to disadvantages on multiple levels [43,44]. One such level is the structural integrity of the liposome, required to retain or release its therapeutic cargo in a reproducible fashion. Clearly, this important property may be subject to dramatic alterations, depending on the lipid extraction conditions in vivo, but also depending on the propensity of a given liposomal constituent compound for lipid exchange. While previous work identified cholesterol conjugates as problematic, our current data offer an approximation for the choice of non-steroid lipids capable of reducing the risk of undesired lipid exchange [26].

On another level, the molecular surface of liposomes, especially when decorated with targeting or stealth structures, is likely to suffer from lipid exchange. Here, too, our studies provide important results. In particular, we show that dialkyl lipids with 16C chains are more likely to escape from liposomal membranes than longer-chain lipids. This behavior may be important for the choice of dialkyl lipids as anchors for modifications, such as PEGylation or antibody conjugation, because the number of carbon atoms in dialkyl chains may have an influence on stability and half-life time of a modification in the application of liposomes as drug delivery systems. Particularly after intravenous application, liposomes encounter various moieties capable of lipid exchange, including cellular components of blood such as erythrocytes and thrombocytes, but also various protein–lipid particles involved in lipid transport such as HDLP, LDLP, and the like.

As a final comment, we would like to point out the striking fact that we detect increasing propensity for lipid exchange for lipids with alkyl chains just below the typical length encountered in many biomembranes, namely C18 (e.g., oleic acid, vaccenic acid or stearic acid) [45,46]. While ours is but a single data point of one lipid with C16 chains, it might be worth looking into this border in a more systematic fashion.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4409/9/10/2213/s1>, Scheme S1: Synthesis Route of the Dialkyl PEG-Lipids and Functionalization with propargyl bromide. Scheme S2: Synthesis Route of the Cholesterol-PEG-hbPEG Lipids and Functionalization with propargyl bromide.

Author Contributions: Conceptualization, M.H., H.F. and A.T.; methodology, J.S., M.H. and A.T.; validation, L.G., N.Z. and J.S.; synthesis E.B.; formal analysis, J.S., N.Z. and L.G. writing—original draft preparation, M.H.; writing—review and editing, J.S., L.G., H.F., M.H. and A.T.; visualization, J.S. and L.G.; supervision, M.H. and A.T.; project administration, J.S.; funding acquisition, M.H., H.F. and A.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was founded by German Research Foundation (CRC1066), Project A7 to MH and H.F. and Project B3 to AT, and by the Wilhelm-Sander Stiftung to A.T.

Acknowledgments: We thank the imaging core facility of Gregory Harms in the University Medical Center Mainz for providing the Leica TCS SP8 microscope and excellent technical advice. We also thank Anna-Maria Hofmann, Matthias Worm and Ann-Kathrin Danner, former members of the Frey Group, Johannes Gutenberg-University of Mainz, for the synthesis of lipids used in this study. Matthias Voigt, former member of the Helm group, is acknowledged for technical assistance in initial phases of this study.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Material

S1. Materials and Methods

S1.1. Reagents

Unless mentioned otherwise, all chemicals were obtained from *Sigma Aldrich*, *Acros Organics*, *Fisher Scientific* or *TCI Europe*. Deuterated solvents (pyridine-*d*₅, benzene-*d*₆ or chloroform-*d*) were purchased from *Deutero GmbH*. Ethylene oxide (EO) was received from *Sigma Aldrich* and must be handled with high precaution. Ethoxyethyl glycidyl ether (EEGE) was synthesized according to literature¹ and was dried over CaH₂ for at least 30 min. Subsequently it was freshly cryo-transferred prior to use. For the anionic ring-opening polymerization (AROP) dry THF was stored over benzophenone/sodium.

S1.2. Instrumentation

¹H, ¹³C NMR and 2D spectra were measured on a Bruker Avance III HD 300 (300 MHz, 5 mm, BBFO probe, and B-ACS 60 auto sampler) or rather a Bruker Avance II spectrometer operated at 400 MHz (5 mm BBFO smart probe and SampleXpress 60 auto sampler) at 296 K. Pyridine-*d*₅, benzene-*d*₆, chloroform-*d* or DMSO-*d*₆ was used as a solvent. The NMR spectra were referenced internally to the respective signals of the deuterated solvent. Analysis of all spectra was carried out using the software MestReNova version 9.0.

S1.3. Handling of Ethylene Oxide (EO)

The gaseous, flammable and highly toxic ethylene oxide (EO) must be handled carefully and has to be stored in pressure-proof gas bottles. It must be used only in an adequate fume hood under appropriate safety precautions. Polymerizations in which EO is involved are performed in flame-dried glassware to enable the conversion of EO inside the sealed and evacuated glass apparatus and to guarantee secure handling via cryo-transfer techniques. To avoid abrupt detachment of the septum and hence release of EO the maximum batch-sizes of 8 g EO in a 500 mL flask must not be exceeded.

S2. Synthesis

S2.1. Synthesis of Ethoxyethyl Glycidyl Ether (EEGE)

EEGE was synthesized as described in literature.¹

¹H NMR, COSY (300 MHz, chloroform-*d*, δ): 4.75–4.68 (qd, *J* = 5.3 Hz; 3.4 Hz, 1H, acetal H), 3.86–3.29 (m, 4H, -CHCH₂OCOCH₂-, -CHCH₂OCOCH₂-), 3.13–3.07 (m, 1H, CH₂OCHCH₂-), 2.77–2.74 (ddd, *J* = 5.1 Hz; 4.1 Hz; 1.0 Hz, 1H, CH₂OCHCH₂-), 2.61–2.55 (ddd, *J* = 10.7 Hz; 5.1 Hz; 2.7 Hz, 1H, CH₂OCHCH₂-), 1.29–1.26 (dd, *J* = 5.4 Hz; 4.7 Hz, 3H, -OCHCH₃), 1.18–1.13 (td, *J* = 7.1 Hz; 0.9 Hz, 3H, -OCH₂CH₃).

S2.2. Synthesis of 1,2-Bis-*N*-Octadecyl Glyceryl Ether (BisOD)

The synthesis was carried out according to literature.²

¹H NMR, COSY (400 MHz, chloroform-*d*, δ): 3.74–3.41 (m, 9H, glycerol H), 1.59–1.53 (m, 4H, -OCH₂CH₂-), 1.31–1.25 (m, 60H, -OCH₂CH₂(CH₂)₁₅CH₃), 0.88 (t, *J* = 13.1 Hz; 7.2 Hz, 6H; -O(CH₂)₁₇CH₃).

¹³C NMR, HSQC, HMBC (101 MHz, chloroform-*d*, δ): 78.4 (glycerol C), 72.0 (-OCH₂CH₂-), 71.1 (glycerol C), 70.6 (-OCH₂CH₂-), 63.3 (glycerol C), 32.1 (-OCH₂CH₂(CH₂)₁₇CH₃), 30.3 (-OCH₂CH₂-), 29.9–29.5 (-OCH₂CH₂(CH₂)₁₇CH₃), 26.3 (-OCH₂CH₂(CH₂)₁₇CH₃), 22.9

($-\text{OCH}_2\text{CH}_2(\text{CH}_2)_{17}\text{CH}_3$), 14.3 ($-\text{O}(\text{CH}_2)_{19}\text{CH}_3$).

S2.3. Polymer Synthesis of BisOD-PEG

The synthesis is described for BisOD-PEG81 as a representative example.

1,2-Bis-*n*-octadecyl glyceryl ether (BisOD) (0.2 g, 0.36 mmol, 1 eq.) was placed in a dry Schlenk flask and dissolved in benzene (10 mL). The solution was stirred at 60 °C for 30 min and dried in vacuo for 16 h to remove moisture. Dry tetrahydrofuran (approx. 10 mL) was cryo-transferred to the Schlenk flask to dissolve the initiator. Afterwards, the initiator was deprotonated with a 0.5 M solution of potassium naphthalenide in THF (0.36 mL, 0.18 mmol, 0.5 eq.) while stirring. The solution was cooled down to −80 °C and ethylene oxide (EO) (1.70 mL, 37.57 mmol, 105 eq.) was cryo-transferred using a graduated ampule. The polymerization was carried out at 60 °C for 24 h. In order to quench the polymerization, an excess of ethanol was added. The solvent was removed under reduced pressure, the crude product was dissolved in methanol and precipitated twice in cold diethyl ether to obtain the pure product. Yield: 99%.

¹H NMR, COSY (400 MHz, benzene-*d*₆, δ): 3.67–3.30 (m, 359H, polyether backbone and glycerol H), 1.66–1.56 (m, 4H, $-\text{OCH}_2\text{CH}_2-$), 1.44–1.28 (m, 58H, $-\text{OCH}_2\text{CH}_2(\text{CH}_2)_{15}\text{CH}_3$), 0.92–0.89 (m, 6H, $-\text{O}(\text{CH}_2)_{17}\text{CH}_3$).

¹³C NMR, HSQC, HMBC (101 MHz, benzene-*d*₆, δ): 79.0 (glycerol C), 73.4 (polyether backbone and glycerol C), 72.4–70.9 (polyether backbone and glycerol C), 62.2 (polyether backbone and glycerol C), 32.6 ($-\text{OCH}_2\text{CH}_2(\text{CH}_2)_{14}\text{CH}_2\text{CH}_3$), 31.1–30.1 ($-\text{OCH}_2\text{CH}_2-$ and $-\text{OCH}_2\text{CH}_2(\text{CH}_2)_{14}\text{CH}_2\text{CH}_3$), 27.0 ($-\text{OCH}_2\text{CH}_2(\text{CH}_2)_{14}\text{CH}_2\text{CH}_3$), 23.4 ($-\text{OCH}_2\text{CH}_2(\text{CH}_2)_{14}\text{CH}_2\text{CH}_3$), 14.64 ($-\text{O}(\text{CH}_2)_{17}\text{CH}_3$).

S2.4. Functionalization of BisOD-PEG with Propargyl Bromide

The functionalization is described for BisOD-PEG81-alkyne as a representative example.

BisOD-PEG81 (0.3 g, 0.074 mmol, 1 eq.) was placed in a dry Schlenk flask and dissolved in dry THF. The solution was cooled to 0 °C and sodium hydride (0.005 g, 0.223 mmol, 3 eq.) was added while stirring. Subsequently, propargyl bromide (0.020 mL, 0.223 mmol, 3 eq.) was added and the solution was stirred for 24 h at room temperature. The reaction mixture was filtered and the solvent was slightly reduced under reduced pressure. The remaining solution was precipitated twice in cold diethyl ether and the pure product was dried in vacuo. Yield: 66%.

¹H NMR, COSY (300 MHz, benzene-*d*₆, δ): 3.95–3.94 (d, *J* = 2.4 Hz, 2H, $-\text{OCH}_2\text{CCH}$), 3.77–3.24 (m, 374H, polyether backbone and glycerol H), 2.13–2.12 (t, *J* = 2.4 Hz, 1H, $-\text{OCH}_2\text{CCH}$), 1.69–1.57 (m, 12H, $-\text{OCH}_2\text{CH}_2-$), 1.47–1.30 (m, 60H, $-\text{OCH}_2\text{CH}_2(\text{CH}_2)_{15}\text{CH}_3$), 0.94–0.90 (m, 6H, $-\text{O}(\text{CH}_2)_{17}\text{CH}_3$).

S2.5. Synthesis of 1,2-Bis-*N*-Octadecyl Glyceryl Ether (BisHD)

The synthesis was carried out according to literature.²

¹H NMR, COSY (400 MHz, chloroform-*d*, δ): 3.78–3.36 (m, 9H, glycerol H), 1.63–1.49 (m, 4H, $-\text{OCH}_2\text{CH}_2-$), 1.40–1.17 (m, 52H, $-\text{OCH}_2\text{CH}_2(\text{CH}_2)_{13}\text{CH}_3$), 0.88 (t, *J* = 13.1 Hz; 7.2 Hz, 6H; $-\text{O}(\text{CH}_2)_{17}\text{CH}_3$).

S2.6. Synthesis of 1,2-Bis-*N*-Icosanyl Glyceryl Ether (BisID)

The synthesis was carried out according to literature.³

Dry tetrahydrofuran (THF) was placed in three-necked round bottom flask equipped with Dimroth condenser and sealed precision glass (KPG) stirrer. Under argon atmosphere and stirring 3-benzyloxy-1,2-propanediol (2.7 mL, 0.017 mol, 1 eq.), sodium hydride (1.62 g, 0.068 mol, 4 eq.) and 1-bromoicosane (24.42 g, 0.068 mol, 4 eq.) was added. The reaction mixture was stirred at 80 °C for 9 days. Additional NaH was added (1.00 g, 0.042 mol, 2.5 eq) and the solution was stirred for another 23 days. The solvent was removed under reduced pressure to obtain a total volume of 250 mL. Water

(250 mL) and diethyl ether (250 mL) was added and the mixture was stirred overnight at room temperature. To neutralize the reaction mixture, sulfuric acid (1 mol·L⁻¹, 15 mL, 0.015 mol) was added and again stirred overnight. The organic phase was extracted three times with diethyl ether (150 mL each) and dried over sodium sulfate. The solvent was removed under reduced pressure and the crude product was purified using flash column chromatography (petroleum ether/diethyl ether 40:1). The intermediate 1,2-bis-*n*-icosanyl-3-benzyl glyceryl ether (4.97 g, 0.007 mol) was obtained as colorless solid. Yield: 41%.

1,2-Bis-*n*-icosanyl-3-benzyl glyceryl ether (4.97 g, 0.007 mol, 1 eq.) was dissolved in dichloromethane. Palladium on activated charcoal was added (5 weight percent). Hydrogen was introduced and the mixture was stirred at room temperature for 20 days. The catalyst was removed via filtration over celite®. Afterwards the solvent was removed under reduced pressure to obtain 1,2-bis-*n*-icosanyl glyceryl ether (1.84 g, 0.003 mol) as colorless solid. Yield: 42%.

¹H NMR, COSY (400 MHz, chloroform-*d*, δ): 3.74–3.41 (m, 9H, glycerol H), 1.60–1.52 (m, 4H, -OCH₂CH₂-), 1.34–1.21 (m, 68H, -OCH₂CH₂(CH₂)₁₇CH₃), 0.89–0.86 (m, 6H, -O(CH₂)₁₉CH₃).

¹³C NMR, HSQC, HMBC (101 MHz, chloroform-*d*, δ): 78.4 (glycerol C), 72.0 (-OCH₂CH₂-), 71.1 (glycerol C), 70.6 (-OCH₂CH₂-), 63.25 (glycerol C), 32.1 (-OCH₂CH₂(CH₂)₁₇CH₃), 30.2 (-OCH₂CH₂-), 29.9–29.5 (-OCH₂CH₂(CH₂)₁₇CH₃), 26.3 (-OCH₂CH₂(CH₂)₁₇CH₃), 22.9 (-OCH₂CH₂(CH₂)₁₇CH₃), 14.3 (-O(CH₂)₁₉CH₃).

S2.7. Polymer Synthesis of BisID-PEG

The synthesis is described for BisID-PEG₆₂ as a representative example.

1,2-Bis-*n*-icosanyl glyceryl ether (BisID) (0.2 g, 0.307 mmol, 1 eq.) was placed in a dry Schlenk flask and dissolved in benzene (10 mL). The solution was stirred at 60 °C for at least 30 min and dried in vacuo for 16 h to remove moisture. Dry tetrahydrofuran (approx. 10 mL) was cryo-transferred to the Schlenk flask to dissolve the initiator. Afterwards, the solution was stirred and the initiator was deprotonated with a 0.5 M solution of potassium naphthalenide in THF (0.31 mL, 0.153 mmol, 0.5 eq.). The solution was cooled down to -90 °C and ethylene oxide (EO) (0.95 mL, 20.919 mmol, 68 eq.) was cryo-transferred using a graduated ampule. The polymerization was carried out at 60 °C for 24 h. Subsequently, the reaction mixture was heated to 80 °C for 16 h. To quench the polymerization, an excess of ethanol was added and the solvent was removed under reduced pressure. The crude product was dissolved in methanol and precipitated three times in cold diethyl ether to obtain the pure product. Yield: 95%.

¹H NMR, COSY (400 MHz, benzene-*d*₆, δ): 3.73–3.31 (m, 256H, polyether backbone and glycerol H), 1.67–1.58 (m, 4H, -OCH₂CH₂-), 1.45–1.27 (m, 70H, -OCH₂CH₂(CH₂)₁₅CH₃), 0.93–0.89 (m, 6H, -O(CH₂)₁₉CH₃).

¹³C NMR, HSQC, HMBC (101 MHz, benzene-*d*₆, δ): 79.0 (glycerol C), 73.4 (polyether backbone and glycerol C), 72.4–70.8 (polyether backbone and glycerol C), 62.1 (polyether backbone and glycerol C), 32.6 (-OCH₂CH₂(CH₂)₁₆CH₂CH₃), 31.1–30.1 (-OCH₂CH₂- and -OCH₂CH₂(CH₂)₁₆CH₂CH₃ (BisID)), 27.0 (-OCH₂CH₂(CH₂)₁₆CH₂CH₃ (BisID)), 23.4 (-OCH₂CH₂(CH₂)₁₆CH₂CH₃ (BisID)), 14.6 (-O(CH₂)₁₉CH₃ (BisID)).

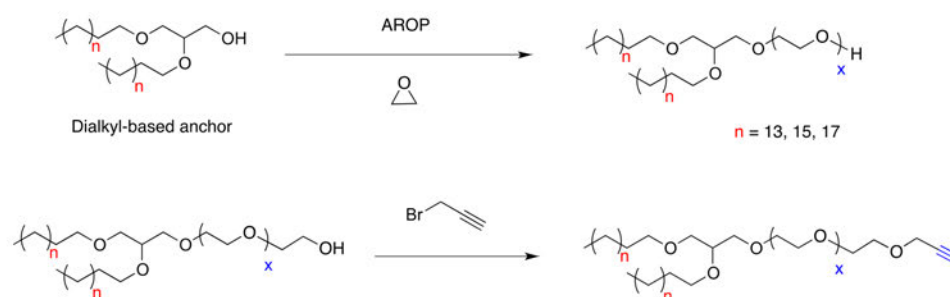
S2.8. Functionalization of BisID-PEG with Propargyl Bromide

The functionalization is described for BisID-PEG₆₂-alkyne as a representative example.

BisID-PEG₆₂ (0.2 g, 0.059 mmol, 1 eq.) was placed in a Schlenk flask and dissolved in dry THF (10 mL). The solution was cooled to 0 °C and sodium hydride (0.004 g, 0.178 mmol, 3 eq.) was added. Afterwards, propargyl bromide (0.016 mL, 0.178 mmol, 3 eq.) was added, and the solution was stirred for 24 h at room temperature. The reaction mixture was filtered, and the solvent was reduced under reduced pressure. The remaining solution was precipitated twice in cold diethyl ether and the pure product was dried in vacuo. Yield: 61%.

¹H NMR, COSY (300 MHz, benzene-*d*₆, δ): 3.95–3.94 (d, *J* = 2.4 Hz, 2H, -OCH₂CCH), 3.77–3.24 (m, 245H, polyether backbone and glycerol H), 2.12–2.11 (t, *J* = 2.4 Hz, 1H, -OCH₂CCH), 1.69–1.57 (m, 4H, -OCH₂CH₂-), 1.49–1.28 (m, 67H, -OCH₂CH₂(CH₂)₁₅CH₃), 0.94–0.90 (m, 6H, -O(CH₂)₁₇CH₃).

Scheme S1: Synthesis Route of the Dialkyl PEG-Lipids and Functionalization with propargyl bromide.



S2.9. Polymer Synthesis of Cholesterol-PEG-PEEGE

The synthesis protocol was described in previous reports.³

¹H NMR, COSY (300 MHz, DMSO-*d*₆, δ): = 5.30 (C=CH cholesterol), 4.63 (br, CHO acetal), 3.72–3.21 (polyether backbone; CHO cholesterol), 2.28–0.82 (br, CH₂, CH cholesterol), 1.18–1.06 (br, CH₃ acetal), 0.64 (br, CH₃ cholesterol).

S2.10. Polymer Synthesis of Cholesterol-PEG-linPG

The synthesis protocol was described in previous reports.³

¹H NMR, COSY (300 MHz, DMSO-*d*₆, δ): = 5.30 (C=CH cholesterol), 4.24 (br, OH), 3.72–3.21 (polyether backbone; CHO cholesterol), 2.28–0.82 (br, CH₂, CH cholesterol), 0.64 (br, CH₃ cholesterol).

S2.11. Polymer Synthesis of Cholesterol-PEG-hbPG

The synthesis protocol was described in previous reports.³

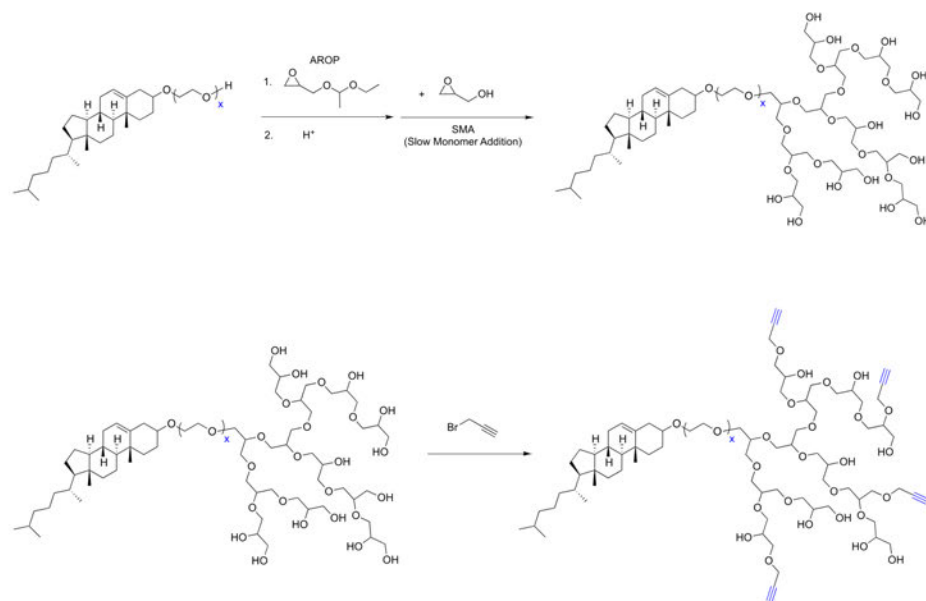
¹H NMR, COSY (300 MHz, DMSO-*d*₆, δ): = 5.30 (C=CH cholesterol), 4.77–4.43 (br, OH, different signals due to hyperbranched PG), 3.76–3.22 (polyether backbone; CHO cholesterol), 2.28–0.82 (br, CH₂, CH cholesterol), 0.64 (br, CH₃ cholesterol).

S2.12. Functionalization of Cholesterol-PEG-hbPG with Propargyl Bromide

The synthesis protocol was described in previous reports.³

¹H NMR, COSY (300 MHz, DMSO-*d*₆, δ): = 5.30 (C=CH cholesterol), 4.77–4.43 (br, OH, different signals due to hyperbranched PG), 4.31–4.17 (OCH₂C≡CH), 3.76–3.22 (polyether backbone; CHO cholesterol), 2.45 (C≡CH), 2.28–0.82 (br, CH₂, CH cholesterol), 0.63 (br, CH₃ cholesterol).

Scheme S2: Synthesis Route of the Cholesterol-PEG-hbPEG Lipids and Functionalization with propargyl bromide.



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4.2 Dual centrifugation-based screening of pH-sensitive liposomes

Stimuli-responsive liposomes are an important approach for tissue-specific tumour therapy, as they reduce damage to healthy tissue. For this purpose, many different strategies have been developed in recent decades, such as the development of thermosensitive, enzymatically degradable and pH-reactive liposomal drug delivery systems.^{224,254,255}

To contribute to this therapeutic approach, Matthias Voigt and his cooperation partner Jonathan Schupp from the Department of Dermatology developed a rational multistep screening protocol for pH-sensitive liposomes that does not depend on the use of specially designed lipids, as often reported in the literature. Instead, Matthias Voigt classified known and common lipids from the literature into stabilising lipids, which can form liposomes themselves, and those that require one or more co-lipids to form particles and were categorised as destabilising lipids. In several screening steps, he and Jonathan Schupp identified and optimised three potential new pH-sensitive liposomes.³

I became involved in the project at the beginning of my PhD, initially focusing on the establishment of quality control procedures based on thin-layer chromatography for lipids. The objective was to guarantee the integrity of the samples throughout the preparation process of the liposomes. Moreover, the influence of human serum albumin on the structural integrity of the liposomal candidates was elucidated through the establishment of an HPLC-coupled incubation assay. The understanding of the particle stability in serum is crucial for the prediction of potential stability in animal models and cell culture.³

In a later study, we conducted a comparative analysis of the three final candidates with a conventional liposome model in mice, in collaboration with Barbara Gräfen from the Department of Dermatology. In this *in vivo* imaging experiment, we analysed the half-life of the particle circulation and the tissue accumulation in mice model. In addition, we validated the results by analysing blood and organ samples taken at the end of the study and used these for the extraction of liposomes to determine concentrations. Overall, Barbara's and my *in vivo* data confirmed particle properties and organ accumulation similar to those of the conventional liposome.³

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Dual Centrifugation-Based Screening for pH-Responsive Liposomes

Lukas Gleue^{+, [a]}, Barbara Graefen^{+, [b]}, Matthias Voigt^{+, [a, g]}, Jonathan Schupp^{+, [b, h, i]}, Dirk Schneider^{, [c]}, Michael Fichter^{, [b, d, e]}, Michael Kuske^{, [b, e, j]}, Volker Mailaender^{, [b, d, e]}, Andrea Tuettenberg^{, [b, f]} and Mark Helm^{*, [a]}

In liposomal drug delivery development, the delicate balance of membrane stability is a major challenge to prevent leakage (during shelf-life and blood circulation), and to ensure efficient payload release at the therapeutic destination. Our composite screening approach uses the processing by dual centrifugation technique to speed up the identification of *de novo* formulations of intermediate membrane stability. By screening binary lipid combinations at systemically varied ratios we highlight liposomal formulations of intermediate stability, what we termed „the edge of stability“, requiring moderate stimuli for destabilization. Supplementation with a pH-sensitive cholesterol derivative (to obtain acid labile liposomes) and renewed assess-

ment with cargo load led to the discovery of three formulations with sufficient shelf-life stability, acceptable cargo retention and efficient pH-responsive cargo release *in vitro*. The „lead candidates“ exhibited promising *in cellulo* uptake with increased intracellular cargo release and revealed *in vivo* performance advantages compared to a control liposome. Our approach filters lipid compositions on „the edge of stability“ that were introduced with a pH-sensitive cholesterol derivative leading pH-responsive liposomes, out of a multidimensional parameter space. Their discovery by rational approaches would have been highly unlikely, thus highlighting the potential of our screening approach.

1. Introduction

The stability of its lipid bilayer membrane is a defining characteristic of a liposome, which governs the retention, transport, and eventual release of an encapsulated cargo in fields ranging from fundamental research^[1] over cosmetics^[2] to medical applications.^[3] Since the first liposomal medical treatment was approved in 1995,^[4] a vast amount of data, clinical and other, became available, which focused on increasing liposome stability to overcome fast clearance within the body^[5] and to decrease side effects.^[6] Major issues being addressed by

current research include drug delivery by active or passive targeting,^[7] liposome penetration of target tissues,^[8] incorporation of cationic lipids for DNA and RNA delivery^[9] and efficient release of small molecule cargo.^[10] This latter aspect in particular is crucially related to stability, and the object of our research.

Many molecular and biophysical concepts and corresponding compounds were put forward to address the partially contradictory requirements for liposomal stability versus release at a therapeutically relevant site.^[11] Among various factors that may contribute to stability and release, the biophysical stability

[a] L. Gleue, M. Voigt, M. Helm
Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg University, Staudingerweg 5, 55128 Mainz, Germany
E-mail: mhelm@uni-mainz.de

[b] B. Graefen, J. Schupp, M. Fichter, M. Kuske, V. Mailaender, A. Tuettenberg
Department of Dermatology, University Medical Center of the Johannes Gutenberg University Mainz, Langenbeckstr. 1, 55131 Mainz, Germany
E-mail: antuette@uni-mainz.de

[c] D. Schneider
Department of Chemistry-Biochemistry, Johannes Gutenberg University, Hanns-Dieter-Hüsch-Weg 17, 55128 Mainz, Germany

[d] M. Fichter, V. Mailaender
Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany

[e] M. Fichter, M. Kuske, V. Mailaender
Paul-Klein-Center for Immunintervention, University Medical Center of the Johannes Gutenberg University Mainz, Langenbeckstr. 1, 55131 Mainz, Germany

[f] A. Tuettenberg
Research Center for Immunotherapy, University Medical Center of the Johannes Gutenberg University Mainz, Langenbeckstr. 1, 55131 Mainz, Germany

[g] M. Voigt
BioNTech SE, An der Goldgrube 12, 55131 Mainz, Germany

[h] J. Schupp
Frankfurt Cancer Institute, Paul-Ehrlich-Straße 42–44, 60596 Frankfurt, Germany

[i] J. Schupp
Goethe University Frankfurt, Institute of Neurology (Edinger Institute), Heinrich-Hoffmann-Straße 7, 60528 Frankfurt, Germany

[j] M. Kuske
Institute for translational oncology Mainz (TRON-Mainz), Freiligrathstraße 12, 55131 Mainz, Germany

[*] authors contributed equally

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cmdc.202400648>

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of the liposomal membrane is an attractive target for rational manipulation, because the membrane lipid order can be described by basic thermodynamic concepts. Below the phase transition temperature (T_m), lipids are in the gel phase, whereas above the T_m they are in the liquid crystalline phase.^[12] Membranes in lipid crystalline phase are more fluid than membranes in gel phase, which are more permeable to small molecules, and are therefore less effective in retaining encapsulated cargo.^[13] The T_m of a pure lipid is specific, and depends on its fatty acid chain length, saturation and head group. In contrast, it can be roughly estimated, but not accurately predicted for binary lipid mixtures, even when the T_m values of the components are known.^[14,15] However, liposomal stability can still be rationally manipulated by certain destabilizing lipids, which do not form lamellar phases on their own. Their addition decreases stability and T_m of lipid membranes. Liposomal membranes can also benefit from a “molecular plug” effect caused by incorporation of cholesterol into the liposomal membrane, which can decrease membrane permeability while increasing its stability.^[16] More recent efforts focused on liposomal formulations, which change membrane permeability in response to therapeutically relevant stimuli such as heat,^[17] or pH.^[10e,18,20]

For our study concept “Liposomal formulations on the edge of stability”, we hypothesized that a stimulus-dependent cargo release requires a liposomal formulation of minimum stability, and that a stimulus must then supply the energy needed to rupture the liposomal membrane. Liposomes would require that minimum stability to retain their cargo until arrival at a therapeutic location, but overly stable liposomes would fail to open upon a stimulus such as heat or pH change. This brings a need for tuning liposomal stability to that delicate balance, which we have taken to call “the edge of stability”.

Many liposomal formulations for classical non-stimulus responsive liposomes share common components, e.g. fully saturated lecithins in combination with cholesterol, while DPPC features in thermo-sensitive liposomes.^[17] A combination of DOPE and DOPC with cholesterylhemisuccinate (CHEMS) leverages its pK_a of ~pH 5.5 as a molecular concept for acid-triggered cargo release from liposomes.^[10e,18b,19–21]

A relatively high variety of modifying lipids, often of experimental/exploratory nature, were reported in liposomal formulations, reflecting the need for improved performance and the potential of a vast ‘lipid-composition-space’, most of which are essentially unexplored.^[22] Therefore, we implemented a combinatorial and largely unbiased approach. We postulated that in liposomes whose membranes are composed of two lipids, membrane stability can be tuned through the lipid ratio. Upon addition of increasing amounts of a destabilizing lipid to a lipid that can sustain liposome formation by itself, increasingly unstable liposomes would form up to the edge of stability, beyond which no more liposome formation is observed. For a given binary lipid combination the goal would then be to determine the amount of destabilizing lipid that would still allow the postulated minimum stability. In the perspective of a molecular concept, the resulting liposomes were to be further

developed by addition of CHEMS^[10e,19a] to confer a pH-sensitive molecular entity.

We needed an experimental approach to determine or approximate liposomal stability, and a medium-to-high throughput formulation technique to produce liposomal formulations for screening in large numbers in small amounts. The latter prerequisite is met by the dual centrifugation technique (DC). DC, an ‘in vial’ homogenization technique, allows fast preparation of up to 40 individual liposome samples in parallel, controllable diameters and variation in batch sizes, starting with single digit milligram amounts.^[23a,24,25] Caveats include polydispersion index (PI) values that are relatively high in comparison to other techniques such as extrusion, thereby limiting perspectives for advanced development of studies *in vivo*. With these limitations in mind, the present work does not directly aim at the development of a ready-to-use formulation of a defined payload drug in a fully defined liposome. Rather, our intention is to demonstrate a screening approach by DC with different payloads, that can identify promising lead candidates. Arguably, a full development of such leads with a defined payload will require reevaluation, and may need a switch in later stages to more controllable process such as extrusion.^[25,26]

Materials and Methods

Consumables, Devices and Cells

Chemicals and Consumables

DMPC (#850345C-200mg), DODAP (#890850C-25mg), DOPC (#850375C-200mg), DOPE (#850725C-200mg), DOPG (#840475C-200mg), DOTAP (#890890C-200mg), DPPC (#850355C-200mg), DPPG (#840455P-200mg) and Laurdan (#850582P) were obtained from Avanti Polar (Alabaster, Alabama, USA). Biocoll® (#L6115) (Bio&Sell GmbH, Feucht, Germany); Chloroform (#37331.2), Ethanol (#5054.3), Methanol (#KK39.2), Paraformaldehyde (#0964.2) and Triton-X100 (#3051.3) were received from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). 100 mm culture petri dishes (#430167), 24 well plates (#3527), 96 well plates (#3598) and round bottom polystyrene test tubes (#352008) were obtained from Corning Inc. (Corning, New York, USA). Sephacryl S500-HR slurry (#GE17-061301) was used purchased from GE Healthcare (Chicago, USA). 50 ml tubes (#227261) and 96-well optical bottom plate black (#10706172) were obtained from Greiner Bio-One GmbH (Frickenhausen, Germany). Ampuwa® (#B230673) was ordered from Fresenius Kabi (Bad Homburg, Germany). 8 well μ -slides (#80826) was obtained from Ibidi GmbH (Gräfelfing, Germany). Recombinant human M-CSF (#11343117) was purchased from ImmunoTools GmbH (Friesoythe, Germany). Primocin® (#ant-pm-2) was obtained from InvivoGen (San Diego, California, USA). PCR tubes 200 μ L (#G001-F) was received from Kisker Biotech GmbH & Co. KG (Steinfurth, Germany). Calcein (#C0875-5G), Cholesterol (#C8667-25G), Doxorubicin hydrochloride (#D1515-10MG), Potassium chloride (#P9541-500G), Resazurin sodium salt (#7017), RPMI1640 (#R7509), Sulfurhodamine B (#230162-5) and Water (ultra pure) (#ZRQSVRSWW) were obtained from Merck KGaA (Darmstadt, Germany). HPLC vials (#EC-1026 + #7-0632 + #7-0693) were obtained from neoLab Migge GmbH (Heidelberg, Germany). Heparin-sodium (#PZN: 16200037) was purchased from PANPHARMA GmbH (Trittau, Germany). Isoflurane (PZN: 09714675) was purchased from Piramal Critical Care (Voorschoten, Netherlands). NeuroDiD (#PK-

CA707-60014) and Hoechst 33258 (#PK-CA707-40046) were obtained from PromoCell GmbH (Heidelberg, Germany). 1.5 ml reaction tubes (#72.706) were purchased from (Sarstedt AG & CO. KG, Nümbrecht, Germany). SiLiBeads® Typ ZY-S 0.3–0.4 mm (#96035-362) was received from Sigmund Lindner GmbH (Warmensteinach, Germany). Accutase® (#00-4555-56), DiD (#D7757), DiR (#D12731), Fetal calf serum (#10500-064), GlutaMAX™ (#35050038), Human serum (#31876), Phosphate buffered saline (#14190094), Trypsin-EDTA (0.05 %) (#25300054) and Viability Dye eFluor™ 780 (#65-0865-18) were ordered by Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Liposome Formulation by Dual Centrifugation

The lipids used for liposome preparation are shown in Table 1. Liposomes were prepared via dual centrifugation with a modified protocol as previously described.^[23b,24a] Lipids were dissolved in absolute ethanol, chloroform or chloroform:methanol:water (2:1:0.1) in stock concentrations of 20 mg/mL and combined in PCR tubes to yield 2 and 5 μmol of the intended compositions for the laurdan fluorescence and all other experiments, respectively. 1 mg/mL laurdan in methanol and membrane dye DiD or DiR in ethanol were added for laurdan and cell experiments to yield 0.1 mol-% of the total lipid amount, respectively. Combined lipids were pre-dried in a SpeedVac® (Eppendorf SE, Hamburg, Germany) vacuum centrifuge at 30 °C overnight and then lyophilized with Alpha 2–4 LD (Christ Gefriertrocknungsanlagen GmbH, Osterode im Harz, Germany) for at least 48 h to completely remove solvents. The integrity of the lipid compounds was tested by thin-layer chromatography (Figures S1 and S2, Section Sup. 2). Thoroughly dried lipid compositions were kept at –20 °C until usage. For preparation of a vesicular phospholipid gel, following volumes were used and scaled linearly with the lipid amount. 5 mmol dried lipids were incubated for 10 minutes with 9.3 μL of cargo solution. As cargo, 50 mM sulforhodamine B (SRb), 20 mM calcein (CAL) or 50 mg/mL doxorubicin hydrochloride (DXR) in water were used. After addition of 350 mg of 0.3–0.4 mm ceramic SiLiBeads®ZY, samples were subjected to dual centrifugation in Zentrifuge 380R (Hettich GmbH, Tuttlingen Germany), at 2500 RPM and 25 °C for 20 min. The resulting vesicular phospholipid gel was diluted with 77.2 μL phosphate buffered saline (PBS) and subjected to dual centrifugation again at 2500 RPM and 25 °C for 2×2 min while turning the sample tray between the runs to form liposomes.

Evaluation of Liposome Stability Using Laurdan Fluorescence Spectroscopy

10 μL of liposome stock solutions with PBS as cargo were diluted 5x in PBS and subjected to a black 96-well polypropylene optical bottom plate. Fluorescence measurements were performed in a M200 Pro multiplate reader (Tecan, Männedorf, Switzerland) at 388/440 nm (Ex./Em.) and 388/490 nm (Ex./Em.) to determine the fluorescence emission intensities at 440 nm (I_{440}) and 490 nm (I_{490}), respectively. The laurdan generalized polarization value (LGP)^[12] was calculated using Equation (1).

$$LGP = \frac{(I_{440} - I_{490})}{(I_{440} + I_{490})} \quad (1)$$

The obtained LGP-values were normalized to simplify the resulting graphs. The LGP value of liposomes with 100 mol-% lipid 1 (Table 2) was set to 0% deviation and the LGP value of the respective liposomes with 10 mol-% lipid 1 and 90 mol-% lipid 2 was set to 100% deviation. The % of deviation from lipid 1 (%dev) was plotted against increasing mol-% of lipid 2 and fitted with a sigmoidal, 4PL, X is concentration fit with least squares as fitting method (see Equation (2)) using GraphPad Prism 9 (GraphPad Software, Boston, Massachusetts, USA).

$$y = \text{bottom value} + \frac{(\text{top value} - \text{bottom value})}{(1 + 10^{-(\log EC_{50} - x) \cdot \text{HillSlope}})} \quad (2)$$

Determination of Encapsulation Efficiency, Cargo Release and Retention by Size-exclusion Chromatography

To remove non-encapsulated cargo, liposome stock solutions were subjected to 1.5 mL glass vials for automated size-exclusion chromatography by HPLC (1100 Series, Agilent Technologies, Santa Clara, California, USA). The body of an UNO Q1 column (Bio-Rad, Hercules, California, USA), cleared and self-packed with 2 mL Sephacryl S500-HR slurry was used as chromatography column. Purification resulted in 600 μL of purified liposomes and a free cargo fraction. Encapsulation efficiencies (EE) were calculated by dividing absorption intensity of the used cargo (calcein & doxorubicin at 490 nm, sulforhodamine b at 550 nm) within the purified liposome fraction (L) by the total absorption intensity multiplied by 100% (Equation (3)).

Table 1. Abbreviation list for used lipids and membrane supplements.

Abbreviation	Full name	Abbreviation	Full name
CHEMS	cholesterylhemisuccinate	DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
CHOL	cholesterol	DOPE	1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine
EPC	egg phosphatidyl choline	DOPG	1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt
DiD	1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate salt	DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride
DiR	1,1'-dioctadecyl-3,3',3'-tetramethylindotricarbocyanine-iodide	DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine	DPPG	1,2-dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt
DODAP	1,2-dioleoyl-3-dimethylammonium-propane	laurdan	1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine

Table 2. Lipids and lipid combinations (L) used for liposome screening. **A** Binary lipid composition mixtures. Information of a sigmoidal progression of lipid mixture (laurdan assay, see Figure 2) is listed by check symbol. Candidates identified in Section 2.3.2., are highlighted in color (L2, L11, L15). **B** and **C**: Lipid properties are displayed as lipid netto charges at pH 7.4 (pH 5.5), fatty-acid chain lengths (carbon atoms:double bonds) and phase transition temperatures T_m s for stabilizing lipids in **B** and modifying lipids in **C**. For abbreviations of lipids, see methods section Table 1.

