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The Biomolecular Corona of Lipid Nanoparticles for Gene Therapy

30th Anniversary Review

Valentina Francia,* Raymond M. Schiffelers, Pieter R. Cullis, and Dominik Witzigmann*

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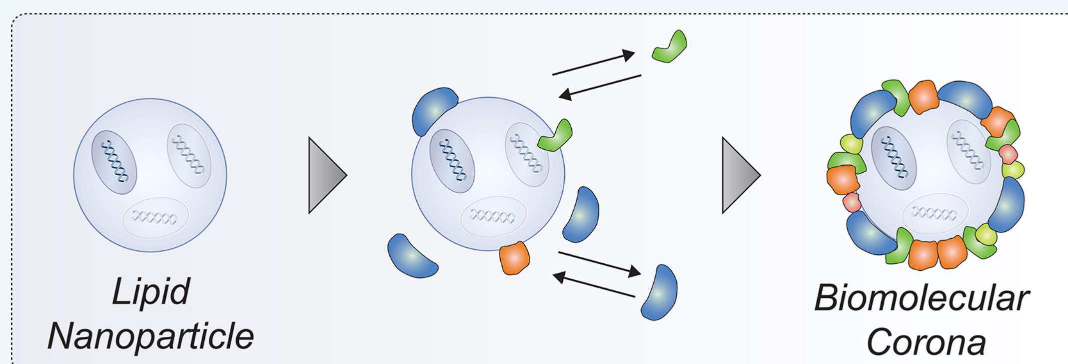

ABSTRACT: Gene therapy holds great potential for treating almost any disease by gene silencing, protein expression, or gene correction. To efficiently deliver the nucleic acid payload to its target tissue, the genetic material needs to be combined with a delivery platform. Lipid nanoparticles (LNPs) have proven to be excellent delivery vectors for gene therapy and are increasingly entering into routine clinical practice. Over the past two decades, the optimization of LNP formulations for nucleic acid delivery has led to a well-established body of knowledge culminating in the first-ever RNA interference therapeutic using LNP technology, i.e., Onpattro, and many more in clinical development to deliver various nucleic acid payloads. Screening a lipid library *in vivo* for optimal gene silencing potency in hepatocytes resulted in the identification of the Onpattro formulation. Subsequent studies discovered that the key to Onpattro's liver tropism is its ability to form a specific "biomolecular corona". In fact, apolipoprotein E (ApoE), among other proteins, adsorbed to the LNP surface enables specific hepatocyte targeting. This proof-of-principle example demonstrates the use of the biomolecular corona for targeting specific receptors and cells, thereby opening up the road to rationally designing LNPs. To date, however, only a few studies have explored in detail the corona of LNPs, and how to efficiently modulate the corona remains poorly understood. In this review, we summarize recent discoveries about the biomolecular corona, expanding the knowledge gained with other nanoparticles to LNPs for nucleic acid delivery. In particular, we address how particle stability, biodistribution, and targeting of LNPs can be influenced by the biological environment. Onpattro is used as a case study to describe both the successful development of an LNP formulation for gene therapy and the key influence of the biological environment. Moreover, we outline the techniques available to isolate and analyze the corona of LNPs, and we highlight their advantages and drawbacks. Finally, we discuss possible implications of the biomolecular corona for LNP delivery and we examine the potential of exploiting the corona as a targeting strategy beyond the liver to develop next-generation gene therapies.

■ INTRODUCTION

Gene therapy is a promising therapeutic option for a wide range of disorders allowing us to tackle a disease at its root by inactivating or replacing mutated genes or by producing new therapeutic proteins directly on-site. Many technological advances have been made over the past 30 years, starting from the delivery of naked genetic material to its encapsulation into non-viral delivery vectors.[1] Among these technologies, lipid nanoparticles (LNPs) have proven to be an excellent delivery platform for various nucleic acid payloads such as DNA, messenger RNA (mRNA), or small interfering RNA (siRNA).[2−5] In fact, directly administering naked, chemically unmodified nucleic acids is highly inefficient due to degradation by nucleases and potentially dangerous due to host immune response.[6] Encapsulating genetic material into LNPs (composed of a cationic or ionizable lipid for nucleic

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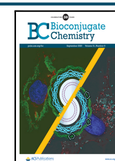

