Phase separation in lipid-based nanoparticles: exploring the nano-bio interface

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CHAPTER 6

Summary and Closing remarks

"Αυτός ο κόσμος, ο μικρός, ο μέγας" – Odysseas Elytis, 1959

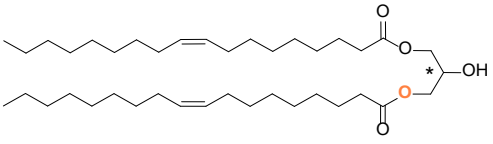
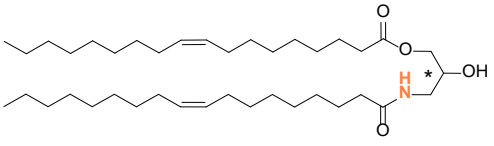
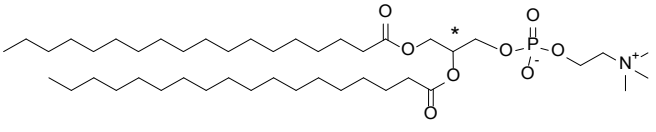
Lipid-based nanoparticles are the most clinically advanced drug delivery systems; however, a persistent and limited understanding of their assembly properties and the key nano-bio interactions hampers their clinical translation, which mainly relies on trial-error approaches and labor-intensive empirical screenings. In this doctoral dissertation, lipid chemistry, composition and morphology, are all connected to provide a comprehensive picture of novel phase separated lipid-based nanoparticles with specific behavior at the nano-bio interface. This behavior is characterized by selective nanoparticle-protein communications and the hijacking of endogenous biological mechanisms for cell specificity *in vivo*. To achieve nanoparticle-mediated targeted therapies, a better understanding on how lipid composition determines morphology and influences (desired) nano-bio interactions is needed. Therefore, the information provided in this thesis broadens current, fundamental knowledge on lipid nanoparticle assembly and *in vivo* behavior, and can be the foundation for rationally optimized nanoparticle designs.

This Chapter summarizes and discusses the key findings and relevance of this thesis followed by future perspectives.

6.1 Summary and outlook

In **Chapter 2** a novel liposomal formulation (named PAP3) with cell-selective *in vivo* behavior is described, and found to specifically accumulate in brain endothelial cells (bECs) of embryonic zebrafish. PAP3 liposomes consist of only two lipids, the natural phospholipid DSPC and the synthetic diacylglycerol (DAG) analogue DOaG, without using traditional targeting functionalities, *e.g.*, antibodies, peptides (**Table 1**). In contrast, cell-specific uptake was mediated by a unique "parachute morphology" which was characterized by a single lipid droplet within each DSPC leaflet, as created by lipid phase-separation. While DSPC has a cylindrical geometry and favors assembly into a flat bilayer, DOaG has a conical geometry preferring non-bilayer phases. As a result, upon mixing, these two lipids phase separate.

Table 1. Molecular structures of lipid components in PAP3 liposomes (compared to natural DAGs).

Lipid	Molecular structure	
DOG Natural DAG		
DOaG synthetic		**PAP3 liposomes (1:1)**
DSPC Natural phospholipid		

Atomic differences between DOG and DOaG are shown in orange. Abbreviations in table 1: DOG=Dioleoylglycerol, DOaG=Dioleoylamidoglycerol, DSPC= Distearoylphosphocholine.

By varying the molar ratio of DOaG in the DSPC formulation, it was revealed that phase-separation only occurs at high DOaG concentrations. At a low DOaG/DSPC ratio the lipids mix, while above a threshold concentration DOaG phase-separates into a lipid droplet surrounded by a DSPC monolayer. Strikingly, it was shown that bEC targeting occurred only when liposomes where phase-separated. Mechanistic studies using zebrafish revealed the involvement of a triglyceride lipase-mediated mechanism in the cell selective uptake. At this developmental stage, zebrafish embryos have high lipid and metabolic demands and therefore lipases, such as endothelial (EL) and lipoprotein lipase (LPL), are highly present in the zebrafish head region. Indeed, in the presence of a small molecule lipase inhibitor (XEN445), liposome bEC uptake was abolished. Although in a different tissue (liver), the involvement of triglyceride lipase (TGL) in the uptake, was also found to be (partially) conserved in mice. Here, competing clearance mechanisms lead to the rapid accumulation of PAP3 liposomes in the liver and spleen.

