Gaining control of lipid-based nanomedicine by understanding the nano-bio interface

Pattipeiluhu, R.

Chapter 1

Introduction

1.1 The concept and design of nanomedicine

In 1900, Paul Ehrlich conceived the scientific concept of the "magic bullet". The idea postulated a therapeutic agent that, like the bullet of a gun, could hit diseased cells without harming healthy cells.[1] Traditionally, antibiotics are often defined as magic bullets, laying the foundation for pharmaceutical research.[2] In modern medicine, the principal concept of a magic bullet has transcended from an ideal binary response and the paradigm continues by the development of 'personalized and tailored drugs' that precisely target the specific molecular defects of a patient.[3] For example, gene therapy in which exclusively the defective gene is repaired is considered as a modern day approach for the development of a magic bullet.

The application of nanotechnology to the field of medicine is considered as one of the technologies that can realize the concept of the "magic bullet". Nanotechnology is a field of research that focuses on engineering functional systems at the molecular scale, typically defined in the range of 1 to 100 nm, in which its lower limit is defined by the size of the individual molecules. These functional systems are commonly achieved through the "bottom-up" assembly of molecular components through chemical principles. In medicinal approaches, the understanding and control over the self-assembly of these molecular components with therapeutics agents allows for the fabrication of nanosized materials (nanoparticles) that can be applied for therapies, defined as nanomedicines.

Nanomedicine spans a variety of medical applications such as therapeutics, diagnostics, imaging and tissue engineering.[4,5] Out of these different nanomedical applications, nanoparticle based drug delivery is one of the most extensively explored.[6] The key principles of nanoparticle-based drug delivery is to obtain one or more benefits over the administered drug alone.[7] Firstly, the strategy allows for the delivery of sensitive cargo that would otherwise be degraded by the body before reaching its target. Secondly, nanoparticles can alter the biodistribution compared to administration of the free drug, allowing the drug to reach novel targets. Thirdly, the nanoparticles can serve as a platform for slow release of the desired therapeutic, prolonging the bioavailability of the drug. Finally, nanoparticles can facilitate the intracellular delivery of cargo that is not capable of passing endogenous barriers by itself.

While nanoparticles are formally defined as particles in the 1–100 nm size range, nanomedicine for drug delivery purposes are typically defined within the 10–450 nm range. Over the past decades, intensive research has yielded a large variety of nanoparticle types (**Figure 1a**). These nanoparticles are typically fabricated from the bottom-up using either

synthetic organic (*e.g.*, polymers, proteins), bio-derived organic (*e.g.*, lipids, proteins) or inorganic materials (*e.g.*, iron oxide, silica). In addition, hybrid nanoparticles that contain a variety of these materials are also extensively investigated.[8] Furthermore, there is an increasing interest in nanoparticles derived from biological systems, such as a weakened viruses or virus-like particles, as well as biomimetic or bio-derived nanoparticles such as engineered extracellular vesicles, lipoprotein mimics and exosomes.[9]

The choice for a certain nanoparticle type depends on the desired purpose. For example, organic nanoparticles are often employed for drug delivery purposes due to their high flexibility in cargo encapsulation and fabrication from non-toxic components. However, inorganic nanoparticles can offer exclusive magnetic, electrical and optical properties for isolation and imaging purposes.[10] These nanoparticles can function as carriers for a variety of cargos, such as DNA/RNA and small molecules (**Figure 1b**). The successful encapsulation of these cargos is dependent on the nanoparticle type and the physicochemical characteristics of its component. Another common strategy that is often employed in nanoparticle design is the chemical conjugation of different (bio)molecules or (bio)polymers to the nanoparticle surface (**Figure 1c**). This can be employed for the stabilisation of the nanoparticle and its encapsulated cargo, or for targeting/shielding strategies in order to manipulate the nanoparticle behaviour *in vivo*.

However, although these principles of nanomedicine design are stated as straightforward, simple and ideal, there are multiple layers of complexity involved with nanomedicine design and application. A successful strategy depends on the proper therapeutic target, the ability to fabricate a fitting nanoparticle carrier and its performance in biological systems.

1.2 Lipid based nanomedicine

Out of the different nanoparticle types, lipid-based nanoparticles were among the first to be investigated and are the most extensively pursued for drug delivery purposes. In addition, the majority of clinically approved nanocarriers for drug delivery belong to the class of lipid-based nanoparticles.[11] They are either completely, or mostly, made from lipid molecules. Lipids are a group of organic biomolecules and one of the most abundant cellular metabolites. The lipids used for nanoparticle fabrication are typically amphiphilic small molecules, possessing both a hydrophobic and hydrophilic region (**Figure 2a**). In nature, these amphiphilic lipids are crucial for the assembly of cellular membranes, in which phospholipids organize themselves into bilayers with a spatiotemporal pattern containing various other lipids. This self-assembly process of amphipathic lipids is a chemical property that enables cells to segregate their internal constituents from the