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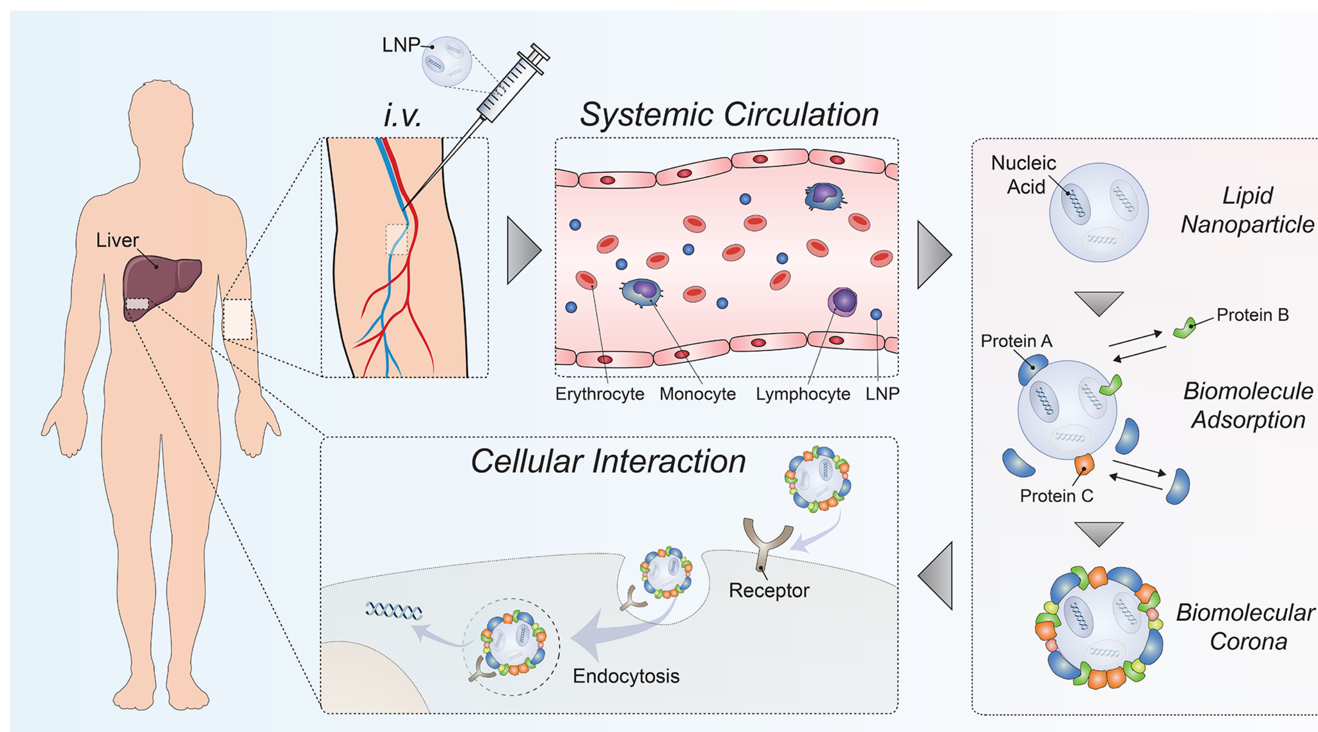

Figure 1. Biomolecular corona of LNPs. Following intravenous (i.v.) administration, LNPs interact with biomolecules present in blood, which adsorb on the LNP surface forming a "corona". Biomolecules with high affinity for the LNP surface are retained, whereas those that are loosely bound continuously exchange with biomolecules present in the environment. Eventually, these LNP−corona complexes are recognized by cell receptors (e.g., on the sinusoidal membrane of hepatocytes), endocytosed, and processed into endosomes, and finally their nucleic acid payload is released into the cytoplasm.

acid complexation and combined with helper lipids) offers a safe and straightforward solution to overcome these issues and to efficiently deliver the nucleic acid payload to target tissue.

Over the past decades, several aspects have been investigated and optimized to develop LNP formulations with clinical utility including (i) scalability and reproducibility of the formulation process;[7] (ii) efficient encapsulation of genetic material;[7] (iii) control over particle physicochemical characteristics (such as size, charge, and hydrophobicity);[8] (iv) pharmacokinetic properties (such as avoidance of premature clearance[9] and accumulation at target tissue[10]); (v) internalization processes; (vi) and mechanisms of nucleic acid release.[11] This LNP optimization process has led to the development of Onpattro (composed of an ionizable cationic lipid, a phospholipid, cholesterol, and a PEG-lipid), the landmark LNP-RNA interference therapeutic for gene regulation in the liver.[10,12,13] Numerous other LNP-based nucleic acid therapeutics have entered clinical development to treat genetic, malignant, or infectious diseases.[14] In the future, these technologies may enable the treatment of any (genetic) disease by simply changing the nucleotide sequence of the encapsulated nucleic acid.[3,15]