Nevertheless, it was important to identify the involvement of TGL as a mechanism for the clearance of PAP3 liposomes by the liver, which has not been described before. Since TGLs are regulated in both time and space and are highly present in certain disease states such as cancer, they are potentially novel targets to achieve selective nanoparticle *in vivo* uptake and be exploited in a therapeutic setting.

In **Chapter 3**, the lipase-liposome interaction was studied in more detail, revealing the DOaG-rich lipid droplet is specifically hydrolyzed by TGLs, leaving the lamellar liposome membrane intact. By combining cryo-transmission electron microscopy (cryo-TEM), mass spectrometry, enzymatic analysis and molecular dynamic (MD) simulations, the exact mechanism of this interaction was discovered. Phase-separation due to DOaG, results in membranes with lipid packing defects – areas where the distance of adjacent phospholipids is increased – exposing hydrophobic patches rich in DOaG to the aqueous environment. These defects facilitate TGL binding to the liposome surface resulting in DOaG hydrolysis. It was also shown that the so-called tryptophan loop – the natural lipoprotein binding domain of the enzyme – acts as a packing defect sensor, implying that TGLs preferentially bind to phase-separated membranes as compared to flat lamellar membranes.

The observed mechanism of TGL-mediated hydrolysis of PAP3 liposomes has similarities to the endogenous mechanism that takes place on lipoproteins: Involved in natural lipid transport and metabolism pathways, TGLs either 1) hydrolyze di- and triglycerides and cholesterol esters in lipoproteins, or 2) facilitate lipoprotein cell uptake.[1–3] In summary, Chapters 2 and 3 show that phase-separated PAP3 liposomes hijack a biological pathway of lipid transport and/or metabolism, mediated by TGLs.

Chapter 3 demonstrated that, *in vitro*, the droplet of PAP3 liposomes undergo lipolysis by TGL; while Chapter 2 described the TGL-mediated endocytosis of PAP3 liposomes *in vivo*. An unanswered question was whether PAP3 liposomes undergo lipolysis before endocytosis *in vivo*. An indication for this, may lie on the inhibition of TGL by the XEN445 inhibitor, which is usually used to inhibit the catalytic activity of TGLs. *In vitro*, XEN445 inhibits the lipolysis of PAP3 droplets (Chapter 3). In zebrafish, XEN445 treatment inhibits the uptake (Chapter 2). Hence, the

lipolysis of PAP3 liposomes could play a role in the cell uptake *in vivo*. Another interesting question posed in Chapter 3 is whether PAP3 liposome metabolites could still target zebrafish bECs. Indeed, preliminary data show PAP3 metabolites still accumulate in bECs (**Figure 1**).