A	Lipid combination (L)	Stabilizing lipid 1	Modifying lipid 2	Sigmoidal LGP?
	L1		DOPE	
	L2	DMPC	DODAP	✓
	L3		DOTAP	
	L4		DOPE	✓
	L5	DPPC	DODAP	
	L6		DOTAP	✓
	L7		DOPE	
	L8	DPPG	DODAP	
	L9		DOTAP	✓
	L10		DOPE	
	L11	DOPC	DODAP	✓
	L12		DOTAP	
	L13		DOPE	
	L14	DOPG	DODAP	✓
	L15		DOTAP	✓
	L16		DOPE	
	L17	EPC	DODAP	
	L18		DOTAP	

B	Stabilizing lipid 1	Chain lengths ¹	T_m (°C) ¹	Lipid charges ¹
	DMPC	14:0	24	0
	DPPC	16:0	41	0
	DPPG	16:0	41	-1
	DOPC	18:1	-17	0
	DOPG	18:1	-18	-1
	EPC	variable	variable	0

C	Modifying lipid 2	Chain lengths ¹	T_m (°C) ¹	Lipid charges ¹
	DOPE	18:1	-16	0
	DODAP	18:1	-	0 (+1)
	DOTAP	18:1	0	+1

[a] Values obtained from.^[31]

$$\%EE = \frac{\text{absorption L}}{\text{total absorption}} \times 100\% \quad (3)$$

For determination of cargo release, 100 μ L of SEC purified liposomes were incubated at pH 5.5 and 37 °C for 4 h to simulate lysosomal conditions and subjected to SEC for purification again. To determine long-term cargo leakage, purified liposomes were kept at pH 7.4 and 5 °C for 1 week and purified again. Cargo release was calculated by dividing the absorption intensity of the respective cargo within the free cargo fraction (FC) by the total absorption intensity multiplied by 100% (Equation (4)). Cargo retention was therefore calculated as 100% minus %release.

$$\%release = \frac{\text{absorption FC}}{\text{total absorption}} \times 100\% \quad (4)$$

For serum stability experiments, liposomes were incubated for 4 h at 37 °C in full human serum.

Liposome Loading Analysis

Doxorubicin-loaded liposomes and unloaded liposomes were each mixed with 5% (v/v) Triton-X100 dissolved in PBS. Doxorubicin absorbance of triplicates was measured at 488 nm using a Sense Beta Plus Microplate Reader in 96 well plates (total volume 200 μ L/well). Unloaded liposome sample values were subtracted from corresponding doxorubicin loaded liposomes. Loading efficacy was then calculated by applying linear regression to a 1:2 dilution series of doxorubicin from 108 μ M to 0.1 μ M in Microsoft Excel.

Total volume needed for treatment with 1 μ M doxorubicin-loaded liposomes was then further calculated for cell treatment assays.

Physicochemical Characterization by Dynamic Light Scattering (DLS)

Size, polydispersity (PDI) and zeta potential (ζ -potential) of 20 μ L purified liposome sample were determined using a Malvern Zetasizer Nano Series (Malvern Panalytical, Malvern, United Kingdom) in a folded disposable capillary cell at 25 °C after an equilibration time of 30 s. Liposome samples were diluted in 1 mL of freshly filtered aqueous 10 mM potassium chloride solution for zeta potential analysis or in phosphate-buffered saline for size and size distribution. Each sample was measured in triplicate in 30 runs. The viscosity of the water was set to 0.8872 cP, with a refractive index of 1.33, a scattering angle of 173° and an automatic selection for the attenuation index and voltage.

Human Macrophage Culture

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy volunteers, with approval by the local ethical committee (Landesärztekammer Rheinland-Palatinat No. 837.019.10 (7028)) via Biocoll density gradient centrifugation. 200 million PBMC were seeded in RPMI1640, supplemented with 1% Glutamax™, 0.2% Primocin®, and 50 ng/mL M-CSF in cell culture petri dishes for 1 h in a 37 °C 5% CO₂ incubator. After 1–2 h non-adherent cells were rinsed off by repeated washing of the petri dishes with pre-warmed to 37 °C PBS checked by microscopy. Adherent cells (monocytes) were cultured in the medium described above containing an additional 1% heat

inactivated human plasma for six days. Macrophages were harvested using Accutase™.

Human Plasma

Human plasma of ten healthy donors was pooled in 2×50 ml tubes and centrifuged for 15 min at 800× g. The supernatant was pooled and centrifuged for 15 min at 1000× g to deplete the cellular contents. The collected supernatant (plasma) was then heat inactivated for 20 min at 56 °C. Insoluble denatured protein was removed by centrifugation at 3000× g for 15 min at room temperature. Heat inactivated plasma was stored at −20 °C until further use.

Melanoma Cell Line UKRV-Mel-15a

Human melanoma cells from cell line UKRV-Mel-15a (obtained from Dr. A. Paschen in 2014 (Essen, Germany)) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, GlutaMAX™ and Primocin®. After reaching approximately 80% confluence, cells were harvested with Trypsin-EDTA solution and reseeded in a higher dilution. This cell line has been validated *via* short tandem repeat (STR) analysis (Eurofins Genomics) last in 08/2022. The resulting STR profiles matched with the online databases of the German collection of microorganisms and cell cultures (DSMZ) (Available online: <https://www.dsmz.de/services/human-and-animal-cell-lines/online-str-analysis>).

Flow Cytometry

Human macrophages were seeded in 24 well plates at a density of 2.5×10^5 cells per well in 1 mL of culture medium. Human melanoma cells (UKRV-Mel-15a) were seeded in 24 well plates at a density of 5×10^4 cells per well in 1 mL of culture medium. Cells were treated in triplicates with 1 μ M doxorubicin loaded within liposomes (volumes were estimated by doxorubicin loading analysis), 1 μ M free doxorubicin or left untreated as control. After 30 minutes and 4 h treatment cells were harvested by using trypsin-EDTA (melanoma) or Accutase™ (macrophages). Dead cells exclusion was achieved by using Fixable Viability Dye eFluor™ 780. Afterwards, cells were fixed by using 4% paraformaldehyde dissolved in PBS for 30 min. Flow cytometry analysis was performed on an LSRII cytometer (BD Biosciences, Heidelberg, Germany). Data were analyzed using Cytobank (Beckman Coulter, Brea, California, USA).^[27]

Cell Viability Assay (Resazurin)

Human macrophages were seeded in 96 well plates at a density of 5×10^4 cells per well in 0.2 mL of culture medium. Human melanoma cells (UKRV-Mel-15a) were seeded in 96 well plates at a density of 1×10^4 cells per well in 0.2 mL of culture medium. Cells were treated in triplicates with either liposomes loaded with 1 μ M of doxorubicin (volumes were estimated by doxorubicin loading analysis) or equivalent volumes of unloaded liposomes, 1 μ M free doxorubicin or left untreated as control. After 24 h and 72 h treatment, medium was replaced with resazurin sodium salt dissolved in PBS at a final concentration of 6 μ g/mL. Cells were incubated for 1 h at 37 °C. Fluorescence was measured at 544/590 nm (excitation/emission) using a Sense Beta Plus Microplate Reader (Hidex Oy, Turku, Finland). Fluorescence values were blank corrected and normalized to untreated sample in Excel (Microsoft Corporation, Redmond, Washington, USA), graphs were created in GraphPad Prism 9.

Intracellular Doxorubicin Release/Staining (Microscopy)

Human macrophages were seeded in 8 well μ -slides at a density of 7.5×10^4 cells per well in 0.3 mL of culture medium. Human melanoma cells (UKRV-Mel-15a) were seeded in 8 well μ -slides at a density of 1.5×10^4 cells per well in 0.3 mL of culture medium. After 30 minutes and 4 h treatment with 1 μ M of loaded doxorubicin (volumina were estimated by doxorubicin loading analysis), 1 μ M free doxorubicin or left untreated, the cells were fixed by using 4% paraformaldehyde dissolved in PBS for 30 min. Hoechst 33258 in PBS at 10 μ M was used to stain the nuclei. Cell membranes were stained using the green-fluorescent cytoplasmic membrane staining kit on fixed cells. Images were acquired with a Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) and analyzed using the Fiji ImageJ (National Institutes of Health, Bethesda, Maryland, USA) distribution.^[28]

Ethical Approval Statement (Mice Experiments)

The biodistribution study of liposomal lead candidates without cargo in mice was carefully evaluated and approved by local authorities (G20-1-123, Landesuntersuchungsamt Rheinland-Palatinat, Germany). *In vivo* biodistribution study was necessary for the evaluation of elimination from blood stream and distribution of the liposomal candidates in a complex organism. A total of 26 female mice of B6 N-Tyrc-Brd/BrdCrJ („B6 Albino“) (Strain code 493) with an average age of ≥ 7 weeks and body weight between 17–20 g were used with each mouse as an experimental unit. Sample size and group size was based on prior experimental studies, without exclusion criteria.^[29] The mice were purchased from Charles River. The mouse were housed under a 12 h dark-light cycle at a constant temperature and with ad libitum feeding on water and standard laboratory mice chow. The experiment was performed after one week of adaptation in the Paul-Klein-Zentrum für Immunintervention (Building 308 A) of the University medical center of the Johannes Gutenberg-University in Mainz after random assignment. Potential confounders were not controlled and the experimenters were aware of the group allocation by housing each treatment group in an own cage. Experiments were performed in accordance with relevant laws and institutional guidelines. Differences between groups with a significance level of $p \leq 0.05$ (student's t-test) were considered statistically significant.

In vivo biodistribution

Background fluorescent signal images of animals were acquired under 2.5% isoflurane oxygen anesthesia in the IVIS Spectrum CT (Perkin Elmer, Waltham, Massachusetts, USA) for 2 seconds exposure time with 745/800 nm (Ex/Em). 5 ml/kg bodyweight lipid-nanocarrier labeled with 0.2 mol-% fluorescence DiR (L0, L2C, L11C, L15C) were then injected intravenously (i.v.) in the tail vein ($n = 5$ for each nanocarrier). The control groups received PBS or DiR dissolved in PBS, respectively (DiR was dissolved in 1% ethanol) ($n = 3$ for each control). After injection, mice fluorescence intensities were imaged first time under 2.5% isoflurane anesthesia at time point “0 h”. 3 h and 24 h after injection 2–3 drops of blood were collected in 1.5 ml tubes (with 2 μ l heparin-sodium) from the mice before imaged, while anesthetized with 2.5% isoflurane oxygen. After the final measurement at 24 h mice were euthanized by cervical dislocation. DiR control was used as an additional control to PBS and measured data for DiR were not significantly varying from PBS control, resulting in the exclusion of the data from all following analyses.

Ex vivo Organ Analysis

The abdomen was opened in one mouse from each group and imaged in the IVIS CT. Fluorescent organs (liver, spleen) were collected in 6 well plates with 2 ml PBS in each well. Organs from each group were then dried, placed on a black plate and imaged with IVIS CT for 0.5 seconds exposure time.

Image Analysis

In vivo images were analyzed using Living Image version 4.5.2. software (Perkin Elmer, Waltham, Massachusetts, USA). For *in vivo* imaging, the abdomen area was defined as area between the sternum and middle of the abdominal area. Region of interest (ROI) was drawn in the defined area with ROI size copied for each picture and kept for all *in vivo* images. The min and max values of epi fluorescence were adjusted to the same min and max values for each *in vivo* image to make them visually comparable (Min: 3.50×10^5 , Max: 1.50×10^9 Radiant Efficacy). ROIs were quantified in the physical, calibrated unit "Total Radiant Efficiency [p/s]/[μW/cm²]". Tissue autofluorescence of the mice was subtracted from the ROI values measured after injection of the labeled lipid-nano-carriers. Total sample number of animals was 26. *Ex vivo* images were analyzed similarly. After adjusting the min and max values for each *ex vivo* image the ROI size was kept for all livers and a separate ROI was kept for all *ex vivo* images for the spleen. Total sample number per organ was 26.

Blood Fluorescence Analysis

Blood samples were taken 3 h and 24 h after sample injection from vena facialis. For fluorescent analysis, 25 μL blood were diluted in 175 μL PBS and fluorescence was measured in a 96-well flat bottom black well with Spark 10 M multiplate reader at 740/780 nm (Ex./Em.). Total sample number for blood samples was 2x26. Fluorescence was measured for each sample in triplicates. Blank corrected values from each time point were normalized to average PBS value. Subsequently 24 h values of treatment groups were normalized to 3 h values of each treatment group.

2. Results & Discussion

2.1. Study Design, Caveats and Limitations

This study followed a workflow (Figure 1) for a physicochemistry-based screening of large sample numbers of liposomal formulations. The latter contained systematically variegated lipid compositions to reveal lipid compositions where membrane stability transitions into membrane instability ("at the edge of stability"). We deliberately omitted cholesterol and its derivatives in Section 2.2., because the equalizing effect of such "molecular plug" compounds on membrane stability were anticipated to impair the detection of membrane transitions at "the edge of stability". Subsequent embedment of CHEMS implemented pH-stimulated release of encapsulated cargo by acidic while sustaining maximum retention upon storage (described in Section 2.3.). Lead formulations identified in Section 2.3. were tested *in cellulo* (described in Section 2.4.) and *in vivo* (described in Section 2.5.).

2.2. Evaluation of Membrane Stability (Stage 1)

Two lipid panels were categorized into "stabilizing" and "modifying" lipids according to literature, ignoring aspects of suitability in clinical studies, known biological effects, and toxicity. Permutations of these two lipid panels lead to 18 "simplified" lipid compositions, i.e. binary lipid mixtures (numbered L1 through L18) (Table 2A). Some of the liposome-forming "stabilizing" lipids in Table 2B and C were chosen such as to represent different properties like charge, T_m and fatty acid chain length. The "modifying" lipids shown in Table 2C have identical fatty acid chain lengths and T_m s below preparation temperature (25 °C), hence an impact on membrane stability was supposed to derive mainly from their charge and miscibility with lipid 1.

Lipid compositions were prepared using dual centrifugation, with ratios varied in 10 mol-% steps, starting from 100 mol-% of stabilizing lipid 1, with stepwise substitution of modifying lipid 2 up to 90 mol-%. Given that the 100 mol-% lipid 1 samples

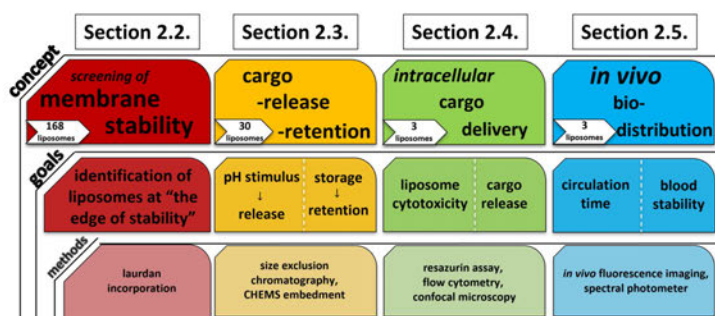


Figure 1. Visual concept of screening workflow with goals and methods. The manuscript sections corresponding to the performed steps are indicated.

were identical in all three series of lipid 2 permutations, the total number amounted to 168 different lipid compositions. To monitor lipid phase transitions upon increasing content of destabilizing lipids, we incorporated 0.1 mol-% of the hydrophobic fluorescent dye laurdan into liposomal membranes during preparation, expecting a characteristic sigmoidal curve. The fluorescent properties of laurdan depend on the polarity of its intimate environment and any changes (e.g. in lipid packing) result in an altered fluorescence emission at two wavelengths. This means properties are significantly affected upon membrane destabilization, e.g. when polarity of the environment increases due to surrounding water molecules.^[30] Changes in the laurdan fluorescence emission are given as laurdan generalized polarization (LGP) value.^[12b,15]

Within a ratio series we observed either constant increase or constant decrease of the LPG value (Figure S3). The properties of lipid 1 might explain some of the values. For example, in liposomes containing DPPC (L4–L6) or DPPG (L7–L9) as main stabilizing lipid with T_m above ambient temperature (25 °C), the lipids are in rigid gel phase and the LGP decreases upon membrane destabilization.^[12b] For the other class-1-lipids (Table 2B), the T_m is lower than 25 °C and these lipids are therefore in the liquid-crystalline phase. As the only type 1 lipid, none of the lipid combinations including EPC led to a sigmoidal curvature in any combination, possibly due to the heterogeneity of the EPC lipid composition.^[12]

After excluding lipid combinations without a sigmoidal shape or with low changes in the overall LGP values we had 7 promising lipid compositions left (namely L2, L4, L6, L9, L11, L14 and L15) for which normalized LGP values are plotted against increasing mol-% of lipid 2 in Figure 2. Liposomes containing 100 mol-% lipid 1 (0 mol-% of lipid 2) were normalized to 0% deviation, and liposomes containing 90 mol-% of lipid 2 corresponded to 100% deviation. Note that, for better visualization, the absolute (rather than the relative) deviation is shown.

Interestingly, the data in Figure 2 suggested that the composition range of 20–40% of lipid 2 is frequently encountered at “the edge of stability”, as highlighted in Figure 2 in grey. For the subsequent studies, we focused on these as potential candidates for further development.

2.3. Determination of Cargo Release and Retention Behavior (Stage 2)

To assess shelf-life, we established a protocol for measuring retention and release, using hydrophilic dyes as model cargos. Data presented below pertain to sulforhodamine b (SRb) as model cargo, but key data were recapitulated with other dyes also representing model cargos for small hydrophilic molecules (vide infra). SRb was the cargo of choice, because its absorption at 550 nm is independent of pH value in buffered medium, which prevents biases when comparing data from pH 7.4 with pH 5.5.^[32] SRb was encapsulated by DC, and non-encapsulated cargo was subsequently removed *via* size exclusion chromatography (SEC). Figure 3 shows an SEC elution profile measured by UV absorption of SRb at 550 nm, revealing two populations, namely encapsulated SRb eluting with the liposomes (L) at ~1 min and free cargo (FC) SRb eluting at ~2–5 min.

Encapsulation efficiencies (EE) were calculated as the ratio of SRb absorption signal in the liposome fraction L relative to that of L plus free cargo fraction, consistently showing EEs of 30–50% (Figure S4). Data are consistent with the vast majority of the cargo being encapsulated, although we cannot exclude that minor amounts might adhere to the liposomal surface.

Furthermore, as an *in vitro* mimic of acidic lysosomal conditions after liposome endocytosis, an aliquot of the purified liposomes was incubated at pH 5.5 and 37 °C for 4 h.^[33] Thereafter, liposomes were submitted to SEC again and the cargo release was determined as the ratio of SRb absorption signal in FC relative to that of L + FC (see Equation (4) in the methods section). The remainders of the purified liposomes were stored

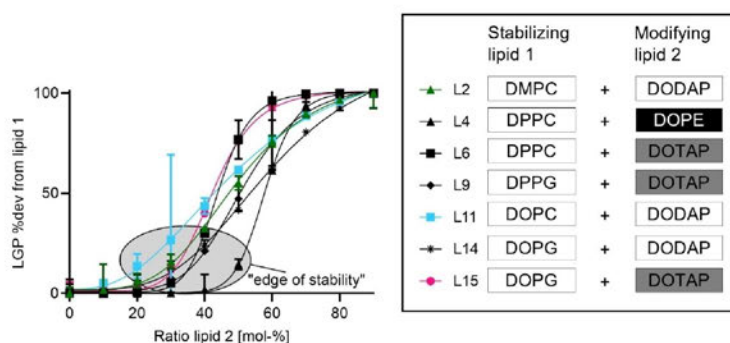


Figure 2. Laurdan assay curve progression of lipid mixtures L2, L4, L6, L9, L11, L14, L15 (n = 3). LGP values as deviation percentage from lipid 1 were plotted against increasing mol-% of modifying (destabilizing) lipid 2 as described in Materials and Methods. Liposomes within the grey circle of the graph were identified as potentially “at the edge of stability” formulations. Lipid compositions are displayed right side of the figure in black box. Lead candidates found in Section 2.3.2. are highlighted in color.

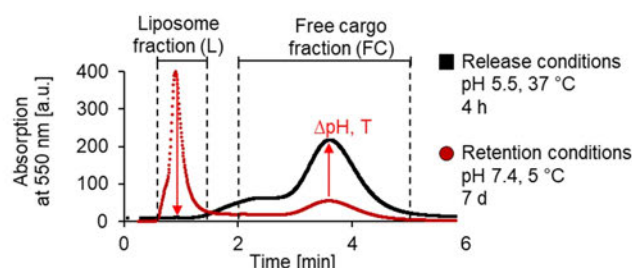


Figure 3. Determination of liposome cargo release and retention by SEC. A typical chromatogram obtained after separation of free cargo from intact liposomes by SEC is shown. Cargo encapsulation efficiency, release after 4 h incubation at pH 5.5 and 37 °C and retention after incubation for 1 week at pH 7.4 and 5 °C were calculated as described in the methods section. Model cargos sulforhodamine b and calcein were quantified by absorption at 550 and 490 nm, respectively.

at 5 °C for 1 week and then again analyzed to determine cargo retention under normal shelf conditions.

2.3.1. Mapping “The Edge of Stability” in Selected Binary Composition Liposomes

From the lipid combinations L2, L4, L6, L9, L11, L14 and L15 identified above, initial characterizations compared performances of liposomes containing 20, 30, and 40 mol-% type-2 lipid and additionally 0 and 10 mol-% for L6 and L9 and for L4 from 0–70 mol-% (Figure 4A).

Overall the optimal performance was observed at 30 mol-% for the 4 lipid combinations L2, L11, L14 and L15. While a content of modifying lipid 2 below the edge (e.g. 20 mol-%) led to more stable liposomes with high cargo retention, it simultaneously caused lower release (exception L2). Inversely, higher amounts of lipid 2 (e.g. 40%) resulted in insufficient stability and retention (see Figure 4A). We set 80% as the minimum release as an arbitrary parameter for selection of formulations, which was reached by L11, L14 and L15 at 40 mol-% of lipid 2.

L6, and L9 presented interesting cases, which did not reach the self-set limitation, since 30 and 40 mol-% type 2 lipid in L6 did not come with an acceptable retention, while L9 came with low release capability. Here we extended the range to 0 mol-% type 2 lipid resulting in high release with higher retention for 10 mol-% of lipid 2, but still leading to exclusion for L9 with release of only 72%. For L4 we applied a similar approach, but were unsuccessful even though increasing type 2 lipid from 0 to 70 mol-% substitution leading to exclusion.

Physicochemical characterization of respective lipid combinations via dynamic light scattering (DLS) indicated the start of liposome destabilization by an increase of sizes except for L9, PDIs (Figure 4B) and a drop in encapsulation efficiency (Figure S5), plausibly going along with the low cargo retention. Additionally, candidate L9 did not show a steady increase of retention with a stepwise increase of lipid 2 in 10 mol-% increments.

Including the narrated “excursions” in the L4, L6 and L9 series brought the liposomal formulations under “advanced scrutiny” to a total of 30 (Figure 4A). Of these 30, 5 formulations showed retention and reached a release of 80%. These formulations, marked with a grey rectangle in Figure 4A, were termed L2e, L6e, L11e, L14e and L15e as identified as “the edge of stability” formulations. Figure 4A clearly shows that the retention characteristics needed further improvement.

2.3.2. Addition of CHEMS as a pH-sensitive “Molecular Plug”

Since most successful developments contain at least three lipids, typically including a cholesterol-derivative, we introduced cholesterylhemisuccinate as a third compound. We started with low concentrations of 5 and 10 mol-%, which were added to the liposome mixtures L2e, L6e, L11e, L14e and L15e. The resulting liposomes, termed L2c, L6c, L11c, L14c and L15c, were tested for cargo release and retention at pH 5.5 and 37 °C. Additionally, we measured retention after 4 h incubation at physiological pH and 37 °C to differentiate between temperature and pH triggered release.

Figure 5A shows the results of the 5 leading candidates containing 5 or 10 mol-% CHEMS (with composition listed in Figure 5B), compared to a conventional and widespread liposomal formulation L0 (55 mol-% EPC and 45 mol-% cholesterol) with limited cargo release.^[34] As expected, CHEMS increased retention but decreased release (compare Figure 4A with minimum 80% and Figure 5A).

Two candidates, L6c and L14c, exhibited poor performance upon CHEMS additions. L6c, primarily composed of stabilizing lipid DPPC, showed minimal cargo retention after 4 h incubation at 37 °C (red boxes in Figure 5A).^[35] While CHEMS improved the long-term cargo retention of L14c to 76%, this value was the weakest among the candidates. Increasing CHEMS from 5 to 10 mol-% did not significantly change retention, but lowered release below 50% (data not shown).

In contrast, adding 5 mol-% CHEMS in L2c and L15c considerably increased retention above the 90% while maintaining release above 50%. For L11c, 5 mol-% CHEMS was

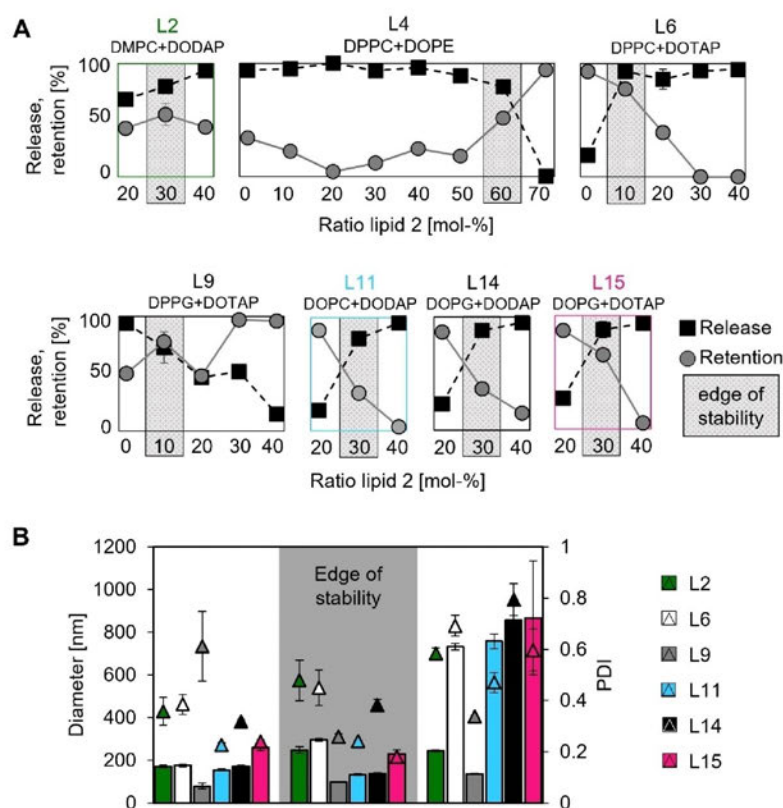


Figure 4. Identification of lipid ratios with increasing modifying lipid that showed high release of SRb at acidic pH and body temperature and corresponding diameter and PDI. **A:** Release (4 h at pH 5.5, 37 °C) and retention of increasing modifying lipid content ($n = 3$ of sample on edge of stability, $n = 1$ for sample next to it). Centered are the liposomes considered at “the edge of stability” (grey) with a release above 80% that in addition showed the highest cargo retention (1 week at pH 7.4, 5 °C) within the corresponding composition. Performance drops in cargo release and retention are apparent for liposomes left and right of “the edge of stability”, respectively. **B:** Physicochemical parameters with increasing mol-% of lipid 2 obtained from dynamic light scattering ($n = 3$). Candidate liposomes L2, L11, L14 and L15 were prepared with 20, 30 and 40 mol-% DODAP & DOTAP, respectively, while L6 and L9 was prepared with 0, 10 and 20 mol-% DOTAP. Destabilized liposomes at higher mol-% stand out by high values for diameter (bars) and PDI (triangles), supporting the classification of liposomes at 30 (10) mol-% lipid 2 as “the edge of stability”. Error bars indicate standard deviation from three individual liposome preparations. Lead candidates found in Section 2.3.2. are highlighted in color.

insufficient to raise cargo retention from 33% (Figure 4A, Section 2.3.1.) to above 90%, but 10 mol-% CHEMS achieved this target. Consistent with the “molecular plug” effect, we set thresholds of minimum 50% release and 90% retention, after one week of storage for one week and incubation for 4 h at 37 °C. Three candidates (L2c, L11c and L15c) met these criteria (Figure 5A), whose final lipid compositions shown in Figure 5B.

We recapitulated the pH-responsive effect of CHEMS with different cargoes by comparing pH-induced release between liposomes containing either cholesterol or CHEMS (Figure S6), supporting the working hypothesis of using CHEMS *versus* unmodified cholesterol for pH-responsive liposomal drug delivery.

2.3.3. Effect of Serum

Because blood components can affect overall vesicle stability, e.g. by lipid extraction of steroid scaffold lipids, retention and release parameters of the L2c, L11c and L15c were again determined after incubation in full human serum for 4 h at 37 °C (Figure 5C). Indeed, serum incubation lead to a 15–30% drop in retention values after 4 h at 37 °C, although the remaining cargo retention was deemed acceptable for further characterization. Varying the CHEMS content did not improve cargo retention in the presence of human serum (Figure S7).

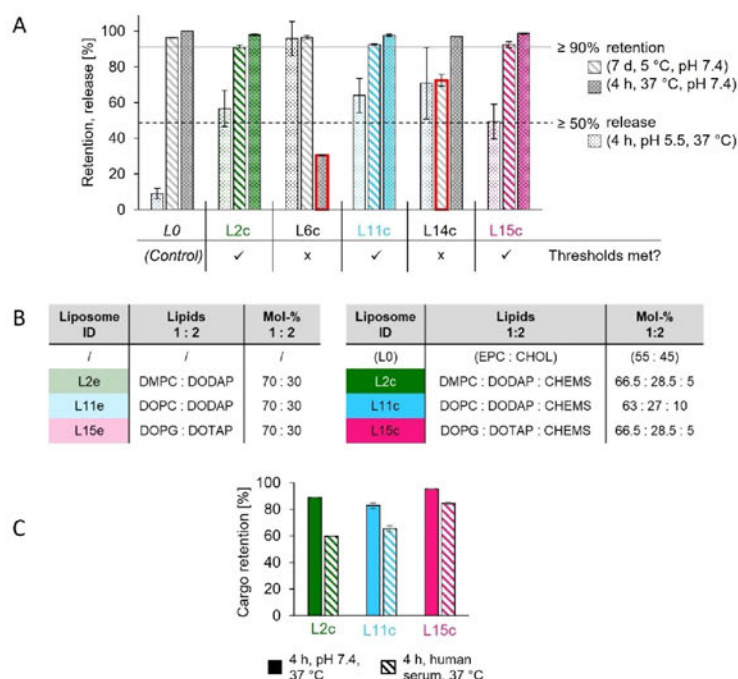


Figure 5. Liposome refinement by incorporation of cholesterylhemisuccinate. **A:** Percentage of cargo release (SRb) and retention for conventional liposome L0 and candidate liposomes L2c, L6c, L11c and L15c with 5 mol-% and L11c with 10 mol-% CHEMS incorporated (n = 3). The dotted lines define the release threshold of at least 50% and the retention threshold of minimum 90%. Note that the control liposome L0 showed only little pH-dependent release. Error bars indicate standard deviation from at least three individual liposome preparations. **B:** Lipid compositions and ratios of candidate liposomes with and without CHEMS and control liposome L0 with cholesterol. **C:** Influence of human serum on cargo retention for lead candidate liposomes after incubation for 4 h at 37 °C in the presence or absence of full human serum (n = 3). Lead candidates found are highlighted in color.

2.3.4. Varying the Molecular Cargo

Properties of the lead candidates L2c, L11c and L15c were also investigated with alternative model cargos of comparable molecular weight, namely calcein (Figure S5) and doxorubicin (DXR) (Figure 6).

DLS reported liposome diameters of about 150 to 250 nm, with PDIs below 0.3, regardless of the type of cargo (Figure 6A). After storage at 5 °C for 4 months, L11c and L15c showed no significant changes in size, zeta potentials and PDIs, while L2c showed a higher PDI and an increase in zeta potential, indicating a destabilization that might potentially be related to protonation (Figure 6B).

Acidic incubation at pH 5.5 and 37 °C for 4 h led to a slight decrease in size and an increase in PDI, as observed by DLS (Figure 6B). The zeta potentials of the liposomes L2c and L11c shifted to positive upon acidification, while the zeta potential of L15c increased only slightly and remained negative. The obtained data suggests an acid induced release mechanism due to an increased membrane permeability, rather than liposome aggregation, which would have resulted in a size increase.^[20] Transmission electron microscopy (TEM) pictures of all candi-

dates as well as a size comparison before and after cargo release are provided in Figure S8.

2.4. Intracellular Cargo Delivery (Stage 3)

The next stage comprised the *in vitro* validation of liposomal formulations using human melanoma cells (UKRV-Mel-15a) and human monocyte-derived macrophages. We took advantage of doxorubicin hydrochloride as model cargo since its DNA-binding ability and the correlated fluorescence are enabling features for the discrimination between total DXR and DXR released inside the cell and thereby the verification of *in vitro* release from different liposomal formulations.

2.4.1. Toxicity Assay

Empty liposomes showed acceptable toxicity after 72 h in both cell types, with the lowest viability being ~65% in melanoma cells, measured in a resazurin assay (Figure 7, plain bars). Empty L2c, L11c and L15c showed cytotoxicity comparable to, or

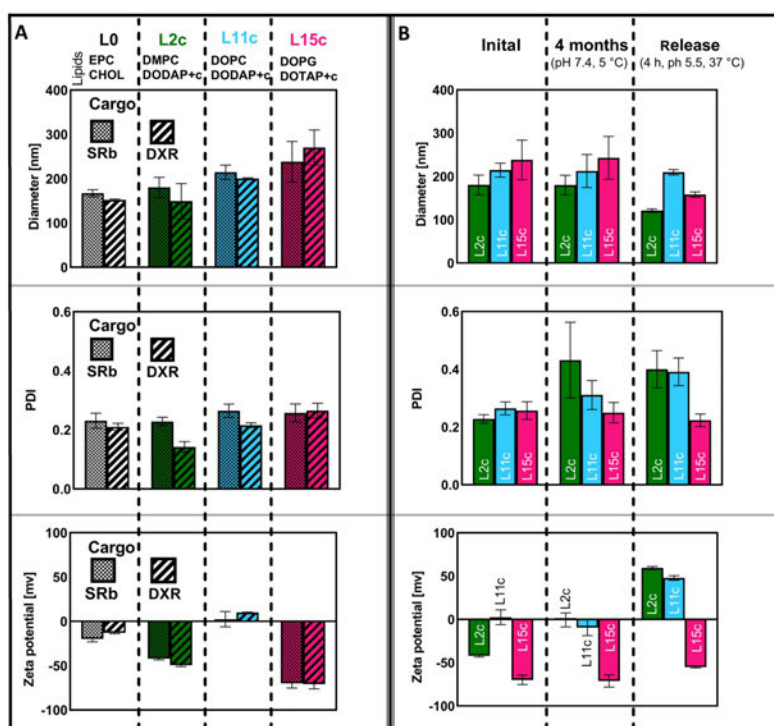


Figure 6. Physicochemical characterization of liposomes by dynamic light scattering. **A:** Diameters, PDIs and zeta potentials of lead candidates L2c, L11c and L15c and control L0 after encapsulation of SRb (checked bars) or DXR (dashed bars) ($n=3$). **B:** Diameters, PDIs and zeta potentials of lead candidates on 1 day or 4 months after preparation (stored at pH 7.4 and 5 °C) and after incubation under release conditions (4 h at pH 5.5 and 37 °C) ($n=3$). Depicted, as diameter is the calculated z-average. Error bars indicate standard deviation from three individual liposome preparations.

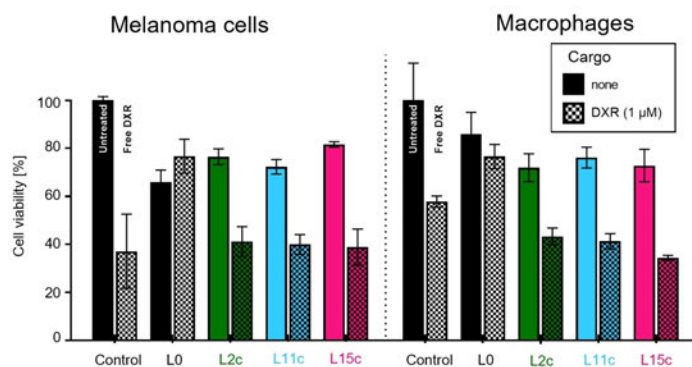


Figure 7. Resazurin assay to determine liposome toxicity after 72 h. Reduction of resazurin to resorufin was used as an indicator for cell viability. Human melanoma (UKRV-Mel-15a) cells and human monocyte-derived macrophages were incubated for 72 h with 1 μM free DXR as positive control, the conventional liposome L0 and the three lead candidates L2c, L11c and L15c all containing DXR equal to 1 μM (checked bars) or no cargo as control group (plain bars) ($n=9$, from 3 independent experiments). Results were normalized to untreated cells.

slightly better than, the control liposome (L0) which is widely regarded as non-toxic (shown in *in vivo* studies^[36]).

1 μ M DXR-loaded candidate liposome treatment (Figure 7, checkered bars) exhibited toxicity equivalent to 1 μ M free DXR treatment in melanoma (~40% viability), and even stronger toxicity than free DXR in macrophages. Toxicity of DXR-loaded lead candidates was ~40% higher compared to the conventional liposome (L0) in both cell types. Together, these findings indicate a successful cargo release in lead candidates. Cell viability after 24 h treatment showed comparable results (Figure S9), although cell viability of DXR containing samples was overall higher due to a shorter incubation period.

2.4.2. Cellular Uptake and Cargo Release Assessed by Flow Cytometry

To assess uptake and intracellular DXR release of liposomes inside the cells, the overall fluorescent signal of DXR per single cell was monitored using flow cytometry.

Melanoma cells (left) showed lower fluorescence signals after 4 h (Figure 8, plain bars), compared to macrophages (right). Also in melanoma cells, candidate liposomes caused fluorescence intensity comparable to free DXR, indicating DXR release, in contrast to control liposome L0. In macrophages, the whole cell fluorescence for candidate liposomes was around three times higher than for L0, but did not reach the level of free DXR. 30 minutes incubation showed comparable results, indicating uptake and accumulation of DXR in all samples, albeit lowest for L0 (Figure S10). Release of DXR from liposomes resulted in fluorescence increase through unquenching, while DNA intercalation decreases fluorescence.^[37] Therefore, after a first rough estimation of DXR release from liposomes inside the cells by flow cytometry, we followed up with spatial inves-

tigation of different cellular compartments by confocal microscopy.

2.4.3. Model Cargo Accumulation in the Nucleus

Confocal microscopy was used to quantify DXR fluorescence in the nucleus, as DXR intercalates into nuclear DNA only after release from liposomes and cellular organelles, such as endosomes. Nuclei were counterstained with Hoechst 33258. To minimize fluorescence crosstalk, plasma membrane labeling was omitted during these experiments. However, in control experiments where membranes were labeled, we observed that the vast majority of membrane labeled (DiD) liposomes had been taken up by the cell rather than adhering to the outside after sample preparation (Figure S11) and that liposomes did not seem to be able to cross the nuclear membrane. Consistent with the investigations above (2.3.3.), there was no evidence that serum contained in cell culture media (10%) might destabilize liposomes prior cellular uptake, since intact liposomes were visible in confocal microscopy images after 4 h, and no staining of the cellular membrane with liposomal membrane dye DiD was observed.

DXR accumulation in the cell nuclei was quantified, and the results shown in Figure 8 (checkered bars) confirm the conclusions drawn from the flow cytometer analyses: Macrophages displayed higher DXR fluorescence compared to melanoma cells. L0 liposomes showed decreased cargo release compared to L2c, L11c and L15c after 4 h. DXR released from pH-responsive liposomes accumulates as fast as free DXR in the nucleus and reaches the same levels. Data obtained after 30 min of incubation showed the same trends (Figure S10).

Although transfer of lipophilic fluorescent probes, such as DiD, between intact membranes was reported to be

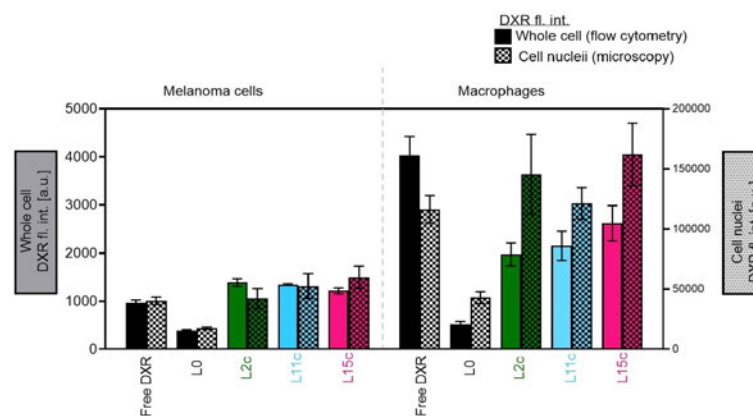


Figure 8. Determination of DXR fluorescence in whole cells by flow cytometry and in cell nuclei by confocal laser microscopy. Liposomal and free DXR fluorescence (dashed bars, left y-axis) in live cells was measured by flow cytometry ($n = 11$ from 3 independent experiments). DXR fluorescence of nuclei after DXR release from liposomes was measured by confocal microscopy (checkered bars, right y-axis) ($n = 3$, from 3 independent experiments). UKRV-Mel-15a cells (left) and human monocyte-derived macrophages (right) were incubated for 4 h with 1 μ M free DXR as positive control, the conventional liposome L0 and the three lead candidates L2c, L11c and L15c all containing DXR equal to 1 μ M. Cell nuclei were counterstained using Hoechst 33258.

negligible,^[38] we observed lateral diffusion of DiD within the plasma membrane, visible as staining of the entire cell (Figure 9, pictures 2C–E and 4C–E). Of note, this observation applied exclusively to lead formulations L2c, L11c and L15c, but not to the conventional liposome L0. This effect likely resulted from a decomposition or a change in membrane permeability of the pH responsive candidate liposomes or at least from a change in their membrane permeability within the observed 24 h time span.

2.5. *In vivo* Bio Distribution (Stage 4)

We undertook a limited characterization of the *in vivo* distribution of our lead candidates by live imaging after injection into albino black 6 mice. As a simplistic model, we used phosphate buffer as cargo and limited the label to incorporation of DiR, an NIR dye known to stably integrate into liposomal membranes where it fluoresces strongly, whereas its free form does not.^[39] Importantly, our objective was to observe stability during circulation reported by DiR. We complemented the analysis with characterization of the general distribution in the body, and determined detailed amounts in liver and spleen. Fluorescence images and intensity data taken 0 h, 3 h and 24 h after tail vein injection showed similar distributions for the candidates compared to L0 in the abdomen area, shown in Figure 10A and B. Worth mentioning is the strong drop in fluorescence intensity of candidate L11c below 50% of the starting value between 3 h and 24 h. Interestingly, the same formulation showed in the earlier measurement (3 h) the highest fluorescence signal for our three candidates. On the

other hand, almost no drop in fluorescent intensity could be observed between 3 h and 24 h for L2c.

The comparison of blood samples taken at 3 h and 24 h showed a relatively lower elimination from bloodstream, for L2c, L11c and L15c compared to L0, suggesting increased blood circulation (Figure 10C).

