



Universiteit
Leiden

The Netherlands

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

Papadopoulou, P.

Citation

Papadopoulou, P. (2023, November 7). *Phase separation in lipid-based nanoparticles: exploring the nano-bio interface*. Retrieved from <https://hdl.handle.net/1887/3656645>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/3656645>

Note: To cite this publication please use the final published version (if applicable).

CHAPTER 1



Introduction to phase separation in lipid-based nanoparticles: exploring the nano-bio interface

Parts of this chapter were used for the review article:

Escalona Rayo, O.[#]; **Papadopoulou, P.**[#]; Slutter, B.; Kros, A.* Biological recognition and cellular trafficking of targeted RNA-lipid nanoparticles. Current Opinion in Biotechnology 2023.

denotes equal contribution
under revision

“There is plenty of room at the bottom” - Richard Feynman, 1959

1.1 Nanotechnology in life sciences

Nanotechnology (from the Greek *νανοτεχνολογία*: *νάνο*, lit. 'dwarf', *-τέχνη*, lit. 'craft, art' and *-λογία*, lit. 'study, knowledge') is a multidisciplinary field that involves the engineering of materials at the nanoscale level, typically ranging from 1 to 100 nanometers (nm). At this scale, materials exhibit unique properties attributed to surface and quantum effects, that differ from those from the bulk scales allowing for the development of novel materials, devices, and systems. Richard Feynman is considered the father of Nanotechnology, as he was the first to introduce the concept of manipulating matter even at an atomic level. In an iconic presentation with the title “There is plenty room at the bottom” Richard Feynman suggested the concept of scaling down bulk materials, such as copying the whole encyclopedia in the size of a headpin.¹ Ever since, several pioneers established the term and field of Nanotechnology. Importantly, nanotechnology is an emerging field for several biomedical applications in life sciences, including therapeutics, diagnostics, theranostics, imaging, regenerative medicine, and tissue engineering.

1.2 Nanotechnology in medicine

Nanoparticles as Drug Delivery systems

Nanomedicine is the field of medicine which employs nanotechnology, mainly as a drug delivery system or as a tool in photothermal therapy. Nanomedicines have been proposed to be beneficial as drug delivery systems for several reasons. Firstly, nanoparticles offer the advantage of increasing the solubility of poorly soluble drugs and protecting sensitive cargo from degradation. Additionally, nanoparticles allow for the slow controlled release of drugs, enabling sustained drug delivery and lowering the required drug dose and frequency. Therapeutics delivered using nanoparticles typically have different biodistribution, pharmacodynamics and pharmacokinetics compared to the free drug. Nanoparticle-based therapeutics also have reduced toxicity, improved bioavailability and drug half-life, and sometimes

the ability to cross biological and cellular barriers enabling drugs to reach difficult, inaccessible targets. Additionally, a desired concept around nanomedicines is targeted drug delivery, which proposes nanoparticles to act as “magic bullets”, targeting predominantly specific (diseased) tissues or cells, while leaving other (healthy) tissues unaffected.² By delivering medicines directly to the desired site, the therapeutic window is increased and off-target effects are minimized, leading to better outcomes, a reduced required dose, and fewer adverse side effects. Targeted nanomedicine will be extensively discussed in a separate section.

1.3 Nanoparticle design

Nanoparticles used in life sciences consist of inorganic, organic or hybrid materials. Inorganic nanoparticles based on gold, silver, iron oxide or others – such as quantum dots – have unique plasmonic, magnetic and electronic properties influenced by their shape and size.^{3–5} Inorganic nanoparticles are employed in drug delivery, however their properties mostly render them attractive for imaging, diagnosis and photothermal therapies, *i.e.*, photodynamic therapy and hyperthermia. In contrast, organic nanoparticles show the biggest potential in drug delivery, due to their high drug encapsulation capacity and biocompatibility. Organic nanoparticles can be divided in lipid-based, polymer-based, carbon and hybrid (*i.e.*, lipid-polymer, lipopeptide-based)⁶ (**Figure 1a**). Additionally, bio-mimetic or bio-derived materials such as virus-like particles (VLPs),⁷ extracellular vesicles (EVs),⁸ or apolipoprotein-based nanomaterials,⁹ are studied as drug delivery systems. Different nanocarrier designs – *i.e.*, micellar, disc, sphere, fiber – are suitable for specific therapeutic cargo encapsulation depending on cargo properties – *i.e.*, size, hydrophobicity, charge –. Therapeutic cargos can be small molecule drugs, nucleic acid-based therapeutics, or protein/peptide based (**Figure 1b**).¹⁰ Additionally, surface coating on nanocarriers with a small amount of the polymer polyethylene glycol (PEG) is a common strategy for stabilization and prevention of opsonization and rapid nanoparticle clearance.^{11,12} Other surface functionalities – *i.e.*, antibodies, cell-penetrating peptides, sugar moieties – are utilized to enhance the therapeutic potential by mainly recognizing cell receptors and promoting active cell targeting (**Figure 1c**).

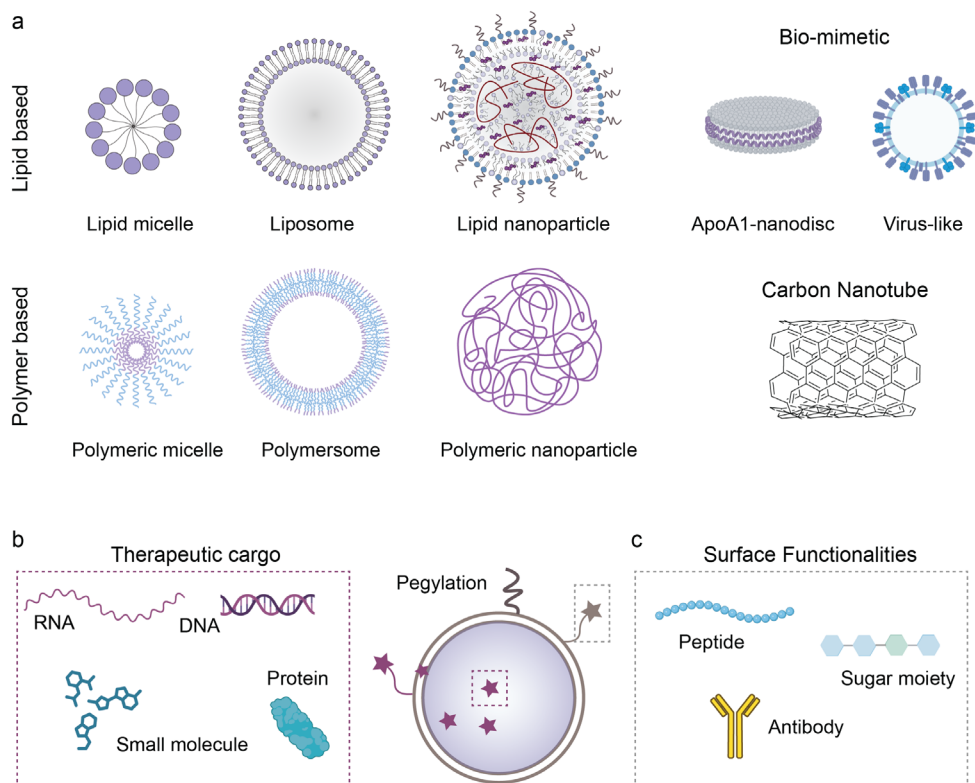


Figure 1. A selection of organic-based nanoparticle designs. **a)** Depending on the component they mainly consist of, organic-based nanoparticles can be lipid-based (e.g., micelles, liposomes, lipid nanoparticles), polymer-based (e.g., polymeric micelles, polymersomes, polymeric nanoparticles) or lipid-polymer hybrids, biomimetic nanoparticles (e.g., apolipoprotein-based nanodiscs or virus-like particles) and carbon-based (e.g., carbon nanotubes). **b)** Therapeutic cargos usually incorporated in drug nanocarriers are small molecule drugs, nucleic-acid therapeutics and protein/peptide-based therapeutics. **c)** Surface modification of nanoparticle designs are mainly employed for stability (e.g., PEGylation) or targeted performance (e.g., antibodies, sugar moieties and peptides). Polymer based and biomimetic nanoparticle cartoons, therapeutic cargo and surface functionalities cartoons were adapted from Biorender.

1.4 Lipid-based nanoparticles

General description and properties

Lipids are naturally derived molecules which constitute cell membranes and organelles, but are also utilized in energy storage and signaling.¹³ Being amphiphilic, lipids self-assemble in physiological environments to create nano- or microstructures. Controlling the natural self-assembly of lipids with various nanofabrication methods (e.g., extrusion, nanoprecipitation, microfluidic mixing) leads to nanomaterials with tunable size and morphology. Due to their biocompatibility, lipid-based nanoparticles typically exhibit low toxicity rendering them suitable as drug carriers. Main lipid classes which can be used for the assembly of lipid-based nanoparticles are phospholipids, sterol lipids, sphingolipids – or other non-phosphate containing lipids *i.e.*, natural diacylglycerols (DAGs) – and synthetic lipids, such as ionizable (amino)lipids (**Figure 2**). Lipid-based nanoparticles are typically in the range of ~20-500 nm in size and can be characterized by i) microscopy techniques – mainly cryo-transmission electron microscopy (cryo-TEM) – for size distribution and morphology, ii) dynamic light scattering (DLS) for size and colloidal stability, and iii) zeta potential measurements for surface charge.^{14–18} These physicochemical properties should be the minimum reported on nanoparticle characterization.^{19,20}

Nanoparticle complexity in assembly and lipid arrangement is dependent on the number of individual lipid components. One or two-component lipid-based systems usually result in simple liposomal or micellar assemblies. Multicomponent systems exert more intricate morphologies and properties. In general, due to the large variety of all individual lipid components and their combinations, lipid-based nanoparticles are endlessly tunable. A plethora of nanoparticle formulations with different physicochemical properties can be generated, designed to exert specific properties.²¹ For example, saturated lipids usually have a high transition temperature (typically ~30-80 °C), therefore these lipids exist in a liquid ordered (gel) phase (L_o) at room or body temperature. This physicochemical property induces tight packing and stiffness in lipid membranes.

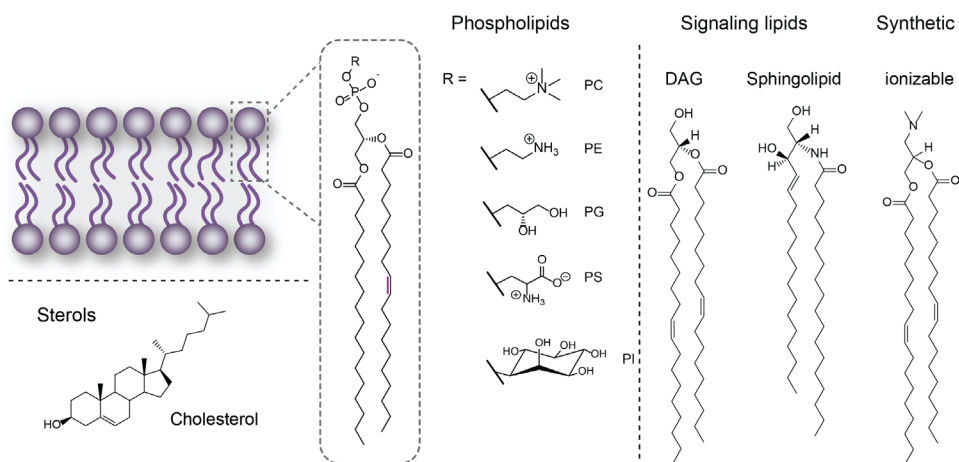


Figure 2. Main lipid classes used in lipid-based nanoparticles. Phospholipids are one of the main lipid components in cell membranes, as they naturally self-assemble into a lipid bilayer. The phospholipid's acyl chains can be i) short, medium or long depending on the number of carbons, and ii) saturated or mono, di- and polyunsaturated depending on double bonds. Their hydrophilic domain consists of a phosphate group along with a polar headgroup – usually a choline, ethanolamine, glycerol and others –. Additional lipids that can be used for lipid-based nanoparticle designs are sterols (*i.e.*, cholesterol), signaling lipids (*i.e.*, diacylglycerols and sphingolipids) or synthetic non-natural lipids (*i.e.*, ionizable lipids). Abbreviations: PC = phosphatidylcholine, PE = phosphatidylethanolamine, PG = phosphatidylglycerol, PS = phosphatidylserine, PI = phosphatidylinositol, DAG = diacylglycerol.