external environment. The first type of lipid-based nanomedicine, liposomes, relied on this process of vesicle self-assembly. These vesicles, formed from mainly phospholipids, were discovered in the early 1960s and initially used as model systems for cellular membranes.[12] This gave researchers an unique tool to study multiple cellular mechanisms, such as diffusion across membranes.[13,14] It was not until the early 1970s that liposomes were pursued as possible carriers for therapeutically relevant agents.[15–17]
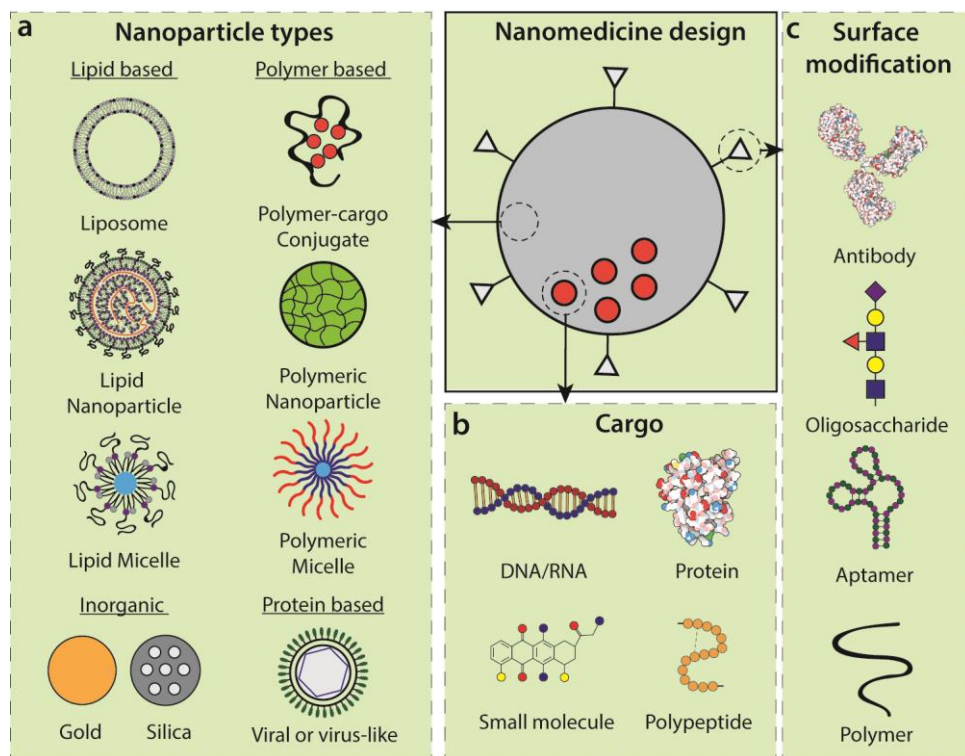

Figure 1. Schematic representation nanomedicine design. (a) Commonly used nanoparticle variants for drug delivery purposes **(b)** encapsulated cargos and **(c)** types of surface modifications.

Lipid-based nanomedicine such as liposomes possess several benefits that are still valid to this date. Firstly, due to the biological nature of lipids, the components of lipid-based nanomedicine are often non-toxic and biodegradable. Secondly, the assembly of lipid-based nanomedicine is controllable at the nanometre scale. Finally, the wide variety of lipid molecules gives rise to a plethora of formulations with varying lipid compositions. Identification of lipid species has shown that there are over 40,000 unique known lipid structures that can be classified by structural similarity to 8 different categories.[18,19] Out of

these categories, phospholipids, sterol lipids and sphingolipids are currently most common for the fabrication of lipid-based nanoparticles (**Figure 2b**).[20]

The versatility of lipid molecules also allows for a variety of physicochemical properties of lipid-based nanoparticles (**Figure 2c**). The saturation of alkyl chains in the lipid backbone can modulate the stiffness of lipid mixtures, whereas the preferred geometry of lipid molecules can determine the morphology and size of the nanoparticles. Furthermore, the surface charge of lipid-based nanoparticles depends on the formal charge and organization of the lipids in their composition. There has been a large improvement in understanding the influence of lipid properties (*e.g.*, charge, geometry) on self-assembly, as well as development of production (*e.g.*, thin-film hydration, microfluidics) and characterization techniques (*e.g.*, light scattering, electron microscopy).[21,22] This allows for the fabrication of different variants of lipid-based nanoparticles beyond liposomes, such as (filled) lipid nanoparticles (LNPs), lipid micelles and lipid emulsions (**Figure 1a**). Finally, the chemical structure of lipid molecules can be altered through chemical conjugation or bottom-up synthesis of these molecules, allowing for the fabrication of novel lipids and the fabrication of hybrids of lipids with other biomolecules (*e.g.*, glycans, peptides).
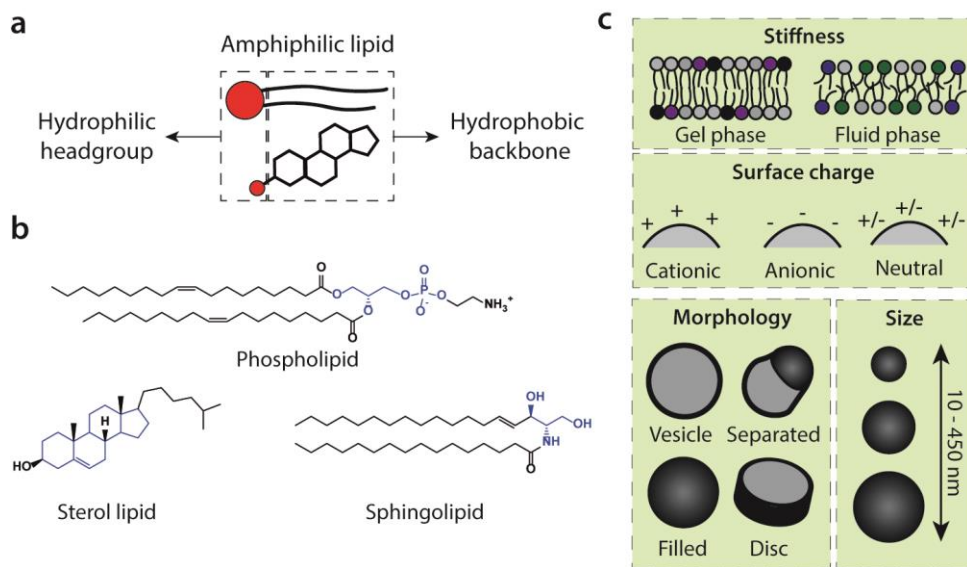

Figure 2. Building blocks and characteristics of lipid-based nanomedicine. (**a**) Schematic representation of common amphiphilic lipids used for the self-assembly of lipid-based nanoparticles. Amphiphilicity is caused by the presence of both a hydrophobic and hydrophilic domain within the same molecule. (**b**) Common lipid groups used for the self-assembly of lipid-based nanoparticles.