Subsequently, the mice were sacrificed after 24 h, and liver as well as spleen were fluorescence imaged *ex vivo*. As shown in Figure 10E, substantial DiR fluorescence was found in the liver and spleen for all liposomal formulations. Fluorescence intensities in the liver were roughly comparable among formulations (Figure 10G), whereas values for spleen (Figure 10F) showed significant differences, with particularly low values for L2c and L15c relative to L0 and L11c. These results were confirmed by analysis of extracted DiR from organ slices (Figure S12). At the same time, with the highest drop in blood fluorescence for L0, the DiR signal was not significantly stronger in both organs.

As a summary of the *in vivo* analysis we conclude, that “the edge of stability” formulations were distributed in a fashion mostly similar to the conventional inspired liposomes L0. Exceptions of interest include lower accumulation in the spleen and concomitantly lower elimination capability from bloodstream for L2c and L15c.

3. Conclusions

General aspects: The concept presented allows the screening of large numbers of liposomal formulations to identify lipid compositions that position the resulting liposomes properties

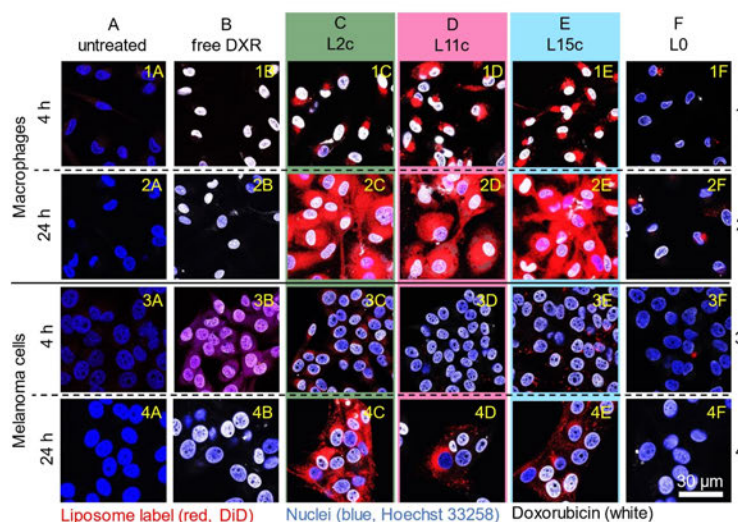


Figure 9. Confocal laser microscopy images of stained nuclei (blue, Hoechst 33258). Human monocyte-derived macrophages (rows 1 and 2) and human melanoma cells (UKRV-Mel-15a, rows 3 and 4) after incubation for 4 and 24 h with free DXR or DXR loaded liposomes ($n = 3$ from 3 experiments). DXR fluorescence is depicted in white, and liposomes were membrane labeled with membrane dye DiD (red).

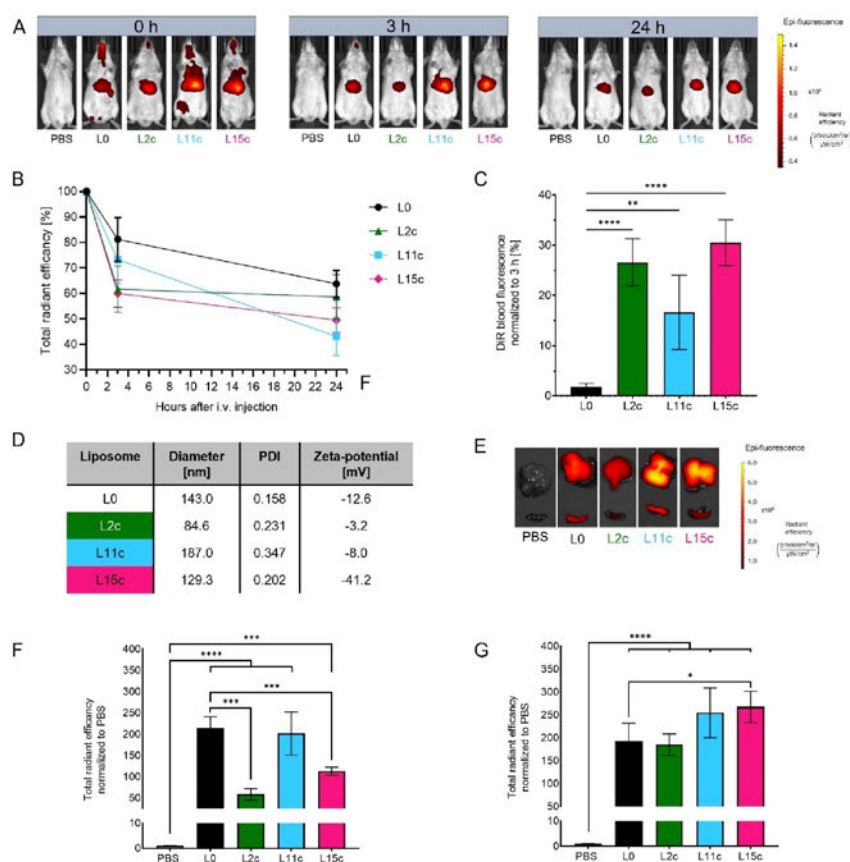


Figure 10. *In vivo* and *ex vivo* analysis after 0 h, 3 h and 24 h after tail vein injection. **A:** Representative DIR fluorescence images of treatment groups. Albino black 6 mice were treated with DiR-labeled lead candidates or PBS as control (L2c, L11c, L15c, L0 (n = 5) at 5 mg/kg bodyweight or only phosphate buffer saline (n = 3)). Signals in the area of mouth and nose are caused by wound care of the animal after injection. **B:** Percent of total radiant efficiency of pH-responsive lipid-nanocarriers in abdomen area displayed for the different timepoints of liposomal candidates and control (n = 5). Total radiant efficiency at 0 h after injection in the abdomen area (lower belly) was set to 100%. **C:** Blood fluorescence intensity at the experimental end normalized to the 3 h value (phosphate buffer saline group not included) (n = 5). Significances were calculated comparing to L0 (black) via ordinary one-way ANOVA with $r^2 = 0.8622$ for with a 95% confidence interval. **D:** Table of physicochemical characteristics of liposomal formulations used for biodistribution study. **E:** Fluorescence images of liver (top) and spleen (bottom) taken by *in vivo* imaging 24 h post injection (hpi). Representative images of liver and spleen of treatment groups (L0, L2c, L11c, L15c (n = 5) at 5 mg/kg bodyweight or phosphate buffer saline control (n = 3)) are displayed after normalizing epi-fluorescence to the highest values. **F:** Epi-fluorescence from E in spleen (n = 5 for liposomes, n = 3 for control, total of 23 samples) and liver (**G**) (n = 5 for liposomes, n = 3 for control, total of 23 samples) normalized to PBS treatment signal. Significances were calculated to phosphate buffer saline (grey) or L0 (black) via ordinary one-way ANOVA with $r^2 = 0.9091$ (liver) and $r^2 = 0.9060$ (spleen) with a 95% confidence interval. P-values of ≤ 0.05 are displayed with *, $p \leq 0.01$ is equal to **, while *** and **** show a p-value of ≤ 0.001 and ≤ 0.0001 .

at “the edge of stability”. This term refers to parameters which were experimentally verified such as shelf-life stability and stimulus-dependent release narrowing the liposomal selection down to 3 out of several hundred possible lipid compositions (binary lipid compositions (Table 2) plus formulations with CHEMS at different mol-%). Our concept has proven itself for hydrophilic small molecules as model cargo, which so far appeared interchangeable among sulforhodamin b, calcein, and

doxorubicin, but may have to be rerun for small molecule drugs with higher lipophilicity.

“The edge of stability” is experimentally accessible: The use of the fluorescent dye laurdan into the liposomal membrane enabled the identification of binary lipid compositions that form liposomes at “the edge of stability” showing a sigmoidal curve. While it is clear, that sigmoidal behavior does not *per se* reflect a suitable binary lipid compositions, we employed this method as a rationale first step to narrow down the 168

combinations. This selection criterium significantly impacted the outcome, offering potential for alternative strategies. Our approach appears to work for certain lipids, while others, such as EPC, lack dynamic ranges in this readout, presumably because of inhomogeneity in lipid composition.^[40] Implications from these experiments are that (i) certain lipid compositions might escape detection by this assay although being suitable for the task at hand, and (ii) homogenous materials likely show better defined responses to outside stimuli. Other solvatochromic fluorescent membrane probes such as Nile Red or Prodan may help to reveal even more liposomes at “the edge of stability”.^[41] We further refined the candidate pool from 30 accentuated lipid combinations down to 5 by employing the self-imposed 80% cargo release cut off and simultaneously acceptable stability despite our efforts to minimize rationalism. This logical threshold was implemented with the understanding that stability could be theoretically enhanced in subsequent steps through the molecular plug effect of cholesterol derivatives, allowing to prioritize release characteristics at this stage.

Typical liposome destabilization occurred around 40 mol-% lipid 2 (Figure 2, Section 2.2.) while “the edge of stability” was located at lower amounts of destabilizing lipid 2 relative to the inflection point, typically at 30 mol-%. The final lead candidate selection process after implementation of CHEMS necessitated two additional cut-offs, aligning with our primary liposomal design objectives, namely sufficient membrane stability (min. 90%) and efficient payload release (min. 50%, pH 5.5).

Unsuspected pH-responsiveness and helper lipid CHEMS: Most pH-responsive formulations include known pH-sensitive lipids, typically containing ionizable amino groups. L15e (DOPG/DOTAP) exhibited pH-responsiveness without containing ionizable lipids, presumably due to its position at “the edge of stability”. Embedment of 5 mol-% CHEMS improved stability for all formulations while maintaining pH-dependent cargo release, but the zeta potential upon acidification was clearly distinct in L15c from those formulations containing ionizable lipids, namely L2c and L11c (both containing DODAP). The zeta potential of L15c remained mostly unchanged at acidic pH, while the latter both showed a positive shift. Diameters of candidate liposomes did not increase upon acidification, suggesting an increased membrane permeability as an acid induced release mechanism. These findings suggest that liposomes at “the edge of stability” might show unsuspected responsiveness to slight changes in pH or temperature.

Increased performance compared to conventional liposome L0: The final candidates showed comparable toxicity to the control liposome L0, which we take as a potential predictor for plausible systemic side effects in *in vivo* applications.^[42] DXR-loaded candidates exceeded performance of conventional liposome L0 in terms of cargo release (demonstrated by increased cell death), higher intracellular DXR fluorescence (flow cytometry) and co-localization of DXR in the nuclei (confocal laser microscopy). Quantification of DXR fluorescence in nuclei revealed a three times higher release capability (amount and speed) of candidate liposomes in comparison to L0. We also observed that the liposomal “lead”-formulations distributed *in vivo* in a fashion comparable way compared to L0 with a

slight indication of lower elimination from the bloodstream for all three candidates. We see this as a proof-of-principle that should be applicable to a range of lipids, including such that have been in human and have been found non-toxic.

Aspects of intracellular fate: Observations suggest changes in membrane permeability or decomposition of liposomal membranes and even endosomal escape of not only cargo, but also membrane dye, within 24 h for lead candidates, as seen for DiD-labeled liposomes inside the cell after 4 h (without membrane staining, Figure 9). We concluded that negatively charged liposomes might be endocytosed and did not fuse with the cellular membrane, which is in line with reports by Straubinger *et al.*^[33a]

Perspectives despite caveats: The presented approach may be applied to liposomes consisting of more than two lipids, allowing for the design and/or development of liposomes with increased complexity and added functionalities influencing stability and cargo release (lipid anchors for stealth-like surface decorations, clickable functionalities for labelling or targeting, stimulus-responsive surface decorations).^[24a,43] Future studies are necessary for transition from model-cargo containing lead-candidate liposomes to formulations with dedicated drugs, as the behavior may vary for different cargoes. Also fine-tuning of individual formulations require switching from dual centrifugation to other formulation methods to achieve lower PDI values.

Overall, this study provides a promising approach for identifying liposomal formulations with desirable properties and highlights the potential for further development and optimization.

Author Contributions

Mark Helm: Conceptualization, Methodology, Resources, Writing – Original Draft, Funding acquisition, Writing – review & editing. **Andrea Tuettenberg:** Conceptualization, Methodology, Resources, Writing – Original Draft, Funding acquisition, Writing – review & editing. **Lukas Gleue:** Methodology, Validation, Data Curation, Visualization, Formal analysis, Investigation, Writing – Original Draft, Writing – review & editing. **Barbara Graefen:** Methodology, Validation, Data Curation, Visualization, Formal analysis, Investigation, Writing – Original Draft, Writing – review & editing. **Matthias Voigt:** Conceptualization, Methodology, Validation, Data Curation, Visualization, Formal analysis, Investigation, Writing – Original Draft, Writing – review & editing. **Jonathan Schupp:** Conceptualization, Methodology, Validation, Data Curation, Visualization, Formal analysis, Investigation, Writing – Original Draft, Writing – review & editing. **Dirk Schneider:** Conceptualization, Methodology, Writing – Review & Editing. **Micheal Fichter:** Resources, Writing – Review & Editing, Project administration. **Micheal Kuske:** Resources, Writing – Review & Editing, Project administration. **Volker Mailaender:** Resources, Writing – Review & Editing, Project administration. Final approval of the version to be published was given by all authors and they agreed to be accountable for all aspects of the work.

Acknowledgements

This work was supported by the DFG research center SFB1066, project B14N to MH and AT, Wilhelm-Sander-Foundation (2020.132.2) to AT and Hiege-Stiftung against skin cancer (200504) to AT. DS was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, CRC1552 Project Nr 465145163). We thank the imaging core facility of the cell biology unit of Krishnaraj Rajalingam in the PKZI Mainz for providing the Leica SP8 microscope. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interests

Mark Helm is a consultant for Moderna Inc. Andrea Tuettenberg is the CEO of ActiTrexx GmbH. The other authors report no competing interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Screening concept · Dual centrifugation · Intracellular release · Liposomes · Drug delivery

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Manuscript received: August 19, 2024
 Revised manuscript received: September 20, 2024
 Accepted manuscript online: September 27, 2024
 Version of record online: November 12, 2024

ChemMedChem

Supporting Information

Dual Centrifugation-Based Screening for pH-Responsive Liposomes

Lukas Gleue, Barbara Graefen, Matthias Voigt, Jonathan Schupp, Dirk Schneider, Michael Fichter, Michael Kuske, Volker Mailaender, Andrea Tuettenberg,* and Mark Helm*

1 Supplementary data

2 Journal: ChemMedChem

3 Title: Dual Centrifugation-based Screening for pH-responsive Liposomes

4 Authors: Lukas Gleue[#], Barbara Graefen[#], Matthias Voigt[#], Jonathan Schupp[#], Dirk Schneider, Michael Fichter,

5 Michael Kuske, Volker Mailaender, Andrea Tuettenberg^{**} and Mark Helm^{**} [#] authors contributed equally

6 ^{*} corresponding author, E-Mail: mhelm@uni-mainz.de and antuette@uni-mainz.de

7 Sup. 1 Materials and Methods

8 Sup. 1.1 Materials, Devices and Cells:

9
10 Iodine (#X864.1), Potassium permanganate (#8004.1) and Sodium hydroxide (#1CCX.2) were obtained
11 from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Uranyl acetate solution (#22400-4) was
12 purchased from Electron Microscopy Sciences (Hatfield, Pennsylvania, USA). Formic acid (#10596814)
13 was received from Fisher Scientific, Hampton, New Hampshire, USA). Thin-layer chromatography slides
14 (#818333) was obtained from Macherey-Nagel (Düren, Germany). Ammonium hydroxide (#221228),
15 Chloroform (#366927-1L), Cholesterol (#C8667-25G), Cyclohexane (#650455-1L), Ethylacetate
16 (#34858-1L), Methanol (#34860-2.5L-R), Myristic acid (#M3128-10G), Oleic acid (#4954-1GM) were
17 purchased (Merck KGaA, Darmstadt, Germany). 1.5 ml reaction tube (#72.706) and 6 well-plate
18 (#83.3920) were received from Sarstedt (Nümbrecht, Germany). DiR (#D12731), Phosphate buffered
19 saline (#14190094) and 96 well-plate, flat bottom (#137101) were obtained from Thermo Fisher
20 Scientific (Waltham, Massachusetts, USA). Cell strainer, 40 µm (#732-2757) and Pestle (#AXYGPE-
21 15-B-SI) were obtained from VWR International (Radnor, Pennsylvania, USA).

22
23

24 Sup. 1.2 Methods

25 **Sup. 1.2.1 Transmission electron microscopy (TEM)** Corresponding liposome samples with a volume
26 of 2 µL were placed and embedded in 1% trehalose with 4% uranyl acetate on a lacey grid. A FEI Tecnai
27 F20 transmission electron microscope (FEI Houston Inc., Hillsboro, Oregon, USA) was used for
28 measurement at a working voltage of 200 kV. The electron micrographs were taken by an Ultrascan
29 1000 (Gatan) charged-coupled device camera, while the DigitalMicrograph (Gatan, Pleasanton,
30 California, USA) collected the images.

31

32 Sup. 1.2.2 DiR extraction and fluorescence measurement from organs

33 15-30 mg per spleen and 95-170 mg per liver were collected and based on the whole weight of the
34 organ a dilution factor was calculated. Slices of organ tissue were passed with a pestle through a 40 µm
35 cell strainer for each sample and organ per well of a 6 well plate. Cell strainers were washed with 750 µl
36 chloroform:methanol (1:1) as solvents and subsequently collected in 6 well plates. Plates with single cell
37 suspensions were treated for 30 seconds with ultrasound. Single cell suspension were transferred in
38 1.5 ml tubes and centrifuged at 400x g for 5 min at RT. Supernatant was transferred to a new 1.5 ml
39 tube. 150 µl of each sample were transferred to a black 96 flat bottom well plate in triplicates. For DiR
40 calibration samples, defined concentrations for DiR were dissolved in chloroform:methanol (1:1). DiR

41 Stock solution was 10 mg/ml and dilutions with 50 nM, 10 nM, 5 nM, 1 nM, 0.5 nM, 0.1 nM were
 42 transferred in triplicates to the same wellplate. One plate was used for organs of liver, the other one for
 43 the spleen with calibration samples for each plate. Plates were measured with Spark 10 M multiplate
 44 reader (Tecan, Männedorf, Switzerland) at 740/780 nm (Ex./Em.). Total sample number for each organ
 45 was 26. Calibration curve was calculated by Excel to and measured values were inserted to obtain DiR
 46 concentration. This value was multiplied by the dilution factor for the chloroform:methanol total volume
 47 (750 µl – used for each well 150 µl; dilution factor = 5). The resulting DiR concentration is equal to the
 48 DiR concentration in the tissue sample and was multiplied by the dilution factor of the whole organ.
 49 Graphs were made with GraphPad Prism 9 and significances were calculated with an ordinary one-way
 50 ANOVA compared to PBS treatment samples. DiR sample values were not significantly altering to PBS
 51 and were excluded from the graph.

52 **Sup. 1.2.3 Lipid quality control**

53 To analyze the integrity of the lipids after solvent separation, the lipids were individually dried as
 54 described in the previous paragraph and resolved in chloroform. Subsequently, the dissolved lipid, the
 55 stock lipid, and the potential corresponding degradation product were analyzed on the thin-layer
 56 chromatography (TLC) slide using the mobile phase composition from Sup. Tab. 1. For spot
 57 visualization, the slides were subsequently stained either in an iodine chamber or with an aqueous
 58 potassium staining reagent (60 mM potassium permanganate, 550 mM sodium hydroxide).

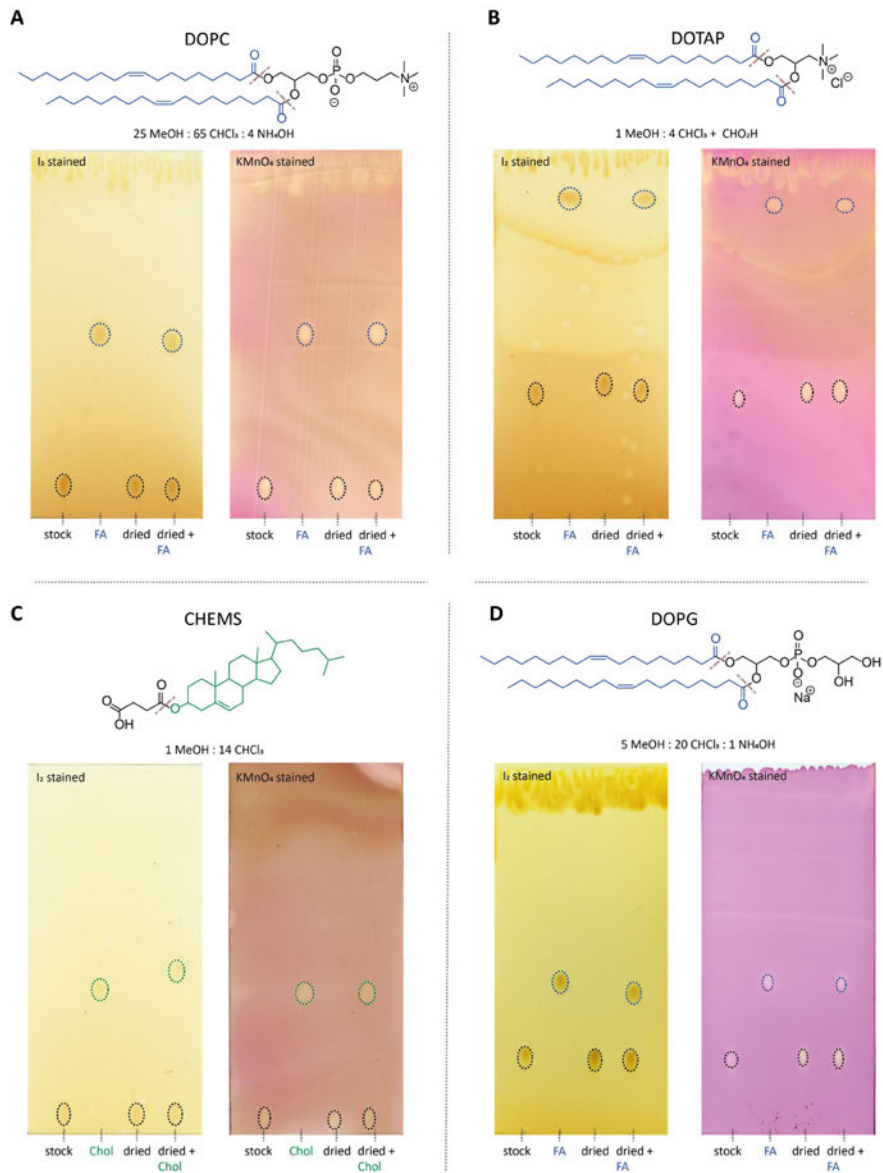
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 60 **Sup. Tab. 1: composition of mobile phase for thin-layer chromatography analysis**

Lipid	Mobile phase composition (v-% : v-%)
DOPC, DMPC, DODAP, DOPE	25 methanol : 65 chloroform : 4 ammonium hydroxide
DOTAP	1 methanol : 4 chloroform + formic acid
CHEMS	1 methanol : 14 chloroform
DOPG	5 methanol : 20 chloroform : 1 ammonium hydroxide
CHOL	1 ethylacetate : 4 cyclohexane

61

62

Sup. 2 Figures



64

65 **Fig. S1: Quality control of used lipids after drying process:** Verification of the lipid integrity was performed
66 by thin-layer chromatography. Here, all plates were stained with both iodine (I₂) and an alkaline
67 potassium permanganate (KMnO₄) solution after separation. **A:** DOPC; **B:** DOTAP; **C:** CHEMS; **D:**
68 DOPG

69 stock = lipid; FA = free fatty acid; chol = cholesterol; dried = dried lipid; dried + FA = dried lipid + free
70 fatty acid; dried + chol = dried lipid + cholesterol

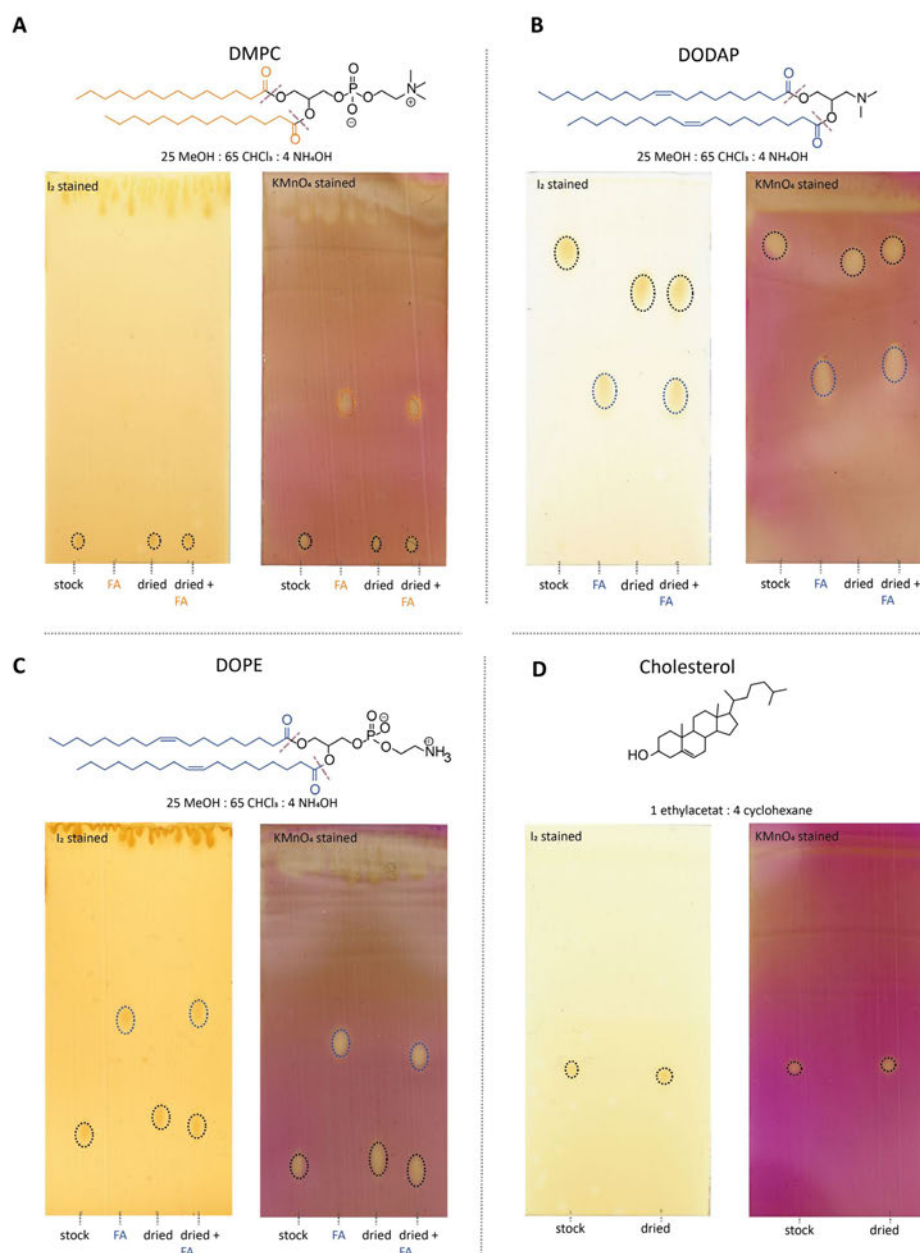
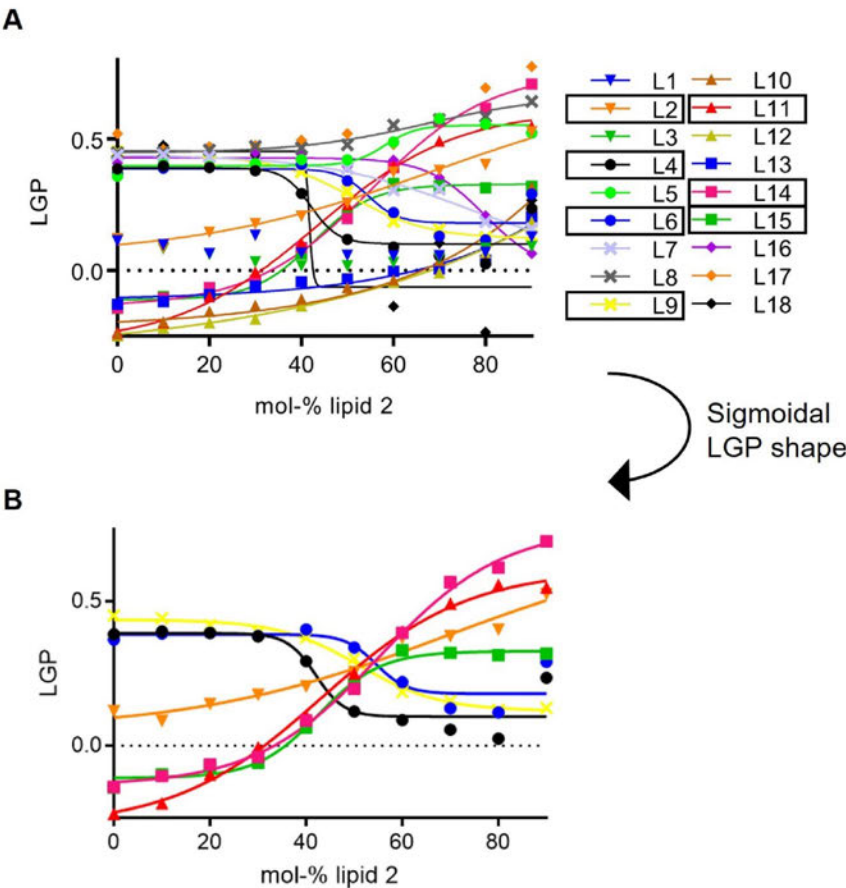


Fig. S2: Quality control of used lipids after drying process: Verification of the lipid integrity was performed by thin-layer chromatography. Here, all plates were stained with both I₂ and an alkaline KMnO₄ after separation. **A:** DMPC; **B:** DODAP; **C:** DOPES; **D:** cholesterol

stock = lipid; FA = free fatty acid; chol = cholesterol; dried = dried lipid; dried + FA = dried lipid + free fatty acid

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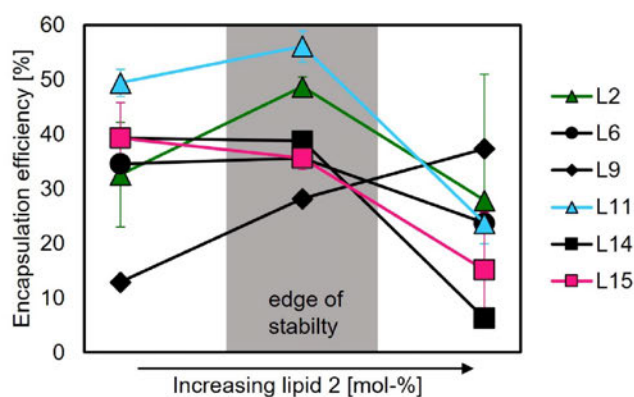


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79 Fig. S3: LGP values of tested lipid combinations. Obtained data were fitted with a sigmoidal 4PL curve
80 with least squares using Graphpad Prism 7. **A:** Laurdan assay of all lipid combinations (n=1). **B:** Laurdan
81 assay of lipid combinations that showed a sigmoidal LGP value (n=1). For the description of the method,
82 please check out the corresponding manuscript.

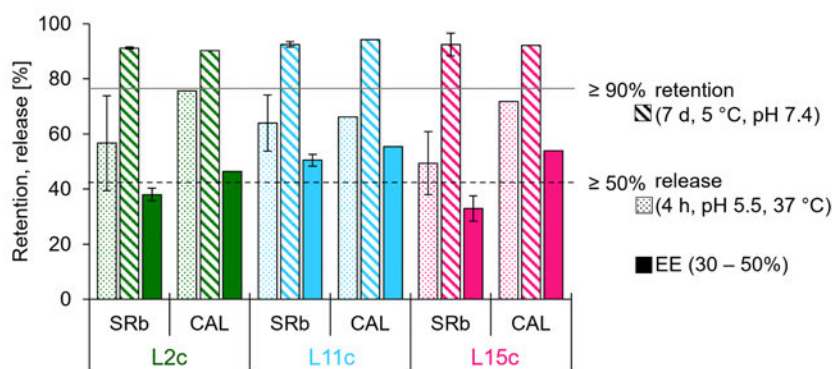
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85

86 Fig. S4: Encapsulation efficiencies of candidate liposomes (n=3). Increasing mol-% of type-2 lipid led to
 87 a drop in encapsulation efficiency of sulforhodamine b past the considered "edge of stability" (grey).
 88 Error bars indicate standard deviation of three individual liposome preparations. For the description of
 89 the method, please check out the corresponding manuscript. Data for L4 not shown.



90

91 Fig. S5: Release (4 h at pH 5.5, 37 °C), retention (1 week at pH 7.4, 5 °C) and EE for lead candidates
 92 with SRb or CAL as a cargo (n=3). Threshold parameters were successfully reached by both model
 93 cargos. Error bars indicate standard deviation from three individual liposome preparations. For the
 94 description of the method, please check out the corresponding manuscript.

95

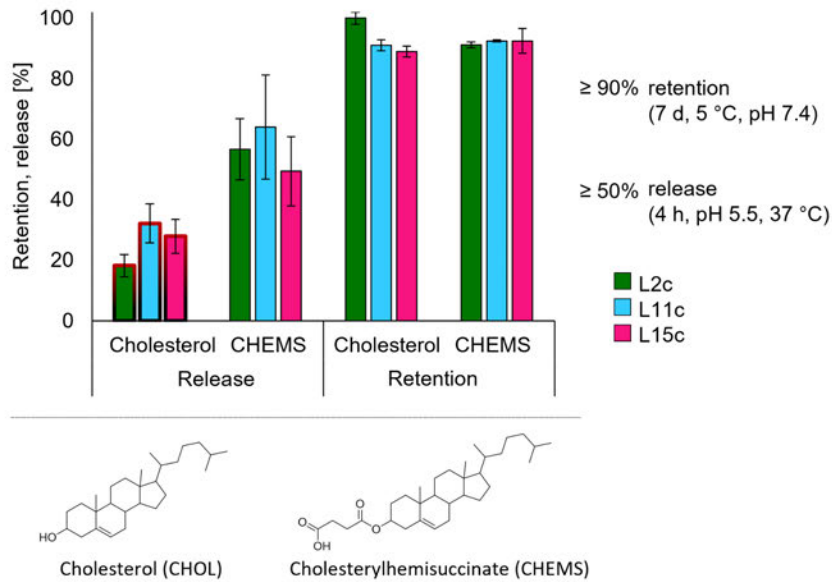


Fig. S6: Cargo release and retention for lead candidates either prepared with CHOL or CHEMS in the respective mol-% (n=3). Lead candidate L11c was prepared with 10 mol-%, L2c and L15c with 5 mol-% of CHOL or CHEMS, respectively. Error bars indicate standard deviation from three individual liposome preparations. Structure of CHOL and CHEMS are displayed. For the description of the method, please check out the corresponding manuscript.

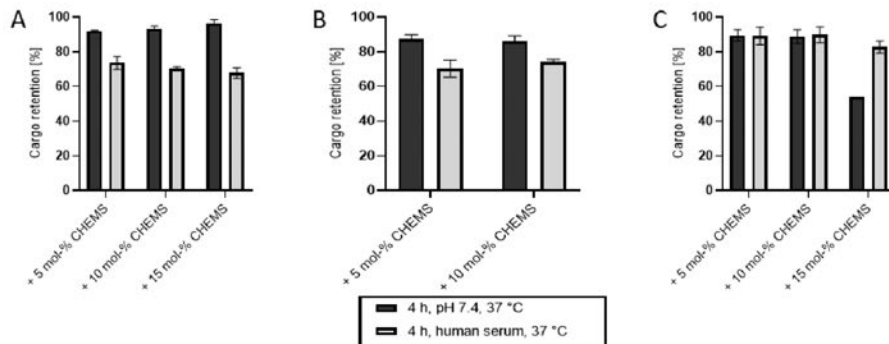
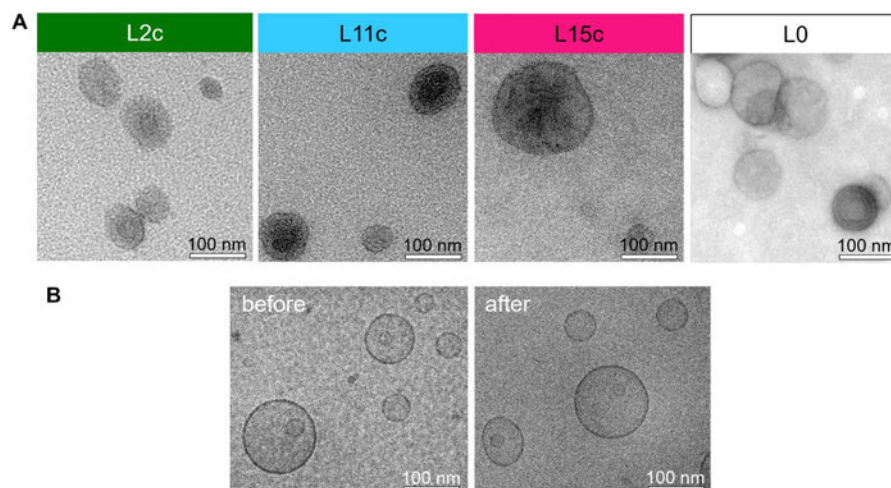


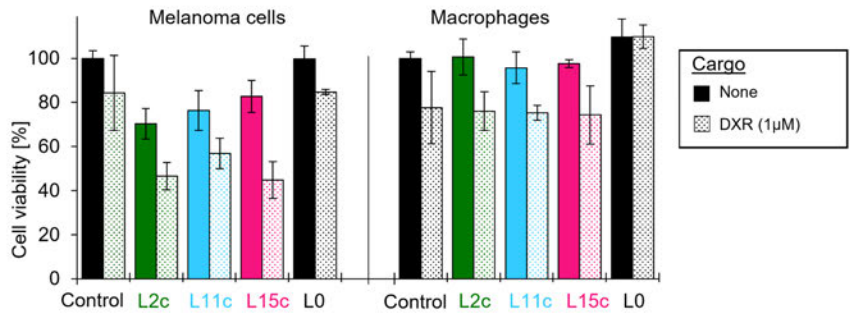
Fig. S7: Influence of human serum on sulforhodamine b cargo retention for lead candidate liposomes L2c (A), L11c (B) and L15c (C) with increasing CHEMS content (n=3). Retention was determined after incubation for 4 h at 37 °C in the presence or absence of full human serum. For the description of the method, please check out the corresponding manuscript.



110

111 **Fig S8: Transmission electron microscopy (TEM) pictures.** **A:** TEM pictures after trehalose-
 112 uranylacetate staining of final candidate liposomes L2c, L11c and L15c as well as control liposome L0.
 113 **B:** Cryo-TEM pictures of candidate L15c before and after incubation for 4 h at pH 5.5 at 37 °C.

114



115

116 Fig. S9: Resazurin assay to determine liposome toxicity after 24 h (n=3). Reduction of resazurin to
117 resorufin was used as an indicator for cell viability. UKRV-Mel-15a cells and human monocyte-derived
118 macrophages were incubated for 24 h with 1 µM free DXR as positive control, the conventional liposome
119 L0 and the three lead candidates L2c, L11c and L15c all containing DXR equal to 1 µM (dotted bars) or
120 no cargo as control group (plain bars). Results were normalized to untreated cells. Error bars indicate
121 standard deviation from three individual assays. For the description of the method, please check out the
122 corresponding manuscript.

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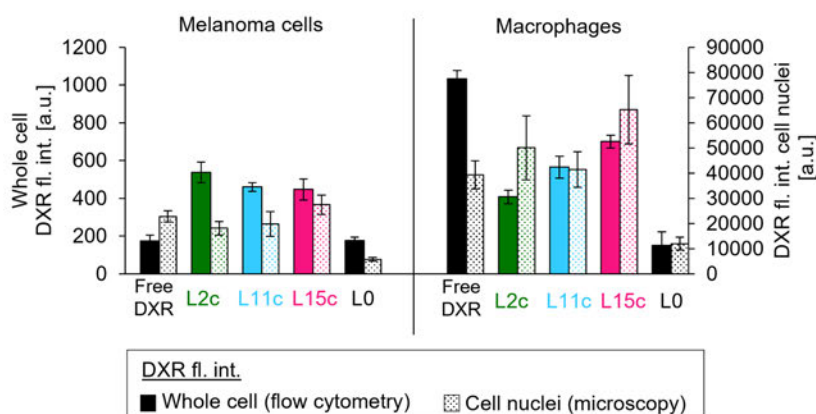
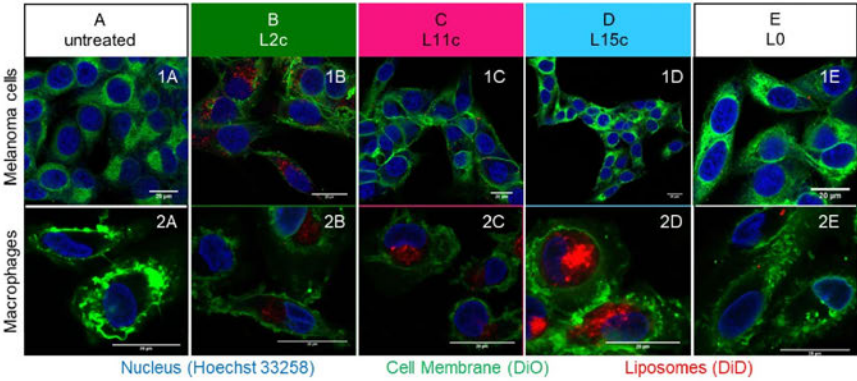


Fig. S10: Determination DXR fluorescence in whole cells by flow cytometry and in cell nuclei by confocal laser microscopy (n=3). Liposomal and free DXR fluorescence (dashed bars, left y-axis) in live cells was measured by flow cytometry. DXR fluorescence of nuclei after DXR release from liposomes was measured by confocal microscopy (dark grey bars, right y-axis). UKRV-Mel-15a cells (left) and human monocyte-derived macrophages (right) were incubated for 0.5 h with 1 μ M free DXR as positive control, the conventional liposome L0 and the three lead candidates L2c, L11c and L15c all containing DXR equal to 1 μ M. Cell nuclei were counterstained using Hoechst 33258. Error bars indicate standard deviation from three individual cell-experiments. For the description of the method, please check out the corresponding manuscript.