In contrast, unsaturated lipids form more fluid assemblies existing in liquid disordered phase (L_d), giving fluidity on the lipid membrane (**Figure 3a**). Additionally, for all lipids, polar head groups can be zwitterionic, anionic, or cationic therefore different net surface charge can be presented on the nanoparticle (**Figure 3b**). Lipids also display polymorphism, a biophysical property which gives the ability of lipid systems to exist in various phases depending on lipid geometry (**Figure 3c**).²² Lipids with a cylindrical geometry, such as phosphatidylcholines (*i.e.*, both hydrophobic and hydrophilic domains are of similar size) prefer a planar bilayer assembly. In contrast, conical lipids, such as phosphatidylethanolamines (*i.e.*, their polar head group is smaller than the acyl chains) create non-bilayer inverted phases some of which are crystalline *i.e.*, inverse hexagonal (H_{II}) or cubic. Finally, inverse conical lipids, such as lysophosphatidylcholines (*i.e.*, their polar

head group is larger than the acyl chains) induce micellar phases. Conical and inverted conical lipids, when mixed with phospholipids, can respectively decrease, or increase the spontaneous membrane curvature of bilayers (**Figure 3d**).

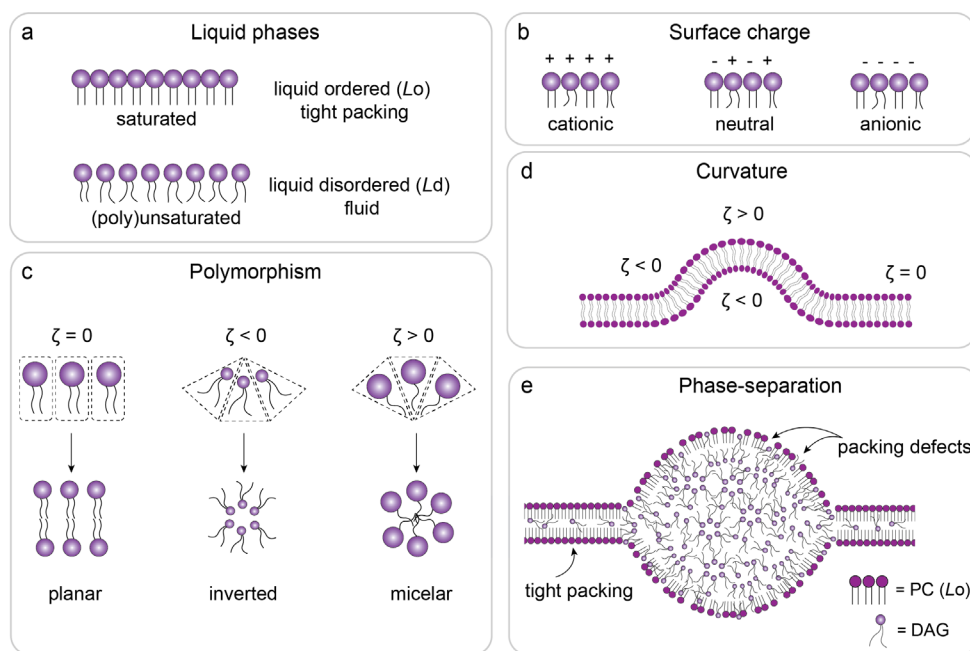


Figure 3. Lipid and membrane properties influencing nanoparticle properties. **a)** Liquid phases in lipid membranes depend on the degree of (un)saturation and length of individual lipids. Saturated lipids result in tightly packed lipid membranes existing in the liquid ordered phase (L_o). Unsaturated lipids result in a liquid disordered (L_d) phase, making the lipid membranes more fluid. **b)** Net surface charge of nanoparticles (cationic, neutral, anionic) depends on individual lipid charge (cationic, zwitterionic, anionic). **c)** Lipid polymorphism leads to lipid assemblies with various phases and morphologies. Cylindrical lipids tend to assemble in planar bilayers, while conical or inverted conical lipids tend to assemble in inverted or micellar phases, respectively. **d)** Membrane curvature as influenced by lipid polymorphism. **e)** Lipid phase-separation as described when DAGs exceed the miscibility threshold within lipid bilayers. Tight packing of phospholipids – especially of those existing in L_o phase – is impaired and lipid packing defects emerge. Abbreviations: ζ = membrane curvature, PC = phosphatidylcholine, L_o = liquid ordered phase, DAG = diacylglycerol.

DAGs as potential lipid components in lipid based nanoparticles

DAGs are endogenous conical lipids mostly found in cell membranes and lipoproteins, and due to their small polar headgroup and hydrophobicity they embed in lipid bilayers, avoiding exposure to the surrounding environment.^{23,24} Upon the miscibility threshold within the lamellar bilayer, DAGs tend to induce distinct, non-bilayer phases within the phospholipid leaflet, resulting in lipid phase separation (**Figure 3e**).^{25–28} This phenomenon increases the spacing between adjacent phospholipid headgroups in a lipid membrane, an effect that is amplified by membrane curvature – especially for membranes in L_o phase where high curvature is unfavorable.^{29–31} The transient areas resulting from such packing frustrations exposing the apolar domain of the lipid membrane, are known as lipid packing defects.³² Therefore, the distance between adjacent phospholipids is increased and tight packing is impaired, leaving DAGs exposed to the surrounding environment (**Figure 3e**). Some membrane peripheral proteins have been proposed to rely on these hydrophobic lipid packing defects, – generally caused by factors such as phase separation, lateral tension, or membrane curvature – for membrane binding and activation.^{31,33–36}

In cell membranes, DAGs are metabolite products from the hydrolysis of the phospholipid phosphatidylinositol (PI). Their local accumulation in the membrane induces non-bilayer phases potentiating the recruitment and enzymatic activation of Protein Kinase C, which further controls functions of other proteins.^{24,37–39} The toxin Equinatoxin-II28 and several lipases also sense DAG-induced phase separation and packing defects, which facilitate membrane binding and subsequent endogenous activity.^{40–42} Additionally to their signaling and protein recruitment properties, DAGs act as fusogens due to their ability to increase the negative curvature of lipid membranes. As conical lipids, DAGs have the propensity and ability to induce inverted liquid crystalline phases, especially hexagonal and cubic phases, which contribute to membrane fusion.^{43–46}

Finally, DAGs have been found to be main lipoprotein components, especially of high-density lipoproteins (HDLs).^{23,47} DAGs, but also triglycerides (TGs) and cholesteryl esters (CEs), are transported in the form of solid lipoprotein particles via endogenous pathways responsible for lipid transport and metabolism.

In summary, DAGs are involved in several biological phenomena, including membrane phase separation, fusion, protein recruitment and communication, signaling, and lipid metabolism. Therefore, DAGs are potentially interesting lipid components for lipid-based nanomedicines, but surprisingly have hardly been studied in depth.^{48,49} Fusogenic lipid-based nanoparticles have been proposed to enhance the efficient delivery of therapeutics into cells.^{50–52} The same has been proposed for phase-separation.^{52–54} Specific nanoparticle-protein communications could be exploited for targeting nanoparticle delivery and signaling could be used for therapeutic purposes.

Evolution of lipid-based nanoparticles

Liposomes are considered the earliest form of lipid-based nanoparticles (**Figure 4a**). They were discovered in the early 1960s, when lipid membrane vesicular assemblies were found to spontaneously form in water.^{55,56} Initially, liposomes were used as model membranes to study biological mechanisms including ion transport, fusion and drug-membrane interactions.^{57,58} However it became apparent that liposomes could be used to entrap drugs and their potential as drug delivery platforms was established.^{59,60} Already by the early 1970s, liposomes were being tested as drug delivery carriers, showing their potential in enzyme entrapment and prevention of immunological reactions.^{59,61–63} Later, *in vivo* biodistribution studies revealed their rapid clearance from blood circulation, however further studies showed that their size and unilamellarity, as well as composition and charge, can be tuned to generate liposomes with longer circulation lifetimes.^{64–66} Moreover, the addition of cholesterol, as well as PEGylation, increased liposome circulation.^{67,68} Almost three decades after their discovery, the first liposomal drug formulations were approved for clinical use (**Table 1**). Examples include the liposomal form of amphotericin B – with the trade name Ambisome® – used to treat severe fungal infections, and the liposomal form of doxorubicin – with the trade name Doxil®/Caelyx® – used for the treatment of several cancer types.

Table 1. A selection of lipid-based nanoparticle therapeutics approved by FDA and/or EMA.^{69,70}

Year	Brand name	Formulation	Lipid Composition	Active ingredient	Indication for use
1997	AmBisome®	Liposome	HSPC, DSPG, CHO (2:0.8:1)	amphotericin B	fungal infections
1995	Abelcet®	Liposome	DMPC, DMPG (7:3)	amphotericin B	fungal infections
2021	Comirnaty®	mRNA-LNP	DSPC, CHO, ALC-0315, ALC-0159	tozinameran	vaccine against SARS-CoV-2
1995	Doxil®/Caelyx®	Liposome	HSPC, CHO, DSPE-PEG (5.6:3.9:0.5)	doxorubicin	Kaposi's sarcoma, breast cancer
1996	DaunoXome®	Liposome	DSPC, CHO (2:1)	doxorubicin	Kaposi's sarcoma
2012	Marquibo®	Liposome	Sphingomyelin, CHO (6:4)	vincristine	acute lymphoblastic leukemia
2009	Mepact®	Liposome	DOPC, DOPS (3:7)	mifamurtide	Osteosarcoma
2000	Myocet®	Liposome	EPC: CHO (5.5:4.5)	doxorubicin	breast cancer
2018	Onpattro®	RNAi-LNP	DSPC, CHO, DLin-MC3-DMA, DMG-PEG2k	patisiran	hATTR amyloidosis
2021	Spikevax®	mRNA-LNP	DSPC, CHO, SM-102, DMG-PEG2k	elasomeran	vaccine against SARS-CoV-2
2000	Visudyne®	Liposome	EPG, DMPC (3:5)	verteporphin	macular degeneration

Abbreviations in Table 1: FDA = Food and Drug Administration, EMA = European Medicines Agency, CHO = cholesterol, HSPC = hydrogenated phosphatidylcholine (soy), DSPG = distearoyl-phosphatidylglycerol, DMPC = dimyristoyl-phosphatidylcholine, DMPG = dimyristoyl-phosphatidylglycerol, DSPE-PEG = distearoyl-phosphoethanolamine-polyethylene glycol, DSPC = distearoyl-phosphatidylcholine, DOPC = dioleoyl-phosphatidylcholine, DOPS = dioleoyl-phosphatidylserine, EPC = phosphatidylcholine (egg), EPG = phosphatidylglycerol (egg), DMG-PEG2k = dimyristoyl-glycerol-polyethylene glycol-2000.