The major challenge for developing LNP-based gene therapies, however, is finding effective, tissue-specific delivery strategies (beyond the liver). In fact, many nanomedicines have limited accumulation in extrahepatic tissues or tumors, with only a small percentage of the injected dose actually reaching its target.[16] Many approaches have been devised to enhance tissue- or cell-specific delivery, particularly for targeting tumors,[17] including grafting targeting moieties onto the nanoparticle surface or modulating nanoparticle surface properties.[18] Both strategies assume that LNPs will directly interact with the cell surface once administered; however, this is not the case.[19] In fact, LNPs first interact with biological fluids, whose nature depends on the administration site: from lung surfactants when inhaled, to interstitial fluid when locally injected, to blood plasma following intravenous administration. The biomolecules in these fluids include electrolytes, lipids, and most importantly proteins, which adsorb on the LNP surface, forming a layer that defines the new biological identity of an LNP: the "biomolecular corona" (Figure 1).[19,20] This surface coating can be formed by hundreds of biomolecules including apolipoproteins, immunoglobulins, coagulation factors, and many others (Table 1).[21−23] Some of these biomolecules might associate almost irreversibly to the LNP surface, in either their native or denatured form,[24] affecting, *de facto*, all subsequent interactions. It has been proposed that the corona comprises both these tightly bound proteins ("hard" corona), which presumably bind directly to the LNP surface with high affinity, and also a looser, more dynamic layer ("soft" corona) which constantly exchanges with proteins in the environment. The hard and soft corona are both considered to be relevant in determining LNP interactions with cells.[25]

The biomolecular corona can be seen as an LNP surface modification: it can affect particle physicochemical characteristics[26] and therefore stability, blood residency, biodistribution, immune system recognition, and cell binding.[18,27,28] Moreover, the corona can confer, *per se*, (endogenous) targeting capabilities to the LNP (Figure 1)[29−31] since the presence of certain biomolecules in the LNP corona can facilitate interactions with specific receptors.[31] This phenomenon enabled the success of Onpattro, which efficiently targets

Table 1. Protein Corona Composition of a Lipid-Based Nanoparticle System[47a]

protein name	entry	average ppm
Serum albumin	ALBU	166624
Complement C3	CO3	125283
Immunoglobulin heavy constant mu	IGHM	42156
Apolipoprotein E	APOE	38054
Serotransferrin	TRFE	20767
Alpha-2-macroglobulin	A2MG	18900
Immunoglobulin heavy constant alpha 1	IGHA1	11451
Apolipoprotein A-IV	APOA4	10705
Apolipoprotein A-I	APOA1	9582
Clusterin	CLUS	6738

[a]A limited number of recent articles has analyzed the protein coronas of LNPs.[22,48] Most studies, however, have used sub-optimal conditions, such as 10% human serum or non-human serum, to analyze the protein corona. Therefore, we list here the top 10 most abundant proteins present in the corona of AmBisome (a liposome-based system) after incubation under (optimized) physiological conditions in 100% human serum and isolation by centrifugation.[47]

hepatocytes due to the retention of one particular protein, namely, apolipoprotein E (ApoE), in its corona.[10,32,33] While the *in vivo* journey of LNPs depends on their corona, the nature and dynamics of the biomolecular corona highly depend on the LNP physicochemical characteristics.[34−36] *De facto*, any change in LNP composition may affect the biomolecular corona's composition. In addition, biofluid type (serum, plasma, blood),[37−40] concentration,[41,42] temperature,[43] and origin (animal, human, disease)[44−46] are all critical parameters that can influence the corona and therefore the subsequent nanobio interactions. Thus, even the same LNP tested in different animals, in different patients, or in the same individual over time might acquire different coronas, with potentially important implications for LNP clearance, target tissue accumulation, and efficacy.

In this review, we address LNP stability, biodistribution, and targeting capabilities with regard to the biological environment following intravenous administration. We summarize recent discoveries about nanomedicines' biomolecular coronas, translate these studies to LNPs for gene therapy (of note, only a limited number of studies specifically investigated the impact of the LNP corona for nucleic acid delivery), and use Onpattro as a case study describing the successful development of an LNP-based nucleic acid therapeutic. Moreover, we summarize available techniques to isolate and analyze LNP coronas, highlighting the advantages and drawbacks of each. Lastly, we discuss the possible implications of the biomolecular corona for LNP delivery, and we explore the possibility of utilizing the corona as a targeting strategy for tissue-specific gene therapy.