136
137 **Fig. S11: Confocal laser microscopy images** of plasma membranes and nuclei of UKRV-Mel-15a (row
138 1) and human monocyte-derived macrophages (row 2) stained with DiO (green) and Hoechst 33258
139 (blue), respectively. Cells were incubated for 4 h with three lead candidate liposomes L2c (**B**), L11c (**C**)
140 and L15c (**D**) and conventional liposome L0 (**E**) or with phosphate buffer as control condition (**A**), all
141 containing doxorubicin (not shown) equal to 1 μ M and fluorescent membrane label DiD (red). Scale bars
142 refer to 20 μ m. For the description of the method, please check out the corresponding manuscript.
143

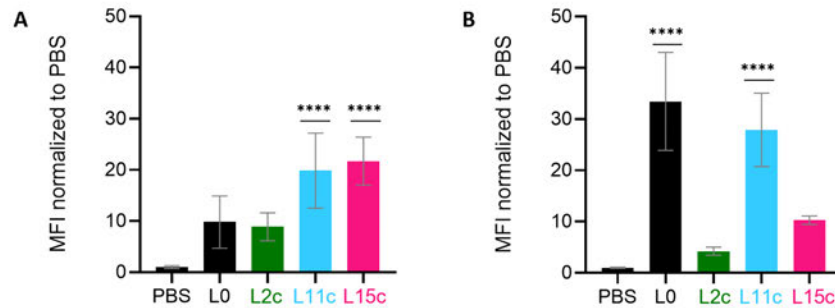


Fig. S12: Determination of uptaken DiR fluorescence in organ slices after tissue extraction. Measured fluorescence in liver (A) and spleen (B) by organs measured photometrically and normalized to PBS. Mice were treated with either L0, L2c, L11c, L15c (n=5) and PBS or DiR in PBS as control (n=3) 24 h before sacrificing. Each organ tissue was measured in triplicates. Significance was calculated comparing to phosphate buffer saline via ordinary one-way ANOVA with $r^2 = 0.8789$ (spleen) and $r^2 = 0.7768$ (liver) with a 95% confidence interval. P-values of ≤ 0.0001 are displayed with ****.

4.3 *In cellulo* delivery of potential DNMT2-inhibitors

Liposomal-Mediated Transfection System for Non-Cell-Permeable Small Molecules

Lukas Gleue^a, Jan Laurenz Meidner^a, Ganna Podoprygorina^a, Zarina Nidoieva^a, Andrea Tuettenberg^b, Tanja Schirmeister^a and Mark Helm^{a*}

* corresponding author, E-Mail: mhelm@uni-mainz.de

^a Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University, Staudingerweg 5, 55128 Mainz, Germany

^b Department of Dermatology, University Medical Center of the Johannes-Gutenberg University Mainz, Langenbeckstr. 1, 55131 Mainz, Germany

Abstract

This study investigated FTAD, a compound structurally similar to S-adenosyl-L-methionine, as a potential DNMT2 inhibitor. Although FTAD has demonstrated inhibitory activity against recombinant enzymes *in vitro*, its cellular effects and potential cross-inhibition of other methyltransferases remain undetermined. Previous *in cellulo* studies have been unsuccessful, and it remains unclear whether this is due to the inefficacy of FTAD or a lack of cellular uptake. To address these limitations, we developed a novel liposomal delivery system using LFT1, a liposome designed to mimic the lipid composition of naturally occurring exosomes.⁷ By encapsulating FTAD within LFT1, we aimed to exploit the fusogenic properties of the liposome to

facilitate cellular entry and to determine its biological effects. The efficiency of the encapsulation and cellular uptake process was assessed using FDP, a fluorescent phosphatase substrate, as a model compound. To evaluate the ability of the system to inhibit tRNA methylation levels, sinefungin, a pan inhibitor of methyltransferases, was used as a positive control. In this study, sinefungin as a liposomal formulation and as a free drug effectively reduced several levels of tRNA methylation in total tRNA. In contrast, FTAD, when administered at equivalent concentrations and conditions, had no detectable effect on tRNA methylation levels, indicating minimal or no inhibitory activity. These results highlight the potential of the LFT1 system as a delivery platform for *in cellulo* activity studies of non-cell permeable small molecules. Furthermore, the results demonstrated the efficacy of sinefungin in reducing multiple tRNA methylation levels in HEK293 cells.

1 Introduction

The early stages of drug development primarily focus on cell- and organ-free methods to assess the activity and affinity of potential enzyme inhibitors. Techniques such as isothermal titration calorimetry (ITC), microscale thermophoresis (MST), and enzyme activity assays are commonly employed to study pharmacokinetic properties like enthalpy, entropy, and affinity.^{256–258} However, translating these findings to *in cellulo* experiments often faces challenges due to poor cell permeability or aqueous solubility of small molecules.

To address these issues, pharmacological properties are typically optimised by masking polar groups as prodrugs. Modifications such as esters or amides can be cleaved enzymatically or chemically after cellular entry to form the active compound.²⁵⁹ However, this approach requires time-consuming synthetic modifications for each candidate, limiting its practicality in screening applications.

An alternative to structural optimisation involves using drug delivery systems that transport candidates across cell membranes and release them into the cytosol. Liposomes, first described in 1964, have emerged as one of the most promising lipid nanoparticle systems. By 2024, the U.S. Food and Drug Administration (FDA) had approved 15 liposomal and lipid particle based drugs.^{260,261} In accordance with their lipophilic character, small molecules are either encapsulated into the aqueous core or

enclosed within the phospholipid bilayer of the liposomes.^{121,262} This approach has demonstrated improved biodistribution, enhanced tissue uptake, and reduced side effects compared to non-liposomal applications in clinical practise.^{95,263}

Our approach utilised a lipid composition inspired by exosomes, the body's natural lipid nanoparticles, which was described by Lu *et al.* in 2018. Their lipid particles demonstrated three times higher delivery efficiency of siRNA than traditional lipid nanoparticles based on cholesterol and phosphocholine.⁷

We combined this liposome, named LFT1, with our dual centrifugation technique to produce up to 40 liposome batches per run under sterile conditions. This method generates lipid nanoparticles with consistent physicochemical properties without requiring an additional extrusion step, making it ideal for larger screening studies, as recently demonstrated for pH-sensitive liposomes.^{1-3,264}

This study investigated 5-FAM-triazolyl-adenosyl-diaminobutyric acid (FTAD), a potential methyltransferase inhibitor designed to mimic S-adenosylmethionine (SAM). *In vitro* experiments demonstrated the enzymatic binding and inhibitory effect of FTAD on DNA methyltransferase 2 (DNMT2), with a half inhibitory concentration (IC₅₀) of 26.7 µmol (data not shown).²⁴² DNMT2 is an enzyme responsible for the methylation of cytosine to 5-methylcytosine (m⁵C) at position 38 in human tRNA^{Asp(GUC)} (transfer RNA), tRNA^{Gly(GCC)} and tRNA^{Val(AAC)}.^{231,237,265} However, when tested in HEK293 cells, FTAD showed no effect on tRNA modification levels, possibly due to poor cell permeability or lack of inhibition of cellular methyltransferases. To overcome this challenge, we investigated the potential application of the fusogenic liposome LFT1 as a cellular delivery system for FTAD to facilitate activity studies.

2 Materials and Methods

Consumables

DOPC (#840035C-25mg), DOPE (#840035C-25mg), DOPS (#840035C-25mg) and sphingomyelin (#860061C-200mg) were obtained from Avanti Polar (Alabaster, Alabama, USA). Fluorescein diphosphate, tetraammonium salt (#11600) was purchased by Biomol (Hamburg, Germany). UNO Q1 column was obtained from (Bio-Rad, Hercules, California, USA). Sephacryl S500-HR slurry (#GE17-061301) was ordered from GE Healthcare (Chicago, USA). Ethanol (#5054.3) and isopropanol (#AE73.2) were ordered from Carl Roth (Karlsruhe, Germany). Acetonitril (#10001334) and black half-area 96-well plate (#10762394) were received from Fisher Scientific (Hampton, NH, USA). 2.2 mL centrifugation tubes twist-top (#3225) were obtained from Hettich GmbH (Tuttlingen, Germany). Folded capillary zeta cell (#DTS1070) and glass cells 12 mm (#PCS1115) were purchased by Malvern Panalytical (Malvern, UK). Sinefungin (HY-101938) was ordered from MedChemExpress (Monmouth Junction, NJ, USA). TC-platte 6-Well (#83.3920.005) was ordered from Sarstedt (Nümbrecht, Germany). Aammomium acetate (#73594-25G-F), benzonase (#E1014-25kU), bovine intestine phosphatase (#P0114-10kU), chloroform (#C2432-500ML), cholesterol (#C8667-25G), dynasore (#324410-10mg), magnesium chloride solution (#63069-100mL), nuclease P1 (#E1014-25kU), pentostatin (#SML0508-5MG), TRI-reagent (#T9424), triton x-100 (#X100) and 12-well plate (#CLS3513) were ordered from Sigma Aldrich (Burlington, MA, USA). SiLiBeads Typ ZY-S 0.3-0.4 mm was received Sigmund Lindner (Warmensteinach, Germany). DMEM (#31966021), FastAP (#EF0651), fetal bovine serum (#A5256801), glycogen (#R0551), penicillin-streptomycin (#15140122), phosphate buffered saline (#14190094), RIPA buffer (#89900), 1M tris pH 7.0 (#AM9850G), trypsin-EDTA (#25300054) and Viability Dye eFluor™ 780 (#65-0865-18) were purchased from Thermo Fisher (Waltham, MA, USA). Snake venom phosphodiesterase (#LS003926) was ordered from Worthington Biochemical (Lakewood, NJ, USA). RNA clean & concentrator-5 (#R1016) was received from Zymo Research (Orange, CA, USA).

Liposome formulation by dual centrifugation

For the preparation of liposomes by dual centrifugation, a total amount of 5 μ mol lipids (21 mol-% DOPC, 17.5 mol-% sphingomyelin, 14 mol-% DOPS, 30 mol-% cholesterol, 17.5 mol-% DOPE) dissolved in chloroform were collected in a 2.2 mL centrifuge tube according to their molar ratio.⁷ The solvent was removed under reduced pressure for 2 h in a SpeedVac (Eppendorf SE, Hamburg, Germany). Next, 9.3 μ L of 50 mM cargo dissolved in buffer, and 325 mg of 0.3-0.4 mm ceramic SiLiBeads®ZY were added and homogenised by dual centrifugation for 20 min. Then 77.2 μ L of phosphate buffer (PBS) was added, the sample was mixed for 4 min and the liposomal suspension was isolated. Lastly, 77.2 μ L of phosphate buffer was added and the sample was mixed again for 4 min.

Liposome purification and quantification of cargo encapsulation

To separate the non-encapsulated Cargo, the liposome suspension was centrifuged at 145,000 $\times$ g for 75 min at 4 °C (Optima + 60 TI fixed angle centrifuge rotor, Beckman Coulter, Brea, CA, USA). The supernatant was replaced with phosphate buffer and the liposome pellet was carefully resuspended by pipetting. For quantification, the sinefungin content in the supernatant was determined by high-performance liquid chromatography (HPLC) (Waters Corporation, MA, USA) and then subtracted from the total amount of used inhibitor.

FTAD and fluorescein diphosphate (FDP) concentrations were quantified by addition of 0.1 vol-% triton X-100 to the purified sample to disrupt the nanoparticles and the fluorescence intensity was then measured in a plate reader (Spark, Männedorf, Switzerland) at 488/525 nm (excitation and emission). External calibration was used to calculate concentrations for all three cargoes.

Dynamic light scattering

The physicochemical properties of the particles were characterised by dynamic light scattering using a Malvern Zetasizer Nano Series (Malvern Panalytical, Malvern, UK). Particle size and size distribution (PdI) of diluted liposomes (1:1000, v:v) were measured in a 12 mm glass cell at 25 °C after a 30 s equilibration time and the zeta potential was analysed in a folded capillary zeta cell under the same conditions. The

water's viscosity was adjusted to 0.8872 cP, with a refractive index of 1.33 and a scattering angle of 173°. Additionally, the system automatically selected the appropriate attenuation index and voltage settings. All samples were measured in technical triplicates.

Size-exclusion chromatography (SEC) coupled high-performance liquid chromatography (HPLC)

15 µL of the cleaned or uncleaned loaded LFT1 (57 µmol / mL) was separated using a UNO Q1 column packed with S500-HR size exclusion chromatography resin. The absorbance was measured at 230 nm by HPLC by a flow rate of 0.5 mL/min phosphate buffer.

Cell cultivation

HEK293 cells (DSMZ, Germany) were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum and 10 U/mL penicillin-streptomycin at 37 °C and 5% CO₂. To detach the cells, they were first washed with phosphate buffer and then treated with trypsin-EDTA.

Flow cytometry

HEK293 cells were seeded in 24-well plates at a density of 2x10⁵ cells per well with 1 mL culture medium. The experiment was performed in triplicate for each experimental condition. To inhibit particle uptake, the cell culture medium was additionally supplemented with 80 µM dynasore 30 min before experimental start. After the incubation period, the medium was carefully removed, the cells were washed with phosphate buffer and then detached using trypsin-EDTA. For differentiation between viable and non-viable cells, the viability dye eFluo 780 was used and then the cells were fixed overnight in 1% paraformaldehyde-PBS solution. Flow cytometric analysis was performed using a BD Biosciences LSRII cytometer (BD Biosciences, Heidelberg, Germany) and data were subsequently processed using flow cytometry platform (Beckman Coulter, Brea, Ca, USA).²⁶⁶

FDP extraction and digestion

200k HEK293 cells in 1 ml DMEM per well were seeded into 24-well plates the day before. The cells were then incubated with LFT1-FDP or free FDP for 21 h. The following steps must be carried out very carefully to prevent the loss of cells. After incubation, the medium was removed, the cells were washed three times with PBS and 400 μ L of RIPA buffer was added per well. To prevent evaporation, the plate was sealed with foil and agitated at 75 °C for 45 min to lyse the cells and deactivate the phosphatases. The plate was then centrifuged at 400 x g for 5 min before the foil was removed and the cell lysates were transferred to 1.5 mL reaction tubes. The tubes were centrifuged at 15,000 x g for 15 min to pellet the cell fragments. 50 μ L of each sample was then transferred to a black half-microwell plate and the fluorescence intensity at (ex. 488 nm / em. 525 nm) was quantified using a plate reader (Spark, Männedorf, Switzerland). For the analysis of uncleaved FDP, each well was then supplemented with 1000 U FastAP, a commercially available phosphatase, incubated for 30 min at 37 °C and the fluorescence intensities were measured at (ex. 488 nm / em. 525 nm) again.

tRNA-sample preparation

500,000 HEK293 cells were resuspended in 1 mL of DMEM and seeded in 12-well plates one day prior to the start of the incubation. On the experimental day, the medium was replaced with a fresh medium and liposome suspension or free cargo was added. After a period of 48 h, the medium was replaced, the cells were washed once with phosphate-buffered saline and then detached from the surface by trypsin-EDTA. The cell suspensions were then transferred to 6-well plates containing fresh DMEM and allowed to grow for a further two days before harvesting. For RNA extraction, the detached cells were added to DMEM and centrifuged at 400 x g for 5 min. The supernatant was discarded, and the pellet was washed once with phosphate buffer. The cell pellet was then resuspended in 1 mL TRI-reagent and stored at -20 °C.

Total RNA was extracted from harvested cell pellets using TRI reagent. The cell pellets were resuspended in TRI reagent according to the manufacturer's protocol and mixed thoroughly. Chloroform (half the volume of TRI reagent used) was added, mixed and the samples were centrifuged at 15,000 g for 15 min at 4 °C. The aqueous phase was

carefully transferred to a new tube and RNA was precipitated by adding isopropanol (half the volume of TRI reagent used) and 1 μ L of glycogen.

The mixture was centrifuged again at 15,000 g for 15 min at 4 °C and the supernatant was discarded. The RNA pellet was washed with 500 μ L of 70% ethanol and centrifuged at 15,000 g for 15 min at 4 °C. After removal of the supernatant, the RNA pellet was briefly air dried, resuspended in RNase-free water and the RNA concentration was determined by measuring absorbance using a NanoDrop One (Thermo Fisher, Waltham, MA, USA). Zymo Research's RNA Clean and Concentrator-5 kit was used to isolate tRNA from total RNA according to the manufacturer's protocol for small RNAs and then stored at -20 °C.

Quantification of RNA modification levels by LC-MS

100 μ g tRNA samples were enzymatically digested to nucleosides using a mixture of enzymes in a buffered solution. The digestion was performed at 37 °C for 2 h using nuclease P1, snake venom phosphodiesterase, bovine intestine phosphatase, benzonase and pentostatin in a tris-magnesium chloride buffer. After digestion, the samples were spiked with ^{13}C -labelled nucleosides from *S. cerevisiae* as internal standards. Liquid chromatography–mass spectrometry (LC-MS) analysis was conducted using an Agilent 1260 series liquid chromatograph coupled to an Agilent 6460 Triple Quadrupole mass spectrometer with an electrospray ionisation source (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved using a Synergi Fusion column (250 $\times$ 2.0 mm, 4 μ m particle size, 80 Å pore size) (Phenomenex Inc., Torrance, CA, USA) with a gradient elution of ammonium acetate buffer (pH 5.3) and acetonitrile. The flow rate was set at 0.35 mL/min, with the gradient increasing from 0% to 40% acetonitrile over 20 min. Mass spectrometry was performed in positive ion mode using dynamic multiple reaction monitoring. Quantification was accomplished through a combination of external and internal calibration methods.²⁶⁷

Statistical analysis

Statistical significance was assessed using biological triplicates for all conditions except 4 h 10 μ M LFT1-FDP with dynasore in Figure 4.2 (duplicate). The statistical analysis was evaluated using an ordinary two-way ANOVA with Tukey's multiple-comparisons test for comparisons between multiple measurement groups. In the case of comparisons between two conditions, Welch's t-test was used. p-values of ≥ 0.05 were considered non-significant (ns); $p \leq 0.05$ is indicated with *; $p \leq 0.01$ is marked with **; $p \leq 0.001$ is shown with *** and $p \leq 0.0001$ is displayed with ****. The analyses were conducted using GraphPad Prism 9 (GraphPad Software, Boston, MA, USA).

3 Results and Discussion

Characterisation of LFT1 and influence of the purification process

The exosome-mimicking liposome LFT1 was evaluated as a transfection reagent for small molecules using three model drugs: fluorescein diphosphate (FDP), FTAD, and sinefungin (SFG). To ensure reproducibility, the impact of drug loading on size, size distribution, and zeta potential was assessed. Dynamic light scattering experiments revealed homogeneous particle populations, indicating maintained particle stability, no aggregate formation, and absence of diverse size populations (**Figure 4.1 A, B and C**). The purification of LFT1, by centrifugation showed no effects on sample homogeneity or increased aggregation (**Figure 4.1 D, E and F**). Consistent physicochemical properties were observed, with average particle sizes ranging from 125-150 nm and PDIs of 0.15 (**Figure 4.1 G and H**). Nevertheless, variations of the zeta potential among encapsulated cargos indicate the small molecules' impact on LFT1's electrostatic properties (**Figure 4.1 I**). This analysis demonstrates LFT1's potential as a versatile and stable transfection reagent for various small molecules, with minimal impact on its key physicochemical properties.

The purification process was validated using size exclusion chromatography (SEC) coupled to HPLC, which separated non-encapsulated cargo from the liposomal fraction. Results showed no detectable free cargo in purified LFT1-FDP and LFT1-SFG samples (**Figure 4.1 J and L**). However, small amounts of free FTAD remained in LFT1-FTAD samples after centrifugation (**Figure 4.1 K**).

These findings suggest that purification efficiency depends on the properties of the incorporated small molecule. The varying results across different cargos highlight the importance of continuous quality control to ensure comparability of experimental data. This cargo-dependant efficacy of the purification process underscores the need for ongoing monitoring such as high performance liquid chromatography (HPLC) and dynamic light scattering (DLS) when working with different small molecules in liposomal formulations, ensuring consistent and reliable results.

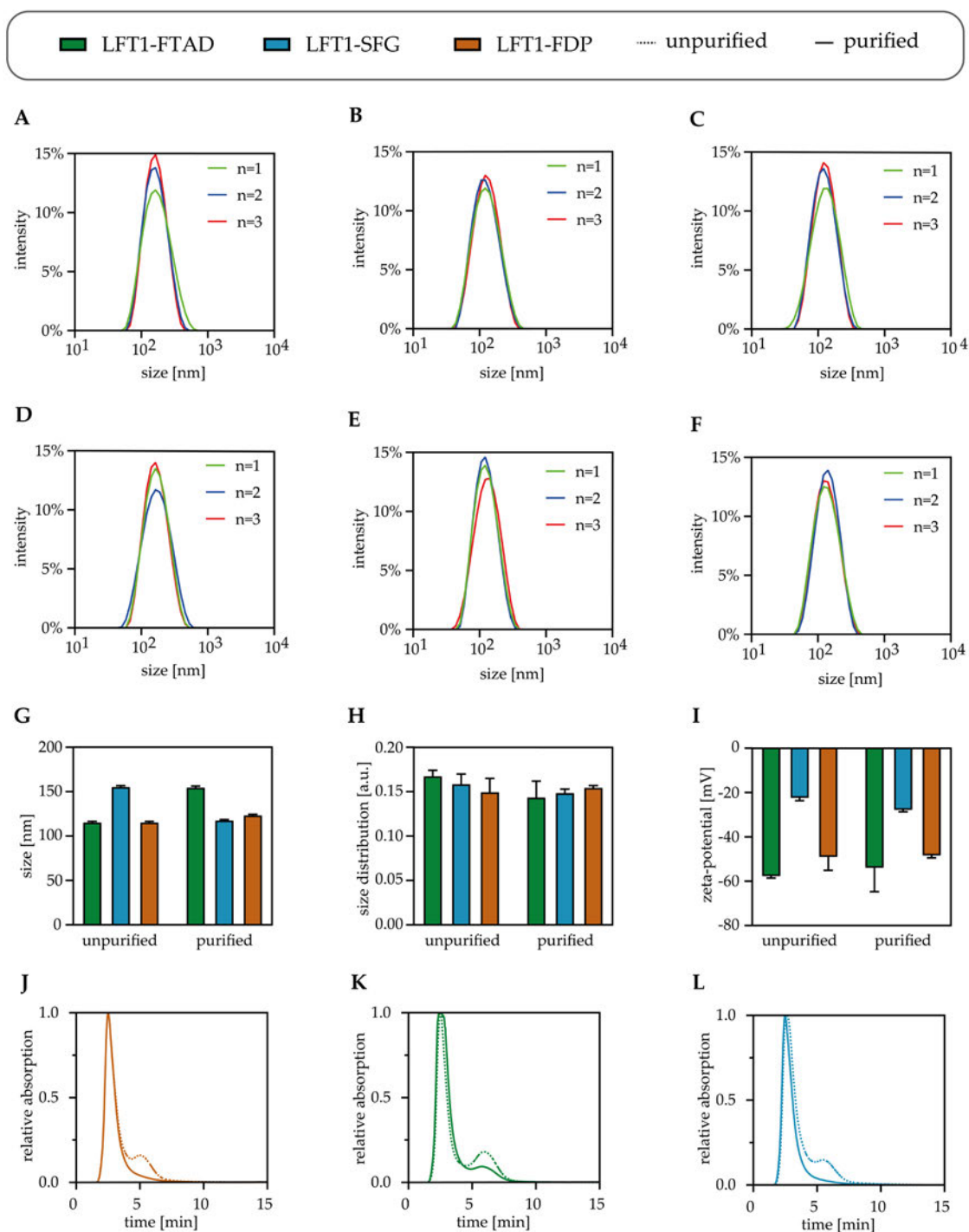


Figure 4.1: Physicochemical characterisation by dynamic light scattering of loaded liposomes before and after purification in triplicate. Size distribution in unpurified liposome samples of **A:** LFT1-FTAD, **B:** LFT-SFG and **C:** LFT-FTAD as well as for purified **D:** LFT1-FTAD, **E:** LFT1-SFG and **F:** LFT1-FDP. **G:** Average particle sizes, **H:** size distributions and **I:** zeta potentials of loaded liposomes. Chromatogram of unpurified and purified liposome separated by SEC-HPLC of **J:** LFT1-FDP, **K:** LFT1-FTAD and **L:** LFT1-SFG.

Liposomal delivery of phosphatase substrate

To demonstrate biological efficacy, liposomes must release their cargo intracellularly, enabling interaction with target enzymes. LFT1 was loaded with fluorescein diphosphate (FDP), a derivative of the fluorescent dye fluorescein whose two hydroxyl groups have been esterified with phosphate groups to avoid fluorescence. If LFT1 is capable of cargo release, fluorescein would be formed after enzymatic cleavage of the phosphate groups by the cell's own phosphatases (**Figure 4.2 E**).

HEK293 cells were incubated with either LFT1-FDP or free FDP and the uptake rate was measured at the single cell level by flow cytometry. In addition, the experiment was repeated in the presence of 80 μ M dynasore to improve understanding of the uptake process. Dynasore is an inhibitor of clathrin-coated pit-mediated endocytosis and fast endophilin-mediated endocytosis, which are the major uptake pathways in HEK293 cells for nanoparticles <200 nm as reported in the literature.²⁶⁸

The uptake of the particles was studied at cargo concentrations between 0.1 μ M and 10 μ M and at incubation times of 4 h, 24 h and 48 h. After 4 h of incubation (**Figure 4.2 A**), the normalised single cell fluorescence of HEK293 cells treated with 10 μ M LFT1-FDP was up to three times higher than in liposome free conditions. Lower concentrations of FDP and co-incubation with dynasore had no effect on the fluorescence intensity compared to untreated cells. The fluorescence intensity of cells incubated with liposome-encapsulated phosphatase substrate was markedly enhanced at the highest condition, up to 2.5-fold when the treatment time was extended to 24 h (**Figure 4.2 B**). In addition, the longer incubation time resulted in a six-fold increase in the fluorescence intensity of LFT1-FDP treatment compared to free FDP, depending on the concentration used. It is notable that the treatment with FDP alone had no measurable effect on the fluorescence intensity. The addition of dynasore during the incubation period resulted in a reduction in liposome uptake by 30%. Furthermore, the doubling of the incubation period to 48 h did not increase the delivery efficacy compared to the 24 h period (**Figure 4.2 C**).

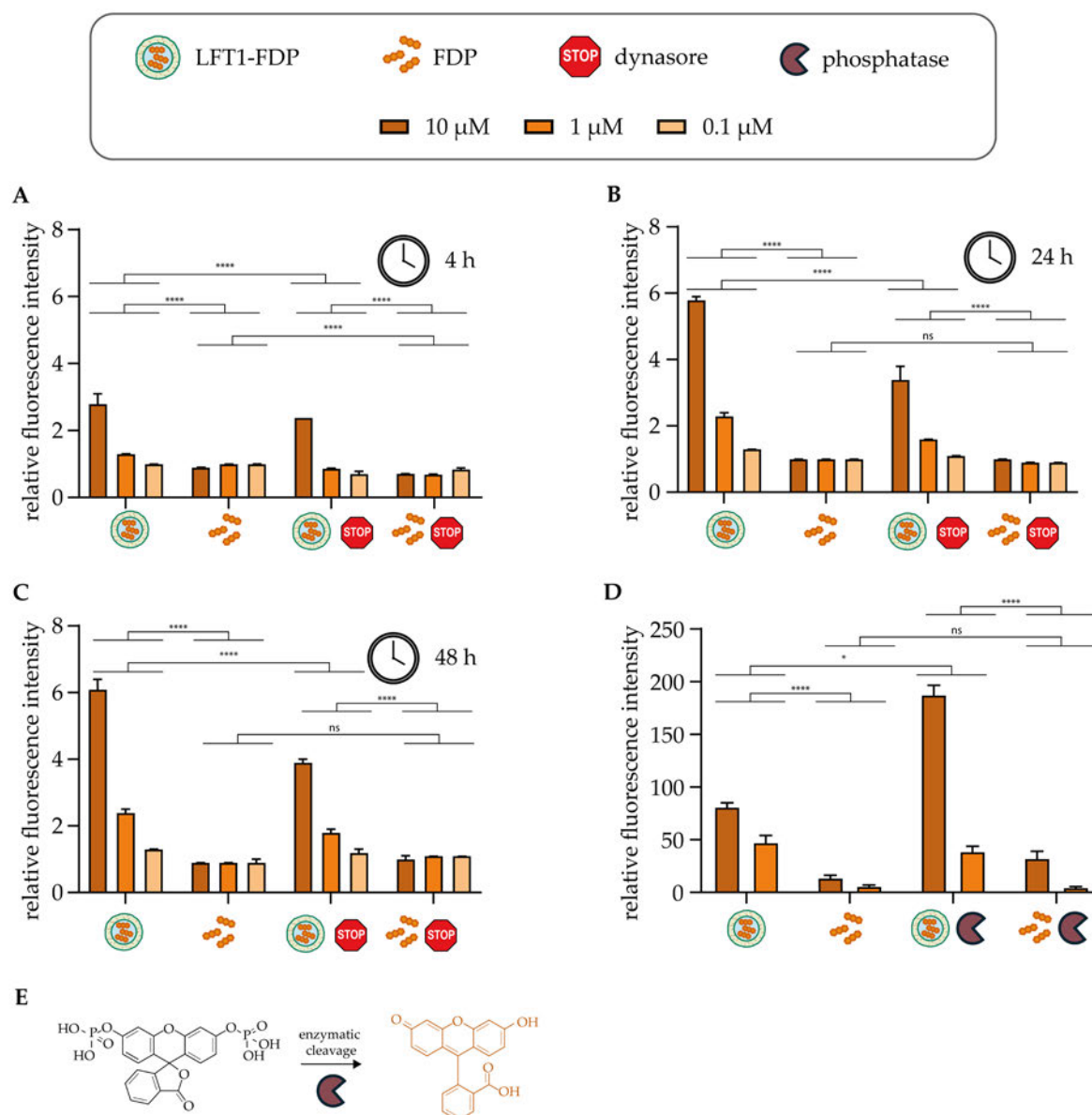


Figure 4.2: Transfection of phosphatase substrate. HEK293 cells were incubated with LFT1-FDP in the absence or presence of the uptake inhibitor dynasore. Delivery efficiency was measured in triplicate (4 h 10 μ M LFT1-FDP with dynasore in duplicate) by flow cytometry. **A:** 4 h, **B:** 24 h and **C:** 48 h incubation time. **D:** LFT1-FDP treated cells were lysed after 21 h incubation and fluorescence was measured. The lysate was then treated with phosphatase and fluorescence was quantified again. **E:** Reaction scheme of the enzymatic cleavage of FDP. Statistical analysis was performed using an ordinary two-way ANOVA with Tukey's multiple-comparisons test. p-values of ≥ 0.05 are not significant (ns); $p \leq 0.05$ is indicated with *; $p \leq 0.01$ is marked with **; $p \leq 0.001$ is shown with *** and $p \leq 0.0001$ is displayed with ****.

Comparison of LFT1-FDP with free FDP showed that the phosphatase substrate was only able to enter the cells when it was combined with liposomes. There was also a correlation between the incubation dose and the particle uptake. Time-dependant changes in relative fluorescence indicated that an optimal incubation time is between 4 h and 24 h. Shorter incubation times resulted in inefficient uptake and release of LFT1-FDP, whereas longer incubation times provided no additional benefit. The fluorescence level was also reduced by the endocytosis inhibitor dynasore, which indicates a reduced uptake of the liposomes. However, further biological processes seem to be involved in the uptake of the nanoparticles, since only a reduction was achieved.

The cellular release rate of liposome-delivered FDP was also evaluated through the extraction of incubated HEK293 cells combined with the addition of phosphatase, which was used to cleave undigested cargo (**Figure 4.2 D**). To enable a comparison between the pre- and post-addition of phosphatase conditions, the fluorescence intensity of the lysate was quantified as a number corresponding to the cargo release. To prevent cleavage of the FDP during lysis, the extraction was conducted at 75 °C to inactivate cellular phosphatases.²⁶⁹

The comparison between LFT1-FDP and free FDP in the absence of phosphatases showed a preferential uptake of the small molecule encapsulated in liposomes. Comparing 10 µM with 1 µM LFT1-FDP resulted in minor changes in fluorescence intensity compared to flow cytometry data (**Figure 4.2 B**). After treatment with phosphatase, the relative fluorescence intensity in all samples, except for 1 µM LFT1-FDP, increased based on previously uncleaved FDP. This suggests limitation of cellular phosphatase, as the ten-fold increase in nanoparticle dosage compared to 1 µM condition may have exceeded the cells' enzymatic capacity to process the cargo. Overall, the results confirmed the successful encapsulation of all three compounds and demonstrated effective cellular delivery of FDP when encapsulated in LFT1. However, the fluorescence changes were minimal compared to controls, suggesting that FDP may not be an ideal cargo for this delivery system.

Delivery of FTAD, a fluorescein linked small molecule

5-FAM-triazolyl-adenosyl-diaminobutyric acid (**Figure 4.3 D**), known as FTAD, is a compound that exhibits a structural resemblance to the methyl donor *S*-adenosyl-*L*-methionine. In a previous study, FTAD demonstrated an inhibitory effect on DNMT2 *in vitro*, an enzyme responsible for the methylation of cytosine to 5-methylcytosine in various tRNA species. Due to its fluorescent properties, the delivery efficiency of FTAD can be quantified using flow cytometry.²⁵⁸

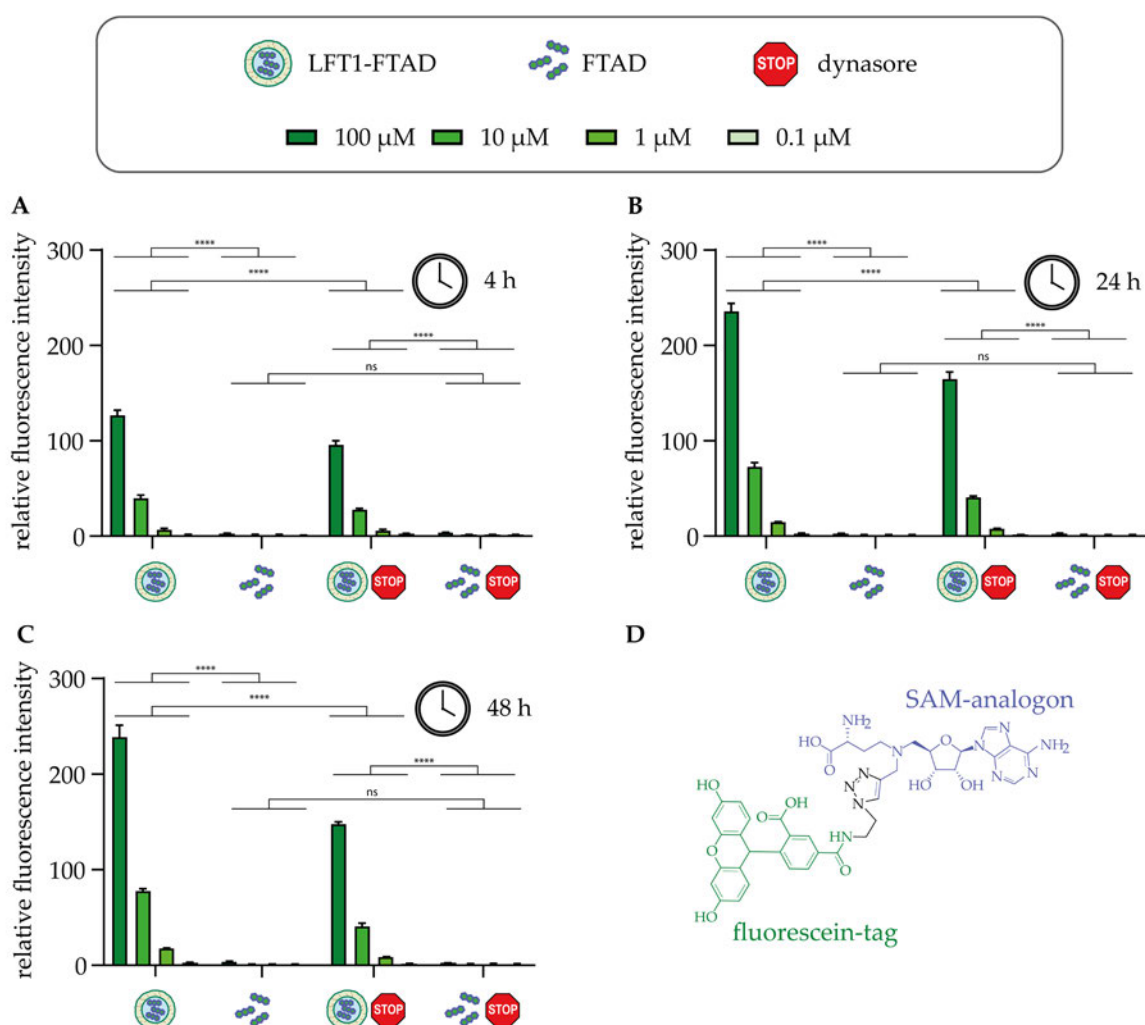


Figure 4.3: Transfection of the fluorescein-conjugated FTAD with potential inhibitory effect on DNMT2. HEK293 cells were treated with liposome-encapsulated FTAD in the absence or presence of the uptake inhibitor dynasore. Single cell delivery efficiency was quantified by flow cytometric fluorescence measurement. The relative fluorescence intensity of triplicates was normalised to untreated cells treated for **A:** 4 h, **B:** 24 h and **C:** 48 h. **D:** Chemical structure of FTAD. Statistical analysis was performed using an ordinary two-way ANOVA with Tukey's multiple-comparisons test. p -values of ≥ 0.05 are not significant (ns); $p \leq 0.05$ is indicated with *; $p \leq 0.01$ is marked with **; $p \leq 0.001$ is shown with *** and $p \leq 0.0001$ is displayed with ****.

HEK293 cells were incubated with either FTAD encapsulated in LFT1 or free cargo at different concentrations and incubation periods ranging from 4 h to 48 h. The uptake was measured at the single-cell level using flow cytometry. Furthermore, dynasore's inhibitory effect on endocytosis of LFT1 was investigated. After the shortest incubation time of 4 h, the normalised fluorescence intensities of the liposome-treated cells increased strongly, especially for conditions with 10 μ M and 100 μ M cargo with relative increases of 127-fold and 97-fold, whereas free FTAD had no effect on the fluorescence intensity of the cells (**Figure 4.3 A**).

By extending the incubation time to 24 h, the efficiency of liposomal FTAD administration to the cells doubled, while the free dye again showed no effect (**Figure 4.3 B**). In contrast, longer incubation times had no effect on particle uptake (**Figure 4.3 C**). In addition, free FTAD was not taken up by cells. The co-incubation of HEK293 cells with dynasore reduced liposome uptake, independent from the incubation time, which was indicated by lower cellular fluorescence intensities compared to the conditions without inhibitor and is in line with the LFT1-FDP finding.

These observations confirmed those from FDP treatment (**Figure 4.2**) concerning incubation time, dose dependence as well as uptake inhibition and demonstrated the potential of LFT1 as a transfection reagent for non-cell permeable small molecules.

Analysis of tRNA methylation levels in treated HEK293 cells

Previous experiments have demonstrated LFT1's capability to transport FTAD and FDP intracellularly. This shuttle system was employed to investigate FTAD's potential inhibitory effects. Given FTAD's structural similarity to the methyl donor SAM, it could potentially inhibit various methyltransferases. Consequently, a wide range of methylations on the four main nucleosides of total tRNA was examined using LC-MS to detect changes in total tRNA modification frequency. To provide a benchmark for comparison and to assess the efficacy of FTAD, the broad spectrum methyltransferase inhibitor sinefungin was also tested as a load for LFT1 (**Figure 4.4 I**).²³⁵

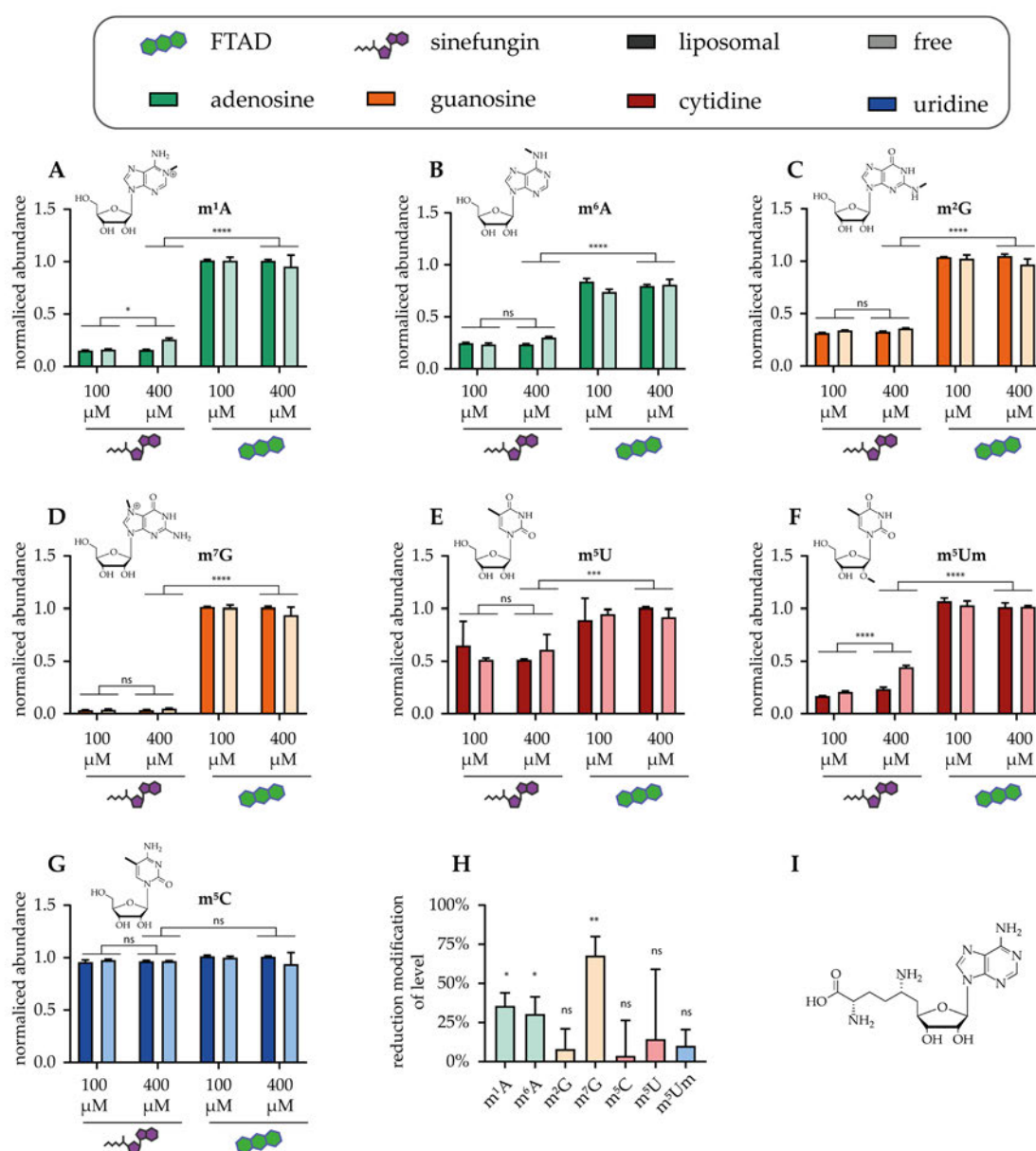


Figure 4.4: Changes in the modification pattern of HEK293 cells treated with liposome-encapsulated FTAD or the methyltransferase inhibitor sinefungin at dosages of 100 μM and 400 μM for 48 h. The abundance of modifications was normalised to untreated cells and each condition was performed in triplicate. The effect of the encapsulated inhibitors was compared with that of free FTAD and SFG. **A:** m¹A, **B:** m⁶A, **C:** m²G, **D:** m⁷G, **E:** m⁵U, **F:** m⁵Um and **G:** m⁵C. **H:** Effect of 48 h incubation with sinefungin and additional 48 h cultivation in DMEM (without sinefungin) on tRNA modification levels. The reduction was normalised to cells incubated for 48 h in the presence of sinefungin. **I:** Chemical structure of sinefungin. The statistical analyses of **A-G** were performed using an ordinary two-way ANOVA with Tukey's multiple-comparisons test whereas the Welch's t-test was used for **H**. For both tests p-values of ≥ 0.05 are not significant (ns); $p \leq 0.05$ is indicated with *; $p \leq 0.01$ is marked with **; $p \leq 0.001$ is shown with *** and $p \leq 0.0001$ is displayed with ****.