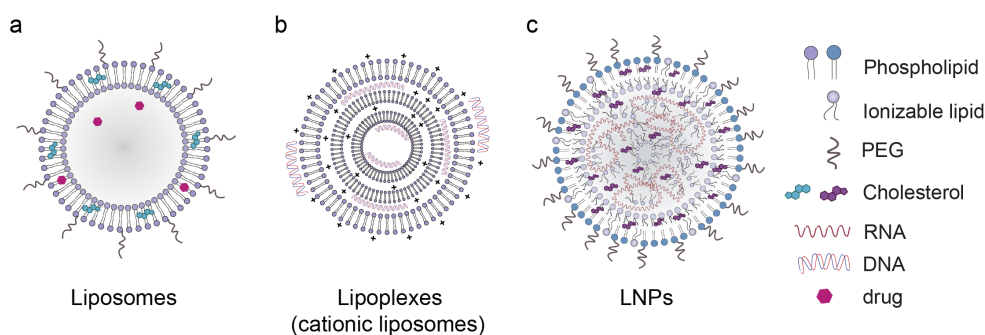


Figure 4. Evolution of lipid-based nanoparticles. **a)** Liposomes are the first lipid-based nanoparticles developed to mainly entrap small molecule drugs. They are usually spherical, consisting of a phospholipid bilayer and their properties can be tuned by lipid composition and addition of cholesterol or PEGylation. **b)** Cationic liposomes were extensively studied in the 1980s as suitable liposomal carriers for the entrapment of polyanionic nucleotides, mainly DNA. Their intrinsic cytotoxicity due to their charge however, led to the development of ionizable aminolipids with low pKa, which can complex with nucleic acids in acidic pH and become neutral at physiological conditions. The development of **c)** RNA-based ionizable LNPs led to stable, robust entrapment of nucleic acids, lower toxicity, and higher transfection efficiency.

In the field of gene therapy, DNA encapsulation and cell transfection using liposomes proved to be a major challenge in the 1980s. Eventually, the development of the synthetic cationic lipid DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane) led to lipofection, where resulting cationic liposomes complex with DNA and transfect cells with high efficiency (**Figure 4b**).⁷¹ Cationic liposomes however, were found to be toxic and rapidly cleared from circulation due to their high surface charge and large size, hindering *in vivo*

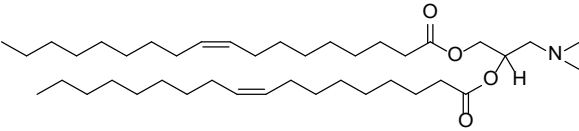
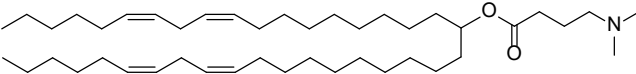
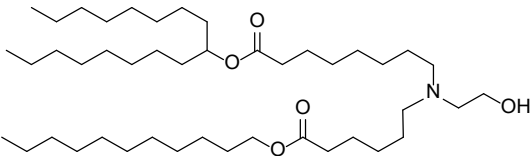
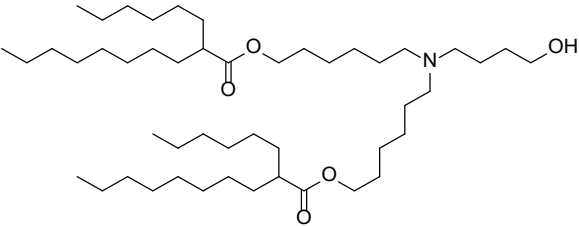
systemic use.^{72,73} This led to the development of ionizable aminolipids such as DODAP (1,2-dioleoyl-3-dimethylammonium propane), DLin-MC3-DMA, SM-102 and ALC-0315 (**Table 2**). The ionizable lipids allowed for the development of more sophisticated lipid nanoparticle assemblies, known as lipid nanoparticles (LNPs), enabling encapsulation of RNA-based therapeutics, lower toxicity and higher transfection efficiency (**Figure 4c**).^{69,74–76} Ionizable amino lipids have an optimal pKa between 6-7 which allows for complexation with RNA in acidic pH, followed by LNP assembly.^{77,78} Subsequently, pH can be adjusted to physiological values enabling the deprotonation of ionizable lipids, giving LNPs a relatively neutral surface and avoiding therefore rapid opsonization or toxicity. LNPs have recently shown tremendous potential in gene therapies, especially after the first clinically approved siRNA-based LNP formulation Onpattro® – which is used to treat polyneuropathies resulting from the hereditary transthyretin (hATTR) amyloidosis – and the mRNA-LNP vaccines against SARS-CoV-2, Spikevax® and Comirnaty® (**Table 1**).^{79–82}

1.5 Targeted drug delivery

The concept of targeted drug delivery

The concept of using lipid-based nanoparticles in targeted delivery has been proposed since the 1970s.^{60,83,84} Nanoparticles can be engineered to exert tailored properties that enable the targeting of specific cells or tissues, hence enhancing their therapeutic efficacy, lowering the dose, and reducing off-target adverse effects. As mentioned previously, nanoparticle modification with targeting ligands – such as antibodies, peptides and sugar moieties – can facilitate nanoparticle recognition by receptors expressed on the surface of specific cell types, to promote active targeting and transport (*i.e.*, receptor-mediated endocytosis). Furthermore, nanoparticles can be designed to respond to specific stimuli such as pH, temperature or light, which allow for spatiotemporal control of drug release and enhanced therapeutic efficacy. Also, a top-down approach is commonly used for targeted lipid nanoparticle discovery, where empirical screenings by altering nanoparticle size and composition, or design of experiment (DOE) methods,⁸⁵ narrow down formulations with optimal *in vivo* behavior.

Table 2. Molecular structures of a selection of ionizable lipids.

Ionizable lipid	Molecular structure and name
DODAP First developed	 1,2-dioleoyl-3-dimethylammonium propane
DLin-MC3-DMA Clinically approved (Onpattro®)	 heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate
SM-102 Clinically approved (Spikevax®)	 Heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy) hexyl) amino)octanoate
ALC-0315 Clinically approved (Comirnaty®)	 (4-hydroxybutyl)azanediylbis(hexane-6,1-diyl)bis(2-hexyldecanoate)

Challenges in targeted drug delivery

Liposomes and LNPs have so far been the most clinically advanced drug delivery platforms. Several liposome drug formulations have been approved for use in patients, mostly because they exhibit lower adverse effects than the free drug. For example, it was found that Doxil[®] reduces the cardiotoxicity otherwise observed during treatment with doxorubicin.^{86,87} Liposomal drug formulations exhibit lower toxicity or slower drug release, however they do not particularly show higher therapeutic potential.⁸⁸ Despite the exciting advancements made in the field of lipid-based nanomedicines, several challenges remain. The biggest challenge continues to be the active targeting of (diseased) tissues and cell types. All clinically approved lipid-based nanomedicines are non-targeted delivery systems; Doxil[®] is a long circulating liposome, PEGylated to prevent rapid clearance, and mainly relies on the enhanced permeation and retention (EPR) effect to accumulate in solid tumors. Other liposomal drug formulations require similar biological mechanisms for accumulation, or they rely on passive uptake due to their size. For example, Mepact[®] (1-5 μm) has been used to activate the complement system and accumulate in macrophages, similarly to other nanoparticles that are cleared from circulation due to size.⁸⁹ Also, the two mRNA-LNP COVID-19 vaccines are administered intramuscularly and therefore immune responses are induced locally.

Consequently, no significant developments leading to selective nanoparticle targeting have yet resulted in a clinical product. Despite five decades of liposome research, discussions reflecting the reasons of the systematic failure to achieve selective therapies is only recently addressed.^{88,90–92} Firstly, rapid clearance of systemically administered nanoparticles from circulation, attributed to their opsonization and uptake by cells of the mononuclear phagocyte (MPS) and reticuloendothelial system (RES), lowers their therapeutic efficiency in tissues and organs other than the liver and kidneys. On the other hand, local nanoparticle administration has been shown to enhance drug delivery in tissues and reduce off-targets.^{93,94} Often however, areas of pathology are not accessible for local administration and when they are, procedures may be invasive, lowering the patient's quality of life. Secondly, targeting ligands are often erroneously assumed

they act as “magic bullets”. They can indeed increase cell specificity within a tissue and actively target cell types, but the poor penetrability of nanoparticles in the tissue – even by local administration – bypassing several cell and tissue barriers, is often ignored. Moreover, the EPR effect, mostly used to target tumors, is often overrated as it shows great potential on animal models due to their faster-growing tumors (especially on xenograft models), but fails to successfully and accurately predict translation in humans.⁹⁵ Taken together with the lack of knowledge on the tumor pathophysiology and diversity, which are not carefully considered during nanoparticle design, studies show that only 0.7% of intravenously (*i.v.*) administered nanoparticles reach solid tumors.⁹¹ Finally, nanoparticle designs utilizing targeting ligands such as antibodies, often exhibit relatively similar therapeutic outcomes to non-targeting formulations, but not higher enough to offset bottlenecks such as developmental costs (manufacturing, quality control *etc.*). The high complexity of such nanoparticle designs, containing multiple biologically active compounds, also requires extensive regulation for authorization and makes them unappealing to regulatory offices.^{88,90} Similarly, uncharted territories in nanomedicine, especially for the more recent LNP-based therapeutics, could lead to several implications in drug development. For example, it is not yet clear whether LNP components should be considered as active ingredients or drug excipients, and as a result regulation can be complex.⁹⁶

1.6 Overcoming challenges in nanomedicine

Bridging the translational gap

The preclinical development pipeline of nanomedicines usually follows that of traditional drug discovery, *i.e.*, physicochemical characterization, *in vitro* evaluation, and finally *in vivo* validation in relevant animal models. However, given the high complexity of nanomaterials – properties are different from a drug molecule – size, morphology, surface charge and surface modifications will profoundly determine the fate of nanomedicines *in vivo*.^{97,98} Taken together that *in vitro* testing fails to reflect a realistic environment, wherein serum proteins, immune cells, clearance mechanisms and dynamic blood flow are present, myriad

preclinical nanoparticle studies achieve very poor translational prediction.⁹⁹ Also, exhaustive large-scale experiments, high costs and ethical considerations involved in animal testing, force to narrow down nanoparticle candidates for evaluation in rodents. Consequently many “poor *in vitro* performers” are disregarded and the potential of a plethora of lipid nanoparticle formulations is not assessed further.

Zebrafish are a robust *in vivo* model that can bridge the existing gap and improve the translational prediction.¹⁰⁰ As an animal model to screen and assess nanoparticle behavior *in vivo*, zebrafish offer several advantages over *in vitro* testing, or organs-on-chips, and can be used prior to (but not replacing) testing in higher animal models. Firstly, zebrafish have 76% homology to human genes, compared to a similar percentage for mice (84%) and many organs and functional systems such as the liver, blood-brain-barrier, vascular and immune system share physiological similarities with mammalian counterparts.^{101–104} Also, plasma proteomes including main apolipoprotein families, as well as lipid transport and metabolism pathways (*i.e.*, several lipases, scavenging and lipoprotein receptors) are conserved.^{105,106} Secondly, zebrafish offer the advantage of optical transparency in early embryonic stages, enabling real-time visualization of nanoparticle behavior *in vivo*, across the whole living embryo and at cellular resolution.¹⁰⁷ Additionally, fast generation of large embryo numbers allows for high-throughput *in vivo* screenings. Another important advantage is the external fertilization, which eases genetic manipulation and generation of numerous transgenic lines (*i.e.*, fluorescent transporter lines, mutants, transparent lines).^{108,109} All these biological features allow for total nanoparticle assessment including toxicity studies, inflammatory responses and biodistribution. More specifically for the latter, embryonic zebrafish lines and biological manipulation (*i.e.*, inhibition of biological pathways) lead to observation of circulation lifetimes and clearance, extravasation, specific cell association, mechanisms of uptake, and transport across biological barriers. They also allow for evaluation of therapeutic outcomes such as gene delivery and cell transfection, gene silencing, drug release or others.