IMPLICATIONS OF THE BIOMOLECULAR CORONA FOR LNP DELIVERY

Efficient translation of LNP formulations relies on rigorous control over physicochemical characteristics such as size, charge, rigidity, and morphology. These parameters are often used to predict the LNP colloidal stability, degradation, biodistribution, and cellular responses. However, these physicochemical properties are altered upon contact with biological fluids.[18,20,26] Therefore, it is crucial that the *in vitro* characterization of LNPs is performed in relevant media to better reflect the fate of LNPs in a physiological environment. In the following sections, we describe how LNP stability, integrity, systemic circulation, immune system activation, biodistribution, and targeting ability are affected by the biomolecular corona.

Stability and Integrity. Maintaining stability and integrity of LNPs is fundamental for preserving particle properties. As demonstrated recently for other nanomedicines, the biomolecular corona can promote or prevent particle agglomeration by altering the surface properties of the particles (Figure 2).[49,50] This might change circulation time, biodistribution, macrophage recognition, toxicity, release profile, targeting, or uptake kinetics.[51−56] For example, it has been demonstrated that the electrostatic interactions between cationic nanoparticles and anionic proteins in the bloodstream cause particle

Chart 1. Definitions

- **Biomolecular Corona:** The sum of all electrolytes, proteins, and lipids (biomolecules), which are adsorbed by the surface of nanomaterials (*e.g.* LNPs) when they make contact with a biological environment. These biomolecules form a *corona* (Latin term for "crown") that associates almost irreversibly with the nanomaterial surface, defining its biological identity.[19,21]
- **Biological Identity**: The physicochemical properties (such as size, shape, charge, aggregation state) of nanomaterials (*e.g.* LNPs) together with their coronas. The biological identity depends on the composition of the surrounding biological environment and determines the subsequent nano-bio interactions. Often mentioned in opposition to "synthetic identity",which represents LNP's composition and its direct implications for LNP's physicochemical properties.[19,21,151]
- **Biodistribution:** The transfer of compounds from one location to another within an organism. In this context, biodistribution represents the LNP localization after administration and depends on LNPs' physiochemical properties and ability to penetrate biological barriers.
- **Gene Therapy:** Therapeutic delivery of foreign genetic material (RNA- or DNA-based) to treat diseases by reducing or increasing the expression of therapeutic proteins or by correcting the genetic defects (genome editing). LNPs are non-viral vectors for efficient delivery of genetic material including siRNA, mRNA, or DNA.

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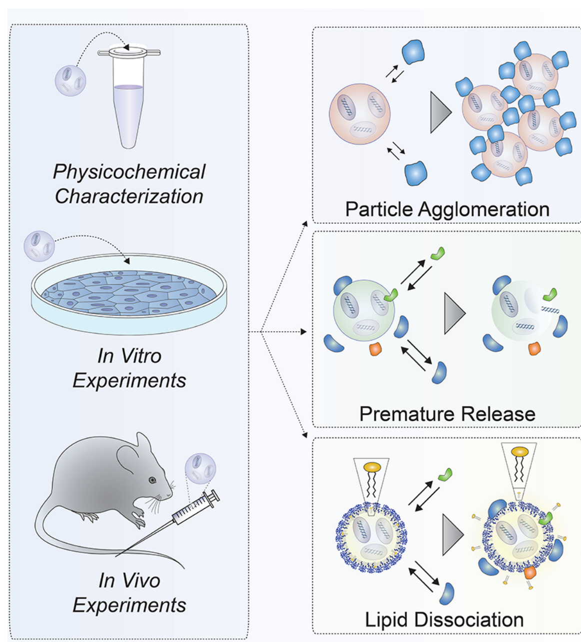

Figure 2. LNP stability and integrity in a biological environment. Upon contact with biological fluids, e.g., during physicochemical characterization or *in vitro* and *in vivo* studies, LNPs adsorb biomolecules present in the environment giving LNPs a new biological identity. The biomolecular corona can alter the characteristics of LNPs and thus can cause particle agglomeration, premature release of LNP content, or lipid dissociation. Consequently, physicochemical characterization and *in vitro* testing of LNPs must be performed in the presence of a biological component that mimics the physiological environment.