Important structural characteristics are displayed in blue: Phospholipids contain a phosphorylated glycerol group; Sterol lipids contain a fused four-ring core structure with varying substituents; Sphingolipids contain a sphingoid base backbone which is substituted to fatty acid amides. (**c**) Lipid-based nanomedicine can vary in multiple physicochemical properties, depending on the choice of lipids. These include, but are not limited to, a difference in morphology, stiffness, surface charge and size.

1.3 Ionizable Lipid Nanoparticles (LNPs) for RNA Therapeutics

Out of the various lipid-based nanomedicines available, the use of the ionizable lipid nanoparticles (LNPs) for RNA therapeutics resembles the current state-of-the-art for lipid-based nanomedicine.[23] In 2018, Onpattro® was the first LNP based RNA interference (RNAi) therapeutic that was accepted in the clinical for the treatment of hereditary transthyretin-mediated amyloidosis, effective through the silencing of transthyretin production in liver hepatocytes after intravenous administration.[24] Furthermore, the onset of the 2020 pandemic due to SARS-CoV-2 has greatly accelerated the development and transition of multiple LNP based messenger RNA (mRNA) prophylactic vaccines into the clinic.[25–28] All together, these successes highlight the bright future of using LNPs for multiple RNA-based therapeutics.

1.3.1 Self-assembly of LNPs

LNPs are self-assembled from multiple lipid components, relying on the electrostatic complexation for the entrapment and protection of fragile RNA molecules using ionizable lipids (ILs) (**Figure 3a,b**). These ILs represent the second generation of lipids used for gene therapy.[29] Initially, permanently cationic lipids were utilized to electrostatically complex negatively charged RNA or DNA molecules, forming so-called lipoplexes.[30] These generated permanently cationic nanoparticles which, due to their surface properties, displayed a limited biodistribution and poor bioavailability, in combination with toxic side effects.[31] ILs circumvent these limitations with tertiary amines, which are predominantly cationic at an acidic pH, while regaining a neutral formal charge at a physiological pH. A combination of ILs and other lipids are mixed with RNA molecules at an acidic pH,[32,33] leading to the spontaneous electrostatic complexation of the RNA by the cationic ILs and nanoprecipitation with the other lipids into intermediate LNPs containing RNA (**Figure 3c**).[34–36] Afterwards, the lipid solvent and acidic buffer are replaced for a physiologically-friendly buffer (PBS, pH = 7.4), which leads to the formation of stable LNPs. Furthermore, any ILs on the surface of the LNP will regain a formal neutral charge at this pH, leading to a zwitterionic neutral surface charge. Throughout this process, the additional lipids used next to ILs serve a variety of purposes. Structural lipids, such as cholesterol, allow for the

formation of a hydrophobic core structure along with the IL-RNA complexes.[37,38] Since the LNPs are assembled in aqueous solution, the helper (phospho)lipids and polyethylene glycol (PEG) conjugated lipids are predominately enriched on the surface of LNPs,[39] and are essential for the colloidal stability of the particles through the formation of a monolayer or bilayer between the hydrophobic core of the LNPs and the aqueous solution. Nevertheless, although solid core structure LNPs are reported most frequently, variation in lipid type, composition and cargo type can lead to the formation of polymorphic or phase separated LNPs.[40–42] For example, the large differences in length and structural organization of siRNA vs. mRNA can significantly impact the complexation state with ionizable lipids.[42] In a similar fashion, there is an increasing number of ILs with diverse chemical structures, such as branched variants and lipidoids.[43–47] The combination of these ILs with the other helper lipids can significantly impact the formation of lipid superstructures and successful RNA encapsulation.

Combining different helper- and PEG-lipids can affect the formation of LNP surface properties.[48,49] The virtually endless variation in possibilities add to the complexity of predicting LNP structures, as well as evaluating their impact on performance in biological systems. Further in-depth biophysical characterization and coupling to efficiency might allow for the elucidation of important structure-activity relationships that will allow for the design of more potent LNPs in the future.
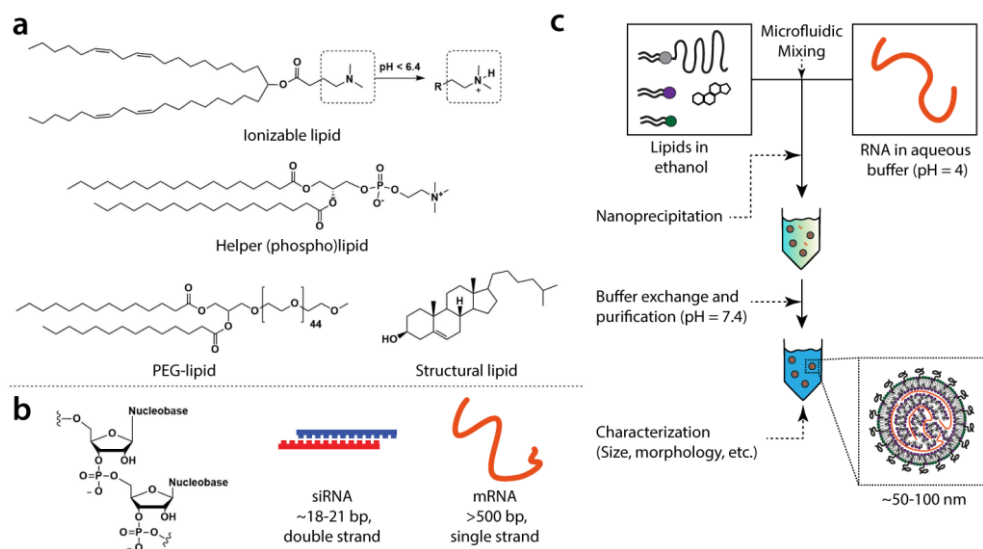
Figure 3. The concept and fabrication of ionizable lipid nanoparticles. (**a**) Examples of lipid components commonly used for LNPs. IL = DLin-MC3-DMA, helper lipid = DSPC, PEG–lipid = DMG-PEG2000, structural lipid = cholesterol. (**b**) Structure of commonly used RNA molecules in LNP fabrication. (**c**) The fabrication process of LNPs.