Due to the half-life of tRNA in growing cells of 60 h, an additional 48 h cultivation phase (without inhibitor) was applied after incubation and before tRNA extraction to obtain a stronger change of tRNA methylations.²⁷⁰ Using this protocol, the level of all modifications in HEK293 cells decreased after sinefungin treatment compared to 48 h incubation alone, especially the levels of m¹A, m⁶A and m⁷G (**Figure 4.4 H**).

To evaluate the efficiency of liposomal delivery, HEK293 cells were incubated with either LFT1-FTAD or LFT1-SFG at concentrations of 100 µM and 400 µM, respectively. The effect was compared to the free inhibitor at the same concentrations. The concentrations used were substantially higher than the IC₅₀ values determined *in vitro*. Specifically, concentrations up to 15.0-fold higher than the IC₅₀ for FTAD (IC₅₀ = 26.7 µmol, data not shown) and up to 30.3-fold higher than the IC₅₀ for SFG (IC₅₀ = 13.2 µmol) were used to maximise the potential for observing any biological effects.²⁵⁸

Treatment with either liposomal or free sinefungin resulted in a uniform reduction in six of the 22 methylation sites in total tRNA, irrespective of the incubation dosage (**Figure 4.4 A-G**). Moreover, the inhibitory effect of liposomal sinefungin was found to be comparable to that of the free cargo. This contrasts with the treatment with FTAD, which was not inhibitory, regardless of whether FTAD was encapsulated in liposomes or administered as a free drug.

The comparable inhibitory effect of liposomal and free sinefungin (which is taken up by the cells themselves) suggests an intracellular release of LFT1-SFG, otherwise no or minor effect of LFT1-SFG treatment on total tRNA methylation sites would be expected. Consequently, the constant modification levels in total tRNA after FTAD treatment indicate a lack of inhibitory activity on tRNA methyltransferases in HEK293 cells under conditions comparable to those of sinefungin. However, it is important to note that during incubation in the medium, some of the particles could become leaky, e.g., due to interaction with protein and released the encapsulated sinefungin and FTAD, making interpretation of the data difficult.

4 Conclusion

This study demonstrates the application of LFT1, an exosome-inspired liposomal delivery system, for transporting small molecules into HEK293 cells. The research focused on screening the biological activity of FTAD, an early-stage methyltransferase inhibitor.

The physicochemical analysis of LFT1 loaded with three different small molecules revealed that cargo nature minimally affected particle size and distribution, while zeta potential varied with molecular polarity. Also, the ultracentrifugation-based purification did not alter the liposomal physicochemical properties. Verification by SEC-HPLC showed complete separation of free SFG and FDP, but incomplete separation of FTAD, suggesting that purification efficiency depends on the loaded molecular properties. This indicates the need for quality control, including the potential influence of the cargo on the physicochemical properties of the particles, which may affect cellular uptake.²⁷¹

The delivery properties of LFT1 were initially demonstrated using the phosphatase substrate FDP, which becomes fluorescent only upon successful release and enzymatic cleavage. HEK293 cells incubated with LFT1-FDP exhibited a significant increase in fluorescence intensity compared to the control group treated with free FDP. This observation strongly indicated successful cellular delivery mediated by the liposomes. The endocytosis inhibitor dynasore partially reduced the liposome uptake, but its effect was less pronounced than achieved by Lu *et al.* in other cell lines.⁷ This suggests the involvement of additional cellular internalisation pathways in HEK293 that are not susceptible to dynasore inhibition.

Experimental results revealed a clear correlation between fluorescence intensity and both incubation time and concentration. The optimal incubation period for maximum delivery efficiency was determined to be between 4 h and 24 h. To validate the flow cytometry results, treated cells were lysed, and the fluorescence of the lysate was quantified. This analysis corroborated the initial findings, showing enhanced FDP uptake by liposomes after incubation.

Further investigation involved the addition of exogenous phosphatase to the cell lysate. This step revealed the presence of non-cleaved substrate in samples containing 10 μ M LFT1-FDP and 10 μ M free FDP. The finding of LFT1-FDP suggests either an incomplete release of FDP from the liposomes post-uptake or insufficient intracellular

phosphatase activity to fully cleave the internalised substrate. The observed increase after 10 μ M FDP treatment may be attributed to minimal cellular uptake without subsequent enzymatic cleavage, or to residual substrate adhering to the cell culture surfaces.

The efficacy of LFT1 in delivering FDP was further corroborated by subsequent experiments using FTAD as cargo. LFT1-FTAD demonstrated high time- and concentration-dependant uptake, whereas free FTAD showed no cellular internalisation.

Looking at the methylation levels of tRNAs by LC-MS analysis after 48 h incubation with LFT1-FTAD and FTAD, both treatments did not affect the methylation levels in tRNAs, whereas SFG and LFT1-SFG reduced six modification sites in total tRNA regardless of the concentration. The comparable inhibitory effects of LFT1-SFG and free SFG suggest successful intracellular delivery and release of the encapsulated inhibitor. We believe that this study represents the first reported inhibitory effects of sinefungin on methyltransferases in HEK293 cells in the absence of liposomal delivery systems. However, it is crucial to consider that during incubation, some liposomes may have released their cargo prematurely due to interactions with media components, potentially complicating data interpretation.

In summary, our experimental data demonstrated the effectiveness of LFT1 as a drug delivery system for investigating the biological activity of small molecules with poor membrane permeability, particularly in early drug development. Using this liposomal transfection system, the biological activity of FTAD was analysed *in cellulo* which was not possible before. However, to confirm the intracellular release of the cargo, further research using fluorescence microscopy is essential to precisely localise the released substances within cellular compartments.

Acknowledgements

This work was supported by project B14N of the german research foundation DFG CRC1066 (project number 213555243) to MH, LG, AT and by TP A01 and TP C03 of the german research foundation DFG TRR-319 (project number 439669440) LM, LG, TS, MH.

Conflict of Interests

Mark Helm is a consultant for Moderna Inc. Andrea Tuettenberg is the CEO of ActiTrex GmbH. The other authors report no competing interest.

Author Contributions statement

Mark Helm: Conceptualisation, methodology, resources, funding acquisition. **Andrea Tuettenberg:** Resources, funding acquisition. **Tanja Schirmeister:** Resources, funding acquisition, conceptualisation. **Lukas Gleue:** Methodology, conceptualisation, validation, data curation, visualization, formal analysis, investigation, writing - original draft. **Laurenz Meidner:** Methodology, validation, chemical synthesis. **Ganna Podoprygorina:** Validation, formal analysis. **Zarina Nidoieva:** Validation, formal analysis.

4.4 Contributing authors

4.4.1 Quantification of tRNA modifications by cDNA hybridisation and next generation sequencing

tRNA plays a central role in protein biosynthesis by acting as the transport system for amino acids, enabling the translational process from mRNA transcript to amino acid chain.²⁷² The rate of translation is largely dependent on the availability of different tRNA species, highlighting their critical importance in this context. Moreover, variations in the abundance of these tRNA species have been linked to the onset of various diseases, including cancer, highlighting the need for advanced diagnostic tools.²⁷³ Consequently, the development of rapid and sensitive methods for quantifying tRNA species is crucial both for understanding these dynamics and for effectively addressing related health issues.²⁷⁴

For this reason, Marco Kristen and Marc Lander from our work group have developed a novel tRNA quantification technique capable of detecting femtomolar concentrations. The method integrates complementary DNA (cDNA) hybridisation with next generation sequencing technology. Marco Kristen and Marc Lander designed complementary oligonucleotides that specifically bind to individual tRNA species. These oligos were introduced into samples for hybridisation, followed by isolation and purification of tRNA-cDNA hybrids by gel excision. After amplification, the purified cDNA oligos were finally quantified by next generation sequencing in collaboration with Virgine Marchand and Yuri Motorin from the University of Lorraine. The composition of the purified cDNA oligos corresponded to the tRNA composition in the samples.²⁷⁴

I joined the project during the method validation phase, using my expertise in eukaryotic cell culture techniques gained from previous liposome characterisation studies *in cellulo*. My main contribution was to grow and maintain a variety of cell lines for Marco Kristen. These cultures were then prepared for RNA extraction. Additional samples for this publication were provided by Lea-Marie Kilz and Marko Jörg, both members of the institute. Mark Helm and Kristina Friedland served as principal investigators for the research project.²⁷⁴

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DORQ-seq: high-throughput quantification of femtomol tRNA pools by combination of cDNA hybridization and Deep sequencing

Marco Kristen¹, Marc Lander¹, Lea-Marie Kilz¹, Lukas Gleue¹, Marko Jörg¹, Damien Bregeon², Djemel Hamdane³, Virginie Marchand⁴, Yuri Motorin^{4,5}, Kristina Friedland¹ and Mark Helm^{1,*}

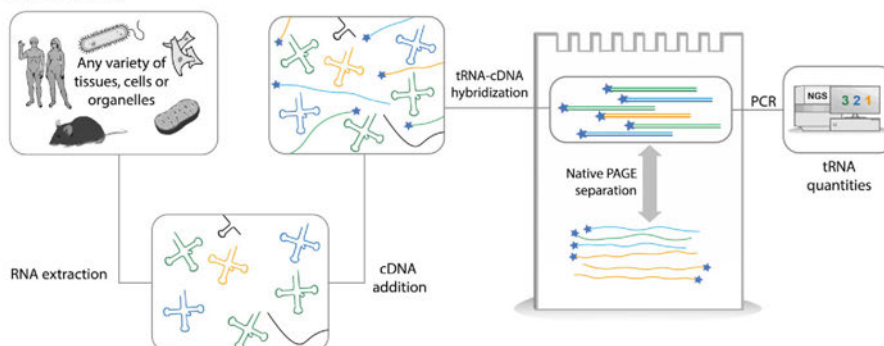
¹Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg University Mainz, Staudingerweg 5, 55128 Mainz, Germany
²IBPS, Biology of Aging and Adaptation, Sorbonne Université, Paris 75252, France
³Laboratoire de Chimie des Processus Biologiques, CNRS-UMR 8229, Collège De France, Université Pierre et Marie Curie, 11 place Marcelin Berthelot, 75231 Paris, Cedex 05, France
⁴Université de Lorraine, IMoPA UMR7365 CNRS-UL, BioPole, 54000 Nancy, France
⁵Université de Lorraine, Epitranscriptomics and RNA Sequencing (EpiRNA-Seq) Core Facility, UAR2008 IBSLor (CNRS-UL)/US40 (INSERM), 54000 Nancy, France

*To whom correspondence should be addressed. Tel: +49 6131 3925731; Fax: +49 6131 3920373; Email: mhelm@uni-mainz.de

Abstract

Due to its high modification content tRNAs are notoriously hard to quantify by reverse transcription and RNAseq. Bypassing numerous biases resulting from concatenation of enzymatic treatments, we here report a hybrid approach that harnesses the advantages of hybridization-based and deep sequencing-based approaches. The method renders obsolete any RNAseq related workarounds and correction factors that affect accuracy, sensitivity, and turnaround time. Rather than by reverse transcription, quantitative information on the isoacceptor composition of a tRNA pool is transferred to a cDNA mixture in a single step procedure, thereby omitting all enzymatic conversions except for the subsequent barcoding PCR. As a result, a detailed tRNA composition matrix can be obtained from femtomolar amounts of total tRNA. The method is fast, low in cost, and its bioinformatic data workup surprisingly simple. These properties make the approach amenable to high-throughput investigations including clinical samples, as we have demonstrated by application to a collection of variegated biological questions, each answered with novel findings. These include tRNA pool quantification of polysome-bound tRNA, of tRNA modification knockout strains under stress conditions, and of Alzheimer patients' brain tissues.

Graphical abstract



Introduction

Transfer RNAs (tRNAs) are universally expressed small non-coding RNA molecules, bearing a distinct secondary structure and extensive levels of RNA modifications. tRNAs are crucial

for protein biosynthesis in translating nucleic acid encoded information from messenger RNA (mRNA) to functional proteins. Alterations in the process of mRNA translation have been associated with various diseases in humans, comprising

Received: March 22, 2024. Revised: July 19, 2024. Editorial Decision: August 16, 2024. Accepted: August 21, 2024

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neurological disorders and cancer (1,2). Approaches to quantify these mRNAs in order to obtain gene expression profiles have recently proven insufficient on their own, missing impact of numerous post-transcriptional factors. At this point, ribosomes, the key player carrying out protein synthesis itself, are highly dependent on availability and abundance of tRNAs, thereby directly influencing speed and efficiency of protein synthesis (3,4). Considering the diverse pool of tRNAs, differing in cognate amino acid and dynamic expression, an accurate, and experimentally simple method for tRNA pool quantification is still highly desirable in biochemical and biomedical sciences.

Despite recent advances, the research field currently lacks an easily accessible and precise high-throughput method for fast and inexpensive characterization of tRNA pools from smallest tRNA quantities. Current methods based on gel electrophoresis offer acceptable accuracy in exchange for the significant effort and time consumption to prepare them, even for single tRNA subtypes. Methods utilizing the distinct sequence of every tRNA subtype to specifically target single tRNAs with an individually tailored nucleic acid counterpart (oligonucleotide), so-called tRNA microarrays, require tremendous hands-on effort and are limited by their fluorescence-based detection (5,6). Due to such restrictions, only few different samples can be analyzed for their full tRNA pool per microarray, thus limiting throughput-capability and therefore applicability for experiments with numerous conditions and replicates. Over the last decade, methodical developments capitalized on high throughput methods, chiefly on Illumina-based next generation sequencing (NGS), allowing to analyze even very rare tRNA subtypes (7,8). Because this method is unable to directly analyze the tRNAs, pertinent RNAseq protocols in general require numerous enzymatic steps during sample preparation. Generally, RNA must be converted to DNA (reverse transcription), a step greatly impeded by structural peculiarities of tRNAs, such as the extensive secondary structure and a vast repertoire of over 160 modified ribonucleotides, each of them idiosyncratically interfering with complete and quantitative reverse transcription. Next, a so-called adapter ligation, used in most methods and based on enzymatic or chemical treatment, varies in efficiency, introducing selection bias and increasing the required amount of tRNA for the method. The same applies to further steps, including the final polymerase chain reaction (PCR) which is required to introduce recognition sequences essential to NGS. The latter may display varying amplification efficiencies and may cause further quantitative bias.

Another important factor is the required input amount of tRNA which ranges around 1–2 µg for microarrays and gel-based approaches (2,9). This amount can be limiting in terms of preparative effort to acquire enough tRNA or biological availability, e.g. when analyzing minor tissue compartments, not yielding sufficient tRNA quantities due to their small size. Current NGS methods lowered the barrier in terms of input requirements, normally ranging between 50 and 500 ng per sample (7,8), an amount achievable in many preparations, however still at the limit of what is feasible when collecting rare or small cells/tissues and considering technical replicates. One more detrimental step in RNAseq based tRNA quantification is the bioinformatic workup of large datasets, which consistently requires sustained user training and is liable to consume significant time and effort.

Clearly, there is a sustained demand for tRNA quantification, as evidenced by the numerous approaches recently developed. While earlier work used yeast tRNAs as a gold standard, more recent methods focus on mammalian tRNA populations, as we will discuss later. Hence, owing in part to these limitations in publicly available datasets, a comprehensive comparison is difficult.

In view of the various obstacles encountered in tRNA quantification, we developed a novel method combining a cDNA hybridization technique with next generation sequencing, thus overcoming RT-derived hindrances while achieving high-throughput capability. We targeted individual tRNA isoacceptors by designing complementary DNA oligonucleotides, the latter already containing adapter sequences required for final library preparation. A tRNA-cDNA hybridization step transfers quantitative information to the cDNA template, thus omitting reverse transcription and any enzymatic step except for a low-cycle index PCR which takes place on the cDNA template. Following NGS, the limited pool of unmodified cDNA reference sequences allows for a greatly simplified bioinformatic analysis which is, in comparison to any other RNAseq approach, almost trivial and therefore extremely fast. Furthermore, preparational effort for 96 samples comprises only around 5 days with 15–20 h of hands-on time and a total cost below 50\$ per sample, considering consumables and NGS, which can be outsourced. Finally, our method allows for tRNA pool characterization with down to 5 ng of tRNA, lowering the RNA input requirements by one order of magnitude compared to current RNAseq methods.

Material and methods

Cell line samples

HEK293- (DSMZ, Braunschweig, Germany, No^oACC 305), HeLa- (DSMZ, No^oACC 57) and T98g- (ATCC, Manassas, VA, USA, No^oCRL-1690) cells were cultivated in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 vol-% fetal bovine serum (Thermo Fisher Scientific) and 0.1% Primocin (InvivoGen, San Diego, CA, USA), GlutaMAXTM (Thermo Fisher Scientific), 4.5 g/l glucose (Thermo Fisher Scientific) and sodium pyruvate (Thermo Fisher Scientific) at 37°C and 5 vol% CO₂. For separation or harvesting, cells were first washed with Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific) and then incubated with trypsin-EDTA (Thermo Fisher Scientific) for 5 min under growing conditions. After detaching, cells were centrifuged for 5 min at 400 g, washed with phosphate buffer and once again centrifuged. Finally, pellets were dissolved in TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) and stored at –20°C. Similarly, *S. cerevisiae* was cultivated in YPD-medium (Carl-Roth, Karlsruhe, Germany) up to an OD of 1.0, centrifuged at 10 000 g for 20 min, supernatant decanted, the cell pellet dissolved in TRI Reagent and stored at –20°C.

Murine tissue samples

Female C57BL/6J mice were used at an age of 16 weeks (for housing conditions and genotyping see Brandscheid *et al.* (10)). All experimental procedures were carried out in accordance with the European Communities Council Directive regarding care and use of animals for experimental

procedures and was approved by local authorities (LUA-Rhineland-Palatinate).

Human tissue samples

Human brain samples were provided by the rapid autopsy program of the Netherlands Brain Bank (NBB, Amsterdam, Netherlands) for high quality specimen from clinically well-documented and neuropathologically confirmed cases. Research on human specimens was performed according to the ethical declaration of the NBB. All cases of Alzheimer's disease were neuropathologically confirmed using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria. The control specimen had no history or symptoms of neurologic or psychiatric disorders and showed no clinical symptoms of dementia. Tissue from the Gyrus frontalis superior 3 + 4 was used for all 12 control and 13 AD specimens. Age varied between 72 to 98 years for AD patients (average 84) and 71 to 102 for control patients (average 85), with an average Braak stage of 4.7 (AD) or 2.5 (Ctr).

RNA isolation from cells or tissue

Total RNA was isolated from either cell pellets or dissected tissue by extraction with TRI Reagent (Sigma-Aldrich). The cell pellet (~2 000 000 cells) or tissue (~50 mg) was suspended in 2 ml TRI Reagent and thoroughly mixed, in case of tissue also crushed with a pestle. Following addition of 400 µl chloroform (Carl Roth) and thorough mixing, samples were centrifuged at 18 000 g for 15 minutes, the aqueous phase transferred to a new tube and 1 ml 2-propanol (Carl Roth), as well as 1 µl Glycogen (Thermo Fisher Scientific), were added. Following another round of centrifugation at 18 000 g for 15 min, supernatant was removed and 1 ml ethanol (75%) (Thermo Fisher Scientific) was added for a final centrifugation step. Finally, supernatant was carefully removed, the RNA pellet briefly airdried and resuspended in RNase free water. The required amount of TRI Reagent varied depending on the number of cells or tissue size used, with respective changes to the other Reagents that were added.

Polysome profiling and tRNA isolation

The *E. coli* wildtype strain Keio parent (BW25113) and the knockout strains $\Delta dusA$ and $\Delta dusB$ were purchased from the *E. coli* Keio knockout collection (GE Healthcare (DharmaconTM, England)). *E. coli* cultures were grown in 100 ml LB medium (Carl Roth) at 37°C and 190 rpm until an optical density of 0.4 at 600 nm. Paraquat dichloride hydrate (Sigma-Aldrich) was added to final concentrations of 0, 0.1 and 0.3 mM and bacterial growth was continued until an OD₆₀₀ of 0.7. Chloramphenicol (100 µg/ml, Carl Roth) was added to the culture which was incubated for 3 min and then harvested by centrifugation (10 min, 10 000 g, 4°C). The pelleted cells were resuspended in buffer (100 mM NH₄Cl (Merck, Darmstadt, Germany), 10 mM MgCl₂ (Carl Roth), 20 mM Tris, pH 7.5 (Carl Roth)), lysozyme (Carl Roth) was added and freeze-thaw cycles in liquid nitrogen were performed. Lysis was completed by adding 10% deoxycholate (Sigma-Aldrich) and cell wall debris was removed by centrifugation (12 000 g, 10 min, 4°C). Cell lysate was loaded on top of sucrose gradients from 5 to 40% using BioComp (BioComp, Fredericton, Canada) gradient station model 108 (settings: time 1.23 min, angle 81.5°, speed 21 rpm). Gradients were ultracentrifuged (150 000 g, 4°C, 2.5 h, Beckman Optima MAX-X Ultracentrifuge, SW40

Ti rotor from Beckman Coulter, CA, USA) and fractionated collecting free RNA fraction (F0) and polysomal fraction (F3). Total RNA was extracted using TRI Reagent (Sigma-Aldrich) and separated subsequently on a 10% denaturing PAGE gel stained with GelRed (Biotium/BIOTREND, Köln, Germany). tRNA bands were excised from the gel after visualization of RNA bands using Typhoon 9400 (excitation wavelength of 532 nm, Amersham Bioscience/GE Healthcare, Chicago, IL, USA) and mashed with a scalpel. tRNA was extracted from the gel adding 300 µl 0.5 M ammonium acetate (Merck) before overnight incubation at 25°C and 750 rpm. After filtration through NanoSep 0.45 µm spin columns (VWR, Darmstadt, Germany), RNA was precipitated using three volumes of 100% ethanol (Carl Roth).

cDOQ design

cDNA oligonucleotides for quantification (cDOQs) were designed from tRNA references retrieved from tRNAdb (11), MODOMICS (12), and gtRNAdb (13). The last 40 nucleotides towards the tRNA 3' end were compiled to non-redundant references utilizing a R-based construction pipeline by Pichot *et al.* (14), collapsing sequences with a levenshtein distance below six nucleotides into one species (Supplementary Figure S1B). Next, the reverse-complement of these retrieved, non-redundant references was merged with the respective P5 (AGACGTGTGCTCTTCCGATCT) and P3 (GATCGTCGGACTGTAGAACTCTGAAC) sequence on the 5' and 3' end. The full-length constructs of 87 nucleotides were finalized with either a 6-FAM (cytosolic tRNAs) or Cyanine-5 (mitochondrial tRNAs) attached to the 5' end. All cDOQs were ordered and synthesized at biomers.net GmbH (Ulm, Germany) or Integrated DNA Technologies (Coralville, IA, USA).

PCR and sequencing primer design

For indexing PCR, twelve i7 and eight i5 primers were designed to allow multiplexing up to 96 samples. To reach full compatibility with Illumina platforms, a custom primer for the i5 index read on Illumina NextSeq platforms was designed and utilized on the sequencing platform. All Primers were ordered and synthesized at biomers.net GmbH.

cDOQ-tRNA hybridization and hybrid purification

Starting from 50–200 ng of total RNA or 5–50 ng of total tRNA, 5x hybridization buffer (150 mM HEPES pH 7.5 (Carl Roth), 500 mM potassium acetate (Carl Roth)) was added. Following addition of a 5-fold excess of an equimolar mixture of cDOQs for the selected targets (e.g. a single tRNA or 43 cytosolic + 22 mitochondrial tRNAs in human), the solution was denatured at 94°C for 2 min and afterwards gradually cooled down to 25°C within 20 min. The sample was then analyzed on a 10% native polyacrylamide-gel in 1x TBE buffer, running at constant power of 60 mA for 45 min. Depending on the experimental context, optional GelRed-staining (Biotium, Fremont, CA, USA) was carried out for 15 min to visualize nucleic acids. cDOQ-attached fluorescent dyes used for stainless visualization comprised 6-FAM or Cy5. Bands of interest (e.g. cDOQ:tRNA hybrid) were excised, crushed and eluted overnight in 300 µl 0.5 M ammonium acetate (Merck) at 750 rpm and 15°C. Afterwards, gel particles were removed with Nanosep 0.45 µm spin columns (VWR), centrifuging at 2000 g for 1 min. Finally, 1 µl glycogen was added to the aqueous

flow-through, together with 2.5 volumes of ethanol (99.5%, Carl Roth), storing the mixture at -80°C for 1 h or -20°C overnight. Afterwards, samples were centrifuged at 18 000 g for 1 h, supernatant removed, the pellet washed with 500 μl ethanol (70%) and again centrifuged at 18 000 g for 30 min. Supernatant was carefully removed, the pellet left to air-dry for 10 min at room temperature and then resuspended in 10 μl of RNase-free water.

Indexing PCR and next generation sequencing

Nucleic acids from excised cDOQ:tRNA hybrids were subjected to index PCR, using half (5 μl) of the previously purified product. For samples with low tRNA input (5 ng or less), complete use of retrieved product was necessary. From a master mix, Taq-Buffer (NEB, Frankfurt am Main, Germany), dNTPs (NEB) and MgCl_2 (Carl Roth) were added to final concentrations of $1\times$ (Buffer), 0.5 mM (dNTP) and 3 mM (MgCl_2). Individual i5 and i7 primer combinations were added to a final concentration of 0.25 μM . Reaction was carried out with Taq-Polymerase (NEB), initially denaturing at 94°C for 30 s, followed by normally 3–6 cycles of denaturation (94°C , 15 s), annealing (62°C , 30 s) and extension (72°C , 15 s), as well as a final extension step at 70°C for 5 min. Next, the reaction mixture was purified on a denaturing 10% PAGE in $1\times$ TBE buffer, running at constant power of 60 mA for 1 hour, excising the product band around 169 nt length. Following overnight elution, ethanol precipitation and resuspension as described previously, samples were sequenced on an Illumina NextSeq 2000 or MiSeq platform. For sequencing on the NextSeq 2000 platform, a custom i5 index read primer (Supplementary Table S1) was used. The achieved read output proved to be dependent on the number of different samples pooled on a flow cell, as well as the amount of sequencing-ready library loaded for each sample. Thus, for samples with low tRNA input (5 ng or less), yielding less DNA in the low-cycle index PCR, adjustments in library amount used and the overall sample number loaded on the flow-cell had to be considered.

RNAseq

Library preparation of tRNA fractions was based on the NEB-next Small RNA Library Prep Set for Illumina (NEB) and is briefly described below. First, tRNA was dephosphorylated with Antarctic Phosphatase (NEB) and afterwards phosphorylated with Polynucleotide Kinase (NEB). Following purification via RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany), a preadenylated 3' adaptor was ligated and the reverse transcription primer hybridized to minimize adaptor-dimer formation. Next, the 5' adaptor was ligated and reverse transcription was performed using SuperScript IV (Thermo Fisher Scientific) for its high sequence fidelity and ability to process sites comprising RNA modifications. Finally, polymerase chain reaction was performed using custom i5 and i7 indexes (Supplementary Table S1) and the PCR product purified via gel excision on a denaturing polyacrylamide gel. Full length product was then subjected to sequencing on an Illumina NextSeq 2000 platform using a custom i5 index read primer (GATCGTCGACTGTAGAACTCTGAAC).

NGS data analysis

cDOQ-related data retrieved from sequencing on Illumina platforms was first quality controlled using FastQC (15) and

reads afterwards trimmed with Trimmomatic (16) to the first 40 nucleotides corresponding to the cDOQ hybridization sequence. Finally, trimmed reads were quantified using the Sailfish tool (17) under standard settings and a reference set including all cDOQ sequences (Supplementary Tables S2–S5). Analysis was performed using the European Galaxy Project platform (usegalaxy.eu) (18).

RNAseq data was first quality controlled using FastQC (15) and afterwards trimmed with Cutadapt (19), thus removing any remaining Illumina-related adapter sequences. Next, the trimmed reads were aligned to the reference sequences (total tRNA references from Modomics (12) and tRNAdb (11)) using Bowtie 2 (20), accounting for the high modification prevalence in tRNA by allowing one mismatch within a 22 nt sequence. Finally, mapped reads were analyzed in the Integrative Genomics Viewer (21).

Results

Method design

For development of an accurate and sensitive high-throughput tRNA quantification method, we focused on reduction of known major bias sources, simple sample preparation, feasibility with equipment available in standard biochemical laboratories, and simple data workflow.

At the core of the method is a hybridization step of the tRNA of interest with an engineered cDNA. This step transmits quantitative information from tRNA to the resulting tRNA-cDNA duplexes in a 1:1 stoichiometry. The duplexes are then physically separated from non-hybridized cDNA. This leaves the quantitative information in single-stranded cDNA from which it is easily read out. In contrast to e.g. RNAseq methods, this foregoes the use of numerous enzymatic conversion steps such as ligation and reverse transcription, thereby avoiding the associated biases. The only remaining enzymatic step is an index PCR for the subsequent quantification by NGS sequencing (Figure 1A).

In practice, an organism-specific mix of some 40 different cDNAs, each one corresponding to a major tRNA family, is hybridized to either total RNA or isolated total tRNA. Following denaturation and subsequent hybridization, the hybridization mixture is purified by native PAGE, thus physically separating tRNA-oligo hybrids from excess oligo and RNA contaminants. Upon elution from the native gel, a mixture of tRNA-oligo hybrids is obtained, in which the initial tRNA abundances are now represented by the abundance of each tRNA-specific oligo in the mixture. Quantification of the ~40 sequences was here developed for Illumina NGS system but may conceivably be adapted to other analytical platforms. Conforming to this system, a bias-aware library preparation and analysis pipeline applies as follows. The oligo-containing gel eluate is directly subjected to low-cycle index PCR to minimize amplification bias. The resulting full-length product is amenable to sequencing on all current Illumina platforms and straightforward analysis requiring a minimum of training or prior knowledge. Data treatment comprises quality control, trimming and alignment. The final output is a quantitative table in which the sum of individual cDOQ reads divided by the total number of reads per sample represent the relative tRNA abundances in the initial RNA sample. Before application to several biological problems, this protocol was extensively scrutinized and validated with respect to

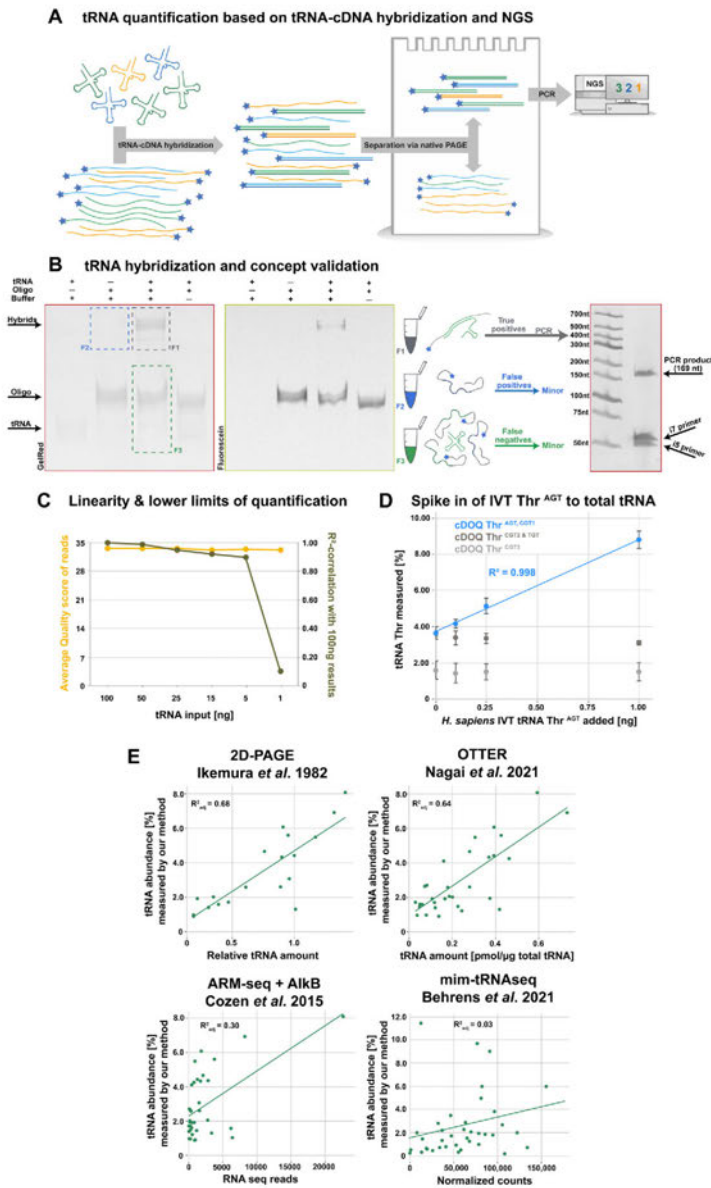


Figure 1. Overview of the hybridization-based quantification method and major performance characteristics. **(A)** Display of major steps in our hybridization-based quantification approach. **(B)** Example of hybridization on a native PAGE gel scanned for tagged fluorescein and GelRed staining. tRNA-cDOQ hybrids (F1) are physically separated from unbound oligo on the gel, afterwards excised and subjected to index PCR yielding full length product at 169 nt. Fraction F2 and F3 were analyzed in separate experiments (Supplementary Figure S2D-F) to characterize potential bias. **(C)** Average read quality scores yielded from samples prepared with decreasing amounts of tRNA displayed in yellow compared to R^2 -values of these samples correlated to a sample prepared from 100 ng tRNA. **(D)** Calibration and absolute quantification by standard addition of *in vitro* transcribed tRNA Thr^{AGT} to 25 ng of total tRNA from HEK 293 cells plotted against the measured tRNA abundance. Biological triplicates, error bars indicate standard deviation. The y-axis interception corresponds to the tRNA abundance of the targeted isodecoders in the sample. **(E)** Correlation plots comparing *S. cerevisiae* tRNA abundances from our method with results obtained from other publications and *H. sapiens* for mim-tRNAseq.

various potential sources for errors and biases, as outlined below.

Method characterization and validation

cDNA design integrates NGS adapters and tRNA hybridization sequences

Probe design for cDNA oligomers (abbreviated „cDOQ“ for cDNA Oligo for quantification) addressed the following criteria, namely (i) facile detection and visualization, (ii) sequence elements for subsequent quantification and (iii) quantitative hybridization of the cognate tRNA. A combination of cDOQs is needed to (iv) capture all known tRNA sequences of a given organism. **Supplementary Figure S1A** shows the general structure of cDOQs, comprising a 40 nt hybridization region complementary to the 3' end of a given tRNA isoacceptor family and framed by primer binding sites for the Illumina i5 and i7 amplification sequences. The main rationale for choosing the 40 nt towards the 3' end was sufficient sequence differentiation while maintaining acceptable cDOQ length. Also, the 3' modification landscape on e.g. human tRNAs comprises few Watson-Crick-impeding modifications, and this design allows separate targeting of 5'-tRFs (*vide infra*). The cDOQ construct is complemented by a 5'-fluorescent dye that aids visualization after PAGE. The process of cDOQ design considers all isodecoders and isoacceptors of a tRNA species, compiling them based on their Levenshtein distance, a metric indicating the number of nucleotides different between two observed sequences, and thus sequence dissimilarity (**Supplementary Figure S1B**). Acknowledging the widespread use of acronyms in the field, we chose DORQ-seq for 'DNA Oligo for RNA Quantification sequencing'.

A surprising PCR amplification bias is minimal at 6 replication cycles

When the composition of an equimolar mixture of ~40 cDOQs, here directed against human tRNAs, was analyzed for relative content after 3, 6, 9 and 12 cycles of index PCR, a significant amplification bias was detected for 9 and 12 cycles. As shown in **Supplementary Figure S1C**, certain sequences were found enriched by 100% while others were decreased to 20% relative to the abundance at 2 cycles. This was unexpected given the identical primer binding sites and was only weakly correlated to the GC content of the hybridizing sequences (**Supplementary Figure S1D**). Interestingly, sequences behaved identically in three experimental replicates (executed on different days), demonstrating that the bias was indeed sequence specific and not random. Similar experiments on cDOQ mixtures covering tRNAs from other model organisms (**Supplementary Figure S1E**) further confirmed this, and consistently showed minimal bias after 6 cycles, which was henceforth the default setting.

cDOQ design is specific to single tRNA sequences

In an application to a first model organism, the 43 known cytosolic tRNA sequences of *Saccharomyces cerevisiae* were collapsed into 38 families, as described previously (14). Within the corresponding 38 cDOQs, the shortest Levenshtein distance was 6 nucleotides, occurring between Gly GCC and Gly CCC. This corresponds to 15.0% of the hybridizing sequence, slightly exceeding the recommendations of 10–12.5% (4–5 nt) by Letowski *et al.* (22). For experimental validation, 4 tRNAs were individually isolated from shifted bands in native

gels after hybridization of total tRNA to their corresponding cDOQs. RNAseq of material isolated with the Gly GCC-cDOQ yielded 99% read alignment to the cognate tRNA Gly GCC family, and only 0.01% aligned to the nearest neighbor Gly CCC (**Supplementary Figure S2A**). Among the 4 tRNAs the average read mapping to the cognate tRNA sequence was 97% and the minimum 95% (**Supplementary Figure S2B**). Similar values were obtained with full-length cDOQs used for isolation of seven different tRNAs. This establishes a Levenshtein distance of 6 nucleotides as efficient in the discrimination of similar sequences by 2–4 orders of magnitude. The cDOQ-Gly GCC also caused near complete depletion of the cognate tRNA from the remaining i.e. unshifted tRNA material (**Supplementary Figure S2C**).

Transfer of quantitative information from tRNA to cDOQs

Moving from single sequences to total tRNA from *S. cerevisiae*, we performed an analogous experiment with a cocktail of 38 cDOQs. The mixture was heated and cooled to room temperature before separation on a native gel, which is shown in **Figure 1B**. The left panel images all nucleic acids as stained by gel red, while the right panel displays only the fluorescein-labeled cDOQs. The resulting tRNA:cDOQ heteroduplexes in lane 3 were well separated due to their retardation relative to tRNAs and cDOQs as evident by comparison with lanes 1 and 2. The duplexes were excised from the region designated by a black rectangle, eluted and further processed in our pipeline as 'true positives' (F1, black sample). The gel region corresponding to duplexes in lane 2 (F2 blue: 'no tRNA' false positive sample, blue rectangle in **Figure 1B**) was similarly treated to determine signals that might have arisen from nonstandard migration of non-hybridized cDOQs. A detailed analysis shown in **Supplementary Figure S2D–E** illustrated that the resulting signals in F2 were negligible. Additionally, the bottom part of lane 3 was excised, and putative residual RNA was submitted to an RNAseq protocol to potentially identify tRNAs that had failed to hybridize to the cDOQs (F3, green sample: 'false negatives'). Its analysis, which is detailed in the supplement (**Supplementary Figure S2F**), involved reverse transcription of the RNA into cDNA, which was then submitted to short PCR-amplification and sequencing alongside the cDOQs. It showed only 0.05% of reads that could be mapped to cytosolic tRNA sequences, with the remainder corresponding to cDOQs, rRNA fragments and mitochondrial tRNA sequences. Finally, hybridization experiments using synthetic tRNA fragments (tRFs) displayed sufficient in-gel separation of mature tRNA species from tRFs, thus indicating a potential of this approach for tRNA fragment quantification and excluding tRFs as potential bias source for mature tRNA quantification (**Supplementary Figure S2G**). We thus concluded that all tRNA molecules were quantitatively hybridized and quantitative information on their composition was contained in the cDOQs of the F1 black sample.

Linearity to quantify the tRNA content of ~2000 cells

To determine the lower limits of our method, a dilution series between 1 and 100 ng of *S. cerevisiae* tRNA was prepared and quantified. **Figure 1C** shows the average quality of reads and their similarity to the 100 ng composition plotted for each amount of input tRNA. Even from the lowest input of only 1 ng tRNA, excellent read quality could be achieved, with an average quality score of 33.8. Within the single-digit nanogram range of tRNA input, several ten-thousands of mapped reads,

up to several million were achieved in our hands, depending on details of the sequencing platform and starting material. For all processed samples, read output proved sufficient, in theory, for relative quantification of the 38 species contained. However, the overall constant read quality, as well as greatly increased similarity to the 100 ng sample at 5 ng input and higher (Figure 1C), suggests an inherent limitation by noise. This places the lower limit for relative quantification around 5 ng tRNA input or slightly less, which, in our experiments, yielded 20000–100 000 mapped reads. This tRNA amount corresponds to the content of ~30 000 yeast cells, or the equivalent of 2000 mammalian cells (23). Conceivably, with reduced sample number, adaptations in sequencing preparation and increased sequencing depth, high quality quantitative data might also be retrievable from lower tRNA amounts.