agglomeration, which reduces circulation times, accumulation in the target tissue, cellular uptake, and/or endosomal escape.[54,56] This protein-induced aggregation in the blood and resulting rapid clearance is one of the major bottlenecks for the efficient use of lipoplexes (composed of permanently cationic lipids), which are characterized by a high positive global net charge.[57] One strategy to increase nanoparticle stability in circulation is surface modification with polyethylene glycol (PEG).[58] Indeed, the degree of PEGylation changes the agglomeration state of LNPs for siRNA delivery in biological fluids,[59] allowing efficient silencing of target cells. Of note, the agglomeration state of the particles greatly depends on the biological environment: while particles might be stable in, for example, blood plasma, aggregation might happen in gastric juice or spleen homogenate.[60] This phenomenon should be carefully considered in the drug discovery process.

The biomolecular corona can also alter nanoparticle integrity (Figure 2), causing premature release of particle content and compromising the drug biodistribution profile and its blood circulation time.[61,62] Therefore, it is possible that the lack of success of some LNP formulations in delivering genetic material *in vivo* might be due to their instability in biological fluids. For example, Sato et al. have recently shown that cholesterol-free LNPs composed only of a pH-sensitive cationic lipid, egg sphingomyelin, and PEG-lipid were unstable in blood circulation.[63] The higher concentration of serum proteins as compared to *in vitro* conditions (10% heat-inactivated fetal bovine serum) resulted in destabilization and premature release of siRNA.

Proteins present in serum or plasma can also result in leakage of fluorophores or radiotracers used for tracking particles *in vitro* or *in vivo*, which can cause misinterpretation of the results (Figure 2). Assessing dye leakage is particularly challenging in biological media *in vivo*. In this case, the standard dialysis method[64] can be substituted by other techniques such as FRET (Förster Resonance Energy Transfer)[65] or FCS (Fluorescence Correlation Spectroscopy),[66] which can quantify dye release both *in vitro* and *in vivo*.[65,66] Dissociation of lipid components from LNPs can be assessed by incorporating radiotracers such as tritiated cholesteryl hexadecyl ether (^{3}H-CHE) or 1,2-distearoyl-*sn*-glycero-3-phosphocholine labeled with ^{14}C (^{14}C-DSPC) into LNPs and isolating dissociated lipids by size exclusion chromatography, as shown by Chen et al.[8]

Unfortunately, LNP stability and integrity in biological fluids are rarely tested, and these data remain unavailable for many nanomedicines currently in the clinic. In light of these considerations, we propose that agglomeration, premature nucleic acid release, and dye leakage should always be tested in relevant biological fluids in order to better predict LNP behavior *in vivo*.

Systemic Circulation and Blood Clearance. The systemic circulation, immune system recognition, and blood clearance of LNPs are fundamental parameters dictating the fate of LNPs within the body. Governed by the physicochemical properties of LNPs that, in turn, can be altered upon protein adsorption on the LNP surface, these pharmacokinetic characteristics determine the applicability for gene therapy.[67,68] In fact, the biomolecular corona can activate coagulation cascades and facilitate binding of opsonins such as immunoglobulins, complement factors, and unfolded fibrinogen, which cause recognition and rapid clearance by the mononuclear phagocyte system (MPS).[69,70] All these phenomena are reported to mitigate efficient nanoparticle delivery into targeted organs and to lower therapeutic efficiency.[22,29,71]

Forming a protein layer composed by opsonins around a nanoscale foreign object can be seen as one of the body's adapted defense mechanisms against infection. *De facto*, nanoparticle formulations, particularly LNPs for gene therapy, closely resemble certain classes of viruses: they are characterized by a lipid envelope, encapsulate genetic material, and have similar size and curvature. A recent paper pointed out that viruses also possess a biomolecular corona that affects their infectivity and ability to activate dendritic cells.[72] We can only assume that viruses evolved this feature in order to evade the immune system and use the corona to their own advantage. Therefore, it is not surprising that one of the biggest challenges in LNP delivery is to similarly elude the immune system.[73−75] Many nanoparticles fail in (pre)clinical trials due to strong immune reactions following parenteral administration. In addition, repeated administrations of the same nanomaterial can induce an adapted immune response.[76,77] The resulting rapid sequestration by the MPS significantly reduces the circulation time of LNPs, thereby hampering their efficient delivery to target tissues. Several strategies have been implemented to prevent immune system recognition and activation by conferring a "stealth effect" to LNPs. One strategy modifies the lipid composition to minimize particle blood clearance. For example, incorporation of cholesterol into liposomes containing phospholipids with high phase transition temperature enhances blood circulation lifetimes.[78,79] It will be interesting to investigate whether these longer circulation times