Zebrafish embryos, however, have an immature adaptive immune system which does not entirely reflect the nanoparticle clearance in higher animal models. They also have a slower blood flow and lower body temperature than rodents and humans, which can affect nanomedicine pharmacokinetics. Also, of particular note is that several biological pathways observed in zebrafish embryos are associated with developmental stages and are not conserved in adult stages. Therefore, a proper evaluation of the desired study and the correct selection of line and experimental setup is needed to effectively exploit these animal models. Since substantial differences in nanoparticle biodistribution has been observed across species,¹¹⁰ a combination of models is desired/should be considered, to evaluate and validate research outcomes.

Overall, zebrafish embryos can bridge the gap between *in vitro* and *in vivo* testing in higher animal models. They present more complex biological conditions and have bigger informative value than *in vitro* testing; at the same time, they are easier to manipulate, and are more applicable to high-throughput screenings, more cost/time effective, and require less regulation than higher *in vivo* organisms.

Understanding nanoparticle behavior

Besides the aforementioned translational gap, the stymied progression on nanoparticle targeting also lies on the lack of fundamental knowledge at the nano-bio interface.^{95,98} A better translation of nanomedicines can be achieved by understanding key molecular interactions – *i.e.*, specific nanoparticle-protein interactions – that drive biological mechanisms underpinning nanoparticle fate, biodistribution and clearance. For example, it was recently shown that surface charge can remarkably affect clearance and uptake of nanoparticles by different cell types.^{111–113} Particularly anionic lipid-based nanoparticles have been found to preferentially accumulate in liver-sinusoidal endothelial cells (LSECs) through an interaction with two transmembrane receptors, Stablin-1 and Stablin-2.^{111,112} Instead of endless nanoparticle screenings, by simply understanding the effect of nanoparticle charge in cell selectivity, and by generating LNPs with anionic surface charge, it was possible to redirect nanoparticle accumulation towards LSECs over hepatocytes.¹¹⁴ This event was found to be conserved across different animal models. Additionally, the formation of a protein corona on lipid-based

nanoparticles profoundly affects nanoparticle fate.^{115,116} Interestingly, the protein corona of several lipid-based nanoparticles has been recently found to be rich in apolipoproteins – the main structural and functional components of lipoproteins which regulate lipid transport and metabolism – as opposed to previous assumptions that larger, more abundant proteins (*i.e.*, albumin) are the main corona components.¹¹⁷ Importantly, many lipid-based nanoparticles have been found to have diverse apolipoprotein classes adsorbed onto their surface, or have no corona at all – depending on lipid composition – diverging therefore nanoparticle biodistribution. Interestingly, personalized nanoparticle protein coronas (*i.e.*, every individual may exhibit different plasma proteomes and therefore coronas) could be exploited in favor of the nanoparticle design and development.^{118,119}

By acquiring a more comprehensive picture on the biological identity of lipid-based nanoparticles influenced by their physicochemical properties, nanoparticle *in vivo* behavior can be more accurately predicted.⁹⁸ In summary, lipid composition, morphology, size, colloidal stability and charge, will all affect *in vivo* performance, corona formation, selective nanoparticle-protein communications, and promote different interactions with endogenous mechanisms. Having therefore a biocentric approach on lipid-based nanoparticle development, considering how physicochemical properties affect the nano-bio interface, could help overcome current challenges on nanoparticle targeting and lead to more precise technologies.

Hijacking endogenous mechanisms of lipid transport and metabolism for targeted drug delivery

In contrast to conventional empirical screenings, exploiting endogenous lipid transport mechanisms to guide lipid-based nanoparticles to specific tissues or cells, is an enticing strategy but largely unexplored. A highlight of this approach is the clinically relevant formulation Onpattro®.⁷⁹ Here, selective recognition and uptake within target hepatocytes rely on the adsorption of soluble apolipoprotein E (apoE) to the surface of circulating siRNA-LNPs. This, in turn, guides LNPs to low-density lipoprotein receptors (LDLr) that are heavily expressed on the sinusoidal surface of hepatocytes.¹²⁰ Similar strategies can employ other

apolipoproteins for enhanced cellular uptake *i.e.*, by scavenger or other apolipoprotein receptors.^{9,121,122} An even more unexplored strategy is employing triglyceride lipase-mediated pathways for nanoparticle cell specific targeting and transport. Triglyceride lipases (TGLs) are extracellular, endothelial lumen-bound hydrolytic enzymes which regulate lipid metabolism throughout the body.^{123–126} TGLs metabolize lipoproteins by hydrolyzing the ester bonds of TGs, DAGs and CEs to release free fatty acids, which in turn are taken up locally by cells; or facilitate lipoprotein margination and holoparticle uptake by the cell (**Figure 5**).^{127–129} The three main members of the TGLs, sharing significant structural homology, are lipoprotein lipase (LPL), endothelial lipase (EL) and hepatic lipase (HL).^{130–135} Once expressed, TGLs are localized on endothelial cells and are mainly connected to cell membranes onto heparan sulfate proteoglycans (HSPG) or the glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP1) via electrostatic interactions.^{136–138} Spatiotemporal regulation of TGLs makes them interesting targets for nanoparticle cell specificity. TGLs are present in organs such as liver, heart, reproductive organs, but also highly present in several cancer types, promoting tumor growth and proliferation through lipid transport.^{139–143}

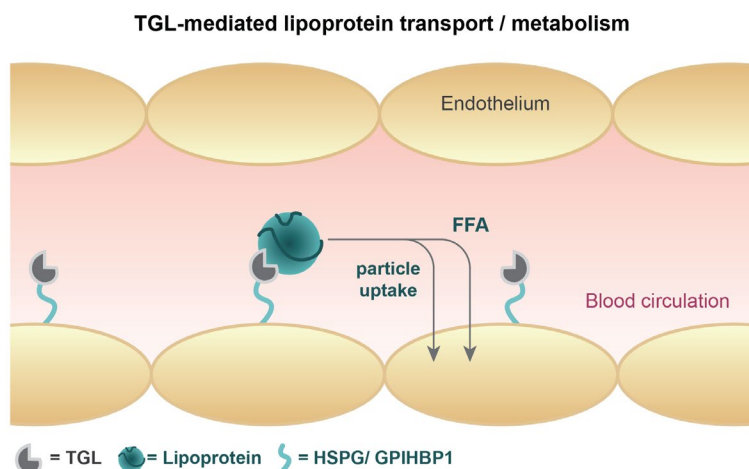


Figure 5. TGL-mediated lipoprotein transport/metabolism. TGLs are electrostatically bound to HSPG or GPIHBP1 and are exposed to the luminal side of endothelial cells. TGLs recognize lipoproteins in blood circulation and facilitate their endocytosis or hydrolyze them to release free fatty acids (FFA) that will be subsequently endocytosed.

1.7 Scope and motivation of this thesis

This doctoral thesis is an effort to understand how lipid phase-separation induced by DAG analogues in lipid-based nanoparticles affects their *in vivo* behavior, leading to specific nanoparticle-protein communications and selective cell targeting. By studying how lipid composition affects morphology and this in turn affects the nano-bio interface, a comprehensive picture and prediction of nanoparticle behavior and cell selectivity is provided.

Chapter 2 of this thesis describes the discovery of a novel liposome formulation consisting of just two lipids, a phospholipid and a synthetic DAG analogue (termed DOaG). This liposome formulation is capable of accumulating within brain endothelial cells of zebrafish embryos, by hijacking a pathway of lipid transport and metabolism mediated by TGL. Cryo-TEM imaging reveals a novel morphology in liposomes, characterized by lipid phase-separation, which is found to be responsible for the selective targeting. Zebrafish embryos are used as the primary *in vivo* model to unravel the biological mechanism underpinning the specific nanoparticle cell uptake, which is also found to be partially conserved in higher animal models (mice).

Chapter 3 describes an in-depth mechanistic understanding of the compositional and morphological changes that the phase-separated liposomes undergo in the presence of TGLs. A combination of cryo-TEM, molecular dynamic simulations and *in vitro* experiments, reveal that liposomes undergo remodeling after liposome-lipase interaction. Additionally, membrane packing defects – which are a result of phase-separation – facilitate the recognition and binding of the lipase onto the liposome surface.

Chapter 4 describes the detailed investigation of the underlying molecular principles underpinning the phase-separation and selective *in vivo* targeting, as induced by DOaG in liposomal membranes. Here, a library of DOaG analogues varying molecular properties is synthesized to investigate the structure- function relationship of DOaG in liposomal formulations. Cryo-TEM and biodistribution studies in zebrafish embryos reveal that only certain analogues can create phase-

separation leading to selective *in vivo* behavior, and that some contribute to the improvement of the formulation in terms of colloidal stability.

Chapter 5 describes the development of a novel mRNA-LNP formulation capable of delivering mRNA preferentially to the brain endothelial cells of zebrafish embryos, with subsequent transfection and protein expression. Guided by the knowledge acquired from the previous chapters, DOaG is used in this study to generate a novel phase-separated LNP formulation, as characterized by cryo-TEM. Zebrafish embryos are used for a biodistribution screening study to determine the exact lipid composition of DOaG-based LNP formulation that leads to specific cell targeting and gene delivery.

Chapter 6 summarizes all main findings generated over the course of this research and reflects on their importance for future studies and applications.

Appendix I describes a developed modified protocol for the *in-situ* formation and encapsulation of gold nanoparticles in phase separated, DOaG-containing liposomes.