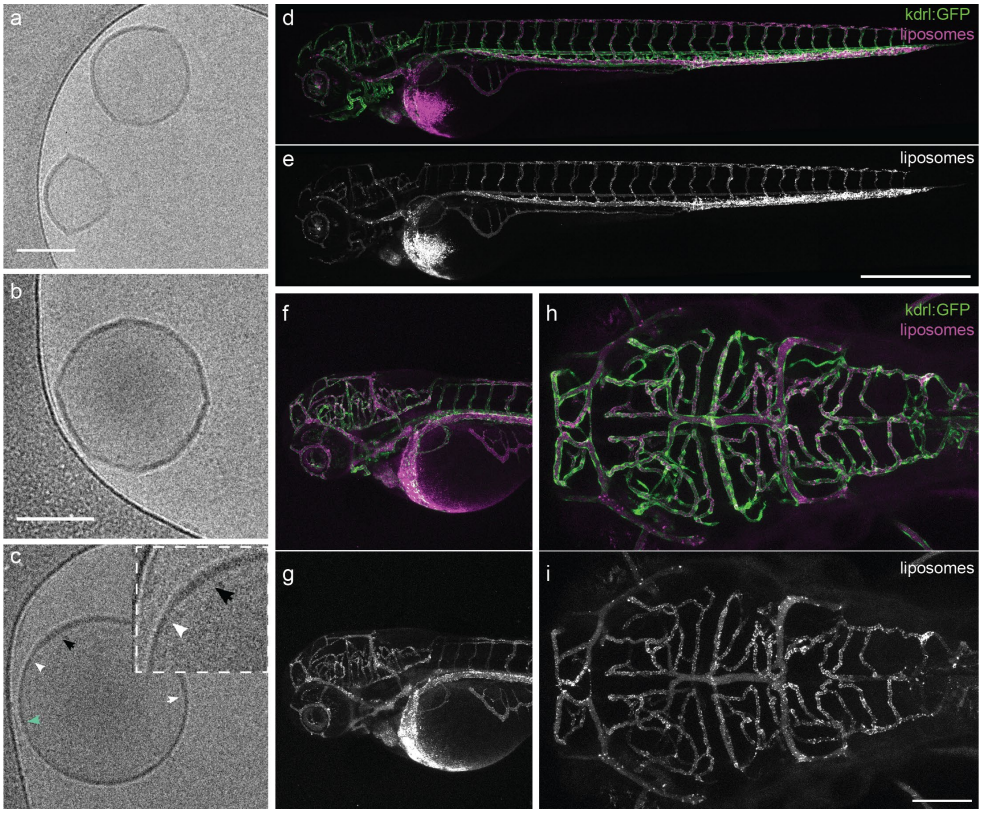


Figure 1. Cryo-TEM images and biodistribution of PAP3 liposomes after droplet hydrolysis in a zebrafish embryo (*kdrl*:GFP, 78 dpf, 2.5 hpi). a-c) Liposomes appear rounder than typical DSPC liposomes and with thicker membranes (8-9 nm compared to 3-4 nm, respectively, as suggested by quantification of N=20). Also, membrane mismatch and co-existence of two phases (gel and fluid) in the membrane can be seen in all images, suggesting the existence of DOaG (or DOaG metabolites). Difference of bilayer thickness is indicated with black (thicker part) or green (thinner part) arrows. The point of thickness mismatch is indicated with white arrows. Biodistribution of PAP3 liposomes after droplet hydrolysis indicating bEC targeting patterns, **d, e**) whole body lateral view**, f, g**) 10x magnification in the head region, lateral view, **h, i**) 10x magnification in the head region, dorsal view. Liposomes prepared by extrusion, at 5 mM total lipid concentration containing 0.2% DOPE-LR for visualization. Scale bars: 100 nm for cryo-TEM, 500 μm for zebrafish lateral view and 100 μm for dorsal view.

This might indicate that lipid droplets are not required for bEC recognition. A small fraction of unhydrolyzed DOaG could still induce packing defects and DOaG exposure throughout the bilayer and could already be enough for TGL recognition and bEC uptake. In Figure 1 such nanodomains can be confirmed: i) the DSPC membrane is observed to be in a liquid disordered (L_d) phase (more fluid) – something uncommon for liposomes made of DSPC only – indicating the existence of a small fraction of DOaG (or DOaG metabolite) that could alter rigid DSPC membranes. ii) A clearly observed membrane thickness mismatch (arrows), have been recently described to be nanodomains in such instances.[4] SANS and cryo-TEM could be used in the future to solidly verify the existence of such domains in the liposome metabolites.[5]

Chapter 4 describes an in-depth investigation of the DOaG's molecular details underpinning the phase-separation and bEC targeting in zebrafish embryos. A library of DOaG analogues was synthesized and the structure-function relationship was investigated. Medium (C16:1 or C18:1) unsaturated chains are necessary to induce phase-separation in liposomes, when DOaG analogues are co-formulated with DSPC, and achieve selective *in vivo* targeting. In contrast, fully saturated DOaG variants did not form liposomes. Variants containing the long acyl chains C20:1 or C24:1, mixed with DSPC, formed liposomes albeit with high instability, or did not form liposomes at all, respectively. Surprisingly, the short acyl chain variant (C14:1) *did* induce phase-separation in DSPC membranes but the resulting liposomes *did not* target bECs.