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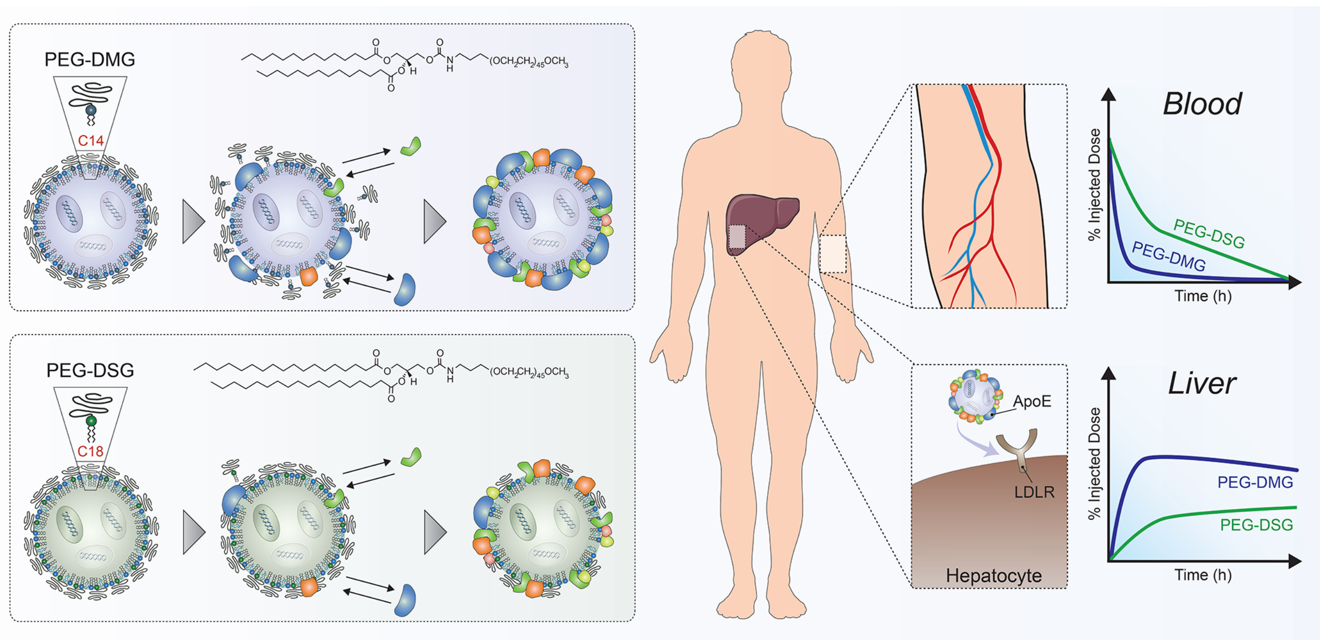

Figure 3. Effect of PEGylation on LNP systemic circulation and biodistribution. To reduce LNP recognition by the immune system and to increase systemic circulation, PEG lipids can be incorporated into LNPs conferring them "stealth" properties. The lipid chain length of the PEG significantly alters LNP blood circulation and biodistribution. Following intravenous LNP injection, detachable PEG C14 lipids (e.g., PEG-DMG) rapidly exchange with proteins present in the blood such as ApoE, allowing efficient uptake into hepatocytes via the LDL receptor. In contrast, PEG C18 lipids (e.g., PEG-DSG) alter LNP biomolecular corona resulting in increased blood residency time and decreased liver accumulation.

can be attributed to distinct protein adsorption onto the nanoparticle surface.