1.8 References

1. Feynman, R. P. There's Plenty of Room at the Bottom. *Resonance* **2011**, 16 (9), 890–905.
2. Tewabe, A.; Abate, A.; Tamrie, M.; Seyfu, A.; Siraj, E. A. Targeted Drug Delivery — From Magic Bullet to Nanomedicine: Principles, Challenges, and Future Perspectives. *J Multidiscip Healthc* **2021**, 14, 1711–1724.
3. Hu, X.; Zhang, Y.; Ding, T.; Liu, J.; Zhao, H. Multifunctional Gold Nanoparticles: A Novel Nanomaterial for Various Medical Applications and Biological Activities. *Front Bioeng Biotechnol* **2020**, 8, 990.
4. Theodosiou, M.; Sakellis, E.; Boukos, N.; Kusigerski, V.; Kalska-Szostko, B.; Efthimiadou, E. Iron Oxide Nanoflowers Encapsulated in Thermosensitive Fluorescent Liposomes for Hyperthermia Treatment of Lung Adenocarcinoma. *Sci Rep* **2022**, 12 (1), 8697.
5. Dreaden, E. C.; Austin, L. A.; MacKey, M. A.; El-Sayed, M. A. Size Matters: Gold Nanoparticles in Targeted Cancer Drug Delivery. *Ther Deliv* **2012**, 3 (4), 457–478.
6. Dong, Y.; Love, K. T.; Dorkin, J. R.; Sirirungruang, S.; Zhang, Y.; Chen, D.; Bogorad, R. L.; Yin, H.; Chen, Y.; Vegas, A. J.; Alabi, C. A.; Sahay, G.; Olejnik, K. T.; Wang, W.; Schroeder, A.; Lytton-Jean, A. K. R.; Siegwart, D. J.; Akinc, A.; Barnes, C.; Barros, S. A.; Carioto, M.; Fitzgerald, K.; Hettinger, J.; Kumar, V.; Novobrantseva, T. I.; Qin, J.; Querbes, W.; Koteliansky, V.; Langer, R.; Anderson, D. G. Lipopeptide Nanoparticles for Potent and Selective siRNA Delivery in Rodents and Nonhuman Primates. *PNAS* **2014**, 111 (11), 3955–3960.
7. Mohsen, M. O.; Bachmann, M. F. Virus-like Particle Vaccinology, from Bench to Bedside. *Cell Mol Immunol* **2022**, 19 (9), 993–1011.
8. Herrmann, I. K.; Wood, M. J. A.; Fuhrmann, G. Extracellular Vesicles as a Next-Generation Drug Delivery Platform. *Nat Nanotechnol* **2021**, 16 (7), 748–759.
9. Schrijver, D. P.; De Dreu, A.; Hofstraat, S. R. J.; Kluza, E.; Zwolsman, R.; Deckers, J.; Anbergen, T.; De Bruin, K.; Trines, M. M.; Nugraha, E. G.; Ummels, F.; Röring, R. J.; Beldman, T. J.; Teunissen, A. J. P.; Fayad, Z. A.; Van Der Meel, R.; Mulder, W. J. M. Nanoengineering Apolipoprotein A1-Based Immunotherapeutics. *Adv Ther* **2021**, 4 (8), 2100083.
10. Ledesma, F.; Ozcan, B.; Sun, X.; Medina, S. M.; Landry, M. P.; Ledesma, F.; Ozcan, B.; Sun, X.; Medina, S. M.; Landry, M. P.; Biohub, C.-Z. Nanomaterial Strategies for Delivery of Therapeutic Cargoes. *Adv Funct Mater* **2022**, 32 (4), 2107174.
11. Papahadjopoulos, D.; Allen, T. M.; Gabizon, A.; Mayhew, E.; Matthay, K.; Huang, S. K.; Lee, K. D.; Woodle, M. C.; Lasic, D. D.; Redemann, C.; Martin, F. J. Sterically Stabilized Liposomes: Improvements in Pharmacokinetics and Antitumor Therapeutic Efficacy. *PNAS* **1991**, 88 (24), 11460–11464.

12. Klibanov, A. L.; Maruyama, K.; Torchilin, V. P.; Huang, L. Amphipathic Polyethyleneglycols Effectively Prolong the Circulation Time of Liposomes. *FEBS Lett* **1990**, 268 (1), 235–237.
13. Van Meer, G.; Voelker, D. R.; Feigenson, G. W. Membrane Lipids: Where They Are and How They Behave. *Nat Rev Mol Cell Biol* **2008**, 9 (2), 112–124.
14. Crawford, R.; Dogdas, B.; Keough, E.; Haas, R. M.; Wepukhulu, W.; Krotzer, S.; Burke, P. A.; Sepp-Lorenzino, L.; Bagchi, A.; Howell, B. J. Analysis of Lipid Nanoparticles by Cryo-EM for Characterizing siRNA Delivery Vehicles. *Int J Pharm* **2011**, 403, 237–244.
15. Stewart, P. L. Cryo-Electron Microscopy and Cryo-Electron Tomography of Nanoparticles. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* **2017**, 9 (2), e1417.
16. Frederik, P. M.; Hubert, D. H. W. Cryoelectron Microscopy of Liposomes. *Methods Enzymol* **2005**, 391, 431–448.
17. Almgren, M.; Edwards, K.; Karlsson, G. Cryo Transmission Electron Microscopy of Liposomes and Related Structures. *Colloids Surf A Physicochem Eng Asp* **2000**, 174 (1–2), 3–21.
18. Bhattacharjee, S. DLS and Zeta Potential – What They Are and What They Are Not? *J Control Release* **2016**, 235, 337–351.
19. Mahmoudi, M. The Need for Robust Characterization of Nanomaterials for Nanomedicine Applications. *Nat Commun* **2021**, 12 (1), 1–5.
20. Faria, M.; Björnmalm, M.; Thurecht, K. J.; Kent, S. J.; Parton, R. G.; Kavallaris, M.; Johnston, A. P. R.; Gooding, J. J.; Corrie, S. R.; Boyd, B. J.; Thordarson, P.; Whittaker, A. K.; Stevens, M. M.; Prestidge, C. A.; Porter, C. J. H.; Parak, W. J.; Davis, T. P.; Crampin, E. J.; Caruso, F. Minimum Information Reporting in Bio–Nano Experimental Literature. *Nat Nanotechnol* **2018**, 13 (9), 777–785.
21. Poste, G.; Papahadjopoulos, D. Lipid Vesicles as Carriers for Introducing Materials into Cultured Cells: Influence of Vesicle Lipid Composition on Mechanism(s) of Vesicle Incorporation into Cells. *PNAS* **1976**, 73 (5), 1603–1607.
22. Cullis, P. R.; Hope, M. J.; Tilcock, C. P. S. Lipid Polymorphism and the Roles of Lipids in Membranes. *Chem Phys Lipids* **1986**, 40 (2–4), 127–144.
23. Coffill, C. R.; Ramsamy, T. A.; Hutt, D. M.; Schultz, J. R.; Sparks, D. L. Diacylglycerol Is the Preferred Substrate in High Density Lipoproteins for Human Hepatic Lipase. *J Lipid Res* **1997**, 38 (11), 2224–2231.
24. Goñi, F. M.; Alonso, A. Structure and Functional Properties of Diacylglycerols in Membranes. *Prog Lipid Res* **1999**, 38 (1), 1–48.
25. Campomanes, P.; Zoni, V.; Vanni, S. Local Accumulation of Diacylglycerol Alters Membrane Properties Nonlinearly Due to Its Transbilayer Activity. *Commun Chem* **2019**, 2 (1), 1–8.
26. Goldberg, E. M.; Lester, D. S.; Borchardt, D. B.; Zidovetzki, R. Effects of Diacylglycerols on Conformation of Phosphatidylcholine Headgroups in Phosphatidylcholine/Phosphatidylserine Bilayers. *Biophys J* **1995**, 69 (3), 965–973.

27. Alwarawrah, M.; Hussain, F.; Huang, J. Alteration of Lipid Membrane Structure and Dynamics by Diacylglycerols with Unsaturated Chains. *Biochim Biophys Acta Biomembr* **2016**, *1858* (2), 253–263.
28. Bilayers Edward Goldberg, P. M.; Lester, D. S.; Borchardt, D. B.; Zidovetzki, R. Effects of Diacylglycerols and Ca^{2+} on Structure of Phosphatidylcholine/Phosphatidylserine Bilayers. *Biophys J* **1994**, *66*, 382–393.
29. Alwarawrah, M.; Dai, J.; Huang, J. Modification of Lipid Bilayer Structure by Diacylglycerol: A Comparative Study of Diacylglycerol and Cholesterol. *J Chem Theory Comput* **2012**, *8* (2), 749–758.
30. Szule, J. A.; Fuller, N. L.; Peter Rand, R. The Effects of Acyl Chain Length and Saturation of Diacylglycerols and Phosphatidylcholines on Membrane Monolayer Curvature. *Biophys J* **2002**, *83* (2), 977–984.
31. Vanni, S.; Hirose, H.; Barelli, H.; Antonny, B.; Gautier, R. A Sub-Nanometre View of How Membrane Curvature and Composition Modulate Lipid Packing and Protein Recruitment. *Nat Commun* **2014**, *5* (1), 1–10.
32. Vamparys, L.; Gautier, R.; Vanni, S.; Bennett, W. F. D.; Tieleman, D. P.; Antonny, B.; Etchebest, C.; Fuchs, P. F. J. Conical Lipids in Flat Bilayers Induce Packing Defects Similar to That Induced by Positive Curvature. *Biophys J* **2013**, *104* (3), 585–593.
33. Cui, H.; Lyman, E.; Voth, G. A. Mechanism of Membrane Curvature Sensing by Amphipathic Helix Containing Proteins. *Biophys J* **2011**, *100* (5), 1271–1279.
34. Drin, G.; Casella, J.-F.; Gautier, R.; Boehmer, T.; Schwartz, T. U.; Antonny, B. A General Amphipathic A-Helical Motif for Sensing Membrane Curvature. *Nat Struct Mol Biol* **2007**, *14* (2), 138–146.
35. van Hilten, N.; Methorst, J.; Verwei, N.; Risselada, H. J. Physics-Based Generative Model of Curvature Sensing Peptides; Distinguishing Sensors from Binders. *Sci Adv* **2023**, *9* (11), eade8839.
36. Bohr, S. S. R.; Thorlaksen, C.; Kühnel, R. M.; Günther-Pomorski, T.; Hatzakis, N. S. Label-Free Fluorescence Quantification of Hydrolytic Enzyme Activity on Native Substrates Reveals How Lipase Function Depends on Membrane Curvature. *Langmuir* **2020**, *36* (23), 6473–6481.
37. Gómez-Fernández, J. C.; Corbalán-García, S. Diacylglycerols, Multivalent Membrane Modulators. *Chem Phys Lipids* **2007**, *148* (1), 1–25.
38. Eichmann, T. O.; Lass, A. DAG Tales: The Multiple Faces of Diacylglycerol—Stereochemistry, Metabolism, and Signaling. *Cell Mol Life Sci* **2015**, *72* (20), 3931–3952.
39. Dawson, R. M. C.; Hemington, N. L.; Irvine, R. F. Diacylglycerol Potentiates Phospholipase Attack upon Phospholipid Bilayers: Possible Connection with Cell Stimulation. *Biochem Biophys Res Commun* **1983**, *117* (1), 196–201.
40. Barlič, A.; Gutiérrez-Aguirre, I.; Caaveiro, J. M. M.; Cruz, A.; Ruiz-Argüello, M. B.; Pérez-Gil, J.; González-Mañas, J. M. Lipid Phase Coexistence Favors Membrane