Chapter 3 illustrates that DOaG lipids induce phase separation in DSPC membranes and TGLs recognize the exposed DOaG molecules due to high membrane packing defects. Since the TGL recognition and binding relies on the exposure of DOaG lipid, the length of DOaG acyl chain may be particularly important. Short chain lipids may not be exposed sufficiently, whereas longer chain DOaG variants may be exposed enough (for TGL recognition), or even too much (potential aggregation risk). The fact that liposomes containing C16:1 or C18:1 variants target bECs, and liposomes containing longer chain DOaG variants (C20:1, C24:1) are colloidally unstable (*i.e.*, massive aggregation or no assembly at all) supports this hypothesis.

In contrast, the shorter C14:1 lipid variant induces phase-separation in stable liposomes, but the chains may be too short to be recognized by TGLs, therefore bEC targeting is not observed. Further investigation is needed to prove this hypothesis.

An alternative explanation why phase-separated liposomes with short C14:1 lipid variants do not target bECs, may lie on the amount of total packing defects they induce throughout the liposome membrane. Due to extreme curvature, the lipid droplet may inevitably induce packing defects exposing the C14:1 lipids locally. However, the lamellar part of DSPC seems to be in a liquid ordered (L_o) phase (gel phase, cornered) in the case of C14:1 liposomes (Figure 3, Chapter 4). This is in contrast to C16:1 and C18:1 liposomes which are more in a L_d phase (Figure 3, Chapter 4). Therefore, the C14:1 liposomes may have less defects throughout due to the shorter chain of C14:1 exposing the lipid less. Hence, in an *in vivo* setting, liposomes with only a small region of C14:1 lipids exposed to the surrounding environment, may have a lower probability to be recognized by TGLs with subsequent bEC uptake, than liposomes with more packing defects throughout the bilayer (*i.e.*, C16:1, C18:1). Besides, liposomes which do not bear a lipid droplet but seem to still be defected throughout retain their bEC targeting, as previously mentioned (Figure 1), supporting this hypothesis.

Other DOaG variants

As previously mentioned (Chapter 3), it is not yet clear whether TGL hydrolysis takes place *in vivo* prior to bEC uptake. Since TGLs endogenously hydrolyze ester bonds in di- and triglycerides to release free fatty acids (FFA), and DOaG contains an ester in the *sn*-3 position, an ether variant of DOaG could be useful to elucidate this mechanism. More specifically, if bEC uptake of liposomes was abolished by replacing the DOaG ester with an ether, then this would indicate hydrolysis prior to PAP3 uptake *in vivo*. However, liposomes containing the ether analogue must have similar physicochemical properties to PAP3 liposomes and not show hydrolysis by TGLs *in vitro*, to prevent false conclusions.

PAP3 Liposome optimization

In Chapter 4, an effort to optimize the PAP3 formulation is described. PAP3 liposomes are only stable for up to ~7 days (Chapter 2) and importantly are not stable when formulated in buffers containing saline (*i.e.*, PBS), which mimic the physiological environment. By assessing the influence of *sn*-isomeric mixtures of DOaG in liposome stability, it is found that DOaG existing in the pure *sn*-1,3 isomer improves long-term stability of PAP3 liposomes. Despite that the presence of a small amount of *sn*-1,2 isomer (20% of the mixture) seems to contribute negatively on the liposome stability, it is important to assess its influence on the liposome assembly when it is in its pure form (*i.e.*, 100% *sn*-1,2). Similar studies have to be performed for other DOaG isomers, such as the *sn*-2,3 (where the amide bond is located on the *sn*-2 position) and racemically pure isomers of DOaG and DSPC.

Additionally, acyl chain length mismatch between DSPC (C18:0) and a DOaG variant (C16:1) improved the stability and ability to assemble the liposomes in PBS with acceptable PDI values and size. This also supports the hypothesis that shorter variants than DOaG (C18:1) are less exposed to the solvent. PEGylation of the liposomes also improved the stability while retaining bECs targeting. Here, it is therefore important to test whether a PEGylated formulation (1% DMPE-PEG2k) with the pure *sn*-1,3 isomer of C16:1 will result in an even more improved, clinically suitable formulation.