The most common strategy for reducing nanoparticle recognition by the immune system is to alter the particle surface during or after synthesis with hydrophilic polymers that have a neutral charge at physiological pH. The most commonly used polymer is polyethylene glycol (PEG). PEGylation has long been considered able to give particles a "stealth effect" against the immune system by preventing protein adsorption and opsonization on the nanoparticle surface (Figure 3). This effectively reduces macrophage uptake and increases nanoparticle blood circulation time.[80−82] However, it is now recognized that it is the presence of certain proteins, such as clusterin (apolipoprotein J), in the corona of PEGylated nanomedicines that confers them "stealth" properties.[83,84] Not only the presence of PEG but also its length and surface density are essential to achieving its function (Figure 3).[85,86] For example, increasing the content of a PEG lipid with C18 acyl chains (e.g., 1,2-distearoyl-*sn*-glycero-methoxypolyethylene glycol 2000, PEG-DSG) from 1.5 to 5 molar % on the surface of LNPs for siRNA delivery (Onpattro) resulted in improved circulation times up to 10 h (Figure 3). When the same molar ratios were used for shorter C14 chain modified PEG (e.g., 1,2-dimyristoyl-*sn*-glycero-methoxypolyethylene glycol 2000, PEG-DMG), this effect was not observed. The lowest concentration of PEG that could still influence LNP pharmacokinetics (and biodistribution) *in vivo* was found to be 1.5 mol % PEG lipid.[9,87] Supposedly, these differences in behavior depend on different corona compositions of LNPs with different PEG lipids, but this has not been tested yet. A study by Chen et al. analyzed the corona composition of LNPs with PEG lipids that varied in lipid chain length (C14 and C18) and molar ratio (6% and 3%). They observed that higher PEG amounts resulted in reduced protein binding and that using PEG with different lipid chain lengths changed the corona composition.[48] This implies that the effects of the PEG density and chain length on LNP behavior depend on corona composition.

It is important to note that PEG (and other stealth modifications) should be carefully evaluated.[88,89] PEGylation has several drawbacks.[90] While on one hand PEGylation can confer longer circulation time (i.e., reduction of rapid clearance by the MPS), on the other it can reduce cellular uptake and endosomal escape (PEG dilemma).[91,92] For example, PEG C18 decreases uptake of LNPs by hepatocytes and thus gene silencing in the liver (Figure 3).[10] A clever way to prevent LNP recognition by the immune system while enabling uptake into hepatocytes is making the PEG lipid detachable in the presence of biological fluids.[9] A systematic study investigated the de-PEGylation kinetics of different PEG lipids in LNPs for siRNA delivery, revealing that PEGs with short and saturated lipophilic tails, such as PEG-DMG, are required for efficient de-PEGylation, uptake, and gene silencing.[93] This is the case for the LNP formulation of Onpattro. Right after injection, the PEG-DMG exchanges with proteins (e.g., ApoE) present in the bloodstream, conferring targeting capabilities toward hepatocytes (Figure 3). This is an excellent example of how corona formation can be exploited to control target tissue accumulation (see section Tissue-Specific LNP Delivery and Gene Therapy for details). In addition to the interference with cellular interactions, PEG can generate unwanted adverse effects such as complement activation, which may result in hypersensitivity reactions[90,94] and even anaphylaxis.[95] PEGylation can trigger the production of antibodies that preclude the possibility of repetitive administrations.[96−98] The use of PEG alternatives such as polysarcosines might be a possible solution to this problem; however, many of those have not been as extensively investigated as PEG regarding tolerability and efficacy.[99,100]

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To summarize, the biomolecular corona is responsible for LNP recognition by the immune system, thereby influencing LNP blood circulation time and biodistribution. Current strategies, such as PEGylation, can alter the LNP corona composition and can confer an increased systemic circulation. In the next section, we discuss in more detail the influence of the corona on tissue-specific targeting and gene therapy.

Tissue-Specific LNP Delivery and Gene Therapy. Selective tissue targeting is key to developing efficient LNPs for gene therapy. Traditionally, targeting is achieved by physically or chemically conjugating ligands for specific receptors onto the nanoparticle surface. However, debate continues about whether this can enhance nanoparticle efficacy, since just a few studies systematically assessed particles with and without a ligand.[101] Among the obstacles to efficient ligand-mediated delivery, the biomolecular corona is crucial. The presence of a corona on a nanomaterial surface can mask the targeting ligands, which might not be able to bind the corresponding receptor.[28] In this situation, a ligand might not be sufficient to reach the target cell: even if the ligand is exposed, corona proteins might still interfere with its targeting. In addition, ligand density can also alter LNP systemic circulation, blood clearance, and targeting, as demonstrated recently,[102] possibly by influencing the protein corona. For these reasons, including a ligand does not necessarily increase targeting capabilities of LNPs, and accurate controls should be performed to verify efficacy.