1.3.2 The intracellular mechanism of LNPs

The use of ILs for the assembly of LNPs serves an additional, and crucial, second purpose. Upon administration, LNPs are typically taken up by cells through a form of endocytosis, which can be facilitated through a receptor-mediated interaction. Upon cellular uptake, the LNPs will end up in endosomal vesicles which are designed for the processing and degradation of foreign compounds by endogenous enzymes.[50] In addition, the pH of the endosomal vesicles is decreased in order to facilitate the degradation processes. Due to the ionizable properties of the LNPs, this leads to protonation of the IL and consequently a cationic surface charge. Since the surface charge of endosomal membranes is typically anionic, this promotes a direct electrostatic interaction between the LNP and the endosomal membrane (**Figure 4**). In this process, destabilization of the LNP as well as the endosomal membrane allows for the de-complexation of the LNPs and consequently for the escape of RNA molecules into the cytosol and further processing by the endogenous machinery.[51] To this end, the formation of non-lamellar and de-stabilizing LNP-membrane intermediates are known to facilitate a more efficient release of RNA molecules into the cytosol.[52,53] However, to date, the absolute efficiency of RNA escape from the LNPs into

the cytosol is estimated to be in the range of ~2%.[54,55] Although sufficient for certain purposes, improvement of this endosomal escape efficiency will pave the way for the delivery of more complex and larger RNA constructs, for example for genome editing. In addition, increased potency will benefit the therapeutic window of LNP-RNA based drugs and decrease costs of production.
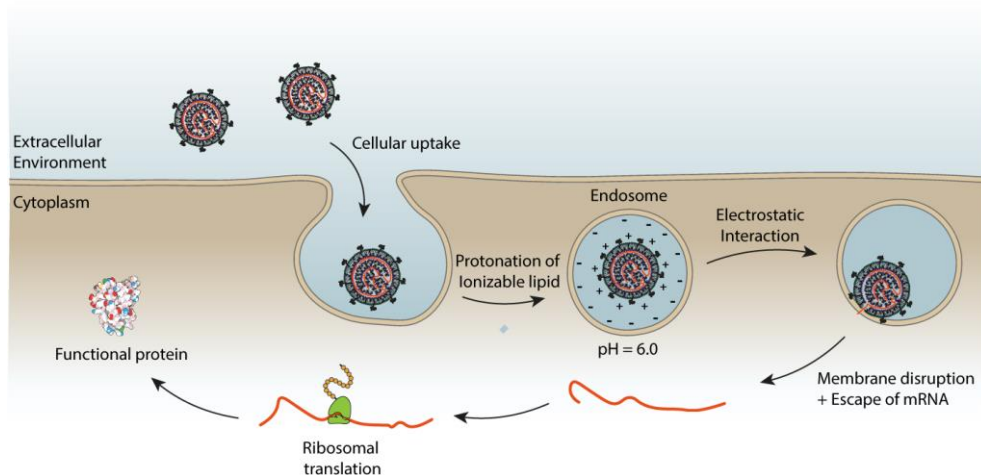

Figure 4. The intracellular mechanism of LNPs containing RNA. Uptake of LNPs is typically mediated through a form of endocytosis. Herein, acidification in the endosome leads to protonation of the ionizable lipids in LNPs and induces an electrostatic interaction between the LNP and endosomal membrane. This interaction leads to membrane disruption and escape of RNA molecules into the cytosol, where they can exhibit their biological effect.

1.4 Clearance of nanomedicine

Although the field of nanomedicine has produced some very promising avenues of therapeutic application, for example by drug delivery to liver hepatocytes or local administration, extra-hepatic drug delivery remains troublesome. Consequently, the body's response to foreign material is one of the most challenging barriers to overcome. For the relevance to this thesis, the focus will be on the clearance of nanoparticles after intravenous administration. However, the mechanisms explained here also affect locally administered nanomedicines as they often reach the bloodstream by, for example, lymphatic drainage.[56–58]

There are three major organs that dominate the clearance of injected nanoparticles: the liver, the spleen and the kidneys (**Figure 5**). The majority of the nanoparticles (30-99%) are

cleared through the liver (hepatic clearance).[59–61] More specifically, early reports established the preferentially accumulation of liposomes in the reticuloendothelial system (RES).[62,63] The RES is the combination of macrophages and specialized cell types, such as scavenger endothelial cells, that are responsible for the clearance of foreign and colloidal material from the blood.[64,65] Within the liver, hepatocytes, liver sinusoidal endothelial cells (LSECS) and Kupffer cells (KCs) orchestrate the uptake and clearance of nanoparticles (**Figure 5**). KCs are known to phagocytose bacteria, viruses and (large) nanoparticles in order to remove them from the bloodstream.[65,66] LSECs are specialized scavenger endothelial cells (SECs) responsible for clearing the blood of colloidal waste, viruses and oxidized macromolecules (*e.g.* oxLDL).[67–69] There are multiple classes of scavenger receptors on their cell surface that perform this function.[68,70] Hepatocytes are not specialized to scavenge the blood of harmfull materials and therefore have a lower endocytic capability and express few scavenger receptors. Instead, they are mostly involved in metabolic processes such as lipid and protein synthesis. Furthermore, in order for nanoparticles to reach the hepatocytes, they have to diffuse through fenestrations of LSECs (<100 nm) and transverse the Space of Disse.[61] Therefore, in general, hepatocyte uptake by nanomedicines is only observed at higher doses, when the scavenger receptors are saturated.