For absolute quantification, a spike-in calibration with an *in vitro* transcript of human tRNA^{Thr AGT} was quantified. The plot in Figure 1D shows an R^2 value of 0.99 that linearly correlates read numbers with three amounts of IVT in 25 ng each of total human tRNA. The value for zero spike corresponds to ~37 000 reads per million, equaling 0.93 ng of the targeted native tRNA Threonine isoacceptors. This comprises Thr AGT and Thr CGT-1, the latter an isodecoder with a single gene and presumably minor expression. Furthermore, this allows for absolute quantification of all other tRNAs analyzed within this sample, based on relative abundance. Additionally, similar experiments in *M. musculus* and prototype prokaryote *E. coli* displayed R^2 -correlation values of 0.99 and 0.97 respectively (Supplementary Figure S2H).

Finally, we compared our results to other published methods (Figure 1E), initially focusing on *S. cerevisiae* as widely used gold standard. Interestingly, we observed poor correlation with RNAseq based methods, but very good correlation with other hybridization-based methods, namely an $R^2_{\text{adj}} = 0.68$ to 2D-gel electrophoresis results by Ikemura *et al.* (24) and an R^2_{adj} of 0.64 with the recently published OTTER method by Nagai *et al.* (9), which utilizes fluorescent labeling and in-gel detection. In contrast, we found rather weak correlations when looking at quantitative RNAseq methods, e.g. an $R^2_{\text{adj}} = 0.30$ for ARMseq (25) data, and, now using HEK293T tRNA, an R^2_{adj} of 0.03 (Supplementary Figure S2I) when comparing to mim-tRNAseq (8). Without being able to offer a molecular rationale, we conclude that hybridization-based methods on one hand, and RNAseq-based methods on the other hand cluster together.

Fast, precise, cost-effective and high-throughput quantification of tRNA

In exploiting the full throughput-capability of NGS, multiplexing was established for parallel analysis of up to 96 samples on any of the current Illumina platforms. A typical run on a Nextseq 2000 platform with 96 samples input yielded over 4 million reads per sample, with an average Q -Score of 33.6. This thus implies less than one error in 2200 base calls, meaning that fewer than 2% of the 40 nt recognition sequences was subject to a single base calling error mutation. Given the minimum Levenshtein distance of 6 between any two cDOQs, misassignments among cDOQs can be considered insignificant for quantification. Indeed, and in contrast to other methods (8,26) we have never observed any multi-mapping. Typically, >98% of the reads mapped to the 38 yeast cDOQ sequences,

providing excellent statistics way above the experimental limit established above (Figure 1C). Of note, the data treatment is, in comparison to any other RNAseq approach, very simple and fast.

The turnaround time of such an analysis, starting from 96 samples of total RNA or total tRNA, is about 5 days, including 15–20 hours of hands-on time and 3–5 h of data analysis. Assuming that the NGS is outsourced, the overall cost including consumables and sequencing is about 50\$ per sample. More importantly, this method evades all problems associated to reverse transcription, including the impact of RNA modifications, which are especially prevalent in tRNA. Consequently, implausible overrepresentation of certain tRNA species, known as ‘jackpot’ effects (7), and presumably arising from a low modification grade and consequently improved reverse transcription rate, do not occur, and the relative distribution of tRNA families is much more physiologically plausible. Because the method easily lends itself to numerous problems in tRNA biology, we have devised and validated species-specific sets of cDOQs for several model organisms including *E. coli*, mouse, and human, wherein we have also included mitochondrial tRNAs when appropriate (Supplementary Tables S1–S5). Among all organisms analyzed, the mapping rate ranged between 92 and 99% uniquely aligned reads (Supplementary Figure S2J).

Mitochondrial tRNA composition in mouse differs from human

An analysis covering both cytosolic and mitochondrial tRNA compositions was conducted in several human cell lines and tissues (vide infra). The mitochondrial tRNA composition consistently showed a ~21% share of mt-tRNA^{Val} (Figure 2A), reflecting the substitution of 5S rRNA in mammalian mitochondria by this tRNA (27,28), which further validates both the method and its application to mammals. Interestingly, the analogous analysis of mouse tissues from brain and liver (29) revealed only 3% abundance of mt-tRNA^{Val}, while the most abundant mitochondrial species was now mt-tRNA^{Ala}, featuring 13%, about 3× as much as in human. We were unable to find any literature on the identity of the 5S rRNA substitute in mouse, and, significantly, a paper summarizing the findings in five (30) mammalian species did not list mouse either. We conclude that an experimental analysis likely failed, and our data indicate that possibly the 5S rRNA role is filled by a mixture of different tRNA species in mouse.

Gender-specific variation of mitochondrial tRNA abundances in Alzheimer patients

In the context of Alzheimer’s disease (AD), we applied our high-throughput quantification approach to a set of 25 human brain samples comprising 14 females and 11 males, both patients and healthy control. Additionally, several frequently used human cell lines were analyzed and compared to results from human tissue. Cytosolic tRNA abundance distributions showed no significant differences between patient groups, though clear differences when compared to human cell lines (Figure 2B). From 43 cDOQs targeting cytosolic tRNAs, 16 (HEK 293) or 21 (T98G WT) showed significant differences compared to human cortex tissue.

Strikingly, the overall abundance of mitochondrial tRNAs was significantly increased by 2-fold in women with AD, relative to the control group (Figure 2C). An increased level in

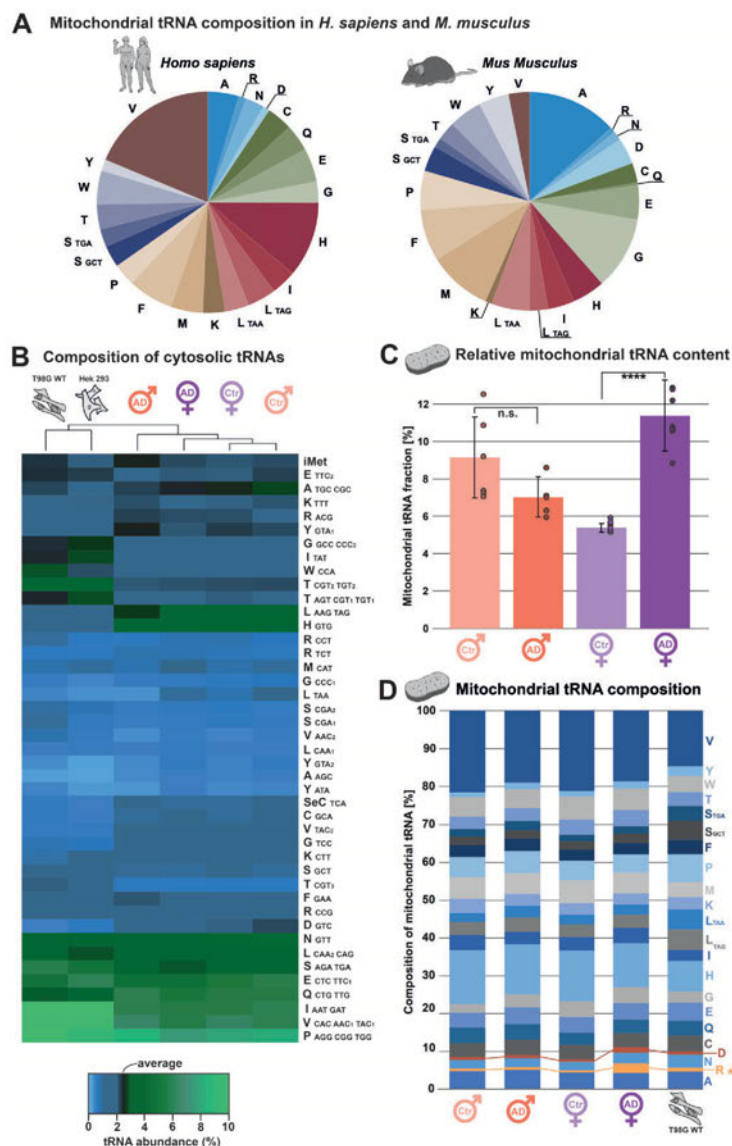


Figure 2. Analysis of tRNA expression in different mammals focusing on mitochondria and Alzheimer's disease. **(A)** Composition profile of all 22 mitochondrial tRNAs in *H. sapiens* and *M. musculus* from frontal cortex tissue. tRNAs are labeled in one-letter amino acid code with anticodons listed for isoacceptors if applicable. **(B)** Composition of 43 cytosolic tRNAs in two human cell lines and human cortex tissue from male and female individuals, either bearing Alzheimer's disease (AD) or being non-dement control (Ctr). The average abundance is visible in black with higher-than-average abundance in green and lesser abundance in blue. tRNAs are labeled in one-letter amino acid code with multiple anticodons listed when cDOQs target multiple isoacceptors at once. Anticodons are additionally numbered in cases where different isodecoders were targeted. Performed in technical triplicates. **(C)** Fraction of mitochondrial tRNA of all tRNAs analyzed in human cortex tissue from male and female individuals, either bearing Alzheimer's disease (AD) or being non-dement control (Ctr). Error bars indicate standard deviation and asterisk indicate a significant difference in one-sided t-test (**** $P \leq 0.0001$) while n.s. indicates no significant difference. Performed in technical triplicates. **(D)** Display of mitochondrial tRNA composition in human cell line and human frontal cortex tissue from male and female individuals, either bearing Alzheimer's disease (AD) or being non-dement control (Ctr). Performed in technical triplicates, asterisks indicate a significant difference in one-sided t-test for the female AD group (** $P \leq 0.01$, *** $P \leq 0.001$).

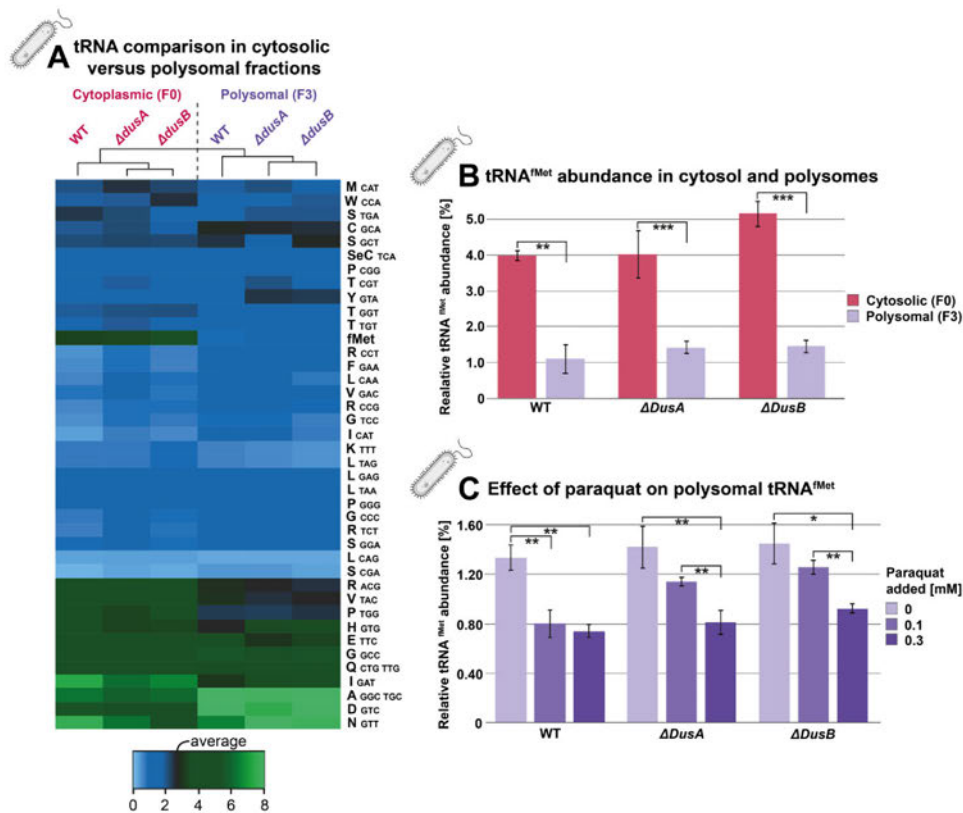


Figure 3. Differing composition of cytosolic and polysomal tRNA pools of *E. coli* mutants under oxidative stress. **(A)** Composition profile of 40 tRNAs in either cytoplasmic (F0) or polysomal (F3) fraction of polysome preparations from *E. coli* K12 wildtype and two mutant strains. tRNAs are labeled in one-letter amino acid code with anticodons listed for multiple isoacceptors if applicable. Asterisks indicate a significant difference (* $P \leq 0.05$) in one-sided t-test between F0 and F3 fraction of Δ DusA tRNA abundance. **(B)** tRNA^{Met} abundance in different fractions (F0/F3) for different mutant strains. Error bars indicate standard deviation and asterisk indicate a significant difference in one-sided t-test (** $P \leq 0.01$, *** $P \leq 0.001$). Performed in technical triplicates. **(C)** Relative abundance of tRNA^{Met} for different mutants in their respective polysomal fractions treated with different amounts of paraquat. Performed in technical triplicates. Error bars indicate standard deviation and asterisk indicate a significant difference in one-sided t-test (* $P \leq 0.05$, ** $P \leq 0.01$).

male patients was not significant relative to the male control group. Investigating the relative abundances of individual mt-tRNAs, two significant differences could be observed for female patients. Both tRNA^{ArgTCT} and tRNA^{AspGTC} displayed significantly increased abundance compared to all other patient groups (Figure 2D). Interestingly, with the exception of the female AD group, no significant differences could be observed between cell lines and human samples on a mitochondrial level.

The increase in mitochondrial tRNAs in female Alzheimer patients is unexpected, yet not implausible. Mitochondrial function is a central focus in Alzheimer research (31,32), and it is also known that the molecular pathogenesis differs significantly between female and male patients. We therefore consider this data an incentive to develop quantification of tRNA in the AD field.

Severe differences in tRNA pool composition of *E. coli* cytosol and polysomes are further impacted by paraquat treatment

As an application that makes use of the low input requirements, *E. coli* tRNAs isolated from a density gradient (Supplementary Figure S3A and B) that separate actively translating polysomes (labeled F3 in Figure 3) from the cytoplasmic pool of tRNAs (F0) were compared. As little as 21 ng of isolated tRNA from F3 fractions was sufficient to prepare technical triplicates. In addition to a wildtype *E. coli* K12 strain, we compared the tRNA composition in two knockout strains of tRNA modification enzymes DusA and DusB. The respective enzymes catalyze dihydrouridine modifications in the namesake D-domain of tRNAs (33,34) and might conceivably influence tRNA fitness and flexibility, possibly including accommodation on the ribosome. We also thought it possible that such an effect might be modulated upon treatment with

paraquat, inducing oxidative stress and potentially altering both tRNA abundances and their usage on actively translating polysomes. A heatmap in Figure 3 gives a detailed view of the results. In summary, no general effects could be observed. Instead, abundances changed for single tRNA species, suggesting a tRNA-specific impact of dihydrouridination on its respective usage in translation. While no general influence on tRNA abundances was visible, pronounced differences were observed between all F0 and their respective F3 fractions, with up to 15 tRNAs significantly under- or overrepresented (Figure 3A). These included tRNA^{Met}, which was far less abundant in all polysomal (F3) fractions compared to the cytosol with differences up to 3.5 fold (Figure 3B), which is highly plausible given that this tRNA is expected to occur only in freshly initiating ribosomes, i.e. the 5'-most ribosome in each given polysome. F3 levels of tRNA^{Met} were similar across mutant strains with only minor differences between F0 fractions. Interestingly, the tRNA^{Met} populations in the F3 fractions decreased upon exposure to paraquat (Figure 3C). The latter was applied at sublethal concentrations to induce oxidative stress, leading to marked growth reduction, which is presumably related to the decreased population of initiator tRNA in translating polysomes.

Discussion

Quantification methods for tRNA are a major driving force in tRNA biology, itself embedded in the larger field of protein translation. While tRNA biology has resurged along with RNA modification research, it has concomitantly become clear that tRNA modifications impose strong limitations to quantification. As a cardinal process in RNA quantification, cDNA hybridization to RNA can be designed to overcome negative effects from RNA modifications, typically by adjusting the cDNA length. Hence, when properly designed, cDNA-based quantification approaches, such as northern blotting, OTTER, MSTand RIP-chips, (see Supplementary Table S6) can avoid biases, albeit at the expense of sequence information. As in the above methods, our approach only reports information corresponding to the employed query sequences, thus missing modification information and details on isoacceptor composition within isoacceptor families covered by a common cDOQ. Among the previous, only chip-based methods tackle multiple sequences in a single experiment, which is otherwise the domain of deep sequencing approaches. However, RNAseq approaches have their intrinsic biases and statistical caveats in the quantification of complex mixtures, which are compounded in tRNA quantification, since modifications compound existing enzyme-based biases and introduce new ones. For example, reverse transcription is known to be hampered by RNA structure (35), which in turn is known to be reinforced by tRNA modifications. Reverse transcription arrest by modifications is known to affect quantification, albeit useful in the detection and quantification of those same modifications (36,37). Removal of a few selected modifications by enzymatic treatment e.g. with AlkB was introduced to improve cDNA synthesis and as a negative control to validate modification detection based on RT-arrest (25,38), yet introducing another enzymatic step prone to bias. Since, on a general level, incubation with any enzyme and the associate workup are not only time-consuming, put at risk additional RNA degradation and associated bias, we designed a streamlined process with a minimum of experimental steps and only a single

enzymatic step. Ultimately, the enabling crucial step resides in the information transfer through hybridization of tRNAs with cDOQ, and subsequent separation. Interestingly, our results align well with other hybridization-based approaches, whereas RT-based approaches align amongst each other. It might arguably benefit the field to conduct a comparative study among labs using the various methods. The strengths of our new approach include very low material input requirements, low turnaround time, low cost and high throughput. While the method only returns information on sequences that are queried by cDOQs, disregarding all other sequences improves the signal-to-noise and leads to extremely simple bioinformatics. The method is thus competitive or leading in any of the above categories (see Supplementary Table S6), and its combined advantages make it possible to analyze complex experimental setups (typically 96 samples) with low abundant samples, e.g. tRNAs from polysomes or brain tissue in just one sequencing run. Furthermore, the method holds great potential considering an application to small RNAs such as microRNAs, siRNAs or any other small non-coding RNAs. Therefore, with further testing and target-dependent optimization, a wide application of our approach seems well feasible. In summary, the method takes tRNA quantification to an advanced level of practical application to low input and high samples numbers. As we have demonstrated with AD biopsies, the method is clearly suited for analysis of clinical samples.

Data availability

The data underlying this article has been deposited in BioProject (<https://www.ncbi.nlm.nih.gov/bioproject/>) with the accession number PRJNA1088289.

Supplementary data

Supplementary Data are available at NAR Online.

Acknowledgements

We are grateful to Valérie Igel-Bourguignon (EpiRNA-Seq Core Facility, UMS2008 IBSLor, CNRS-Université de Lorraine-INSERM, Biopôle, Vandœuvre-lès-Nancy, France) for excellent technical assistance in NextSeq sequencing.

Funding

Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) [TRR-319 TP A05, Project Id 439 669 440; SPP1784, HE 3397/13-2, HE 3397/14-2 to M.H. FRER EpiRNA (Grand Est Region, France to Y.M.); ANR PRCI D-Erase [to M.H., D.B., D.H., V.M.]. The open access publication charge for this paper has been waived by Oxford University Press – NAR Editorial Board members are entitled to one free paper per year in recognition of their work on behalf of the journal.

Conflict of interest statement

Mark Helm is a consultant for Moderna Inc. The method presented is subject to a patent filed.

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Received: March 22, 2024. Revised: July 19, 2024. Editorial Decision: August 16, 2024. Accepted: August 21, 2024

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Supplementary Material

DORQ-seq: High-throughput quantification of femtomol tRNA pools by combination of cDNA hybridization and Deep Sequencing**AUTHORS**

Kristen Marco¹, Lander Marc¹, Kilz Lea-Marie¹, Gleue Lukas¹, Jörg Marko¹, Bregeon Damien², Hamdane Djemel³, Marchand Virginie⁴, Motorin Yuri^{4,5}, Friedland Kristina¹, Helm Mark^{1*}

¹ Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg University Mainz, Staudingerweg 5, 55128, Mainz, Germany.

² IBPS, Biology of Aging and Adaptation, Sorbonne Université, Paris 75252, France.

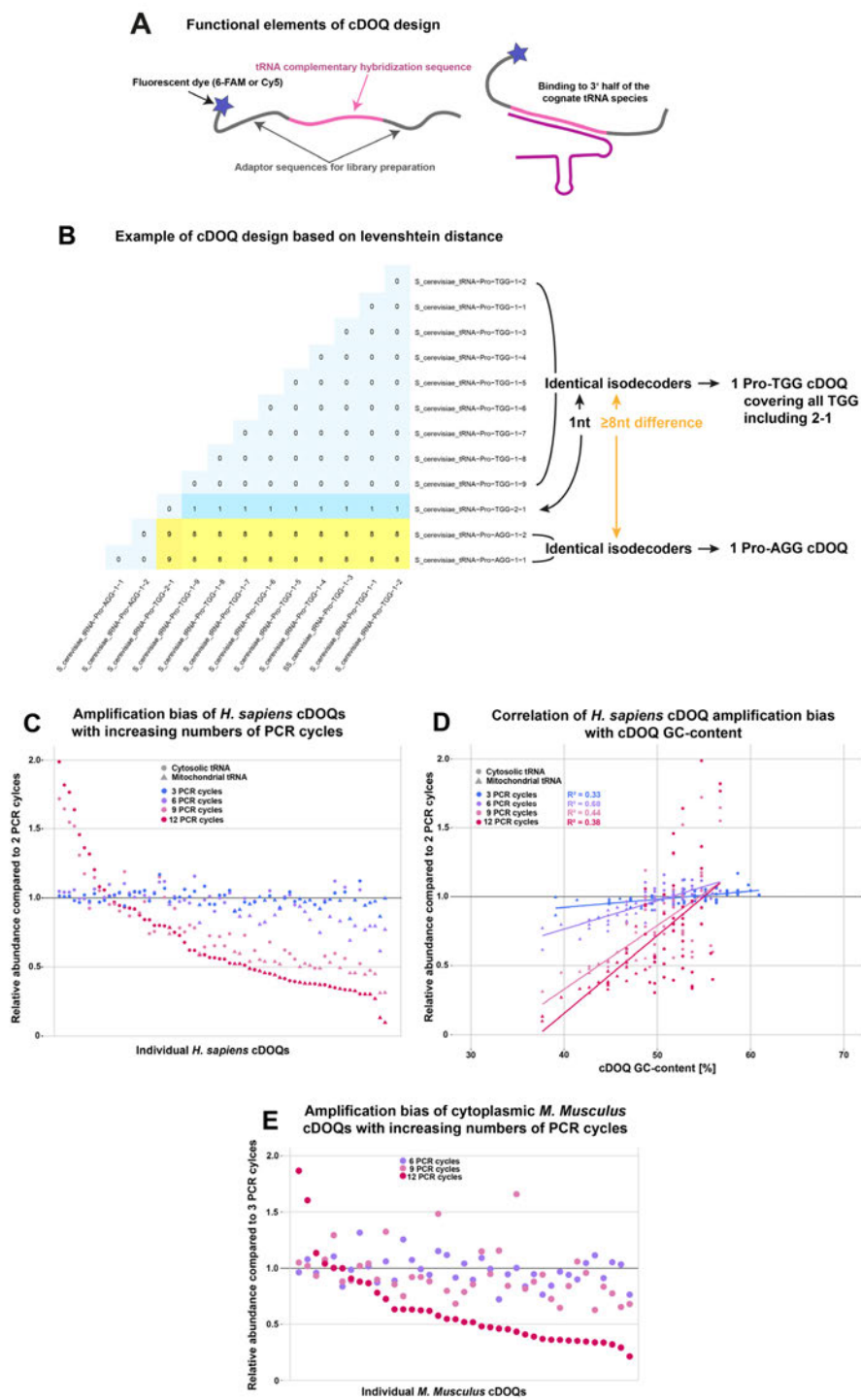
³ Laboratoire de Chimie des Processus Biologiques, CNRS-UMR 8229, Collège De France, Université Pierre et Marie Curie, 11 place Marcelin Berthelot, 75231 Paris, Cedex 05, France.

⁴ Université de Lorraine, IMoPA UMR7365 CNRS-UL, BioPole, 54000 Nancy, France.

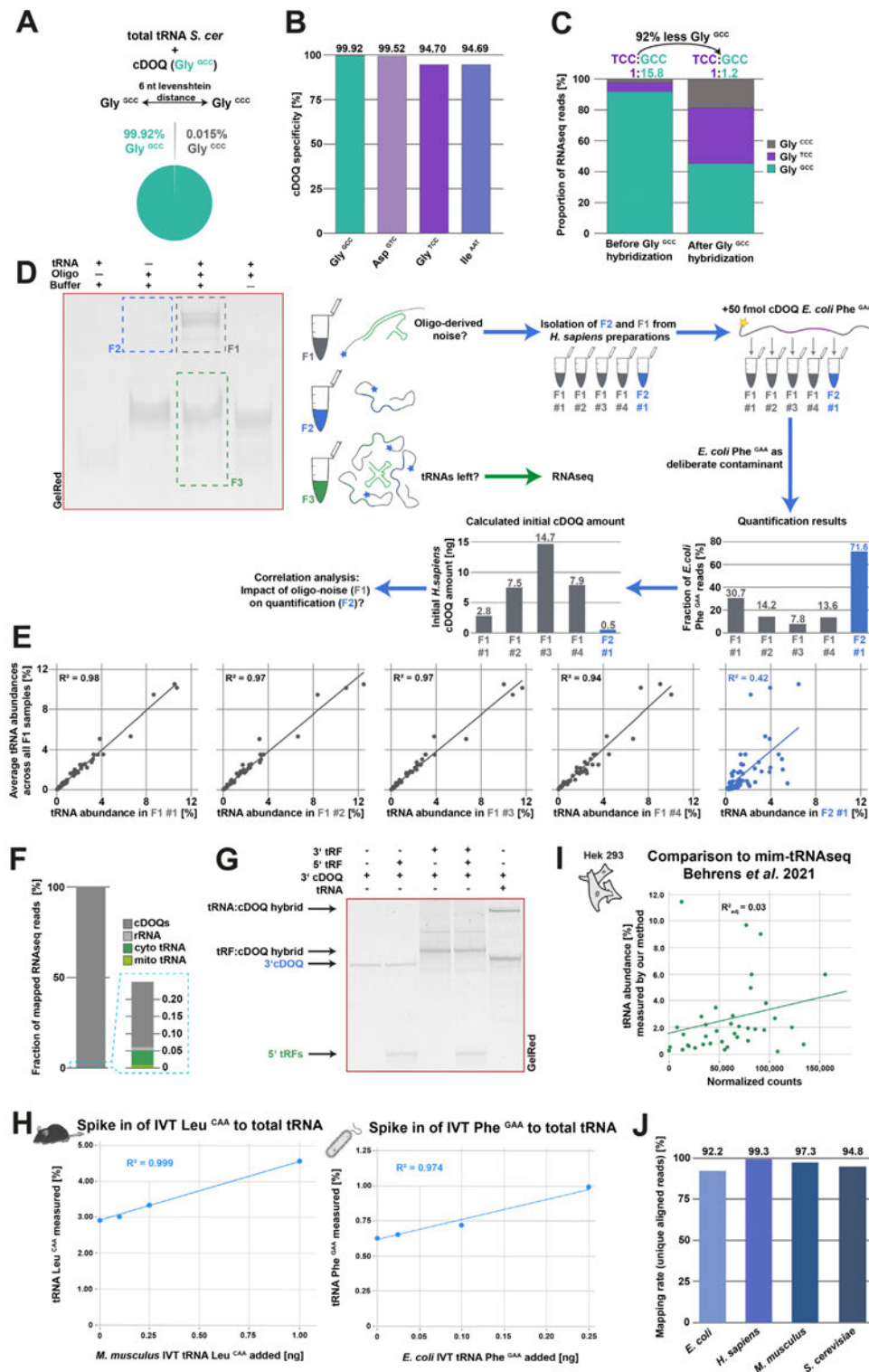
⁵ Université de Lorraine, Epitranscriptomics and RNA Sequencing (EpiRNA-Seq) Core Facility, UAR2008 IBSLor (CNRS-UL)/US40 (INSERM), 54000 Nancy, France.

*Correspondence to mhelm@uni-mainz.de

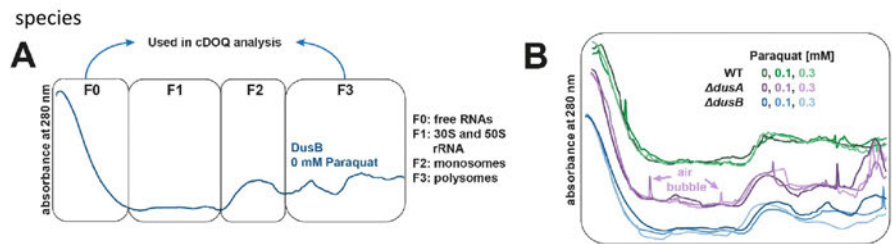
Further characterization of the DORQ-seq method required analysis of potential noise derived from oligo secondary structures and the completeness of hybridization (**Fig. S2 D**). In order to determine potential noise, human tRNA-oligo hybrids (F1) of different human total RNA samples were excised from a native PAGE gel, as well as the corresponding height for samples only bearing oligo (F2), thus excising potential oligo running at hybrid-size. Following elution, 50 fmol of *E. coli* Phe^{GAA} hybridization oligo were added to each sample as deliberate contaminant and internal standard. Following index PCR and Illumina sequencing, the percentage of reads mapped to *E. coli* Phe^{GAA} (corresponding to 50 fmol oligo) was used to calculate the initial amount of *H. sapiens* hybridization oligos before PCR. Calculated oligo amounts ranged between 2.8 ng and 14.7 ng for hybrid (F1) fractions compared to 0.5 ng for the F2 fraction. Correlation analysis of retrieved data (**Fig. S2 E**) compared the individual measured tRNA abundances with the average tRNA abundances calculated from all hybrid (F1) fractions. Adjusted R²-values ranged between 0.94 and 0.98 for F1 fractions and 0.4 for the potential noise, thus indicating great differences in noise composition compared to hybrids. No dependency between R²-value and initial *H. sapiens* oligo amount could be observed as correlation was constantly high across all F1 fractions, implying a very limited impact of potential noise.



Supplementary Figure 1: cDOQ structure and PCR-derived bias in cDOQ quantities. **A** General structure of a cDNA oligo for quantification ("cDOQ") and its binding to the cognate tRNA **B** Design approach for cDOQs as shown for *S.cerevisiae* tRNA Proline. The levenshtein distance of all known isoacceptors and isodecoders is shown in the heatmap with numbers indicating the levenshtein distance between two sequences. Sequences with less than 6nt distance will be grouped as one cDOQ for the respective isodecoders or, if applicable, even isoacceptors. **C** Relative abundance of individual cDOQs in a mixture of *H. sapiens* cDOQs after different numbers of PCR cycles compared to the initial abundance measured after 2 PCR cycles. Data shape indicates cDOQs targeting cytosolic (circle) or mitochondrial (triangle) tRNAs. **D** Correlation the cDOQ GC-content with the relative abundance of individual cDOQs in a mixture of *H. sapiens* cDOQs after different numbers of PCR cycles compared to the initial abundance measured after 2 PCR cycles. Data shape indicates cDOQs targeting cytosolic (circle) or mitochondrial (triangle) tRNAs. **E** Relative abundance of individual cDOQs in a mixture of *M. musculus* cDOQs after different numbers of PCR cycles compared to the initial abundance measured after 3 PCR cycles.



Supplementary Figure 2: Investigation of oligo specificity, potential quantitative bias and completeness of hybridization. **A** Percentage of reads mapped to either glycine tRNA isoacceptor after specifically targeting Gly^{GCC} with the respective cDOQ. The isoacceptors display 6 nt difference in the hybridization sequence (6 nt = 15%). **B** Exemplary cDOQ specificity. **C** Ratio of RNAseq reads from different tRNA Gly isoacceptors before and after specific hybridization of tRNA Gly^{GCC}. **D** In the process of hybridization, the noise derived from oligo secondary structures (F2) and its respective impact on hybrids (F1) and following quantification was investigated. Furthermore, completeness of hybridization was analyzed by RNAseq analysis of tRNA-height post-hybridization (F3). 12 fractions were prepared from four human total RNA samples with F2 running on the same hybridization gel. Following elution, 50 fmol of hybridization oligo targeting *E. coli* Phe^{GAA} was added to all F1 and F2 fractions. Samples were subjected to PCR, sequenced and afterwards analyzed for their *E. coli* Phe^{GAA} oligo read proportion. From this, the initial oligo concentration could be calculated for each sample and used for impact analysis. **E** Correlation plots for all F1 and F2 samples comparing tRNA abundance in the respective sample with the average abundance across all four F1 fractions. **F** Comparison of mapped reads retrieved for cDOQs and RNA species from RNAseq analysis of Fraction 3 (F3). **G** Hybridization and separation of tRNA fragments and mature tRNAs via cDOQs on a PAGE. **H** Standard addition experiments adding in vitro transcribed tRNA Isoacceptors to total tRNA of the respective species. **I** Correlation of tRNA quantification results for Hek293 cells obtained via cDOQs and mim-tRNAseq. **J** Mapping rate of uniquely aligned reads with the novel quantification approach across different



Supplementary Figure 3: Preparation of polysome fractions to characterize tRNA pools involved in translation. **A** Exemplary gradient curve from polysome preparation, indicating the fractions used in cDOQ-analysis. **B** Overview of all polysome profiles generated for *E. coli* WT (K12), Δ dusA and Δ dusB strains under treatment with increasing concentrations of paraquat.

Supplementary Table 1: Custom read and indexing primers

Primer	Sequence
i5 index read	
i 501	5'-GATCGTCGACCTGTGAACTCTGAC-3'
i 502	5'-AATGATACGGCGACCCAGAGATCTACACTATAGCCTGTTCAAGGTTCTACAGTCCAGCATC-3'
i 503	5'-AATGATACGGCGACCCAGAGATCTACACTATAGGCTGTTCAAGGTTCTACAGTCCAGCATC-3'
i 504	5'-AATGATACGGCGACCCAGAGATCTACAGGCTTGAAGTTCAAGGTTCTACAGTCCAGCATC-3'
i 505	5'-AATGATACGGCGACCCAGAGATCTACAGGCGAAGGTTCAAGGTTCTACAGTCCAGCATC-3'
i 506	5'-AATGATACGGCGACCCAGAGATCTACACTATAGTTGTTCAAGGTTCTACAGTCCAGCATC-3'
i 507	5'-AATGATACGGCGACCCAGAGATCTACACAGTCCGTTGTTCAAGGTTCTACAGTCCAGCATC-3'
i 508	5'-AATGATACGGCGACCCAGAGATCTACACGTACTAGACGTTCAAGGTTCTACAGTCCAGCATC-3'
i 701	5'-CAAGCAGAAGACGGCATACGAGATCGAGTATGTGACTGGAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 702	5'-CAAGCAGAAGACGGCATACGAGATCTCCGAGGTGACTGGAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 703	5'-CAAGCAGAAGACGGCATACGAGATATAGCGGTGACTGGAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 704	5'-CAAGCAGAAGACGGCATACGAGATGGAATCTTGACTGGAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 705	5'-CAAGCAGAAGACGGCATACGAGATTCTGAAATGTGACTGGAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 706	5'-CAAGCAGAAGACGGCATACGAGATTCGATTCGATGAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 707	5'-CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 708	5'-CAAGCAGAAGACGGCATACGAGATCGGATTTAGTGACTGGAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 709	5'-CAAGCAGAAGACGGCATACGAGTATAGCGGTGACTGGAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 710	5'-CAAGCAGAAGACGGCATACGAGATTTCGCGAGTGAAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 711	5'-CAAGCAGAAGACGGCATACGAGATGCGGAGAGTGAAGTTCAAGCGTGTGCTTTCGANTCT-3'
i 712	5'-CAAGCAGAAGACGGCATACGAGATCTATCCCTGTGACTGGAAGTTCAAGCGTGTGCTTTCGANTCT-3'

Supplementary Table 2: cDOQs designed for *E. coli*

Anticodon			Sequence
tRNA	Anticodon	Sequence	
Ala	GGC & TGC	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGAGAGCTAAGCGGGATTCGAAACCCGACACCTTCCTCATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Arg	ACG	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGATTCGCGGAGAGATTCGAACTTCGACACCTTCGACGCTGGGTATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Arg	CCG	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGGAGCGGCGCCAGATTCGAAATTCGAAACCTTCGCTGTGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Arg	CCG	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGTCCCGTCCGAGATTCGAACTTCGAACTTATACCTTCGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Arg	TCT	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCGGCGGCGGAGATTCGAACTTCGAACTTCGCTGGCCCGATTCGATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Asn	GTT	5'-AAGAGCTGTGCTTTCOCANCTTGGGCTCTCTGTACTGTGAGCTTCGAACTTCGAACTCAAGTACATACAGATTTATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Asp	GTC	5'-AAGAGCTGTGCTTTCOCANCTTGGGCGGAAAGGAGACGGGAGCTCGACACCCCTCTCTGTATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Cys	GGA	5'-AAGAGCTGTGCTTTCOCANCTTGGAGAGCGGTGCTTCGAGATTCGAAACCGGATATGAGATTTATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
FMET	CAI	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGTGGCGGGGCGCGGATTTGAAACCGAACACACCTTCCTGGGGTTATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Gln	CTG & TTG	5'-AAGAGCTGTGCTTTCOCANCTTGGCTTGGGCTTGAGCTTCGAACTTCGAACTTCGCGAATGCTCGGAAATGATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Glu	TTT	5'-AAGAGCTGTGCTTTCOCANCTTGGAGCGTCCCTTAAGGAGATTCGAAACCCCTTTTACCGCTGGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Gly	GCC	5'-AAGAGCTGTGCTTTCOCANCTTGGAGGGGAGAAACAGATTCGAACTTCGAAACCCCGACCTTGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Gly	CCC	5'-AAGAGCTGTGCTTTCOCANCTTGGAGCGGGGCGCAAGGAAATCCGCTCTATATAGAGTTGGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Gly	TCC	5'-AAGAGCTGTGCTTTCOCANCTTGGAGCGGGGCGACAGCGGAAATCCGACATCATACATGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
His	GTTG	5'-AAGAGCTGTGCTTTCOCANCTTGGGGGTGGGATGGGAAATTCGAACTTCGAACTTGGAATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Ile	GAT	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGAGCCTTGATGGAGCTTGAAACGACGACCTGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Ile	CAI	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGACCTTCTGTGACTTTCGAACTTCGAAACGACCAAGCATTTATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Leu	CAG	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGACGGGGGGGACTTGAACCCCGACAGCTGCTGTAAGAGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Leu	CAA	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCGGAGCGAGCTGACACGGGACATGATTCGAACTTATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Leu	GAG	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGTACGAGAGCGGACTTGAACCCGTTAAACCGTTATGGAATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Leu	TAA	5'-AAGAGCTGTGCTTTCOCANCTTGGTACCCGAGACGGGACTTGAACCCGACACCGCAGACCGATTCGCTGGAGCTGTAGAACTCTGAAC-3'	
Leu	TAG	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGGAGGGGCGAGACTTGAACCTTGAGCTGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Lys	TTT	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGGTTGGTACGAAATTCGAACTTCGAACTCAGCACTTATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Met	CAI	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGGTACGAGCGGGAATTCGAACTTCGAACTGATACCCATATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Phe	GAA	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCCCGGAACTGGAATTCGAACTCGAAACGAGGAGATTCGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Pro	CGG	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCTGATATAGGAATTCGAACTTCGAACTTCGACACCTTCGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Pro	GGG	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCGAGAGAGATTCGAACTTCGAACTTCGACACCCCGACACATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Pro	TGG	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCGGACAGAGGATTCGAACTTCGAACTTCGACACCTGTGTGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Sec	TCG	5'-AAGAGCTGTGCTTTCOCANCTTGGGCGGAAACATACAGGATTCGAACTTCGCGCGGAGCCGCTGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Ser	GGA	5'-AAGAGCTGTGCTTTCOCANCTTGGGCGGAGGGGGGGGATTCGAAACCCCGCACTTCGTCGGAATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Ser	CCA	5'-AAGAGCTGTGCTTTCOCANCTTGGGCGGAGAGGGGGGATTCGAAACCCCGATATGTCGGAATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Ser	GCT	5'-AAGAGCTGTGCTTTCOCANCTTGGGCGGTATGAGCGGGGATTCGAAACCCCGATTCGACTTTTATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Ser	TCG	5'-AAGAGCTGTGCTTTCOCANCTTGGGCGGAACGACGAAATTCGAACTTCGAACTTCGTCGGAATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Thr	CGT	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCATATAGAGATTCGAACTTCGAACTTCGATGCTGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Thr	GGT	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCTATGACCAAGCTTCGAACTTCGAACTTCGATGCTGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Thr	TGT	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCCCATACCCGAAATTCGAACTTCGAACTTCGATGCTGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Trp	CCA	5'-AAGAGCTGTGCTTTCOCANCTTGGGAGGGGCGAGAACTCGAACTTCGCAACCCCGTTTATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Tyr	GTA	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGTGGGGAGATTCGAACTTCGAACTTCGATGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Val	TAC	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGGTATATGAGAGGGAATCGAACCGCGACACCCCTCTCTTATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	
Val	GAC	5'-AAGAGCTGTGCTTTCOCANCTTGGTGGCTTCGAGTGCATCGAAACCAACGCCCCACATGATATGCTGTGGAGCTGTAGAACTCTGAAC-3'	

Supplementary Table 3: cDOQs designed for *S. cerevisiae*

[illegible]

Saccharomyces cerevisiae			
Type	RNA	Anticodon	Sequence
cytosolic	His	GUG ⁺	5'-AGACGTGTGCTCTTCCGATCTCTGGTCCATCTCTAGATCGAACAGGGTTTCATTCGGCCGATCGTGGACTGTAGAACTCTGAAC-3'
	Ile	AAT	5'-AGACGTGTGCTCTTCCGATCTTGGTGGTCTAGAGGATCGAACCGCTGATCCCGGTTGATCGTGGACTGTAGAACTCTGAAC-3'
	Ile	TAT	5'-AGACGTGTGCTCTTCCGATCTTGGTGGTCTCGAGGTTGGGGTTTGAACCCACGACGACCGCTGCGTTGATCGTGGACTGTAGAACTCTGAAC-3'
	Leu	GAG	5'-AGACGTGTGCTCTTCCGATCTTGGTGGTATACCCGCGGGTTTGAACCCGCGCTCCGAAAGAGATCGTGGACTGTAGAACTCTGAAC-3'
	Leu	TAA	5'-AGACGTGTGCTCTTCCGATCTTGGTGAAGATCGAAGATTGAACTCGCGGACCAACCGTGAATCGTGGACTGTAGAACTCTGAAC-3'
	Leu	CAA	5'-AGACGTGTGCTCTTCCGATCTTGGTGGTGGTGGTGAAGATTGAACTCTTGCATCTTACGATCGTGGACTGTAGAACTCTGAAC-3'
	Leu	TAG	5'-AGACGTGTGCTCTTCCGATCTTGGTGAAGCTGAGAGGATTGAAACCTTGCATCCGAAAGATGATCGTGGACTGTAGAACTCTGAAC-3'
	Lys	TTT	5'-AGACGTGTGCTCTTCCGATCTTGGTCCCTCATAGGGGGGCTGAAACCCCTGACATTTGGTTGATCGTGGACTGTAGAACTCTGAAC-3'
	Lys	CTT	5'-AGACGTGTGCTCTTCCGATCTTGGACCCGTGAGAGGAGGCTGAAACCCCTAACCTTATGATTGATCGTGGACTGTAGAACTCTGAAC-3'
	Met	CAT	5'-AGACGTGTGCTCTTCCGATCTTGGTGGTCTCCAGAGAGGTTGAACTCTGACCTTCAGATTGATCGTGGACTGTAGAACTCTGAAC-3'
	Phe	GAA	5'-AGACGTGTGCTCTTCCGATCTTGGTGGCATTTCTGGATCGAACACAGAACTCCAGATCGTGGACTGTAGAACTCTGAAC-3'
	Pro	AGG	5'-AGACGTGTGCTCTTCCGATCTTGGGGGGCGAGCGGGACCTCGGACCTCCGATGATCGTGGACTGTAGAACTCTGAAC-3'
	Pro	TGG	5'-AGACGTGTGCTCTTCCGATCTTGGGGGGCGAGCTGGGAATTGAACCCAGGGCCTCTCGACGATCGTGGACTGTAGAACTCTGAAC-3'
	Ser	GCT	5'-AGACGTGTGCTCTTCCGATCTTGGGGCTACACAGACAGGAATTCGAACTTCGAACTTCGCGAGGTAAACGATCGTGGACTGTAGAACTCTGAAC-3'
	Ser	AGA	5'-AGACGTGTGCTCTTCCGATCTTGGCGACACACAGACTTGAACCAACGCGGGCAGAGCCGATCGTGGACTGTAGAACTCTGAAC-3'
	Ser	CGA & TGA	5'-AGACGTGTGCTCTTCCGATCTTGGCGACACAGACTTGAACCAACGCGGGCAGAGCCGATCGTGGACTGTAGAACTCTGAAC-3'
	Thr	CGT	5'-AGACGTGTGCTCTTCCGATCTTGGTGGTCCCTCTGTGGGAATTTGAACCCGACATCCCGCATTTGATCGTGGACTGTAGAACTCTGAAC-3'
	Thr	AGT	5'-AGACGTGTGCTCTTCCGATCTTGGATCGATCTTGGATCGTCCCATCGGAATTTGAACCCGATTCGATCGTGGACTGTAGAACTCTGAAC-3'
	Thr	TGT	5'-AGACGTGTGCTCTTCCGATCTTGGTCCACCTGTCAGAATTTGAACCTTAACGACCTTTGCATTGATCGTGGACTGTAGAACTCTGAAC-3'
	Trp	CCA	5'-AGACGTGTGCTCTTCCGATCTTGGTGAAGAGCAGAGATTGAACCTGCAACCTTCGATTGATCGTGGACTGTAGAACTCTGAAC-3'
	Tyr	GTA	5'-AGACGTGTGCTCTTCCGATCTTGGTGTTCACACCGAGGATCGAACTCGGACCTTTGGCGTGAATCGTGGACTGTAGAACTCTGAAC-3'
	Val	CAC & TAC	5'-AGACGTGTGCTCTTCCGATCTTGGTGTTCACACCGAGGATCGAACTCGGACCTTTGGCGTGAATCGTGGACTGTAGAACTCTGAAC-3'
	Val	AAC	5'-AGACGTGTGCTCTTCCGATCTTGGTGTTCACACCGAGGATCGAACTCGGACCTTTGGCGTGAATCGTGGACTGTAGAACTCTGAAC-3'

*Of note, the cDOQ His⁶¹⁶, contains 3 mismatches within its 40 nt sequence as a result of database ambiguity. As it still complies to our dogma (≤ 6nt levenshtein distance) for cDOQ design and can target all His-tRNA isodecoders in *S.cerevisiae*, we consider potential influences as negligible.