Chapter 2-4 illustrates that phase-separation induced by DOaGs is a novel, functional *in vivo* targeting modality, that can be of interest for other lipid-based nanoparticle systems. **Chapter 5** illustrates the applicability of DOaG lipid in mRNA-based lipid nanoparticle formulations and serves as a proof-of-concept on the employment of DOaG in LNPs, to achieve specific cell targeting and mRNA delivery. In this study, four mRNA-LNP formulations were created, and their *in vivo* behavior was assessed in zebrafish embryos. A phase-separated mRNA-LNP containing DOaG, in combination with the ionizable lipid DODAP, was successfully formulated encapsulating sufficient amount of mRNA. This formulation specifically targeted and accumulated in bECs of zebrafish embryos, resulting in mRNA delivery and protein expression. Interestingly, the similarly phase-separated LNP formulation with DOaG and MC3 did not target bECs.

The DOaG-MC3 LNP was observed to cluster in circulation, which could indicate a thermodynamically unstable formulation, and therefore its colloidal instability might prevent bEC targeting. This indicates that a simple ionizable lipid switching could massively influence the nanoparticle assembly and stability, by molecular details that are not captured by cryo-TEM, and signifies the complexity of mRNA-LNP assemblies. More experiments are needed to investigate whether DOaG-MC3 LNPs are unstable, or whether phase-separation plays a role in bEC uptake, possibly via a TGL-mediated pathway.

Importantly, this study was able to determine that highly transfecting but not cell-specific formulations result in potent transfections, however not at the desired site; while less potent but more selective formulations achieve the desired cell-specific transfection. This contrasts with an often-used shotgun approach where potent therapeutics are administered in the overall tissue, aiming to target the desired site within this tissue.

The DOaG-LNP formulation exhibiting cell selectivity can be the starting point for future research. Firstly, formulation optimization can be explored to enhance transfection potency. Different molar ratios between DOaG and rest of lipid components, as well as different ionizable lipids, can be used. Additionally, translational studies in healthy and diseased animal models should be performed, as well as studies to reveal the selective uptake mechanism (*i.e.*, investigating the role of TGL in bEC uptake). Finally, a general exploration of the potential of other diacylglycerol lipids in mRNA-LNP developments *i.e.,* in cell specificity or enhanced endosomal escape through fusion, is of interest.

6.2 Other formulations targeting bECs

During this PhD study, some other lipid-based formulations were also observed to target bECs. siRNA-loaded LNPs using DOPE as a helper lipid (DODAP:DOPE:CHO:DMPE-PEG1k_5:1:3.9:0.1), as well as DOPC:DSPC:CHO_5:2:3, (to a lesser extent) were observed to target bECs in zebrafish embryos (data not included). This could suggest that nanoparticles exhibiting lipid nanodomains could utilize the same TGL-mediated mechanism

and/or have similar protein coronas with PAP3 liposomes. DOPE is a lipid with similar conical geometry of that of DOaG, therefore it may induce similar packing defects in lipid membranes. DOPC:DSPC:CHO is a known formulation to create phase-separation in giant unilamellar vesicles (GUVs) [6,7] hence nanodomains may exist in large unilamellar vesicles (LUVs) in a similar fashion. Further investigation is required here to elucidate the exact mechanisms and composition which could be used for predictable TGL-mediated *in vivo* interactions.

6.3 Potential involvement of DOaG in membrane fusion

DAGs are known to decrease the spontaneous negative curvature of lipid bilayers and induce inverse hexagonal liquid crystalline phases due to their conical geometry, perturbing lamellar phases. Therefore, a small amount of DAGs in lipid bilayers could promote fusion.[8–13] Fusogenicity in DOaG-containing lipid nanoparticles (liposomes and LNPs) is an interesting aspect for future investigation. Preliminary results show that membrane docking takes place (**Figure 2**) in a PAP3 varied formulation, containing DSPC:DOaG:CHO (4:4:2). Interestingly, cholesterol increases the L_d phase of DSPC bilayers and make them more fluid, consequently DOaG can mix with a DSPC membrane, and PAP3 liposomes are mostly observed to no longer phase-separate.