In the case of lipid-based systems, hepatocytes play a more profound role in uptake and clearance than for other nanomedicines.[71,72] One of the key functions of hepatocytes is the uptake of lipids from systemic circulation. Endogenous (dietary) fats are transported by lipoproteins (*e.g.*, HDL, LDL, chylomicrons) and taken up by specific lipoprotein receptors (*e.g.*, LDL-receptor family) expressed on hepatocytes. It is known that some lipid-based nanomedicines are taken up and processed by LDL-receptors in similar ways.[72,73] Further downstream, the final fate of the nanoparticle components is typically determined by the material. For example, lipid-based nanoparticles are generally dissociated intracellularly followed by metabolization and biliary secretion of their components via the gall bladder, ending up in the feces.[59] In contrast, inorganic nanoparticles are typically retained in the cells for longer periods prior to biliary secretion.[74]
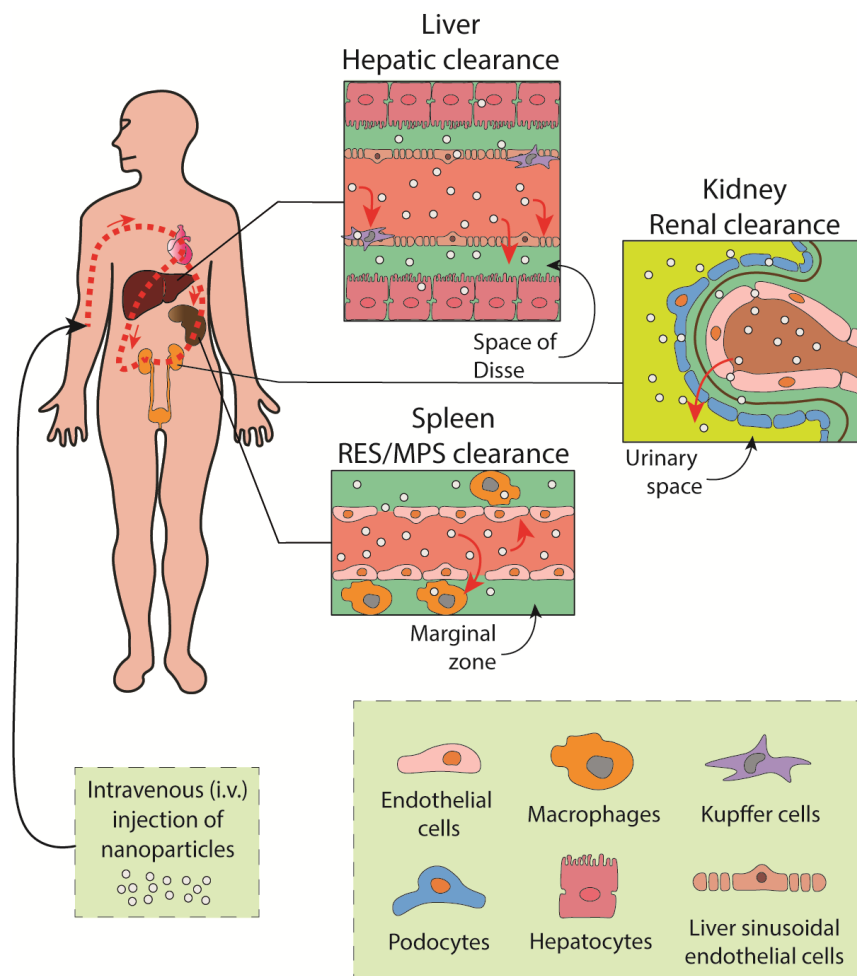
Figure 5. Major organs responsible for nanoparticle clearance upon intravenous (*i.v.*) administration. Nanoparticles in the bloodstream encounter multiple organs during circulation, where the liver, spleen and kidneys are able to remove these particles depending on a variety of nanoparticle characteristics such as size, charge and material. Clearance by the reticuloendothelial system (RES) is most predominant in the liver and spleen, where macrophages, Kupffer cells, macrophages and (liver sinusoidal) endothelial cells remove nanoparticles using a variety of scavenging receptors. Diffusion of nanoparticles through the fenestrations of LSECs in the liver and gaps of ECs in the spleen are largely depend on their size. Renal clearance through the spleen is typically observed for small (<10 nm) nanoparticles, or for metabolised components of nanoparticle reagents and cargo.

Another important organ in the sequestration and clearance of nanoparticles *in vivo* is the spleen.[75] The spleen is responsible for a variety of processes, such as the synthesis of antibodies, production of lymphocytes, sequestration of pathogens, and the regulation of red blood cells and platelets. Similar to the liver, the spleen contains RES cells that are able to scavenge and degrade foreign materials in the blood (**Figure 5**). Splenic macrophages reside in the splenic red pulp and the marginal zone and are the primary regulators of scavenging nanoparticles from the blood sinusoids in the spleen. A process called splenic filtration determines whether nanoparticles will be taken up by macrophages in the marginal zone or in the red pulp.[61] It is typically believed that size is one of the large determinants of this process, although other nanoparticle characteristics such as charge and type of material have been found to be equally important.[76] Furthermore, the spleen has been shown to play a crucial role in the accelerated clearance of lipid-based nanoparticles upon repeated injections.[77,78] Especially in the case of PEGylated or immuno-liposomes, the spleen was found to generate IgM monoclonal antibodies that facilitated the clearance of repeated injections of these nanoparticles by the liver.[79] These results indicate that the spleen is an important regulator of nanoparticle clearance, both by direct and indirect processes.