Supplementary Table 4: cDOQs designed for *H. sapiens*

[illegible]

Homo sapiens			
Type		cytosolic	
tRNA	Anticodon	Sequence	
Ile	GAT & AAT	5'-AGACGTGTCCTTCCGANTCTTGGGCCCTCCAGACCCCGACCTTGGCGTTGATGTCGGACTGTAGAACTCTGAAC-3'	
Ile	TAT	5'-AGACGTGTCCTTCCGANTCTTGGGCTCCAGAGGAGGCTGACATTCGTCGGACTGTAGAACTCTGAAC-3'	
Leu	TAG & AAG	5'-AGACGTGTCCTTCCGANTCTTGGTGGCAGCCGTGGGATTCGAACTCCAGCCGCCCAAGAGGATCGTCGGACTGTAGAACTCTGAAC-3'	
Leu	CAA (1)	5'-AGACGTGTCCTTCCGANTCTTGGTGGCCCTCTGAGAGCTTGAACCTCAGAGACCAATTCAGATGATCGTGGAACTGTAGAACTCTGAAC-3'	
Leu	CAG & CAA (2)	5'-AGACGTGTCCTTCCGANTCTTGGTGGTACAGAGAGTGGGATTCGAACTCCAGCCGCAACACAGTGAATCGTCGGACTGTAGAACTCTGAAC-3'	
Leu	TAA	5'-AGACGTGTCCTTCCGANTCTTGGTGGCCCAACGTGGGGCTCGAACCCAGACCTTGAGATTGAATCGTCGGACTGTAGAACTCTGAAC-3'	
Lys	CTT	5'-AGACGTGTCCTTCCGANTCTTGGAGCCCTGAGACAGGGACTTGAACCTTGAGATTCGATCGTCGGACTGTAGAACTCTGAAC-3'	
Lys	TTT	5'-AGACGTGTCCTTCCGANTCTTGGAGCCCTGAGACAGGGACTTGAACCTTGAGATTCGATCGTCGGACTGTAGAACTCTGAAC-3'	
Met	CAI	5'-AGACGTGTCCTTCCGANTCTTGGAGCCCTCTTGAGGCTCGAACTCAGGAACCTTCAGATTGATCGTCGGACTGTAGAACTCTGAAC-3'	
Phe	GAA	5'-AGACGTGTCCTTCCGANTCTTGGTGGCCAAACCCGGGATTCGAACTCAGGGACCTTTAGATCGATCGTCGGACTGTAGAACTCTGAAC-3'	
Pro	TGG & CGG & AGG	5'-AGACGTGTCCTTCCGANTCTTGGGGGCTCGTCGGGGATTTGAACCCGGGACCTCTCGACGATCGTCGGACTGTAGAACTCTGAAC-3'	
Sec	TCA	5'-AGACGTGTCCTTCCGANTCTTGGGCGCCGAAAGTGGATTTGAACCACTGTGCTAGAGATCGTCGGACTGTAGAACTCTGAAC-3'	
Ser	TGA & AGA	5'-AGACGTGTCCTTCCGANTCTTGGGCGTGAAGCGGAGATTCGAACTCGGCGGGGAGACCCGATCGTCGGACTGTAGAACTCTGAAC-3'	
Ser	GGA (1)	5'-AGACGTGTCCTTCCGANTCTTGGCGCTGTGAGCAGGATTTGAACCTGCGCGGGAGACCCGATCGTCGGACTGTAGAACTCTGAAC-3'	
Ser	GGA (2)	5'-AGACGTGTCCTTCCGANTCTTGGCGTACAGAAAGGATTCGAACCTGCGGGGAAACCCGATCGTCGGACTGTAGAACTCTGAAC-3'	
Ser	GCT	5'-AGACGTGTCCTTCCGANTCTTGGCGACAGGATTCGAACCCAGCGCTGCAAGACAGATCGTCGGACTGTAGAACTCTGAAC-3'	
Thr	AGI & TGI (1) & GGI (1)	5'-AGACGTGTCCTTCCGANTCTTGGAGGCCCGCGTGGGATTCGAACCCAGGATCTCTGTTGATCGTCGGACTGTAGAACTCTGAAC-3'	
Thr	TGI (2) & CGI (2)	5'-AGACGTGTCCTTCCGANTCTTGGAGGCCCGCGAGATTTGAACCTGCGAACCCCTGTTGATTCGTCGGACTGTAGAACTCTGAAC-3'	
Thr	CCA	5'-AGACGTGTCCTTCCGANTCTTGGAGGCCCGAGGATTCGAACCCGATCTTCGATTCGTCGGACTGTAGAACTCTGAAC-3'	
Tyr	ATA	5'-AGACGTGTCCTTCCGANTCTTGGTCTTCAAGCTGSAATCGAACGCAACCTTAAGACCGATCGTCGGACTGTAGAACTCTGAAC-3'	
Tyr	GTA (1)	5'-AGACGTGTCCTTCCGANTCTTGGTCTTCAAGCGGAAATCGAACGACGACCTTAAGATCGATCGTCGGACTGTAGAACTCTGAAC-3'	
Tyr	GTA (2)	5'-AGACGTGTCCTTCCGANTCTTGGCTCTCAAGTCGGAATCAACGACACTTAAGATCTGATCGTCGGACTGTAGAACTCTGAAC-3'	
Val	CAC & TAC (1) & AAC (1)	5'-AGACGTGTCCTTCCGANTCTTGGGTGGATTCGAACTCAGAGGACCTTCATGATGATTCGTCGGACTGTAGAACTCTGAAC-3'	
Val	AAC (2)	5'-AGACGTGTCCTTCCGANTCTTGGGTGGATTCGAACTCAGAGGACCTTCATGATGATTCGTCGGACTGTAGAACTCTGAAC-3'	
Val	TAC (2)	5'-AGACGTGTCCTTCCGANTCTTGGGTGGATTCGAACTCAGAGGACCTTCGATGATTCGTCGGACTGTAGAACTCTGAAC-3'	

Supplementary Table 5: cDOQs designed for *M. musculus*

[illegible]

Type		Anticodon (1° = Same cDOQ as <i>H. sapiens</i>)	Sequence
tRNA			
RNA			
Gly	CCC (1)*	5'-AGACGTGTGCTCTTCCGATCTTGTGGCCGCCGCGGAAATCGAACCCGGGTGCGAAGATGATGCTGGAACTGTAGAACTGTGAAC-3'	
Gly	TCC & CCC (2)*	5'-AGACGTGTGCTCTTCCGATCTTGTGGCAATTTGGAGATTTGGACCCGGGACCTCCGCGTGGATGTCGGAACTGTAGAACTGTGAAC-3'	
Gly	GCC	5'-AGACGTGTGCTCTTCCGATCTTGTGGTTCGCGCCGGTTTCGAAACCCGGGACCTTTCGGTTGATGTCGGAACTGTAGAACTGTGAAC-3'	
His	GTC*	5'-AGACGTGTGCTCTTCCGATCTTGTGGCCGTGACATTCGAACCAAGGTTGCTGGGCCGATGCTGGAACTGTAGAACTGTGAAC-3'	
Ile	AAT*	5'-AGACGTGTGCTCTTCCGATCTTGTGGACCGGATCGAACCCGGACCTTGGCGTTGATGTCGGAACTGTAGAACTGTGAAC-3'	
Ile	TAT	5'-AGACGTGTGCTCTTCCGATCTTGTGATTCACATCGGGGCTCAACACTGAGGACTCTTGATGATGTCGGAACTGTAGAACTGTGAAC-3'	
Leu	TAG & AAG*	5'-AGACGTGTGCTCTTCCGATCTTGTGGGACGGTGGATTCGAACCCAGCCGCCCGGAAGGATGTCGGAACTGTAGAACTGTGAAC-3'	
Leu	CAG & CAA*	5'-AGACGTGTGCTCTTCCGATCTTGTGTACAGATGGGATTCGAACCCACGCTCCATCGAGATGTCGGAACTGTAGAACTGTGAAC-3'	
Leu	TAA	5'-AGACGTGTGCTCTTCCGATCTTGTGACTGGAGTTCGAACCCAGGACCAAGCATGTCGGAACTGTAGAACTGTGAAC-3'	
Lys	TTT*	5'-AGACGTGTGCTCTTCCGATCTTGTGGCCTTAATATGGGGCTCGAACCCATGACCTTGAGATTGATGTCGGAACTGTAGAACTGTGAAC-3'	
Lys	CTT	5'-AGACGTGTGCTCTTCCGATCTTGTGGCCCTGAACAGGAACTTGAACTCGACCTTCAGATTGATGTCGGAACTGTAGAACTGTGAAC-3'	
Met	CAT	5'-AGACGTGTGCTCTTCCGATCTTGTGCCCGCGTGTGAGGATCGAACTGACGACTTCAGATTGATGTCGGAACTGTAGAACTGTGAAC-3'	
Met	GAA*	5'-AGACGTGTGCTCTTCCGATCTTGTGGCCGAACCCGGATTGAACAGGGACCTTTAGATCGATGTCGGAACTGTAGAACTGTGAAC-3'	
Pro	TGG & CGG & AAG*	5'-AGACGTGTGCTCTTCCGATCTTGTGGGGCTGTCCGGGATTTGAACCCGGGACCTTCGACGATGTCGGAACTGTAGAACTGTGAAC-3'	
Pro	TCA*	5'-AGACGTGTGCTCTTCCGATCTTGTGGCCGACGAAGGTGGAAATTGAACCACTGTGTGCTAGAGATGTCGGAACTGTAGAACTGTGAAC-3'	
Ser	TGA & AGA*	5'-AGACGTGTGCTCTTCCGATCTTGTGGCGTAGTCGGCAGGATTCGAACCTGGCGGGGAGACCCGATGTCGGAACTGTAGAACTGTGAAC-3'	
Ser	CGA (1)*	5'-AGACGTGTGCTCTTCCGATCTTGTGGCGCTGTGAGCAGAAATTTGAACCTTGGCCGGGGAACCCGATGTCGGAACTGTAGAACTGTGAAC-3'	
Ser	CGA (2)*	5'-AGACGTGTGCTCTTCCGATCTTGTGGCTACGAACAGAAATTCGAACCTGTGCGGGGAACCCGATGTCGGAACTGTAGAACTGTGAAC-3'	
Ser	GCT	5'-AGACGTGTGCTCTTCCGATCTTGTGGCAGAGATGGGATTCGAACCCAGCGTGCAGAAAGCAGATGTCGGAACTGTAGAACTGTGAAC-3'	
Thr	AGT & TGT (1) & CGT (1)*	5'-AGACGTGTGCTCTTCCGATCTTGTGAGGCCCCCGCTGGGATTCGAACCCAGGATCTCTGTTGATGTCGGAACTGTAGAACTGTGAAC-3'	
Thr	TGT (2) & CGT (2)*	5'-AGACGTGTGCTCTTCCGATCTTGTGAGGCCCCAGCGAATTCGAACCTGCGACCCCTGGTTGATGTCGGAACTGTAGAACTGTGAAC-3'	
Thr	CCA*	5'-AGACGTGTGCTCTTCCGATCTTGTGAGGACGAGACGGGTTCGAACCCGATCTTCGGTTGATGTCGGAACTGTAGAACTGTGAAC-3'	
Tyr	GTA*	5'-AGACGTGTGCTCTTCCGATCTTGTGCTCTTGAGCCCGGAATTCGAACCAAGCACTTCTAGAGATCGATGTCGGAACTGTAGAACTGTGAAC-3'	
Val	CAC & TAC (1) & AAC (1)*	5'-AGACGTGTGCTCTTCCGATCTTGTGGTGTTCGCGCCGGATTCGAACCCGGGACCTTTCGCTGATGTCGGAACTGTAGAACTGTGAAC-3'	
Val	AAC (2)	5'-AGACGTGTGCTCTTCCGATCTTGTGGCTAAGGTAAGTGAACCCAGGACCTTTTGTCATGATGTCGGAACTGTAGAACTGTGAAC-3'	
Val	TAC (2)*	5'-AGACGTGTGCTCTTCCGATCTTGTGGTTCACATGGGGCTCGAACCCAGGACCTTTCGCTGATGTCGGAACTGTAGAACTGTGAAC-3'	

Mus musculus

cytosolic

Supplementary Table 6: Comparative table of exemplary tRNA quantification methods

Name, if applicable	Author	Published	Method	Amount	Parallel quantification of all tRNAs?	Enzymatic steps	DOI
min-tRNAseq	Behrens <i>et al.</i>	2021	RNAseq	50-200 ng total tRNA	Yes	6	10.1016/j.molcel.2021.01.028
OTTER	Nagai <i>et al.</i>	2021	In gel fluorescence	2 µg total RNA	No	1	10.1261/jrna.076489.120
AORNAseq	Hu <i>et al.</i>	2021	RNAseq	50 ng total tRNA	Yes	10	10.1038/s41587-021-00874-y
QuantM-RNA-seq	Pinkard <i>et al.</i>	2020	RNAseq	1 µg deacetyl. total RNA	Yes	5	10.1038/s41467-020-17879-x
MST	Jacob <i>et al.</i>	2019	Microscale Thermophoresis	1-2 µg tot. tRNA	No	0	10.1002/anie.201814377
tRNA microarray	Polte <i>et al.</i>	2018	Microarray	1-2 µg fluoro labeled tRNA	No	1	10.1186/s12864-019-5864-1
Hydro-tRNAseq	Gogakos <i>et al.</i>	2017	RNAseq	20 µg total RNA	Yes	6	10.1016/j.jcelrep.2017.07.029
YAMAT-seq	Shigematsu <i>et al.</i>	2017	RNAseq	1 µg deac. total RNA	Yes	3	10.1093/nar/gkx005
DM-TGIRT-seq	Zheng <i>et al.</i>	2015	RNAseq + alkB	100 ng demethyl. tRNA or 1 µg tot. RNA	Yes	4	10.1038/nmeth.3478
ARM-seq	Cozen <i>et al.</i>	2015	RNAseq + alkB	1 µg total RNA	Yes	7	10.1038/nmeth.3508
tRNA-seq	Pang <i>et al.</i>	2014	RNAseq	-	Yes	6	10.1093/nar/gkx945
tRNA-microarrays	Dittmar <i>et al.</i>	2006	Microarray	1 µg fluoro labeled total RNA	No	1	10.1371/journal.pgen.0020221
-	Dong <i>et al.</i>	1996	2D gel radiolabel	5 µg total RNA	No	0	10.1006/jmbi.1996.0428
-	Ikemura <i>et al.</i>	1982	2D gel radiolabel	-	No	0	10.1016/0022-2836(82)90250-9
Our method	Kristen <i>et al.</i>	-	Hybridization + NGS	5-25 ng total tRNA	Yes	1	-

4.4.2 In-Depth Immune-Oncology Studies of the TME in mice model

The prognosis of cancer patients is strongly influenced by the distribution of immune cells within the tumour microenvironment. Of particular interest are both the spatial relationships between tumour and immune cells and the phenotypic characterisation of these cells.²⁷⁵ Traditional *ex vivo* analysis methods, such as flow cytometry and immunohistochemical staining, have limitations in simultaneously providing information on both cellular phenotypes and their spatial arrangement within the tissue. To overcome these limitations, the application of chip cytometry developed by Zellkraftwerk GmbH was investigated and compared with established techniques. This novel method uses iterative staining and bleaching cycles on tissue samples, allowing the analysis of multiple markers on a single sample, potentially providing a more comprehensive view of the tumour microenvironment.²⁷⁵

For the comparative study, Jonathan Schupp and Niklas Zimmer from the Department of Dermatology at the University Hospital in Mainz, used a humanised mouse model. This model was created by injecting melanoma cells and human immune cells into mice to mimic the human immune system. In collaboration with Arne Christians from Zellkraftwerk GmbH, they analysed tissue samples using chip cytometry and conventional methods. In parallel, the project investigated tasquinimod, a potential immunomodulator known to alter the polarisation of tumour-associated macrophages from the M2 to the M1 phenotype.^{275,276}

My contribution to this project took place during the project preparation and development phase and focussed on the preparation of a liposomal drug delivery system for tasquinimod. I evaluated the compatibility of tasquinimod with established liposomal formulations and assessed its impact on the liposomal properties. I also developed a liquid chromatography–mass spectrometry-based quantification method to determine the entrapment efficiency of liposomal tasquinimod. The results revealed that tasquinimod compromised particle stability, making it incompatible with the liposomal formulations tested (data not shown). Consequently, Niklas Zimmer and Jonathan Schupp administered tasquinimod without liposomal drug delivery. Mark Helm from the IPBW in Mainz, Andrea Tüttenberg and Helmut Jonuleit, both from the Department of Dermatology at the University Hospital in Mainz, are the principal investigators of the research project.²⁷⁵

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Article

In-Depth Immune-Oncology Studies of the Tumor Microenvironment in a Humanized Melanoma Mouse Model

Jonathan Schupp¹, Arne Christians², Niklas Zimmer¹, Lukas Gleue³, Helmut Jonuleit¹ , Mark Helm³ and Andrea Tuettenberg^{1,*}¹ Department of Dermatology, University Medical Center, Johannes Gutenberg University Mainz, 55131 Mainz, Germany; jonathan.schupp@kgu.de (J.S.); Niklas.Zimmer@unimedizin-mainz.de (N.Z.); jonuleit@uni-mainz.de (H.J.)² Zellkraftwerk GmbH, 04103 Leipzig, Germany; Arne.Christians@bruker.com³ Institute of Pharmacy and Biochemistry, Johannes Gutenberg University Mainz, 55128 Mainz, Germany; lugleue@uni-mainz.de (L.G.); mhelm@uni-mainz.de (M.H.)

* Correspondence: antuette@uni-mainz.de



Citation: Schupp, J.; Christians, A.; Zimmer, N.; Gleue, L.; Jonuleit, H.; Helm, M.; Tuettenberg, A. In-Depth Immune-Oncology Studies of the Tumor Microenvironment in a Humanized Melanoma Mouse Model. *Int. J. Mol. Sci.* **2021**, *22*, 1011. <https://doi.org/10.3390/ijms22031011>

Academic Editor: Peter C. Hart

Received: 30 December 2020

Accepted: 18 January 2021

Published: 20 January 2021

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Abstract: The presence and interaction of immune cells in the tumor microenvironment is of significant importance and has a great impact on disease progression and response to therapy. Hence, their identification is of high interest for prognosis and treatment decisions. Besides detailed phenotypic analyses of immune, as well as tumor cells, spatial analyses is an important parameter in the complex interplay of neoplastic and immune cells—especially when moving into focus efforts to develop and validate new therapeutic strategies. Ex vivo analysis of tumor samples by immunohistochemistry staining methods conserves spatial information is restricted to single markers, while flow cytometry (disrupting tissue into single cell suspensions) provides access to markers in larger numbers. Nevertheless, this comes at the cost of sacrificing morphological information regarding tissue localization and cell–cell contacts. Further detrimental effects incurred by, for example, tissue digestion include staining artifacts. Consequently, ongoing efforts are directed towards methods that preserve, completely or in part, spatial information, while increasing the number of markers that can potentially be interrogated to the level of conventional flow cytometric methods. Progression in multiplex immunohistochemistry in the last ten years overcame the limitation to 1–2 markers in classical staining methods using DAB with counter stains or even pure chemical staining methods. In this study, we compared the multiplex method Chipcytometry to flow cytometry and classical IHC-P using DAB and hematoxylin. Chipcytometry uses frozen or paraffin-embedded tissue sections stained with readily available commercial fluorophore-labeled antibodies in repetitive cycles of staining and bleaching. The iterative staining approach enables sequential analysis of a virtually unlimited number of markers on the same sample, thereby identifying immune cell subpopulations in the tumor microenvironment in the present study in a humanized mouse melanoma model.

Keywords: Chipcytometry; multiplex immunohistochemistry; melanoma; humanized mice; tumor microenvironment; flow cytometry; immunohistochemistry

1. Introduction

The tumor microenvironment (TME) is a complex network of interactions between immune cell populations, cancer cells, and vascular and stromal components, which play a critical role in cancer cell growth, disease, prognosis, and therapeutic approaches. Beside detailed phenotypic analysis of cell types in the tumor microenvironment, including immune, as well as stroma and tumor cells, spatial analysis is also important to unravel the complex interplay of neoplastic and immune cells with and without therapy and to develop and validate new therapeutic strategies [1–4].

Whereas in vitro systems allow functional analysis on a single cell level, the complex interplay of immune reactions in the TME and especially the investigation of new thera-

peutic approaches warrant (i) an efficient *in vivo* system and (ii) suitable monitoring of the TME. In the present study, we used inoculation of immunodeficient mice with a melanoma cell line to establish *in situ* immunomonitoring with the future goal to validate the effects of different therapeutic approaches *in vivo*. Although the use of tumor models in immunocompetent mice can indicate biologic activity of different therapies, many approaches, such as therapies using monoclonal antibodies (mAb), are species- and epitope-specific and require the expression of human receptors. Thus, the utilization of humanized mouse models seems to be reasonable. Several groups used immunodeficient mice transplanted with human tumors and subsequent transfer of human immune cells and therapeutic mAb [5–7]. Although anti-tumor responses can be assessed in these systems, xenoreactivity, and onset of graft-versus-host disease (GvHD) conceal which cellular component or immunological event mediates observed effects. Advanced humanized mouse systems, including transgenic expression of relevant human molecules in immunodeficient mice, are a possible solution to improve the robustness of these models [8]. Expression of human major histocompatibility complex (MHC) class I and/or II molecules on murine cells possibly reduce xenoreactivity and delay onset of GvHD mediated by human leukocyte antigen (HLA)-matched CD8⁺ and CD4⁺ T cells.

Thus, in our study, we used immunodeficient NOD/Scid mice transgenic for HLA-A2 molecules as *in vivo* model system with the focus to perform *in-depth* immunomonitoring to analyze the tumor microenvironment using different approaches, such as flow cytometry, multiplex analysis, and immunohistochemistry.

Using flow cytometry, multiple parameters can be detected on the cell surface and in other cellular compartments [9]. However, concrete localization of cells within the tumor tissue cannot be analyzed using this technique as for analysis, tissues need to be disrupted into single cell suspensions. Additionally, flow cytometry lacks certain flexibility. Samples have to be run in real-time (within 1–3 days). This does not allow for storage and repeated analysis of samples—which is especially important when using valuable clinical study samples. The set of markers for analysis needs to be predefined and has to be established before the sample is processed. Thus, flow cytometry cannot be easily adjusted if new clinical questions or hypotheses arise during the period of analysis or the long term follow up of patients. In addition, as tumor tissue has to be digested before analysis, the cellular content may be affected in general, making it difficult to obtain sufficient cell numbers for detailed analysis. Furthermore, cell viability can be affected through sample preparation methods leading to a rapid decline in cell numbers and viability after sample collection.

Conventional IHC is commonly used for diagnostic issues conserving spatial information, but suffers from certain limitations, such as only permitting the labeling of single markers on one tissue section. So far, the number of cluster of differentiation (CD) markers that can be assessed on one tissue section by routine immunohistochemistry (IHC) is limited, making multiplex analysis, particularly of rare cells with a distinct phenotype, such as regulatory T cells or macrophages difficult. Often it is not possible to stain human immune cell subpopulations in tissue samples with the full marker profile needed for detailed phenotyping.

In contrast, multiplex-immunofluorescence is a technique that strains more than 100 biomarkers consecutively on the same cell [10,11].

Slide-based cytometry was first introduced as an alternative to flow cytometry. This method is based on automated epifluorescence microscopy of cells that are immobilized on a solid surface. Since then, this concept has been enhanced with iterative restaining and photobleaching of fluorochromes as newly introduced components. The herein used platform (Chipcytometry) is a modified approach to slide-based cytometry developed at the Hannover Medical School [12]. Based on microfluidic chips containing cell-adhesive surfaces that allow long-term storage and repeated staining and washing steps by simple fluid exchange, Chipcytometry allows for iterative multiplex immunofluorescence staining and analysis of a virtually unlimited number of biomarkers on a single tissue section, including cell–cell interactions and cellular functions. AI-based cell recognition of stained

cells allows for the generation of quantitative, “flow-like” single cell data, while also considering the spatial information of tissue structure, like in classical immunohistochemistry methods. As the tissue is preserved during and after the staining procedure, the sample can be stored and re-analyzed for up to 20 months with additional markers at any time, even if new or additional questions are to be addressed.

So far, Chipcytometry has been applied several times, especially to study circulating tumor cells, cerebrospinal fluid cells, and mesenchymal stromal cells [13–15]. Nevertheless, recently, this technique has been proved to be also suitable to analyze tissue samples originating from the colon, lung, and brain [16–18]. Of note, skin cancer, especially melanoma, has not been studied so far. Whereas the treatment success with checkpoint inhibitors, especially in melanoma, has been ground-breaking both for progression-free and overall survival, only part of the patients show a good response, whereas others do not benefit from this treatment. Many efforts are made to investigate biomarkers to identify and characterize parameters in peripheral blood or tumor microenvironment from melanoma patients to solve this question [19]. In the present study, we investigated immunophenotyping of melanoma, and report on its comparison to flow cytometry and IHC in a relevant preclinical humanized mouse model of melanoma.

Being aware that the three different methods described here are different in the preparation of tissues and cells, the present study wants to point out the advantages and disadvantages of every single method and its possible application in preclinical and clinical studies to study the TME. Whereas the standard immune histochemistry allows the detection and co-localization of cells and is a cost- and time effective and applicable method for standard diagnostic although displaying a high inter-observer variability, mostly only a few markers are stained at a time [11]. In contrast, flow cytometry allows a broader panel of phenotypic markers and detailed analysis of cellular subpopulations. Nevertheless, after tissue sample preparation, spatial information and cellular co-localization are lost. Herein, a modified multiplexed approach to slide-based cytometry might be a useful and complementary tool for analysis of tumor tissue, due to the possibility of iterative staining, imaging, and bleaching cycles, especially in the context of translational research.

2. Results

2.1. Humanized Mouse Melanoma Model

In the present study, we used the inoculation of immunodeficient mice with a human melanoma cell line grown in the presence of a humanized immune system, as shown before to perform comparative analyses of the tumor microenvironment by multiplex staining, immunohistochemistry, and flow cytometry (Figure 1) [11]. This mouse model is particularly relevant, because tumor progression critically depends on the state of human immune cells. The latter were PBMC preparations from HLA-A2⁺ healthy human donors. Tissues of interest for analysis were the melanomas induced. Characterization of molecular markers in these tissues is of high interest for the characterization of various immunotherapeutic treatments [20,21].

Human Ma-Mel-19 melanoma cells injected s.c. into the back of immunodeficient NOD Scid tgHLA-A2.1 mice substituted with human immune cells led to the formation of a xenograft tumor over the course of more than 40 days (Figure 2).

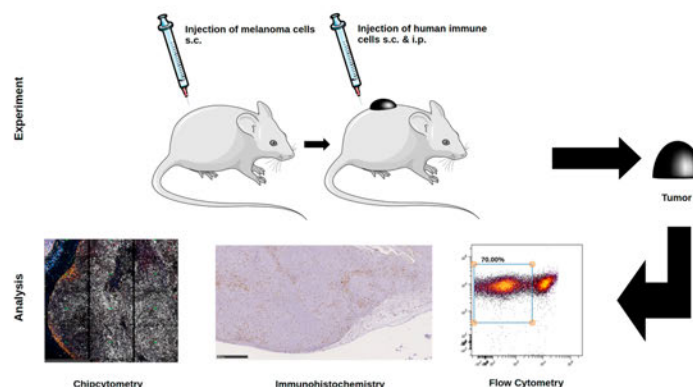


Figure 1. Schematic of the melanoma model in the humanized mice. After subcutaneous injection of human Ma-Mel-19 melanoma cells in NOD Scid tgHLA-A2.1 mice, human immune cells were injected at the tumor site s.c. and i.p. After three more weeks, ex vivo analysis of tumor was performed using Chipcytometry, immunohistochemistry, and flow cytometry.

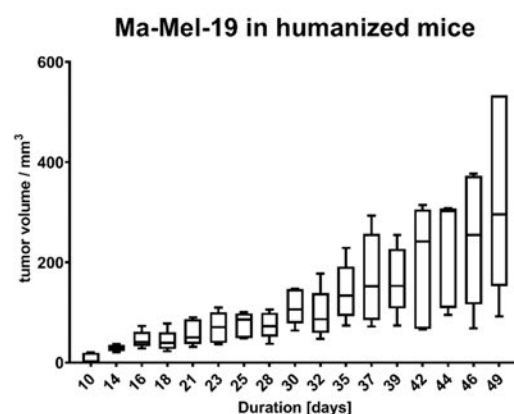


Figure 2. Tumor growth of Ma-Mel-19 cells in humanized mice. Mice were humanized on day 23. Data are shown as box plots with whiskers at min/max. six animals per group.

2.2. Immunomonitoring Using Different Approaches

In a next step, we compared classical immunohistochemistry to two multiplex methods (Figures 3, A2 and A3). Analysis of the tumor tissue by Chipcytometry, flow cytometry, and IHC-P revealed no significant differences in the broad classification of human immune cells (huCD45⁺) and human T cells (CD45⁺CD3⁺), albeit a high variation in cell percentages was detected by IHC-P (Figure 4A). It is important to mention, that only Chipcytometry and IHC-P can focus exclusively on the tumor mass (Figure 3A,B), whereas in flow cytometry, digestion of the whole tumor sample might lead to detection of PBMC also from the injection site or non-tumor tissue. However, flow cytometry gates on live cells exclusively to avoid false positive marker expression of dying cells or cell remnants (Figure 3C). Further and additional sub-classification into CD3⁺CD4⁺ or CD3⁺CD8⁺ double positive T cells and macrophages (CD45⁺CD68⁺) was only possible by chip and flow cytometry (Figures 4C and 5). There was a significant discrepancy in T cell subset distribution detected when comparing of both methods. Whereas, overall human leukocytes (huCD45⁺),

T cells (CD45⁺CD3⁺) and macrophages (CD45⁺CD68⁺) were comparable, in Chipyctometry 30.9% (SD 6.7%) of all T cells were CD8⁺ and 31.1% (SD 11.7%) were CD4⁺. On the contrary, 67.4% (SD 15.2%) of T cells detected by flow cytometry were CD8⁺ and only 17.5% (SD 7.2%) were CD4⁺. Of note, only three mice could be analyzed via Chipyctometry, due to limitation of material, whereas the sample size in the flow cytometry and IHC-P was ten and twelve, respectively, due to tumor slices with more than one distinct tumor.

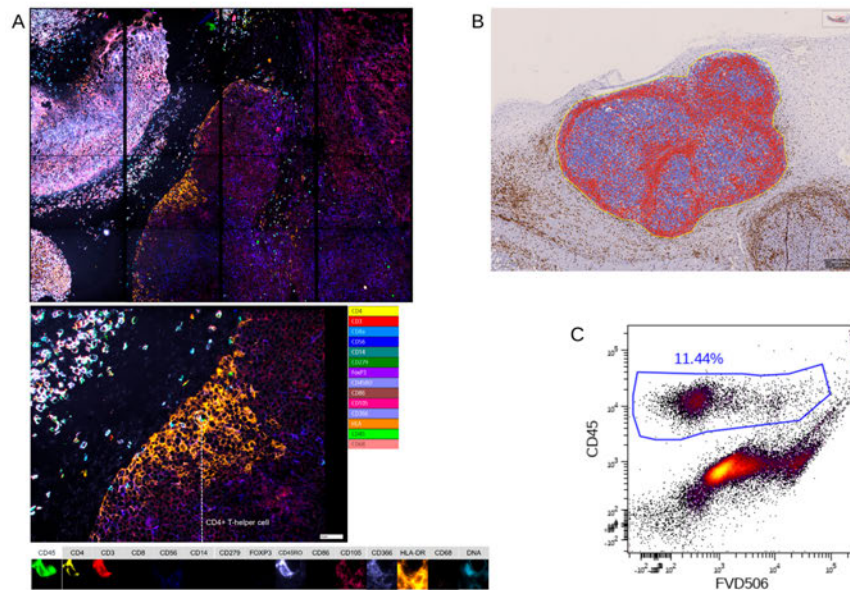


Figure 3. Multimarker analysis of the whole tumor, as well as a single cell in Chipcytometry (A), data analysis of CD45⁺ (positive cells in red, negative cells in blue) cells in immunohistochemistry (B), and gating for all CD45⁺ against a live/dead stain, human immune cells in flow cytometry (C). One typical experiment is shown.

Taking spatial information of Chipcytometry and immunohistochemistry into account, the cell number per square millimeter (Figure 4B) is comparable to cells counted as the percentage of all cells (Figure 4A).

Nonetheless, further analysis of immune cell distribution in the tumor mass and its environment, as well as detection of cell-cell contacts, is feasible using these approaches. As an example, further subpopulations of T cells, such as PD-1⁺/TIM-3⁺ or activated CD45RO⁺ T cells, could be classified by Chipcymetry (see Table 1 and Figure 5). Especially the presence and quantification of PD-1⁺ and TIM-3⁺ T cells may be of future importance, with PD-1 and TIM-3 being important targets molecule in current immunotherapeutic approaches. Thus, changes of the cellular composition in the tumor microenvironment with a focus on CD8⁺PD-1⁺ T cells following, for example, anti-PD-1 therapy can be quantified when using multiplex methods such as Chipcymetry in future experimental setups.

In conclusion, we learned from these data that for further and deep investigations of the TME multiplex analysis would be favorable to investigate in more detail, especially the influence of different systemic treatment approaches with immunomodulatory functions on the composition, but also on the spatial distribution of immune cells in the TME.

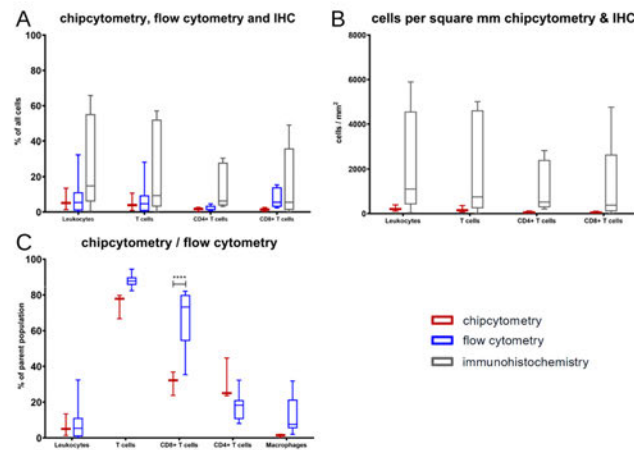


Figure 4. Data comparison of immune cell composition in the tumor microenvironment obtained by Chipcytometry (red), flow cytometry (blue), and immunohistochemistry (grey). Since gating is possible in flow cytometry and Chipcytometry percentages of all cells, respectively, immune cells can be shown (A,C), whereas spatial information is only preserved in Chipcytometry and IHC (B). Data are shown as box plots with whiskers at min/max. **** $p < 0.0001$; Statistical significance was determined using the Šidák's multiple comparison test.

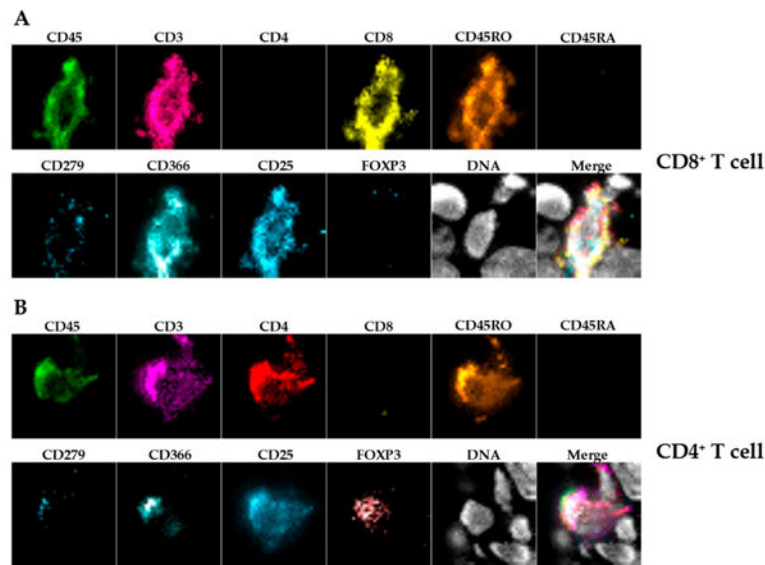


Figure 5. Chipcytometry analysis showing exemplarily the identification of tumor-infiltrating CD8⁺ (A) and CD4⁺ (B) T cells within their spatial context. The lower right picture shows the merged channels.

Table 1. Immune cell subpopulations in untreated tumor-bearing animals detected by Chipcytometry (n = 3).

Marker Definition	Parent Population	Cell Type	% of Parent Population	SD
CD45+	All	immune cells	6.7	5.7
CD3+	CD45+	T cells	74.8	7.1
CD4+	CD45+CD3+	T helper cells	31.1	11.7
CD8+	CD45+CD3+	cytotoxic T cells	30.9	6.7
CD45RO+CD45RA-	CD45+CD3+CD4+	activated/memory t cells	85.2	20.1
CD45RO+CD45RA-	CD45+CD3+CD8+	activated/memory t cells	84.0	24.2
CD4+FOXP3+CD25+	CD45+CD3+CD4+	regulatory T cells	4.8	3.3
CD279+	CD45+CD3+CD4+	PD1+ T cells	24.8	10.3
CD366+	CD45+CD3+CD4+	TIM-3+ T cells	59.2	29.8
CD279+	CD45+CD3+CD8+	PD1+ T cells	16.5	9.7
CD366+	CD45+CD3+CD8+	TIM-3+ T cells	54.7	5.1
CD68+	CD45+	macrophages	1.3	0.9

2.3. Immunomonitoring of the Melanoma Tumor Microenvironment after Tasquinimod Treatment

Previous reports showed that the immunomodulator Tasquinimod is a small molecule with pleiotropic effects on the tumor microenvironment. Besides its influence on neo-angiogenesis, it can modulate tumor-associated macrophages by switching their rather tolerogenic M2-phenotype into an inflammatory M1-phenotype [22,23]. To analyze the effects of Tasquinimod on immune cells and tumors in vivo using our comparative approach for immunomonitoring, tumor-bearing mice were treated with Tasquinimod in different doses (5 mg/kg body weight (high dose) and 1 mg/kg body weight (low dose)). Weekly treatment significantly hampered tumor growth in both doses applied when compared to untreated mice after three weeks of therapy (Figure 6).