- Insertion of Equinatoxin-II, a Pore-Forming Toxin from *Actinia Equina*. *J Biol Chem* **2004**, 279 (33), 34209–34216.
41. Ahyayauch, H.; Sot, J.; Collado, M. I.; Huarte, N.; Requejo-Isidro, J.; Alonso, A.; Goñi, F. M. End-Product Diacylglycerol Enhances the Activity of PI-PLC through Changes in Membrane Domain Structure. *Biophys J* **2015**, 108 (7), 1672–1682.
 42. Dawson, R. M. C.; Irvine, R. F.; Bray, J.; Quinn, P. J. Long-Chain Unsaturated Diacylglycerols Cause a Perturbation in the Structure of Phospholipid Bilayers Rendering Them Susceptible to Phospholipase Attack. *Biochem Biophys Res Commun* **1984**, 125 (2), 836–842.
 43. Basáñez, G.; Nieva, J. L.; Rivas, E.; Alonso, A.; Goñi, F. M. Diacylglycerol and the Promotion of Lamellar-Hexagonal and Lamellar-Isotropic Phase Transitions in Lipids: Implications for Membrane Fusion. *Biophys J* **1996**, 70 (5), 2299–2306.
 44. Siegel, D. P.; Epand, R. M. The Mechanism of Lamellar-to-Inverted Hexagonal Phase Transitions in Phosphatidylethanolamine: Implications for Membrane Fusion Mechanisms. *Biophys J* **1997**, 73 (6), 3089–3111.
 45. Nieva, J. L.; Alonso, A.; Basáñez, G.; Goñi, F. M.; Gulik, A.; Vargas, R.; Luzzati, V. Topological Properties of Two Cubic Phases of a Phospholipid: Cholesterol: Diacylglycerol Aqueous System and Their Possible Implications in the Phospholipase C-Induced Liposome Fusion. *FEBS Lett* **1995**, 368 (1), 143–147.
 46. Siegel, D. P.; Banschbach, J.; Alford, D.; Bentz, J.; Ellens, H.; Lis, L. J.; Quinn, P. J.; Yeagle, P. L. Physiological Levels of Diacylglycerols in Phospholipid Membranes Induce Membrane Fusion and Stabilize Inverted Phases. *Biochemistry* **1989**, 28 (9), 3703–3709.
 47. Vieu, C.; Jaspard, B.; Barbaras, R.; Manent, J.; Chap, H.; Perret, B.; Collet, X. Identification and Quantification of Diacylglycerols in HDL and Accessibility to Lipase. *J Lipid Res* **1996**, 37 (5), 1153–1161.
 48. Bae, C. S.; Ahn, T. Diacylglycerol in Cationic Nanoparticles Stimulates Oxidative Stress-Mediated Death of Cancer Cells. *Lipids* **2018**, 53 (11–12), 1059–1067.
 49. Ibarguren, M.; Bomans, P. H. H.; Ruiz-Mirazo, K.; Frederik, P. M.; Alonso, A.; Goñi, F. M. Thermally-Induced Aggregation and Fusion of Protein-Free Lipid Vesicles. *Colloids Surf B Biointerfaces* **2015**, 136, 545–552.
 50. Kim, B.; Sun, S.; Varner, J. A.; Howell, S. B.; Ruoslahti, E.; Sailor, M. J. Securing the Payload, Finding the Cell, and Avoiding the Endosome: Peptide-Targeted, Fusogenic Porous Silicon Nanoparticles for Delivery of siRNA. *Adv Mat* **2019**, 31 (35), 1902952.
 51. Yang, J.; Tu, J.; Lamers, G. E. M.; Olsthoorn, R. C. L.; Kros, A. Membrane Fusion Mediated Intracellular Delivery of Lipid Bilayer Coated Mesoporous Silica Nanoparticles. *Adv Healthc Mater* **2017**, 6 (20), 201700759.
 52. Hafez, I. M.; Cullis, P. R. Roles of Lipid Polymorphism in Intracellular Delivery. *Adv Drug Deliv Rev* **2001**, 47 (2–3), 139–148.

53. Cheng, M. H. Y.; Leung, J.; Zhang, Y.; Strong, C.; Basha, G.; Momeni, A.; Chen, Y.; Jan, E.; Abdollahzadeh, A.; Wang, X.; Kulkarni, J. A.; Witzigmann, D.; Cullis, P. R. Induction of Bleb Structures in Lipid Nanoparticle Formulations of mRNA Leads to Improved Transfection Potency. *Adv mat* **2023**, e2303370.
54. Imam, Z. I.; Kenyon, L. E.; Ashby, G.; Nagib, F.; Mendicino, M.; Zhao, C.; Gadok, A. K.; Stachowiak, J. C. Phase-Separated Liposomes Enhance the Efficiency of Macromolecular Delivery to the Cellular Cytoplasm. *Cell Mol Bioeng* **2017**, *10* (5), 387–403.
55. Bangham, A. D.; Standish, M. M.; Watkins, J. C. Diffusion of Univalent Ions across the Lamellae of Swollen Phospholipids. *J Mol Biol* **1965**, *13* (1), 238–252.
56. Weissig, V. Liposomes Came First: The Early History of Liposomology. *Methods Mol Biol* **2017**, *1522*, 1–15.
57. Bangham, A. D.; Hill, M. W.; Miller, N. G. A. Preparation and Use of Liposomes as Models of Biological Membranes. In *Methods Membr Biol*; Springer, Boston, MA, 1974; 1–68.
58. Bangham, A. D.; Hill, M. W. The Proton Pump/Leak Mechanism of Unconsciousness. *Chem Phys Lipids* **1986**, *40* (2–4), 189–205.
59. Gregoriadis, G.; Buckland, R. A. Enzyme-Containing Liposomes Alleviate a Model for Storage Disease. *Nature* **1973**, *244* (5412), 170–172.
60. Gregoriadis, G. The Carrier Potential of Liposomes in Biology and Medicine (First of Two Parts). *N Engl J Med* **1976**, *295* (13), 704–710.
61. Gregoriadis, G.; Allison, A. C. Entrapment of Proteins in Liposomes Prevents Allergic Reactions in Pre-Immunised Mice. *FEBS Lett* **1974**, *45* (1), 71–74.
62. Allison, A. C.; Gregoriadis, G. Liposomes as Immunological Adjuvants. *Nature* **1974**, *252* (5480), 252–252.
63. Gregoriadis, G. Liposomes in Drug Delivery: How It All Happened. *Pharmaceutics* **2016**, *8* (2), 19.
64. Hwang, K. J.; Luk, K. F. S.; Beaumier, P. L. Hepatic Uptake and Degradation of Unilamellar Sphingomyelin/Cholesterol Liposomes: A Kinetic Study. *PNAS* **1980**, *77* (7), 4030–4034.
65. Gregoriadis, G.; Senior, J. The Phospholipid Component of Small Unilamellar Liposomes Controls the Rate of Clearance of Entrapped Solutes from the Circulation. *FEBS Lett* **1980**, *119* (1), 43–46.
66. Juliano, R. L.; Stamp, D. The Effect of Particle Size and Charge on the Clearance Rates of Liposomes and Liposome Encapsulated Drugs. *Biochem Biophys Res Commun* **1975**, *63* (3), 651–658.
67. Kirby, C.; Clarke, J.; Gregoriadis, G. Effect of the Cholesterol Content of Small Unilamellar Liposomes on Their Stability in Vivo and in Vitro. *Biochemical Journal* **1980**, *186* (2), 591–598.

68. Gabizon, A.; Papahadjopoulos, D. Liposome Formulations with Prolonged Circulation Time in Blood and Enhanced Uptake by Tumors. *PNAS* **1988**, *85* (18), 6949–6953.
69. Cullis, P. R.; Hope, M. J. Lipid Nanoparticle Systems for Enabling Gene Therapies. *Mol Ther* **2017**, *25* (7), 1467–1475.
70. Crommelin, D. J. A.; van Hoogevest, P.; Storm, G. The Role of Liposomes in Clinical Nanomedicine Development. What Now? Now What? *J Control Release* **2020**, *318*, 256–263.
71. Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. Lipofection: A Highly Efficient, Lipid-Mediated DNA-Transfection Procedure. *PNAS* **1987**, *84* (21), 7413–7417.
72. Filion, M. C.; Phillips, N. C. Toxicity and Immunomodulatory Activity of Liposomal Vectors Formulated with Cationic Lipids toward Immune Effector Cells. *Biochim Biophys Acta* **1997**, *1329* (2), 345–356.
73. Allen, T. M.; Cullis, P. R. Liposomal Drug Delivery Systems: From Concept to Clinical Applications. *Adv Drug Deliv Rev* **2013**, *65* (1), 36–48.
74. Semple, S. C.; Klimuk, S. K.; Harasym, T. O.; Dos Santos, N.; Ansell, S. M.; Wong, K. F.; Maurer, N.; Stark, H.; Cullis, P. R.; Hope, M. J.; Scherrer, P. Efficient Encapsulation of Antisense Oligonucleotides in Lipid Vesicles Using Ionizable Aminolipids: Formation of Novel Small Multilamellar Vesicle Structures. *Biochim Biophys Acta* **2001**, *1510* (1–2), 152–166.
75. Semple, S. C.; Akinc, A.; Chen, J.; Sandhu, A. P.; Mui, B. L.; Cho, C. K.; Sah, D. W. Y.; Stebbing, D.; Crosley, E. J.; Yaworski, E.; Hafez, I. M.; Dorkin, J. R.; Qin, J.; Lam, K.; Rajeev, K. G.; Wong, K. F.; Jeffs, L. B.; Nechev, L.; Eisenhardt, M. L.; Jayaraman, M.; Kazem, M.; Maier, M. A.; Srinivasulu, M.; Weinstein, M. J.; Chen, Q.; Alvarez, R.; Barros, S. A.; De, S.; Klimuk, S. K.; Borland, T.; Kosovrasti, V.; Cantley, W. L.; Tam, Y. K.; Manoharan, M.; Ciufolini, M. A.; Tracy, M. A.; De Fougères, A.; MacLachlan, I.; Cullis, P. R.; Madden, T. D.; Hope, M. J. Rational Design of Cationic Lipids for siRNA Delivery. *Nat Biotechnol* **2010**, *28* (2), 172–176.
76. Horejs, C. From Lipids to Lipid Nanoparticles to mRNA Vaccines. *Nat Rev Mater* **2021**, *6* (12), 1075–1076.
77. Patel, P.; Ibrahim, N. M.; Cheng, K. The Importance of Apparent pKa in the Development of Nanoparticles Encapsulating siRNA and mRNA. *Trends Pharmacol Sci* **2021**, *42* (6), 448–460.
78. Hald Albertsen, C.; Kulkarni, J. A.; Witzigmann, D.; Lind, M.; Petersson, K.; Simonsen, J. B. The Role of Lipid Components in Lipid Nanoparticles for Vaccines and Gene Therapy. *Adv Drug Deliv Rev* **2022**, *188*, 114416.
79. Akinc, A.; Maier, M. A.; Manoharan, M.; Fitzgerald, K.; Jayaraman, M.; Barros, S.; Ansell, S.; Du, X.; Hope, M. J.; Madden, T. D.; Mui, B. L.; Semple, S. C.; Tam, Y. K.; Ciufolini, M.; Witzigmann, D.; Kulkarni, J. A.; van der Meel, R.; Cullis, P. R. The