The last major organ responsible for clearance of IV injected is the kidneys. The kidneys are unique compared to the liver and spleen, by the fact that clearance is not predominantly regulated by RES cells. Instead, the kidney is responsible for the renal clearance of compounds and macromolecules from the blood into the urine. This happens through a process called glomerular filtration, in which the kidneys filter excess fluid and waste products out of the blood. This process is highly dependent on a small size of the waste product and is therefore typically observed only for small (<10 nm) nanoparticles (**Figure 5**).[80,81] Since lipid-based nanoparticles are generally bigger than 10 nm, clearance or retention of these particles by the kidneys is only observed in negligible amounts.[76,81] However, the processing of lipid-based nanoparticles by other organs often leads to the excretion of its metabolites into the blood and clearance by the kidneys, which can lead to the determination of these products in the urine.[82,83] Therefore, although kidneys are not always responsible for the direct clearance of lipid-based nanoparticles, they can give important information about the pharmacokinetics and pharmacodynamics of the encapsulated drug and the lipid components.

1.5 Challenges at the nano-bio interface

The current limitations regarding nanoparticle clearance have hampered nanomedicine development for decades and therefore have been at the core of many research topics. More recently, effort is directed to understanding and exploiting the more specific molecular interactions that underpin these mechanisms, often referred to as the "bio-nano" interface.[80,84–86] This topic aims at the understanding the interaction of endogenous molecules and structures (*e.g.,* proteins, membranes, etc.) with the introduced nanomedicines.

1.5.1 The protein corona of nanoparticles

The adsorption of proteins to a nanoparticle surface is of great importance for the biodistribution and clearance of nanoparticles *in vivo*, and has been studied since the 1990s.[87–89] Nowadays, protein adsorption to nanoparticles is better known as the formation of a "protein corona".[85,90] When introduced into a biological system, nanoparticles encounter a complex mixture of cells, endogenous nanoparticles, proteins and small molecules. For example, human blood can be divided into three phases: the erythrocytes (red blood cells), the buffy coat (white blood cells and platelets) and plasma (**Figure 6a**). The latter contains a large collection and diversity of blood proteins. Therefore, upon administration, the "synthetic identity" of the nanoparticle can change and adapt a "biological identity" (**Figure 6b**). To some extent, it is the protein corona and not the underlying synthetic surface of a nanoparticle, which the body "sees" and interacts with.[91]

The composition and extent of the protein corona is dependent on the "synthetic identity" of a nanoparticle. Physicochemical properties of nanoparticles such as size, surface charge and chemical composition can affect the adsorption of proteins (**Figure 6c**).[92,93] Over the past decade, enormous efforts have focused on the generation of protein corona profiles for a myriad of nanoparticle types.[85,90,94–96] However, identification of reliable profiles and important protein-nanoparticle interactions has been challenging due to limitations and drawbacks in separation techniques of protein coronas from biological media.[97–100]

Derived from studies that have explored the affinity and kinetics of protein binding to nanoparticles, the protein corona is often divided in the "hard" and "soft" corona.[92,101] Here, the "hard" corona is considered as the first layer of tightly binding proteins that are directly adsorbed to the nanoparticle surface. The "soft" corona is considered as a group of proteins that interact with the underlying "hard" corona or with low-affinity binding. The general presence and influence of the "soft" corona still originates from heavy speculation and its

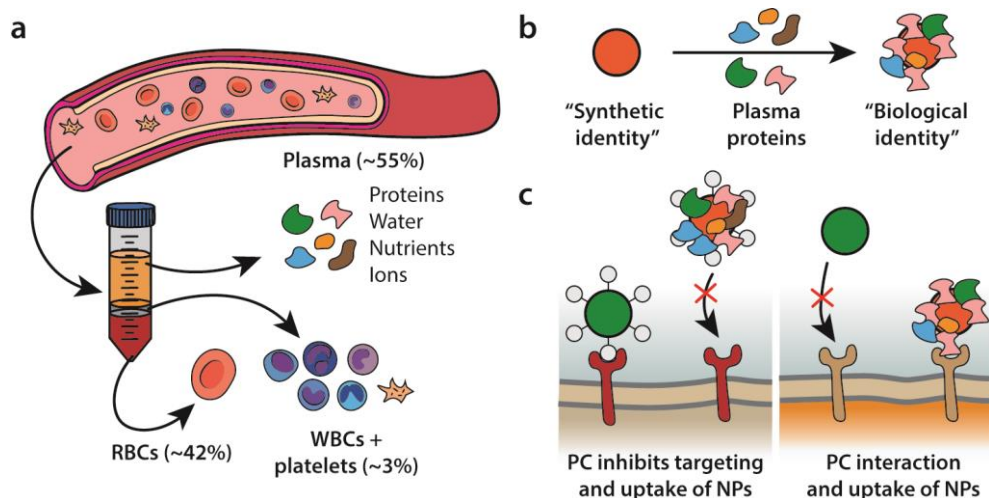presence can be debated on the basis of limitations in protein corona characterisation techniques.[97,98]

Figure 6. The formation of a nanoparticle protein corona. (a) Blood can be divided into three parts: RBCs, WBCs with platelets and plasma. **(b)** Exposure of nanoparticles to plasma proteins leads to the formation of a biological identity. **(c)** The protein corona can influence the behaviour of design nanoparticles by interfering with targeting strategies or by leading to uptake by undesired cell surface receptors. **Abbreviations used:** RBCs = Red Blood Cells, WBCs = White Blood Cells, PC = protein corona, NP = nanoparticle