Also, preliminary data showed high levels of lipid mixing when DOaG is present in a liposome formulation (DOPC:DOaG:CHO_2:1:1 containing the fusion promoting coiled coil lipopeptides CPE_4 and CPK_4[14] – data not included in this thesis –) comparable to lipid mixing levels reported for other fusogenic liposomal formulations (*i.e.*, DOPC:DOPE:CHO_2:1:1 with CPE_4 and CPK_4).[15,16] This result indicates a potential role of DOaG in lipid nanoparticle fusion, which can be particularly important for facilitating endosomal escape or drug delivery. Since the DOaG-based liposome formulations that were observed to dock or undergo lipid mixing also contain cholesterol – which literature has characterized as a potential fusogen [17,18] – further investigation is needed to understand its influence in combination with DOaG.

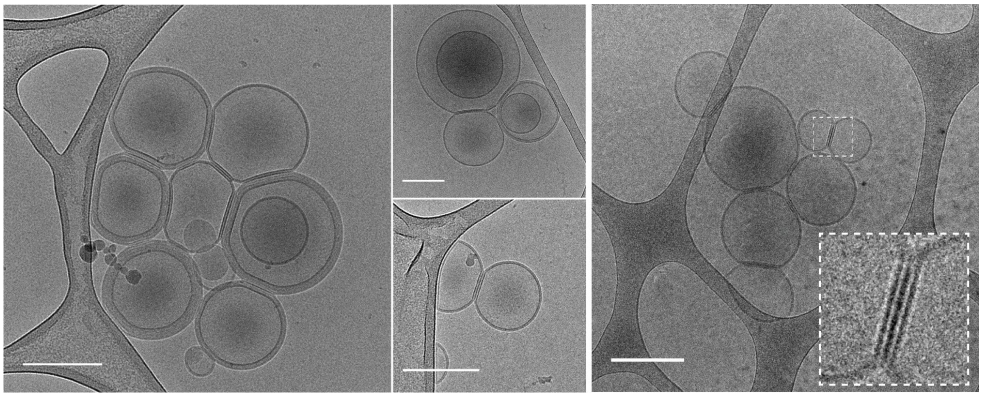


Figure 2. Cryo-TEM images of cholesterol-containing PAP3 liposomes (DSPC:DOaG:CHO_4:4:2) showing membrane docking similarly to other cryo-TEM studies showing docking.[19] Scale bars: 200 nm.

DOaG (or DAGs in general) could be very promising lipid components in mRNA-LNPs and their potential to enhance endosomal escape rates by promoting fusion, should be examined. Molar ratios of DOaG in mRNA-LNPs could vary from the one used in Chapter 5 and can be fine-tuned to promote *i.e.*, liquid crystalline hexagonal phases. It would be interesting to see whether 1-10% mol of DOaG (or variants) in combination (or not) with cholesterol in LNP formulations, may be enough to increase transfection efficiency, retaining ideally specific nano-bio interactions.

6.4 Closing Remarks

Based on the main findings of this thesis and the discussion above, it would be of interest to investigate whether DAG derivatives utilized as lipid components in lipid-based nanoparticles have further potential in selective *in vivo* behavior, nano-protein interactions, fusion and/or enhanced cytoplasmic drug delivery.

Moreover, despite that liposomes have been studied for decades and entered the clinic, this thesis signifies that there is still a large window for exploration at the nano-bio interface, even for simple two-component lipid systems. More importantly, arising multicomponent lipid systems with more intricate supramolecular assemblies such as the ionizable LNPs – which are successfully utilized in the mRNA vaccines against SARs-CoV-2 - [20,21] cannot follow a one-fits-all approach. Rather, a detailed understanding of the properties of each individual lipid component, is vital for rational design strategies leading to more precise targeted therapies.

Finally, this doctoral dissertation describes the novel concept of cell-specific nanoparticle targeting by directly hijacking an endogenous TGL pathway, based on composition and morphology. TGLs are highly involved in lipid transport and metabolism and constitute a rational strategy to promote nanoparticle selectivity *in vivo*, something that has not been widely explored. In contrast, the current practice for targeted nanoparticle discovery relies on large nanoparticle libraries and empirical screenings. Often however, this practice disregards weak *in vitro* "performers", due to exhaustive large-scale experiments and ethical considerations, and therefore a plethora of formulations is not assessed further. To this end, this thesis offers fundamental knowledge on lipid nanoparticle properties at the nano-bio interface to achieve selective *in vivo* behavior. This knowledge can benefit the prediction, development or clinical translation of cell-selective drug delivery systems.

6.5 References

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