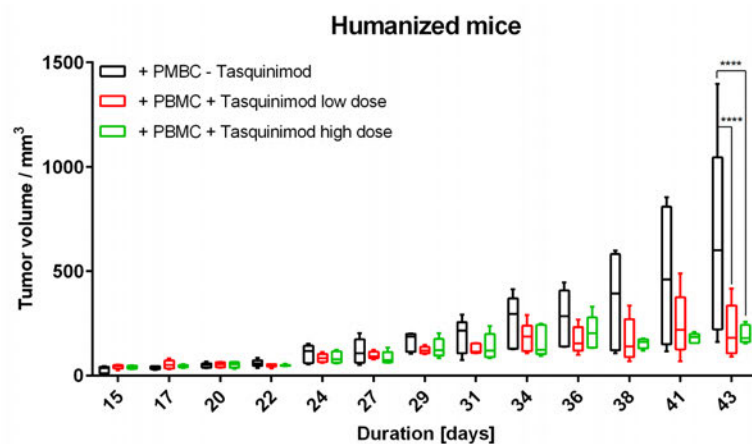


Figure 6. Tumor growth of Ma-Mel-19 cells in humanized mice under treatment with Tasquinimod (high: 5 mg/kg body weight and low: 1 mg/kg body weight). Mice were humanized on day 23. Tasquinimod was applied on day 24, 31, and 37. Data are shown as box plots with whiskers at min/max. **** $p < 0.0001$; Statistical significance was determined using the Tukey's multiple comparison test. Five animals per group.

Since the effects of Tasquinimod were only visible in the presence of human immune cells (Figure A1), we next analyzed the tumor microenvironment for immune cell markers in more detail using multiplex analysis.

Herein we detected significantly higher frequencies of CD8⁺ T cells in tumors of mice treated with Tasquinimod when compared to untreated tumor-bearing mice, which only

showed CD8⁺ T cells in the marginal zones of the tumor (Figure 7A). Furthermore, more CD68⁺ macrophages were present in Tasquinimod treated tumors. In contrast, untreated mice displayed higher frequencies of CD4⁺, HLA-DR⁺, and CD279⁺ T cells in the tumor microenvironment (Figure 7B).

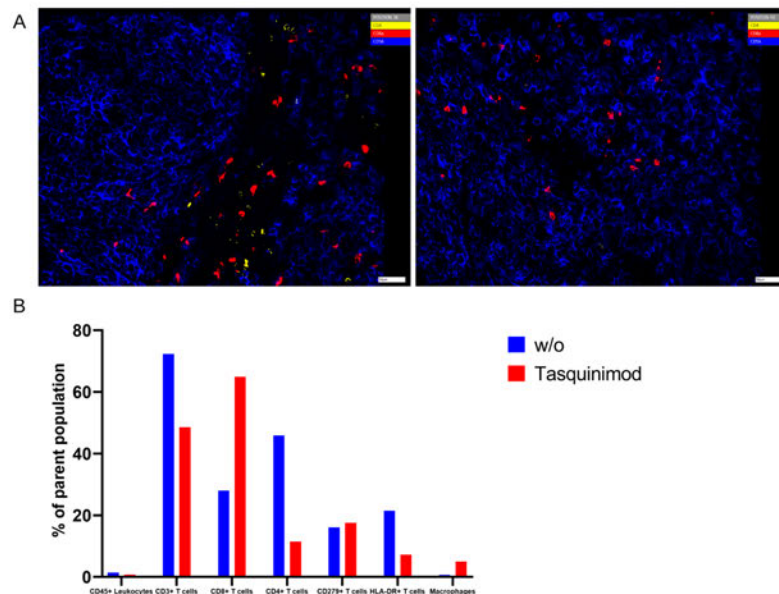


Figure 7. (A) Chipcytometry analysis showing exemplarily the identification of tumor-infiltrating CD8⁺ (red) and CD4⁺ (yellow) T cells within their spatial context with Tasquinimod (right) or without (left). (B) Percentage of cell populations in a representative section of the TME. One experiment out of two (*w/o*) or out of four (treatment) is shown.

These results strengthen the idea, that the modulation of immune cells using systemic therapies is important for effective therapeutic responses, which can be analyzed in a comprehensive way on single cell level, as well as in the spatial context when using multiplex analysis, such as Chipcytometry. Our results are a first proof-of-concept that co-localization of cells and their organization in tissues constitute valuable information, especially in the context of different therapeutic approaches.

3. Discussion

The prognostic and therapeutic value of the tumor microenvironment (TME) in various cancer types is of major interest. Nevertheless, the complexity of the tumor microenvironment acts as a major obstacle in understanding immune regulation of anti-tumor responses, as well as responses to different immunotherapeutic strategies. As the number of clinical trials significantly increased in the last years and the development of markers predictive of therapy response continuously expands, there will be a growing need for (i) relevant preclinical models and (ii) adequate and comprehensive immunomonitoring of tissue specimens in preclinical, as well as in clinical studies.

Characterization of numerous immune cell subpopulations in a tissue sample is only possible with multiplex methods using 20 or more markers. Although fluidics-based systems like flow cytometry or mass cytometry do offer the required depth of markers, simple number games or intensity of markers do not offer the complete perception of cellular processes. Co-localization of cells and their organization in tissues constitute valuable

information that is routinely lost during sample preparation. Multiplex immunohistochemistry or immunofluorescence methods leave spatial information of cells intact and deliver sufficient markers per cell at the same time.

Another aspect is the rarity of tissue samples derived either from animal experiments or from patients. Long time storage, labeling for multiple immunostainings, and several cycles of analysis in the very same cell or tissue section are gaining, thereby inestimably in value.

The herein used Chipcytometry technique offers those two characteristics mentioned and can be conveniently conducted with fluorescence-labeled antibodies that are used for flow cytometry or immunofluorescence. Of note, there are several other platforms, such as PerkinElmer or Roche, which use multi-epitope ligand cartography (MELC) to subject samples to multiple cycles of fluorescent staining, imaging, and photobleaching, very similar to the proposed technique.

In this work, we compared data obtained from three different methods in their ability to identify human immune cells in melanoma *in vivo* in a relevant preclinical model. Being aware that the three different methods described here are different in terms of sample preparation, all methods use the xenograft tumors as the tissue source. Chipcytometry uses frozen or paraffin-embedded tissue, flow cytometry uses viable or fixed cells, and IHC uses formalin-fixed paraffin-embedded tissue. The different preparation and fixation are mandatory to employ the three methods described in this study without changing the cell composition of the tissue. The main objective of the current study was to focus on the ability of each method to resolve human immune cells in the tumor microenvironment to identify the most suitable method for future studies using immunotherapeutic strategies and/or nanoparticle-based therapies.

In the present proof-of-concept study, we concentrated mainly on T cells and on macrophages being important effector cells in the tumor microenvironment. Nevertheless, this panel will be extended in future approaches.

IHC-P was limited to immune cells (huCD45⁺) and T cells (huCD3⁺) because staining using two different anti-human CD68 antibodies caused unspecific staining in the control xenografts without human immune cells (data not shown), showing the limitations of this approach. Furthermore, we stained for huCD4 and huCD8, but refrained from including those single positive cells in the further analysis as CD4⁺ or CD8⁺ T cells, since CD4 and CD8 expression is not exclusive for T cells [24,25].

The comparison showed that, in general, the results of those three methods are comparable without differences among those few markers that are accessible by all methods. At the end of the experiments in mice, T cells were the major immune cell type extracted. Those changes in contrast to the cell composition at the time of PBMC injection arose over time in the xenograft model.

Nevertheless, there was a significant discrepancy between flow cytometry and Chipcytometry regarding the ratio of CD8⁺ and CD4⁺ T cells. This ratio was far higher in flow cytometry than in Chipcytometry. One advantage of Chipcytometry is the preservation of spatial information. As in IHC-P, cell analysis is only performed in the tumor mass and not in the surrounding tissue. This is especially important as it is shown that T effector cells are rather localized in the marginal zones of the tumor and do not enter the tumor tissue itself. Detected immune cells in Chipcytometry, thus have been migrating actively into the tumor. In contrast, in flow cytometry, the whole tumor (including mouse skin, tissue and possibly the PBMC injection site) is digested and analyzed. In sum, Chipcytometry rather shows the T cell ratio inside the tumor, whereas flow cytometry shows the T cell ratio in the whole tissue sample.

Several different multiplexed tissue imaging techniques besides the fluorescent-based strategy (PerkinElmer, Roche, Zellkraftwerk), such as DNA barcoding-based (NanoString, Ultivue, Akoya) or metal-based (Fluidigm or IonPath) approaches, have been developed in the last years all of them providing a comprehensive view of the composition of the TME by labeling multiple markers on limited samples, thus also providing insight into tumor

pathogenesis, as well as responsiveness to therapy [11]. Besides this, every technology has its limitations, such as being limited in the visualization of co-localized biomarkers, being time-consuming or costly, and thus, not applicable for every day.

The multiplex technique used in this study combines the advantages of flow cytometry and microscopy, offering the possibility to analyze more than 100 biomarkers consecutively on the same cell, also allowing the consecutive use of different staining protocols. In addition, it provides a platform that delivers the complete pipeline from staining to high dynamic range imaging to analysis. This allows the analysis of cells that can only be identified by marker combinations and the locating of rare cells in tissue samples. Although several new techniques of cell stabilization have been published in recent years, marker stability on and in cells remains very limited (maximum 12–72 h after sampling). Conventional flow cytometry requires fresh samples for biomarker development, and the cryopreservation of immune cells leads to significant changes in biomarker signatures. In Chipcytometry, the cells are fixed on coated glass surfaces, thereby yielding remarkable long-term stability of the surface and intracellular biomarkers (minimum 24 months after sampling), which is very useful for biobanking of precious or rare samples. In contrast to conventional flow cytometry, where samples are lost during analysis, samples are stored on small microfluidic chips, where they can be reused and assayed several times again with new and additional markers at any time new or additional questions are to be addressed.

Limitations of our study clearly includes the small sample size, as well as its exploratory nature. To analyze melanoma tumor micromilieu, the tissue had to be prepared in different ways. Due to this and the limited material, tumors were used from the same experimental groups. Nevertheless, we could only compare the same samples for flow cytometry and immunohistochemistry. We also did not assess further therapy outcomes with Tasquinimod on cellular levels in more depth. As tumor volume was significantly reduced in Tasquinimod treated animals, we were not able to extract enough tissue to perform a comprehensive comparative analysis for cellular composition and spatial distribution, which is a question we would like to expand on in future studies with adequate sample sizes.

To sum up, depending on the goal of the clinical or research questions, all techniques have their pros and cons. Thus, for each study or experiment, it is necessary to carefully consider which method is suited best. The present study wanted to point out the advantages of every single method and its possible application in preclinical and clinical studies.

Clearly, in routine pathology (where few single cell markers can be enough to identify certain malignancies) classical IHC-P is sufficient. This method is commonly used as an important diagnostic, highly practical, available, and cost-effective diagnostic and prognostic device by staining one or two markers, being able to deliver spatial information. Nevertheless, IHC does allow for multiplex staining. Without an extensive marker panel, it misses the opportunity to gain important information from patient samples and their complex immune microenvironment also in the long term run in both research and clinical settings.

Flow cytometry has its power in cell throughput, the possibility to sort and to detect rare cells with a high marker resolution when spatial information is not mandatory, e.g., blood samples. In tissues, it does not consider the tissue architecture. Thus, an in-depth analysis of different areas of interest in the tumor microenvironment is not possible.

Each time spatial information must not be lost, and high amounts of cellular markers are needed simultaneously, like in tumor biopsies, multiplex methods that can be employed on tissue samples are superior to IHC-P regarding multiplex and to flow cytometry in regard to spatial information.

Chipcytometry delivers as a single method more information in the very same tissue section than both of the other methods, IHC-P and flow cytometry, combined (Figure 3), and thus, is a powerful discovery tool with the ability to investigate the phenotype of rare immune cells in the TME, thus allowing comprehensive studies of cell composition, cell–cell interaction and cellular function with a potential diagnostic benefit. It offers quantitative data for tissue analysis comparable to flow cytometry, while also providing an

“IHC-like”, histological view of the tissue structure. It is, therefore, suitable for in-depth immuno-oncology studies, including different therapeutic approaches. Another advantage compared to flow cytometry is that cells once fixated can be analyzed for up to another 20 months with additional markers at any time. Chipcytometry is mainly limited by its availability and cost limitations since staining, and data acquisition requires automation.

Importantly, in the current study, we did not want to address different treatment modalities. We wanted to compare different analysis methods to study in-depth the tumor microenvironment in an adequate preclinical model, which subsequently, in further studies, allows translation into the clinical settings.

To our knowledge, this is the first approach to combine a relevant preclinical humanized mouse model with different methods to analyze TME. In our hands, the introduced human melanoma cells establish a tumor environment relatively close to the known environment of human melanomas. Cytokines and chemokines produced within the TME attract human immune effector cells being the targets for immunological treatment strategies. First hints are given by the results of the Tasquinimod treatment group, herein only mice substituted with human PBMC showed tumor regression. In further studies, this approach will help to further validate different treatment modalities, especially in the context of immunotherapeutic strategies, where cellular compositions, and more importantly, their distribution (cold and hot tumors) play an important role. Of note, tumors can have the same overall cellular composition (e.g., shown in flow cytometry), but in cold tumors immune cells do not invade the tumor itself in contrast to hot tumors. Therefore, the spatial information, in our opinion, is of great relevance to validate therapeutic responses, and thus, was the focus of our study.

Further development of the multiplexed techniques resulting in high-throughput and standardized quantitative analysis for high reproducibility, as well as better cost-effectiveness, will result in methods with great potential in translational research defining prognostic or predictive cellular biomarkers in different tumor entities with or without treatment.

4. Materials and Methods

4.1. Mice

Animal experiments were approved by local authorities (G 15-1-070, Landesuntersuchungsamt Rheinland-Palatinate, Germany). NOD.Cg-Mcph1^{Tg(HLA-A2.1)1Enge}Prkdc^{scid}/DvsJ mice (#006609) acquired from The Jackson Laboratory (Bar Harbor, USA) were injected subcutaneously (s.c.) with 2×10^6 Ma-Mel-19 melanoma cells at an age older than 8 weeks. Tumor volume was measured using a caliper and the formula $V = (\text{length}/2) \times (\text{width}^2)$. After randomization 20×10^6 human PBMC were injected s.c. and intraperitoneally (i.p.) each. Tasquinimod (#S7617, Selleckchem, Munich, Germany) was applied at doses of 5 mg/kg or 1 mg/kg weekly s.c. at the tumor site in a 1:4 mixture of DMSO (#D2650, Merck, Darmstadt, Germany) and NaCl solution (0.9%) (#3200910, B. Braun, Melsungen, Germany). The control group received the mixture without the compound. After 3 weeks of treatment, the animals were sacrificed, and ex vivo analysis of the tumors was performed. All animals were housed under specific pathogen-free conditions in the central animal facility of the Johannes Gutenberg-University in Mainz, and experiments were performed in accordance with relevant laws and institutional guidelines.

4.2. Cell Lines and Cell Culture

The Ma-Mel-19 cell line originates from a (sub)cutaneous biopsy of stage IV superficial spreading melanoma from a 62-year-old female patient, harbors a B-Raf V600E mutation, and is N-Ras wild-type [26]. The cell line has been tested and authenticated at Leibniz Institute (DSMZ) in Braunschweig, Germany, using DNA profiling lastly in May 2020. Generated STR profiles were matching the STR reference profile of respective parental cell lines from cell banks ATCC, HPACC, JCRB, RIKEN, KCLB, EMBL, and DSMZ. The cell line was cultured with RPMI-1640 (#31870, Thermo Fisher Scientific, Waltham, MA,

USA) supplemented with 10% FBS (#10500064, Thermo Fisher Scientific, Waltham, MA, USA), 1% GlutaMAX™ (#35050038, Thermo Fisher Scientific, Waltham, MA, USA) and 0.1% primocin (#ant-pm-2, InvivoGen, San Diego, CA, USA). Cells were detached via Trypsin-EDTA (#T3924, Merck, Darmstadt, Germany) for 5 min every 3 to 4 days. The Ma-Mel-19 cell line was authenticated at Eurofins Genomics (Ebersberg, Germany) in May 2020. The resulting STR profiles were matched with the online databases of the German collection of microorganisms and cell cultures (<http://www.dsmz.de/de/service/services-human-and-animal-cell>) and Cellosaurus database (<https://web.expasy.org/cellosaurus/>) references.

4.3. PBMC Isolation

Buffy coats were obtained from healthy volunteers, with approval by the local ethical committee (Landesärztekammer Rhineland Palatine No. 837.019.10 (7028), approved on 4 March 2010). PBMC were isolated using density gradient centrifugation with Biocoll Separating Solution (#L6115, Merck, Darmstadt, Germany).

4.4. Flow Cytometry

Tissue samples were digested by incubation with Accumax (#00-4666-56, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature followed by manual disruption and passage through a 40 µm Cell strainer (#732-2757, VWR International, Radnor, PA, USA). Single cell suspensions were treated either with Foxp3/Transcription Factor Staining Buffer Kit (#00-5523-00, Thermo Fisher Scientific, Waltham, MA, USA) (T cell panel) or fixed with 1% PFA (#0335.1, Carl Roth, Karlsruhe, Germany) in DPBS (#14190-094, Thermo Fisher Scientific, Waltham, MA, USA) and stained in 20 µL/sample FACS buffer containing 0.5% HSA (#10530a/96, CSL Behring, Marburg, Germany), 1 mM EDTA (#A3553, AppliChem, Darmstadt, Germany), 10 µg/mL human IgG (#EU/1/08/446/001, CSL Behring GmbH, Marburg, Germany) in DPBS (#14190-094, Thermo Fisher Scientific, Waltham, MA, USA).

The following antibodies were used to stain cells in flow cytometry experiments: Anti-human CD3-APC-Fire750 (#344840, BioLegend, San Diego, CA, USA), anti-human CD4-PE-Vio770 (#130-100-454, Miltenyi Biotech, Bergisch Gladbach, Germany), anti-human CD45-VioBlue (#130-092-880, Miltenyi Biotech, Bergisch Gladbach, Germany), anti-human CD68-Brilliant Violet 711 (#565594, BD Biosciences, Franklin Lakes, NJ, USA), anti-human CD8-BV711 (#344734, BioLegend, San Diego, CA, USA) and fixable viability dye eFluor™ 506 (#65-0866-14, Thermo Fisher Scientific, Waltham, MA, USA). All antibodies were used in 20× dilutions, the viability dye in 200× dilution.

Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed using Cytobank [27].

4.5. Immunohistochemistry

Tumor specimens were immunohistochemically analyzed for infiltration with CD45⁺ and CD3⁺ immune cells. Tumor sections (4 µm) were prepared from formalin-fixed, paraffin-embedded tissue. After de-paraffinization and rehydration, slides were boiled. For CD45 stains, slides were boiled in Target Retrieval Solution, Low pH (#K800521-2, Agilent, Santa Clara, CA, USA). For CD3, CD4, and CD8 stains, slides were boiled in Target Retrieval Solution, High pH (#K800421-2, Agilent, Santa Clara, CA, USA). Endogenous peroxidase activity was blocked by Peroxidase-Blocking Solution (#S2023, Agilent, Santa Clara, CA, USA) for 5 min. Sections were blocked with Normal Horse Serum Blocking Solution (#S-2000-2, Vector Laboratories, Burlingame, CA, USA). Anti-human CD45 (clone M0701, Agilent, Santa Clara, CA, USA), anti-human CD3 (#NCL-LCD3-565, Leica Biosystems Nussloch GmbH, Nussloch, Germany), anti-human CD8 (clone M7103, Agilent, Santa Clara, CA, USA) and anti-human CD4 (#104R-26, Merck, Darmstadt, Germany) were applied as primary mAb. For CD3 and CD45, slides were incubated with a secondary biotinylated Horse Anti-Mouse IgG Antibody (#BA-2000-1.5, Vector Laboratories, Burlingame, USA). The antigen detection by a color reaction with 3,3'-diamino-benzidine (#K346711-2, DAB+,

Agilent, Santa Clara, CA, USA) catalyzed by VECTASTAIN® Elite ABC-HRP Reagent, Peroxidase, R.T.U. (#PK-2000-2, Vector Laboratories, Burlingame, CA, USA). For CD4 and CD8, samples were stained with EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse (#K5007, Agilent, Santa Clara, CA, USA). Both methods were counter stained with Mayer's hemalum solution (#109249, Merck, Darmstadt, Germany).

Tissue samples were imaged by the tissue bank of the University Medical Center Mainz using a Hamamatsu NanoZoomer 2.0HT. IHC data were analyzed using QuPath [28].

4.6. Multiplex Staining

Tissue sections with 7 µm thickness were prepared at a standard cryostat and mounted on glass coverslips. The sections were fixed by immersion in ice-cold 100% acetone (#9372.1, Carl Roth, Karlsruhe, Germany) for 5 min, followed by serial immersion in 90% ethanol (#5054.1, Carl Roth, Karlsruhe, Germany), 70% ethanol, and PBS for 3 min at 4 °C, respectively. The glass coverslips with the fixed tissue samples were loaded onto ZellSafe™ Tissue Chips (#28050606/02-010, Zellkraftwerk, Leipzig, Germany), chips were filled up with storage buffer (Zellkraftwerk, Leipzig, Germany) and stored at 4 °C until and in between staining cycles.

The analysis was performed on a ZellScanner One® instrument (Zellkraftwerk, Leipzig, Germany). Each of the 3 tissue samples was stained and imaged with an iterative multiplex staining assay summarized in Table 2. Tissue chips were rinsed with 5 mL of wash buffer before starting an imaging cycle consisting of photobleaching for 40 s per scanned position, followed by imaging of tissue autofluorescence and subsequently antibody staining and imaging of fluorescence signal. Of note, there is no antibody or fluorophore detachment step. Background fluorescence is recorded before each acquisition step. Staining was performed by diluting the antibodies in the storage buffer and pipetting the working solution into the chip flow chamber. After incubation for 15 min at room temperature, the chip was rinsed thoroughly with wash buffer before imaging. This process was repeated until all biomarkers were stained and imaged. In one of the cycles, the cell nuclei were stained by incubating with 0.05 µg/µL Hoechst 33342 (#H3570, Thermo Fisher Scientific, Waltham, USA) in storage buffer for 5 min at room temperature.

Table 2. Multiplex staining Chipcytometry.

No.	Cycle	Marker	Clone	Catalog Number	Fluorophore	Vendor
1	1	CD25	M-A251	555432	PE	BD Biosciences
2	2	FOXP3	236A/E7	12-4777-42	PE	Thermo Fisher Scientific
3	3	CD3	UCHT1	563546	BUV395	BD Biosciences
4	3	CD4	RPA-T4	300530	PerCP-Cy5.5	Biologend
5	3	CD8	RPA-T8	301008	PE	Biologend
6	4	CD14	HCD14	325622	PerCP-Cy5.5	Biologend
7	4	CD56	AF12-7H3	130-113-307	PE	Miltenyi Biotect
8	4	CD68	KP1	sc-20060	AF488	Santa Cruz
9	5	CD45	HI30	304028	PerCP-Cy5.5	Biologend
10	5	CD45RO	REA611	130-113-559	PE	Miltenyi Biotect
11	5	CD45RA	HI100	740298	BUV395	BD Biosciences
12	6	DNA	—	H3570	BUV395+BV421	Thermo Fisher Scientific
13	7	HLA-DR	L243	307606	PE	Biologend
14	8	CD279	EH12.1	560795	PE	BD Biosciences
15	9	CD86	2331 (FUN-1)	555658	PE	BD Biosciences
16	10	CD105	43A3	323206	PE	Biologend
17	11	CD366	7D3	563422	PE	BD Biosciences

In total, analysis on tumor tissue using Chipcytometry was performed six times ($n = 2$ tumor without treatment, $n = 4$ tumor with treatment).

The resulting images were analyzed with ZellExplorer data analysis software (Zellkraftwerk, Leipzig, Germany). Net-fluorescence images were generated by subtracting the autofluorescence from the staining fluorescence image for each cycle and position. Cell segmentation was performed based on the nuclear DNA stain, and for each segmented cell, the fluorescence values for each marker stained in the multiplex assay were calculated, resulting in a single cell resolution quantitative data set. This data set was analyzed by employing a gating strategy to identify cell populations of interest based on biomarker expression [12].

4.7. Statistics

Statistical calculations were performed by GraphPad Prism V6 (GraphPad Software, San Diego, CA, USA). Results were normalized to the untreated samples as indicated. Box and whiskers plots display median with the 25th and 75th percentiles and min to max whiskers. Statistical significance was determined using Tukey's multiple comparison test and Šidák's multiple comparison test as indicated in the figure legends with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5. Conclusions

The present study demonstrates the advantages and disadvantages of each method, and its possible application in preclinical and clinical studies. The great and complementary potential of multiplex marker analysis of tumor tissue should be considered for both scientific questions and diagnostic purposes. As the cellular composition in a tumor, and its microenvironment, is of significant importance for disease progression, prognosis and therapeutic approaches, these techniques help develop, monitor, and validate new therapeutic strategies.

Author Contributions: Conceptualization, J.S., M.H. and A.T.; methodology, J.S., M.H., H.J. and A.T.; validation, J.S. and N.Z.; formal analysis, J.S. and A.C.; investigation, J.S., L.G. and N.Z.; resources, A.T. and A.C.; data curation, J.S. and A.C.; writing—original draft preparation, J.S.; writing—review and editing, J.S., N.Z., M.H., A.C., L.G., H.J. and A.T.; visualization, J.S. and A.C.; supervision, A.T.; project administration, J.S.; funding acquisition, A.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by German Research Foundation (CRC1066, TP B3 (A.T.) and TP A7 (M.H.)) and Wilhelm-Sander Stiftung to A.T.

Institutional Review Board Statement: Animal experiments were approved by local authorities (G 15-1-070, Landesuntersuchungsamt Rheinland-Palatinate, Germany). Buffy coats were obtained from healthy volunteers (excess material from blood donation), with approval by the local ethical committee (Landesärztekammer Rheinland Palatine No. 837.019.10 (7028), approved on 4 March 2010).

Informed Consent Statement: Not applicable (excess material from blood donation).

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: We thank the tissue bank of the University Medical Center Mainz for providing the Hamamatsu NanoZoomer 2.0HT and the core facility histology of the Research Center for Immunotherapy (FZI) Mainz for staining tissue samples. Data are part of the doctoral thesis of Jonathan Schupp.

Conflicts of Interest: A.C. is employee of Zellkraftwerk GmbH, which has developed and is selling Chipcytometry instrumentation and services.

Appendix A

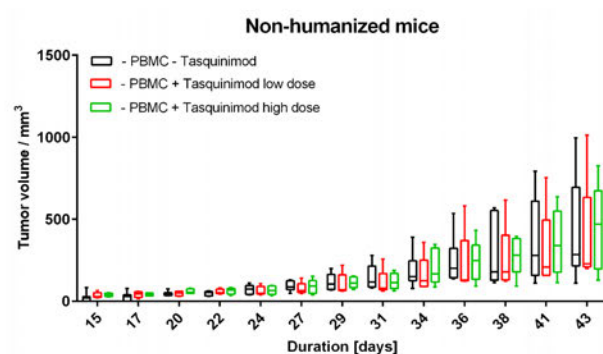


Figure A1. Tumor growth of Ma-Mel-19 cells in immunodeficient non-humanized mice under treatment with Tasquinimod. Tasquinimod was applied on day 24, 31, and 37. Data are shown as box plots with whiskers at min/max. seven animals in control group, five animals in both treated groups.

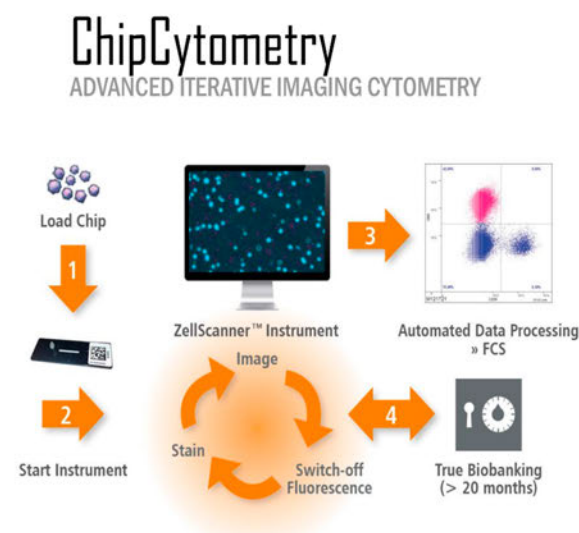


Figure A2. Workflow of ChipCytometry. Cells or tissue samples are loaded onto ZellSafe™ chips (1) and fixed. After starting the process (2), repetitive cycles of staining up to five markers and photobleaching afterwards generates multiplex data that can be analyzed via the ZellExplorer Software and exported as Flow Cytometry Standard files (3). Furthermore, storage times of more than 20 months enable biobanking (4) and reanalysis if necessary or wanted (5).

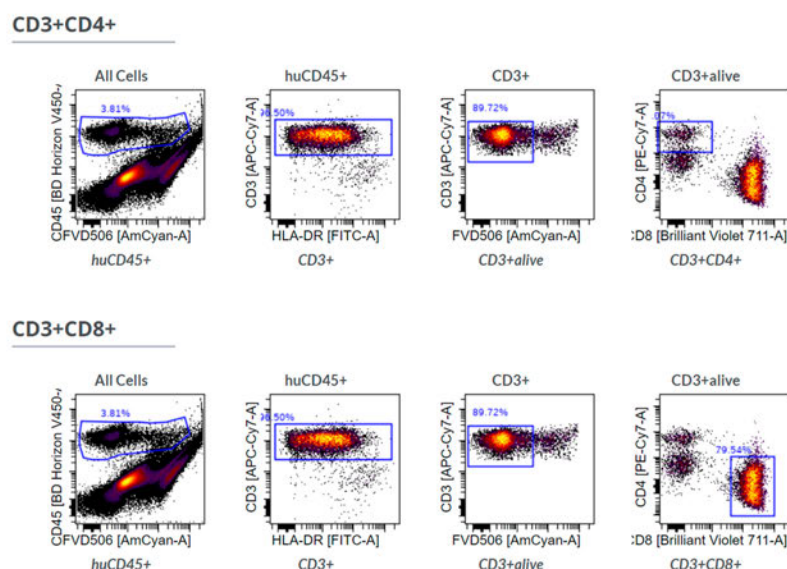


Figure A3. Gating strategy in flow cytometry to identify living CD4⁺ and CD8⁺ T cells in the digested tumor tissue. First, human immune cells are identified by human CD45 expression. Afterwards non-T cells (CD3⁻) and dead cells (FVD506 high) are excluded. Finally, cells are classified by either CD4 or CD8 expression and double negative or double positive cells are excluded.

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5 Conclusion and Perspectives

In the first study, we extended the earlier work of Fritz *et al.* who investigated the fast exchange of liposomal cholesterol with cell membranes. The aim was to analyse the impact of dialkyl chain length on anchoring in liposomal membranes to achieve a deeper understanding of the factors which affect lipid retention in liposomal formulations. *In cellulo* studies have shown a significantly higher membrane anchorage of dialkyl lipids with carbon chain lengths of 16-20 carbon atoms compared to cholesterol derivatives. This result was consistent with the structural characteristics of cholesterol, which has a polycyclic, planar structure and was therefore weaker anchored in the membrane than dialkyl lipids. In contrast, dialkyl lipids carry two fatty acid chains that enabled stronger interactions in the membrane, which contributed to their stronger membrane integration. Our subsequent analysis revealed slightly weaker membrane anchoring of dialkyl lipids with C16 chain lengths in the liposome membrane compared to their C18 and C20 counterparts, although this difference was less pronounced than for cholesterol derivatives. We attributed this effect to a weaker or less favourable interaction of the C16 lipids with the phospholipids of the liposome membrane, which predominantly contained lipids with C18 fatty acids and cholesterol. This hypothesis is supported by the observed inverse relationship between the exchange rate of modified C16 dialkyl lipids and the chain length of the phospholipids in the lipid composition. In this case, the exchange rate decreased when shorter-chain phospholipids were replaced by their longer-chain counterparts.⁶

The observed differences in anchor stability between dialkyl lipids and the influence of lipid composition are crucial for the design of tumour-specific liposomes. This process involves the conjugation of antibodies, peptides and other tumour ligands to modified lipids, and rapid replacement of these components could result in a loss of

cell-specific properties. In addition, the stability of liposomes in the bloodstream is greatly enhanced using PEGylated lipids. Consequently, rapid loss of lipids compromises membrane integrity and shortens the *in vivo* half-life of liposomal drug delivery systems. These findings highlight the importance of a better understanding of lipid interactions and lipid composition for the design of functionalised liposomes.⁶

In our second publication, we presented a methodology for high-throughput screening of novel pH-responsive liposomes. The process started with the preparation of liposomes composed of varying ratios of stabilising and destabilising lipids. Laurdan, a fluorescent dye, was incorporated into the liposome membrane to monitor membrane stability and to identify formulations at 'the edge of stability'. This critical point describes the strong destabilisation of the liposomes due to a small increase in destabilising lipids and is characterised by a sigmoidal change in the fluorescence of laurdan. While this approach is not exhaustive in identifying all potential candidates, it provided a practical method for selecting promising formulations from the original pool of 168 candidates. This is evidenced by the fact that certain lipid compositions seemed highly compatible with this screening approach, whereas other formulations, such as the EPC3 containing liposomes, did not yield suitable candidates. The limited efficacy observed with EPC3 may be due to its heterogeneous composition of different phospholipids. This observation suggests improved sensitivity and reliability of laurdan when applied to homogeneous lipid mixtures.³

The selected two-lipid compositions were characterised for pH sensitivity and retention of the encapsulated model cargo sulforhodamine B, resulting in the identification of five candidates matching the criteria set. Remarkably, all candidates contained the same amount of 30 mol-% destabilising lipid. CHEMS, an ionisable cholesterol derivative, was then added to the candidate formulations. In subsequent incubation experiments, three of the five candidates achieved the predetermined thresholds of 90% retention and 50% release, demonstrating the stabilising effect of CHEMS on liposomal formulations at 'the edge of stability' while maintaining their pH sensitivity. In terms of physicochemical properties, the candidates exhibited the expected changes in zeta potential under acidic conditions, correlating with their lipid composition. Candidates containing the ionisable lipid DODAP showed a shift towards positive values after incubation under acidic conditions, whereas L15c, containing the permanently cationic DOTAP, remained unaffected. Furthermore, acidification did not induce major changes in particle diameters, suggesting an increase in membrane permeability without inducing liposomal disintegration. However, the observed increase in size distributions for L2c and L11c suggests a

correlation with DODAP protonation, in contrast to L15c containing DOTAP, which showed no comparable increase.

In cellulo, the final candidates L2c, L11c, and L15c displayed toxicity levels similar to the reference liposome L0. Furthermore, these candidates improved doxorubicin delivery, achieving superior cellular penetration in both cytosolic and nuclear regions compared to the control L0 formulation. In *in vivo* experiments on mice, all three candidates demonstrated equal blood circulation compared to L0. While the findings indicated promising potential for the liposomal formulations, the candidates are considered primarily as a proof-of-concept approach. Future studies could include exploring alternative lipid compositions and novel stimuli-responsive liposome release mechanisms, including magneto-, light- or heat-controlled release. Other surface modifications, such as the integration of PEG or clickable lipids, offer potential opportunities for improvement. However, it is important to note that the introduction of any new requirement will progressively increase the overall complexity of the system and will require careful experimental design.

The third project investigated the use of LFT1, a liposome with an exosome-like lipid composition, as a transfection system for cell membrane-impermeable small molecules. A potential DNMT2 inhibitor, FTAD, with fluorescent properties and poor membrane permeability, was selected as the primary research focus. Physicochemical measurements showed minimal influence of the entrapped cargos on particle size and size distribution, whereas the zeta potential was influenced by the polarity of the entrapped molecules. The impact on the zeta potential must be taken into account in broader screenings, as electronic interactions between liposomes and the cell membrane are crucial for particle uptake and therefore reduce the comparability of results. Furthermore, residual free cargo after purification emphasises the need for careful monitoring of sample purity.

The drug delivery and release properties of LFT1 were demonstrated using the phosphatase substrate FDP, which becomes fluorescent after enzymatic cleavage. HEK293 cells showed minimal uptake of free FDP, whereas LFT1-encapsulated FDP resulted in a clear increase in fluorescence intensity, indicating successful delivery and release of the cargo. Liposomal uptake was reduced when cells were co-incubated with the uptake inhibitor dynasore, but its effect was less pronounced than achieved by Lu *et al.* in other cell lines.⁷ This indicates the involvement of additional cellular internalisation pathways in HEK293 cells which are not inhibited by dynasore. Flow cytometry results were validated by extracting FDP from the treated cells. Data analysis revealed incomplete substrate cleavage by 10 μ M incubation conditions. This observation suggests either incomplete cellular release of LFT1-FDP or that cellular

phosphatases were overwhelmed by the amount of substrate. In addition, the possibility of LFT1-FDP adhering to the cell membrane cannot be definitively excluded on the basis of these findings.

The fluorescent properties of the potential methyltransferase inhibitor FTAD were then used to confirm the cellular delivery by LFT1. While free FTAD showed low cellular internalisation, the LFT1-FTAD formulation was highly internalised in a time- and concentration-dependant manner, consistent with the findings of LFT1-FDP. The data indicate peak uptake within a time window of 4 h to 24 h, which needs to be further validated for optimised incubation conditions. Analysis of tRNA modification levels in total tRNA showed no changes after incubation with liposomal or free FTAD, while the free and liposomal methyltransferase inhibitor SFG, which is taken up by the cells themselves, caused a reduction of six methylation sites. At the same time, the comparable levels of methylation reduction for the SFG and LFT1-SFG treatments suggest successful release of the encapsulated cargo. If the release had not been successful, the level of the six methylation sites in total tRNA species after the LFT1-SFG treatment would not correspond to that achieved with free SFG. The lack of discernible effects in HEK293 cells exposed to FTAD indicates negligible or no inhibitory activity *in cellulo* where sinefungin shows efficacy. However, the possibility of premature cargo release during incubation due to interactions between media components should be considered, as this complicates the interpretation of the SFG findings.

Overall, these results demonstrate the efficacy of LFT1 in delivering small molecules into cells, facilitating the assessment of the inhibitory effects of FTAD *in cellulo* - an assessment that would not have been possible using conventional methods. This delivery system represents a novel approach to determining the activity of new compounds in the early stages of drug development. However, further validation is required through the analysis of additional cargos. In addition, fluorescence microscopy studies are essential to elucidate cellular localisation and distribution patterns, providing a deeper insight into *in cellulo* behaviour and uptake mechanisms. To the best of our knowledge, this is also the first study in which the addition of sinefungin to the cell culture medium has resulted in the inhibition of multiple methyltransferases *in cellulo*, which may have future applications in nucleic acid research.

In addition to primary projects, collaborative research was a particular focus. A collaborative project with Marco Kristen and Marc Lander focused on developing rapid, sensitive methods for quantifying tRNA species using a combined cDNA hybridisation and next-generation sequencing approach, which is named DORQ-seq

(DNA Oligo for RNA Quantification sequencing). This project involved culturing eukaryotic cell lines, in particular HEK293 cells, to analyse tRNA profiles. Comparisons between HEK293 cells and tissue samples from male and female donors revealed significant deviations in 16 out of 43 cytosolic cDOQs, a pattern also observed when these groups were compared with Alzheimer's disease patient samples. The sequenced cDOQ pool reflected the tRNA composition of the samples. Given the critical role of tRNA in protein synthesis, identifying differences between HEK293 cells and donor tissues may improve translational insights from cell culture to complex biological systems.^{272,274} Furthermore, the samples helped to validate the utility of DORQ-seq for tRNA composition analysis.²⁷⁴

The research presented in this thesis has improved our understanding of stable surface functionalisation, liposome screening and in-cellular drug delivery. Future investigations could extend the screening approach for pH-responsive liposomes to include alternative stimulation mechanisms. The inclusion of modifiable dialkyl lipids in the initial screening process would allow subsequent conjugation with antibodies and other ligands, potentially leading to the discovery of novel, potent and selective liposomal drug delivery systems.

The use of LFT1 in an initial pilot drug discovery study would provide early insight into the biological activity of candidates and would also provide further understanding of the applications and limitations of the liposome as a delivery system. In addition, the screening approach could be adapted to evaluate advanced versions of LFT1 for intracellular activity assays of membrane-impermeable drugs.

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Appendix

Table A.1: Declaration of the use of artificial intelligence tools and software.

Artificial intelligence tool / software	Used for	Why	When
Deepl Translate	Translation of texts from german into english and reverse	Translation of text where wording is unclear	Throughout the writing process
Deepl Write	Reformulation of the self-written text	Improvement of the text structure and grammatical correction	Throughout the writing process except chapter 4.1
Perplexity	Reformulation of the self-written text	Improved text structure and native english style	Throughout the writing process, except chapter 4.1
GoTranscript	Spelling check british and american english	For consistent british english	Throughout the writing process
Gemini	Spelling and grammar check	Correction of grammatical mistakes	Throughout the writing process
Google & Google Scholar	Literature research	Research of scientific publications and reference data	Throughout the writing process
Microsoft Office 365	Writing and layout	Creation of an electronic document	Throughout the writing process

Adobe Illustrator	Creation of illustrations	Visualisation	Throughout the writing process
GraphPad Prism	Statistical evaluation and visualisation of data	Visualisation and statistical calculations	Section 4
Chemdraw	Drawing chemical structures	Visualisation of chemical structures	Sections 1 and 4

Curriculum Vitae

Persönliche Daten

Name Lukas Gleue

Geburtsdatum XXXXXXXXXX

Adresse [REDACTED]

Ausbildung