Besides nanoparticle characteristics, the biological medium can also impact the formation and composition of the protein corona. Since the majority of nanoparticles are administered intravenously, plasma or serum are generally the most investigated biological fluids. Working with whole blood in *in vitro* conditions is problematic due to aggregation without supplementation with anticoagulants. Other biological fluids, such as cerebrospinal fluid or intramuscular fluid, are not yet widely investigated for protein corona formation. However, these fluids are interesting for elucidating the protein corona formation for local administration routes of nanomedicine.[102,103]

The general presence a protein corona is known to influence the behaviour of nanoparticles in various ways. One of the most profound effects is the possibility of inhibiting targeting strategies (**Figure 6c**). For example, it was found that nanoparticles functionalised with transferrin ligands had a significantly reduced uptake with the desired transferrin receptor in the presence of plasma proteins.[104] Similar effects have been reported for other functional ligands such as antibodies, aptamers and click-chemistry handles.[105–107]

Therefore, protein corona formation is often stated as one of the reasons for the limited translation of targeting strategies (**Figure 6**). The protein corona is also known facilitate bodily clearance of nanoparticles (**Figure 6c**),[108,109] in particular by enhancing phagocytic uptake by macrophages.[110–113] Studying specific (classes of) proteins is considered as the next crucial step for mechanistic understanding of the protein corona. Recent efforts have elucidated the influence of apolipoprotein E,[109,114] clusterins[115] and complement components[116] on the behaviour of nanoparticles *in vitro* and *in vivo*. Further exploration of these avenues will allow for the development of rational strategies in manipulating nanoparticle behaviour.

1.5.2 Receptor mediated uptake and endosomal processing

Crucial for the eventual cellular uptake of nanomedicine from the bloodstream is the interaction between nanoparticles, or their protein corona, with cell-surface receptors. Scavenger receptors (SRs) are a group of cell surface receptors that play a crucial role in maintaining homeostasis and immunity, for example by facilitating the clearance of harmful, degraded or foreign material.[117] Throughout the body, SRs are predominantly expressed on macrophages and scavenging endothelial cells (SECs).[67,118–120] SRs are separated into 10 families (classes A-J) according to sequence and further classified based on the variations of the sequence.[121,122] Out of these, several SRs have shown to be involved in the cellular uptake of nanoparticles (**Figure 7a**). A portion of these receptors (MARCO, SR-A1 and SR-B1), are known to facilitate the internalization of a large variety of nanoparticle types in macrophages, without apparent specificity.[72,123–126] However, stabilin receptors expressed on KCs and LSECs are found to take up a large variety of anionic nanoparticles, in which the preference for *stab1* or *stab2* is dependent on nanoparticle size.[127,128] For lipid-based nanomedicine specifically, various lipoprotein receptors (LDLr and LRP1) are known to facilitate uptake (**Figure 7a**).[72,73,129] In these cases, the presence of apolipoprotein E (apoE) in the nanoparticle protein corona is required for efficient uptake. Identification and understanding of the behaviour of SRs in nanoparticle clearance and uptake is important for the safe and efficient development of nanomedicine.[130] This knowledge can provide new strategies for avoiding or inhibiting rapid clearance by undesired cell types, or by the targeting of specific cell types through these receptors.[131,132]

In all cases, nanoparticles are trapped within membrane vesicles (endosomes, phagosomes, pinosomes) that mature into lysosomes and where the particles are exposed to a variety of degradation proteins (*e.g.*, nucleases, proteases) that aim to process the internalized components (**Figure 7b**). In addition to lysosomal degradation, transcytosis and endocytic

recycling have also been found to limit the intracellular delivery of the nanomedicine cargo (**Figure 7c,d**).[55,133]

The crucial step for delivery of the nanomedicine cargo is the escape from these vesicular bodies before degradation or exocytosis can occur.[134] Therefore, research has focused on generating nanoparticle variants that can promote endosomal escape, for example through the introduction of membrane destabilization or fusogenic properties.[135,136] Nevertheless, efficient endosomal escape remains among one of the most challenging hurdles in nanomedicine delivery. Further understanding of the molecular details of intracellular processes and to what extent nanoparticle properties influence endosomal escape of their cargo can yield new strategies to improve endosomal escape and provide more efficient nanomedicines.
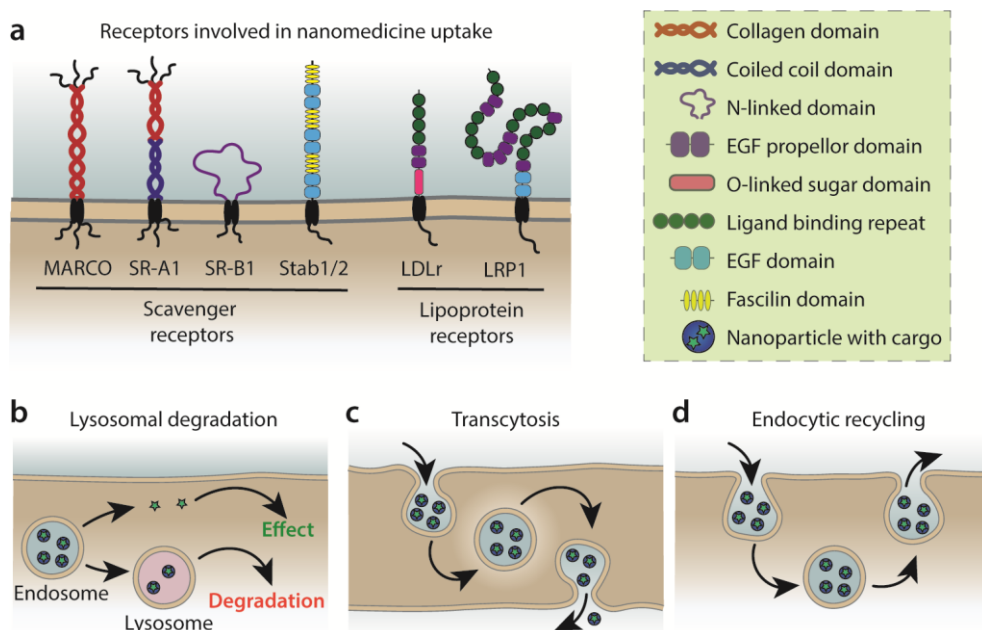
Figure 7. Cellular receptors and mechanisms involved in uptake of lipid-based nanomedicine. (**a**) Common examples of scavenger receptors involved in nanoparticle uptake (**b-d**) Intracellular mechanisms known to limit delivery of NP cargo: lysosomal degradation, transcytosis and endocytic recycling. **Abbreviations:** MARCO = macrophage receptor with collagenous structure, Stab = stabilin receptor, SR = scavenger receptor, LDLr = low-density lipoprotein receptor, LRP1 = Low density lipoprotein-related protein 1, NP = nanoparticle.