- Onpattro Story and the Clinical Translation of Nanomedicines Containing Nucleic Acid-Based Drugs. *Nat Nanotechnol* **2019**, *14* (12), 1084–1087.
80. Schoenmaker, L.; Witzigmann, D.; Kulkarni, J. A.; Verbeke, R.; Kersten, G.; Jiskoot, W.; Crommelin, D. J. A. mRNA-Lipid Nanoparticle COVID-19 Vaccines: Structure and Stability. *Int J Pharm* **2021**, *601*, 120586.
81. Szabó, G. T.; Mahiny, A. J.; Vlatkovic, I. COVID-19 mRNA Vaccines: Platforms and Current Developments. *Mol Ther* **2022**, *30* (5), 1850–1868.
82. Khurana, A.; Allawadhi, P.; Khurana, I.; Allwadhi, S.; Weiskirchen, R.; Banothu, A. K.; Chhabra, D.; Joshi, K.; Bharani, K. K. Role of Nanotechnology behind the Success of mRNA Vaccines for COVID-19. *Nano Today* **2021**, *38*, 101142.
83. Gregory Gregoriadis, E.; Neerunjun, D. Homing of Liposomes to Target Cells. *Biochem Biophys Res Commun* **1975**, *65* (2), 537–544.
84. Gregoriadis, G. Targeting of Drugs. *Nature* **1977**, *265* (5593), 407–411.
85. Bevers, S.; Kooijmans, S. A. A.; Van de Velde, E.; Evers, M. J. W.; Seghers, S.; Gitz-Francois, J. J. J. M.; van Kronenburg, N. C. H.; Fens, M. H. A. M.; Mastrobattista, E.; Hassler, L.; Sork, H.; Lehto, T.; Ahmed, K. E.; El Andaloussi, S.; Fiedler, K.; Breckpot, K.; Maes, M.; Van Hoorick, D.; Bastogne, T.; Schiffelers, R. M.; De Koker, S. mRNA-LNP Vaccines Tuned for Systemic Immunization Induce Strong Antitumor Immunity by Engaging Splenic Immune Cells. *Mol Ther* **2022**, *30* (9), 3078–3094.
86. O'Brien, M. E. R.; Wigler, N.; Inbar, M.; Rosso, R.; Grischke, E.; Santoro, A.; Catane, R.; Kieback, D. G.; Tomczak, P.; Ackland, S. P.; Orlandi, F.; Mellars, L.; Alland, L.; Tendler, C. Reduced Cardiotoxicity and Comparable Efficacy in a Phase III Trial of Pegylated Liposomal Doxorubicin HCl (CaelyxTM/Doxil[®]) versus Conventional Doxorubicin for First-Line Treatment of Metastatic Breast Cancer. *Annals of Oncology* **2004**, *15* (3), 440–449.
87. Northfelt, D. W.; Dezube, B. J.; Thommes, J. A.; Miller, B. J.; Fischl, M. A.; Friedman-Kien, A.; Kaplan, L. D.; Du Mond, C.; Mamelok, R. D.; Henry, D. H. Pegylated-Liposomal Doxorubicin versus Doxorubicin, Bleomycin, and Vincristine in the Treatment of AIDS-Related Kaposi's Sarcoma: Results of a Randomized Phase III Clinical Trial. *J Clin Oncol* **1998**, *16* (7), 2445–2451.
88. Lammers, T.; Kiessling, F.; Hennink, W. E.; Storm, G. Drug Targeting to Tumors: Principles, Pitfalls and (Pre-) Clinical Progress. *J Control Release* **2012**, *161* (2), 175–187.
89. Hoshyar, N.; Gray, S.; Han, H.; Bao, G. The Effect of Nanoparticle Size on in Vivo Pharmacokinetics and Cellular Interaction. *Nanomedicine* **2016**, *11* (6), 673–692.
90. Guidolin, K.; Zheng, G. Nanomedicines Lost in Translation. *ACS Nano* **2019**, *13* (12), 13620–13626.
91. Wilhelm, S.; Tavares, A. J.; Dai, Q.; Ohta, S.; Audet, J.; Dvorak, H. F.; Chan, W. C. W. Analysis of Nanoparticle Delivery to Tumours. *Nat Rev Mater* **2016**, *1* (5), 1–12.

92. Bae, Y. H.; Park, K. Targeted Drug Delivery to Tumors: Myths, Reality and Possibility. *J Control Release* **2011**, *153* (3), 198–205.
93. Pardi, N.; Tuyishime, S.; Muramatsu, H.; Kariko, K.; Mui, B. L.; Tam, Y. K.; Madden, T. D.; Hope, M. J.; Weissman, D. Expression Kinetics of Nucleoside-Modified mRNA Delivered in Lipid Nanoparticles to Mice by Various Routes. *J control release* **2015**, *217*, 345–351.
94. Bastiancich, C.; Bozzato, E.; Henley, I.; Newland, B. Does Local Drug Delivery Still Hold Therapeutic Promise for Brain Cancer? A Systematic Review. *J Control Release* **2021**, *337*, 296–305.
95. Björnmalm, M.; Thurecht, K. J.; Michael, M.; Scott, A. M.; Caruso, F. Bridging Bio-Nano Science and Cancer Nanomedicine. *ACS Nano* **2017**, *11* (10), 9594–9613.
96. Hemmrich, E.; McNeil, S. Active Ingredient vs Excipient Debate for Nanomedicines. *Nat Nanotechnol* **2023**, *18* (7), 692–695.
97. Dai, Q.; Bertleff-Zieschang, N.; Braunger, J. A.; Björnmalm, M.; Cortez-Jugo, C.; Caruso, F. Particle Targeting in Complex Biological Media. *Adv Healthc Mater* **2018**, *7* (1), 1700575.
98. Nel, A. E.; Mädler, L.; Velegol, D.; Xia, T.; Hoek, E. M. V.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M. Understanding Biophysicochemical Interactions at the Nano–Bio Interface. *Nat Mater* **2009**, *8* (7), 543–557.
99. Paunovska, K.; Sago, C. D.; Monaco, C. M.; Hudson, W. H.; Castro, M. G.; Rudoltz, T. G.; Kalathoor, S.; Vanover, D. A.; Santangelo, P. J.; Ahmed, R.; Bryksin, A. V.; Dahlman, J. E. A Direct Comparison of in Vitro and in Vivo Nucleic Acid Delivery Mediated by Hundreds of Nanoparticles Reveals a Weak Correlation. *Nano Lett* **2018**, *18* (3), 2148–2157.
100. Sieber, S.; Grossen, P.; Bussmann, J.; Campbell, F.; Kros, A.; Witzigmann, D.; Huwyler, J. Zebrafish as a Preclinical in Vivo Screening Model for Nanomedicines. *Adv Drug Deliv Rev* **2019**, *151–152*, 152–168.
101. Howe, K.; Clark, M. D.; Torroja, C. F.; Torrance, J.; Berthelot, C.; Muffato, M.; Collins, J. E.; Humphray, S.; McLaren, K.; Matthews, L.; McLaren, S.; Sealy, I.; Caccamo, M.; Churcher, C.; Scott, C.; Barrett, J. C.; Koch, R.; Rauch, G. J.; White, S.; Chow, W.; Kilian, B.; Quintais, L. T.; Guerra-Assunção, J. A.; Zhou, Y.; Gu, Y.; Yen, J.; Vogel, J. H.; Eyre, T.; Redmond, S.; Banerjee, R.; Chi, J.; Fu, B.; Langley, E.; Maguire, S. F.; Laird, G. K.; Lloyd, D.; Kenyon, E.; Donaldson, S.; Sehra, H.; Almeida-King, J.; Loveland, J.; Trevanion, S.; Jones, M.; Quail, M.; Willey, D.; Hunt, A.; Burton, J.; Sims, S.; McLay, K.; Plumb, B.; Davis, J.; Clee, C.; Oliver, K.; Clark, R.; Riddle, C.; Elliott, D.; Threadgold, G.; Harden, G.; Ware, D.; Mortimer, B.; Kerry, G.; Heath, P.; Phillimore, B.; Tracey, A.; Corby, N.; Dunn, M.; Johnson, C.; Wood, J.; Clark, S.; Pelan, S.; Griffiths, G.; Smith, M.; Glithero, R.; Howden, P.; Barker, N.; Stevens, C.; Harley, J.; Holt, K.; Panagiotidis, G.; Lovell, J.; Beasley, H.; Henderson, C.; Gordon, D.; Auger, K.; Wright, D.; Collins, J.; Raisin, C.; Dyer, L.; Leung, K.; Robertson, L.; Ambridge, K.; Leongamornlert, D.; McGuire, S.

- Gilderthorp, R.; Griffiths, C.; Manthravadi, D.; Nichol, S.; Barker, G.; Whitehead, S.; Kay, M.; Brown, J.; Murnane, C.; Gray, E.; Humphries, M.; Sycamore, N.; Barker, D.; Saunders, D.; Wallis, J.; Babbage, A.; Hammond, S.; Mashreghi-Mohammadi, M.; Barr, L.; Martin, S.; Wray, P.; Ellington, A.; Matthews, N.; Ellwood, M.; Woodmansey, R.; Clark, G.; Cooper, J.; Tromans, A.; Grafham, D.; Skuce, C.; Pandian, R.; Andrews, R.; Harrison, E.; Kimberley, A.; Garnett, J.; Fosker, N.; Hall, R.; Garner, P.; Kelly, D.; Bird, C.; Palmer, S.; Gehring, I.; Berger, A.; Dooley, C. M.; Ersan-Ürün, Z.; Eser, C.; Geiger, H.; Geisler, M.; Karotki, L.; Kirn, A.; Konantz, J.; Konantz, M.; Oberländer, M.; Rudolph-Geiger, S.; Teucke, M.; Osoegawa, K.; Zhu, B.; Rapp, A.; Widaa, S.; Langford, C.; Yang, F.; Carter, N. P.; Harrow, J.; Ning, Z.; Herrero, J.; Searle, S. M. J.; Enright, A.; Geisler, R.; Plasterk, R. H. A.; Lee, C.; Westerfield, M.; De Jong, P. J.; Zon, L. I.; Postlethwait, J. H.; Nüsslein-Volhard, C.; Hubbard, T. J. P.; Crollius, H. R.; Rogers, J.; Stemple, D. L. The Zebrafish Reference Genome Sequence and Its Relationship to the Human Genome. *Nature* **2013**, 496 (7446), 498–503.
102. Isogai, S.; Horiguchi, M.; Weinstein, B. M. The Vascular Anatomy of the Developing Zebrafish: An Atlas of Embryonic and Early Larval Development. *Dev Biol* **2001**, 230 (2), 278–301.
103. Chu, J.; Sadler, K. C. A New School in Liver Development: Lessons from Zebrafish. *Hepatology* **2009**, 50 (5), 1656–1663.
104. Kim, S. S.; Im, S. H.; Yang, J. Y.; Lee, Y. R.; Kim, G. R.; Chae, J. S.; Shin, D. S.; Song, J. S.; Ahn, S.; Lee, B. H.; Woo, J. C.; Ahn, J. H.; Yun, C. S.; Kim, P.; Kim, H. R.; Lee, K. R.; Bae, M. A. Zebrafish as a Screening Model for Testing the Permeability of Blood-Brain Barrier to Small Molecules. *Zebrafish* **2017**, 14 (4), 322–330.
105. Otis, J. P.; Zeituni, E. M.; Thierer, J. H.; Anderson, J. L.; Brown, A. C.; Boehm, E. D.; Cerchione, D. M.; Ceasrine, A. M.; Avraham-David, I.; Tempelhof, H.; Yaniv, K.; Farber, S. A. Zebrafish as a Model for Apolipoprotein Biology: Comprehensive Expression Analysis and a Role for ApoA-IV in Regulating Food Intake. *Dis Model Mech* **2015**, 8 (3), 295–309.
106. Li, C.; Tan, X. F.; Lim, T. K.; Lin, Q.; Gong, Z. Comprehensive and Quantitative Proteomic Analyses of Zebrafish Plasma Reveals Conserved Protein Profiles between Genders and between Zebrafish and Human. *Sci Rep* **2016**, 6 (1), 1–15.
107. Karlsson, J.; Von Hofsten, J.; Olsson, P. E. Generating Transparent Zebrafish: A Refined Method to Improve Detection of Gene Expression during Embryonic Development. *Marine biotechnology* **2001**, 3 (6), 522–527.
108. Nasevicius, A.; Ekker, S. C. Effective Targeted Gene ‘Knockdown’ in Zebrafish. *Nature Genetics* **2000**, 26 (2), 216–220.
109. Wienholds, E.; van Eeden, F.; Kosters, M.; Mudde, J.; Plasterk, R. H. A.; Cuppen, E. Efficient Target-Selected Mutagenesis in Zebrafish. *Genome Res* **2003**, 13 (12), 2700–2707.