1.5.3 Translation from *in vitro* to *in vivo*

Another important parameter are the complicated steps in crossing the *in vitro – in vivo* gap.[86] For example, after the fabrication of nanoparticles, they are typically subject to physiochemical analyses such as size determination, morphology, drug encapsulation, etc. However, the introduction of these nanoparticles into a biological system might change those common parameters. Another layer of complexity is that this type of biological system will significantly determine their effect. For example, nanoparticles are commonly tested in cell culture systems for their toxicity, uptake and efficacy, but typically display different behaviour in *in vivo* systems.[137] For example, due to the presence of scavenger receptors and differences in plasma protein composition.[104] Further translation beyond mice can be limited by genetic differences, non-representative disease models and the changes caused by the use of immunodeficient animals.[138–140]

Therefore, more representative animal models are currently being developed and limitations of animal models are highlighted and accounted for to further the translation of nanomedicines.[141,142] Nevertheless, animal models can be expensive, laborious and require certain expertise and ethics approval, and are not readily available for all researchers. Therefore, simpler and alternative biological systems are investigated for the proper translation of nanomedicine strategies. Whereas cell culture is suitable for the study of intracellular processes such as endosomal escape, they do not provide complex multi- and intercellular processes. Consequently, the use of organoids or other 3D cell culture systems can prove to be useful.[143] Furthermore, the use of embryonic zebrafish embryos has been shown useful for the investigation of nanoparticles *in vivo* as a preclinical model.[127,144–146] These embryos have a relatively low cost of maintenance, small size (0.7 mm – 4 mm), rapid development and an external fertilization that allows for genetic manipulations. Furthermore, their optical transparency enables fluorescent imaging of nanoparticles biodistributions *in vivo* in real-time.[147] These advantages are beneficial for the mechanistic evaluation of nanoparticle behaviour *in vivo* at a molecular level. Drawbacks for translation to murine and human models are its non-mammalian nature, development specific processes, lack of adaptive immunity and not fully developed organs. Nevertheless, zebrafish embryos have proven as good predictors of nanoparticle interactions with the RES cells and their receptors (*e.g.* stabilins) in the translation to mammalian models.[127,145] The implementation and combination of different (animal) models for assessing nanoparticle behaviour can help with the productive translation of new nanomedicines in the coming decades.

1.6 Aim and outline

This thesis focuses on the investigation of and exploitation of fundamental knowledge of lipid-based nanomedicine at the interface with biological systems. An ensemble of chemical, biophysical and biological tools are employed to study the effects of nanoparticle structure, protein corona, nanoparticle-receptor interactions and the intracellular processing of lipid-based nanoparticles.

Chapter 2 describes an alternative method for the protein corona determination of liposomes. Here, photoaffinity based chemoproteomics is used to remove the bias of current methods towards large and abundant proteins and allows for the validation of protein binding in simple mixtures of proteins. In turn, it reveals a more rational protein corona profile for liposomes, dominated by apolipoproteins and less complex than previously thought.

Chapter 3 describes the exploitation of the fundamental understanding of nanoparticle-receptor interactions in order to design LNPs that can preferentially deliver mRNA to the reticuloendothelial system through a stabilin receptor mediated interaction. Here, embryonic zebrafish are used to evaluate and validate the designed LNPs before translation validation in mice.

Chapter 4 describes an in-depth molecular view of key protein-nanoparticle interactions. Here, protein adsorption to anionic and zwitterionic lipid-based nanoparticles is studied using the photoaffinity based method described in Chapter 2. This work expands the method towards the use for lipid nanoparticles encapsulating RNA, as well as underlines the apolipoprotein-dominated but yet simple protein corona profiles generated on a collection of liposomal formulations. Specifically, it is shown that apolipoprotein E binds anionic liposomes through its surface exposed heparin binding site. In contrast, the binding to zwitterionic liposomes and lipid nanoparticles is either absent or occurs through in a non-heparin competitive manner.

Chapter 5 describes the programmable induction of paracrystalline inverse hexagonal lipid phases encapsulating siRNA in the core of lipid nanoparticles. Using a combination of cryogenic electron microscopy, tomography and small-angle X-ray scattering, these structures are characterized and the effects of temperature, RNA content and lipid composition are studied. Furthermore, it is shown that the presence of these paracrystalline phases enhances the intracellular silencing efficiency through a preferred one-step deliver mechanism.

Chapter 6 summarizes the key findings of this thesis and poses important further avenues for the understanding of nanomedicine at the bio-nano interface, such as the (un)importance of the protein corona and the general flow of fundamental nanomedicine research.

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