110. Hatit, M. Z. C.; Lokugamage, M. P.; Dobrowolski, C. N.; Paunovska, K.; Ni, H.; Zhao, K.; Vanover, D.; Beyersdorf, J.; Peck, H. E.; Loughrey, D.; Sato, M.; Cristian, A.; Santangelo, P. J.; Dahlman, J. E. Species-Dependent in Vivo mRNA Delivery and Cellular Responses to Nanoparticles. *Nat Nanotechnol* **2022**, *17* (3), 310–318.
111. Arias-Alpizar, G.; Koch, B.; Hamelmann, N. M.; Neustrup, M. A.; Paulusse, J. M. J.; Jiskoot, W.; Kros, A.; Bussmann, J. Stabilin-1 Is Required for the Endothelial Clearance of Small Anionic Nanoparticles. *Nanomedicine* **2021**, *34*, 102395.
112. Campbell, F.; Bos, F. L.; Sieber, S.; Arias-Alpizar, G.; Koch, B. E.; Huwyler, J.; Kros, A.; Bussmann, J. Directing Nanoparticle Biodistribution through Evasion and Exploitation of Stab2-Dependent Nanoparticle Uptake. *ACS Nano* **2018**, *12* (3), 2138–2150.
113. Arias-Alpizar, G.; Kong, L.; Vlieg, R. C.; Rabe, A.; Papadopoulou, P.; Meijer, M. S.; Bonnet, S.; Vogel, S.; van Noort, J.; Kros, A.; Campbell, F. Light-Triggered Switching of Liposome Surface Charge Directs Delivery of Membrane Impermeable Payloads in Vivo. *Nat Commun* **2020**, *11*, 3638.
114. Pattipeiluhu, R.; Arias-Alpizar, G.; Basha, G.; Chan, K. Y. T.; Bussmann, J.; Sharp, T. H.; Moradi, M.-A.; Sommerdijk, N.; Harris, E. N.; Cullis, P. R.; Kros, A.; Witzigmann, D.; Campbell, F. Anionic Lipid Nanoparticles Preferentially Deliver mRNA to the Hepatic Reticuloendothelial System. *Advanced Materials* **2022**, *34* (16), e2201095.
115. Ritz, S.; Schöttler, S.; Kotman, N.; Baier, G.; Musyanovych, A.; Kuharev, J.; Landfester, K.; Schild, H.; Jahn, O.; Tenzer, S.; Mailänder, V. Protein Corona of Nanoparticles: Distinct Proteins Regulate the Cellular Uptake. *Biomacromolecules* **2015**, *16* (4), 1311–1321.
116. Lundqvist, M.; Stigler, J.; Elia, G.; Lynch, I.; Cedervall, T.; Dawson, K. A. Nanoparticle Size and Surface Properties Determine the Protein Corona with Possible Implications for Biological Impacts. *PNAS* **2008**, *105* (38), 14265–14270.
117. Pattipeiluhu, R.; Crielaard, S.; Klein-Schiphorst, I.; Florea, B. I.; Kros, A.; Campbell, F. Unbiased Identification of the Liposome Protein Corona Using Photoaffinity-Based Chemoproteomics. *ACS Cent Sci* **2020**, *6* (4), 535–545.
118. Corbo, C.; Molinaro, R.; Tabatabaei, M.; Farokhzad, O. C.; Mahmoudi, M. Personalized Protein Corona on Nanoparticles and Its Clinical Implications. *Biomater Sci* **2017**, *5* (3), 378–387.
119. Hajipour, M. J.; Laurent, S.; Aghaie, A.; Rezaee, F.; Mahmoudi, M. Personalized Protein Coronas: A “Key” Factor at the Nanobiointerface. *Biomater Sci* **2014**, *2*, 1210–1221.
120. Akinc, A.; Querbes, W.; De, S.; Qin, J.; Frank-Kamenetsky, M.; Jayaprakash, K. N.; Jayaraman, M.; Rajeev, K. G.; Cantley, W. L.; Dorkin, J. R.; Butler, J. S.; Qin, L.; Racie, T.; Sprague, A.; Fava, E.; Zeigerer, A.; Hope, M. J.; Zerial, M.; Sah, D. W.; Fitzgerald, K.; Tracy, M. A.; Manoharan, M.; Kotliansky, V.; Fougères, A. De;

- Maier, M. A. Targeted Delivery of RNAi Therapeutics with Endogenous and Exogenous Ligand-Based Mechanisms. *Mol Ther* **2010**, *18* (7), 1357–1364.
121. Kadiyala, P.; Li, D.; Nuñez, F. M.; Altshuler, D.; Doherty, R.; Kuai, R.; Yu, M.; Kamran, N.; Edwards, M.; Moon, J. J.; Lowenstein, P. R.; Castro, M. G.; Schwendeman, A. High-Density Lipoprotein-Mimicking Nanodiscs for Chemo-Immunotherapy against Glioblastoma Multiforme. *ACS Nano* **2019**, *13* (2), 1365–1384.
122. Alam, S. B.; Wang, F.; Qian, H.; Kulka, M. Apolipoprotein C3 Facilitates Internalization of Cationic Lipid Nanoparticles into Bone Marrow-Derived Mouse Mast Cells. *Sci Rep* **2023**, *13* (1), 1–16.
123. Watt, M. J.; Steinberg, G. R. Regulation and Function of Triacylglycerol Lipases in Cellular Metabolism. *Biochemical Journal* **2008**, *414* (3), 313–325.
124. Watt, M. J.; Spriet, L. L. Triacylglycerol Lipases and Metabolic Control: Implications for Health and Disease. *Am J Physiol Endocrinol Metab* **2010**, *299* (2), 162–168.
125. Beisiegel, U. New Aspects on the Role of Plasma Lipases in Lipoprotein Catabolism and Atherosclerosis. *Atherosclerosis* **1996**, *124* (1), 1–8.
126. Olivecrona, G.; Olivecrona, T. Triglyceride Lipases and Atherosclerosis. *Curr Opin Lipidol* **2010**, *21* (5), 409–415.
127. Goulbourne, C. N.; Gin, P.; Tatar, A.; Nobumori, C.; Hoenger, A.; Jiang, H.; Grovenor, C. R. M.; Adeyo, O.; Esko, J. D.; Goldberg, I. J.; Reue, K.; Tontonoz, P.; Bensadoun, A.; Beigneux, A. P.; Young, S. G.; Fong, L. G. The GPIHBP1–LPL Complex Is Responsible for the Margination of Triglyceride-Rich Lipoproteins in Capillaries. *Cell Metab* **2014**, *19* (5), 849–860.
128. Strauss, J. G.; Zimmermann, R.; Hrzenjak, A.; Zhou, Y.; Kratky, D.; Levak-Frank, S.; Kostner, G. M.; Zechner, R.; Frank, S. Endothelial Cell-Derived Lipase Mediates Uptake and Binding of High-Density Lipoprotein (HDL) Particles and the Selective Uptake of HDL-Associated Cholesterol Esters Independent of Its Enzymic Activity. *Biochem J* **2002**, *368* (Pt 1), 69–79.
129. Merkel, M.; Kako, Y.; Radner, H.; Cho, I. S.; Ramasamy, R.; Brunzell, J. D.; Goldberg, I. J.; Breslow, J. L. Catalytically Inactive Lipoprotein Lipase Expression in Muscle of Transgenic Mice Increases Very Low Density Lipoprotein Uptake: Direct Evidence That Lipoprotein Lipase Bridging Occurs in Vivo. *PNAS* **1998**, *95* (23), 13841–13846.
130. Connelly, P. W. The Role of Hepatic Lipase in Lipoprotein Metabolism. *Clin Chim Acta* **1999**, *286* (1–2), 243–255.
131. Feng, D.; Huang, Q. Y.; Liu, K.; Zhang, S. C.; Liu, Z. H. Comparative Studies of Zebrafish *Danio rerio* Lipoprotein Lipase (Lpl) and Hepatic Lipase (Lipc) Genes Belonging to the Lipase Gene Family: Evolution and Expression Pattern. *J Fish Biol* **2014**, *85* (2), 329–342.

132. Mead, J. R.; Irvine, S. A.; Ramji, D. P. Lipoprotein Lipase: Structure, Function, Regulation, and Role in Disease. *J Mol Med* **2002**, *80* (12), 753–769.
133. Khetarpal, S. A.; Vitali, C.; Levin, M. G.; Klarin, D.; Park, J.; Pampana, A.; Millar, J. S.; Kuwano, T.; Sugasini, D.; Subbaiah, P. V.; Billheimer, J. T.; Natarajan, P.; Rader, D. J. Endothelial Lipase Mediates Efficient Lipolysis of Triglyceride-Rich Lipoproteins. *PLoS Genet* **2021**, *17* (9), e1009802.
134. Choi, S. Y.; Hirata, K.; Ishida, T.; Quertermous, T.; Cooper, A. D. Endothelial Lipase. *J Lipid Res* **2002**, *43* (11), 1763–1769.
135. Jaye, M.; Lynch, K. J.; Krawiec, J.; Marchadier, D.; Maugeais, C.; Doan, K.; South, V.; Amin, D.; Perrone, M.; Rader, D. J. A Novel Endothelial-Derived Lipase That Modulates HDL Metabolism. *Nat Genet* **1999**, *21* (4), 424–428.
136. Birrane, G.; Beigneux, A. P.; Dwyer, B.; Strack-Logue, B.; Kristensen, K. K.; Francone, O. L.; Fong, L. G.; Mertens, H. D. T.; Pan, C. Q.; Ploug, M.; Young, S. G.; Meiyappan, M. Structure of the Lipoprotein Lipase–GPIHBP1 Complex That Mediates Plasma Triglyceride Hydrolysis. *PNAS* **2019**, *116* (5), 1723–1732.
137. Young, S. G.; Fong, L. G.; Beigneux, A. P.; Allan, C. M.; He, C.; Jiang, H.; Nakajima, K.; Meiyappan, M.; Birrane, G.; Ploug, M. GPIHBP1 and Lipoprotein Lipase, Partners in Plasma Triglyceride Metabolism. *Cell Metab* **2019**, *30* (1), 51–65.
138. Fuki, I. V.; Blanchard, N.; Jin, W.; Marchadier, D. H. L.; Millar, J. S.; Glick, J. M.; Rader, D. J. Endogenously Produced Endothelial Lipase Enhances Binding and Cellular Processing of Plasma Lipoproteins via Heparan Sulfate Proteoglycan-Mediated Pathway. *J Biol Chem* **2003**, *278* (36), 34331–34338.
139. Kuemmerle, N. B.; Rysman, E.; Lombardo, P. S.; Flanagan, A. J.; Lipe, B. C.; Wells, W. A.; Pettus, J. R.; Froehlich, H. M.; Memoli, V. A.; Morganelli, P. M.; Swinnen, J. V.; Timmerman, L. A.; Chaychi, L.; Fricano, C. J.; Eisenberg, B. L.; Coleman, W. B.; Kinlaw, W. B. Lipoprotein Lipase Links Dietary Fat to Solid Tumor Cell Proliferation. *Mol Cancer Ther* **2011**, *10* (3), 427–436.
140. Carter, S. A.; Foster, N. A.; Scarpini, C. G.; Chattopadhyay, A.; Pett, M. R.; Roberts, I.; Coleman, N. Lipoprotein Lipase Is Frequently Overexpressed or Translocated in Cervical Squamous Cell Carcinoma and Promotes Invasiveness through the Non-Catalytic C Terminus. *Br J Cancer* **2012**, *107* (4), 739–747.
141. Gago-Dominguez, M.; Redondo, C. M.; Calaza, M.; Matabuena, M.; Bermudez, M. A.; Perez-Fernandez, R.; Torres-Español, M.; Carracedo, Á.; Castelao, J. E. LIPG Endothelial Lipase and Breast Cancer Risk by Subtypes. *Sci Rep* **2021**, *11* (1), 1–10.
142. Trost, Z.; Sok, M.; Marc, J.; Cerne, D. Increased Lipoprotein Lipase Activity in Non-Small Cell Lung Cancer Tissue Predicts Shorter Patient Survival. *Arch Med Res* **2009**, *40* (5), 364–368.
143. Wang, W.; Bai, L.; Li, W.; Cui, J. The Lipid Metabolic Landscape of Cancers and New Therapeutic Perspectives. *Front Oncol* **2020**, *10*, 605154.