An alternative strategy for improving LNP-based delivery of genetic material to specific tissues is to modulate the lipid composition. In recent years, many attempts have been made to correlate the lipid composition and targeted tissue accumulation by using high-throughput screenings. Cheng et al. developed a strategy to systematically tune LNP tropism for CRISPR-Cas gene editing by incorporating charged lipid components, so-called selective organ targeting (SORT) molecules.[103] When increasing amounts of a permanently cationic lipid were added to the formulation, LNP targeting abilities shifted from the liver to the spleen and finally to the lung. The authors noted that adding this lipid appeared to change LNP apparent pK_a values and possibly provide them with distinct coronas. Similarly, barcoded LNPs have been used to investigate how particle properties could affect biodistribution.[104] Dahlman and colleagues administered 30 different LNP formulations containing DNA barcodes into mice and correlated their chemical properties with delivery to specific tissues. In another high-throughput screening, the same authors developed a strategy to assess how more than 250 LNPs could deliver functional mRNA to multiple cell types *in vivo*.[105] In all these studies, the authors observed changes in the LNPs physicochemical characteristics, such as size and charge, which might be responsible for their different biodistributions. Unfortunately, a correlation between a formulation's physicochemical properties, its biomolecular corona, and the biodistribution was not provided. Future studies should investigate whether changes in the corona composition might be involved in the altered biodistribution of these different LNPs.

The lipid composition that led to the success of Onpattro has been selected in an *in vivo* screening that demonstrated that LNPs containing heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate (DLin-MC3-DMA) in combination with a detachable PEG-lipid, DSPC, and cholesterol were more likely to exert optimal targeting and gene silencing in hepatocytes.[106] Subsequent studies revealed that the targeting abilities of Onpattro depend on the presence of certain biomolecules in its corona. In fact, once injected into the bloodstream, the surface of Onpattro can adsorb ApoE, a serum protein recognized by hepatocyte receptors, including the LDL[10,32] and the HSPG receptor.[107] As ApoE can direct LNPs toward hepatocytes, other components in the corona might be able to direct LNPs toward extrahepatic tissues.[33] Therefore, controlling corona composition may be another strategy to steer nanoparticle targeting. For example, LNPs optimized to have a vitronectin-rich corona showed decreased delivery to human liver-derived HepG2 cells expressing the LDL receptor as compared to LNPs with an apolipoprotein-rich corona.[108] In contrast, the corona of synthetic model lipoproteins enriched in apolipoprotein C-III (ApoC-III) attenuates ApoE-mediated uptake into HepG2 cells.[109] In another study, zwitterionic DOPE-containing nanoparticles with an apolipoprotein-enriched protein corona were able to target prostate cancer cells expressing high levels of scavenger receptor class B type 1,[25] while DOTAP/DNA lipoplexes that form a corona rich in vitronectin can target cells with high levels of the $\alpha\nu\beta3$ integrin receptor such as metastatic MDA-MB-435S cells.[110]

We should note that the corona of an LNP (and of nanomaterials in general) is composed of hundreds of biomolecules that might influence its targeting capabilities (Table 1).[47,48,111] Therefore, the mere presence of a protein in the corona does not necessarily mean it will target a specific receptor. In fact, other important parameters to consider are the availability, orientation, and conformation of the corona components: many of the proteins identified in the coronas of different nanomaterials are not in their native form and therefore cannot exert targeting.[112,113]

Understanding how adsorbed proteins affect recognition of LNP by cells is essential to effectively target specific tissues.[114−116] Seeking a correlation between corona composition and biodistribution, Walkey et al. analyzed the coronas formed in whole blood of a library of 105 gold nanoparticles.[115] They used the corona "fingerprint" to predict the NP cellular uptake and found that this parameter is more accurate than size, charge, or aggregation state in predicting NP cellular associations. A similar approach has been used on a library of 16 LNPs to investigate the relationships between physicochemical properties, protein corona fingerprints, and NP−cell interactions.[117] Interestingly, just a minor fraction of the proteins present in the corona was found to enhance LNP association with HeLa cells.

The examples reported here highlight the fundamental role of the corona in determining the biodistribution and targeting of LNPs for gene therapy. This (observation) emphasizes, once more, the importance of systematic studies to correlate lipid composition, physicochemical properties, and corona composition with LNP targeting capabilities. However, the limited range of techniques available to study the corona composition of LNPs poses an obstacle to these studies. In the next section, we discuss commonly used methods for studying the LNP corona and the related challenges.

■ DECIPHERING THE LNP BIOMOLECULAR CORONA

The first step to correctly characterize LNPs in biological environments is to use appropriate incubation conditions, which should be chosen according to the intended application, disease, and injection site and modality.[118,119] LNPs that will

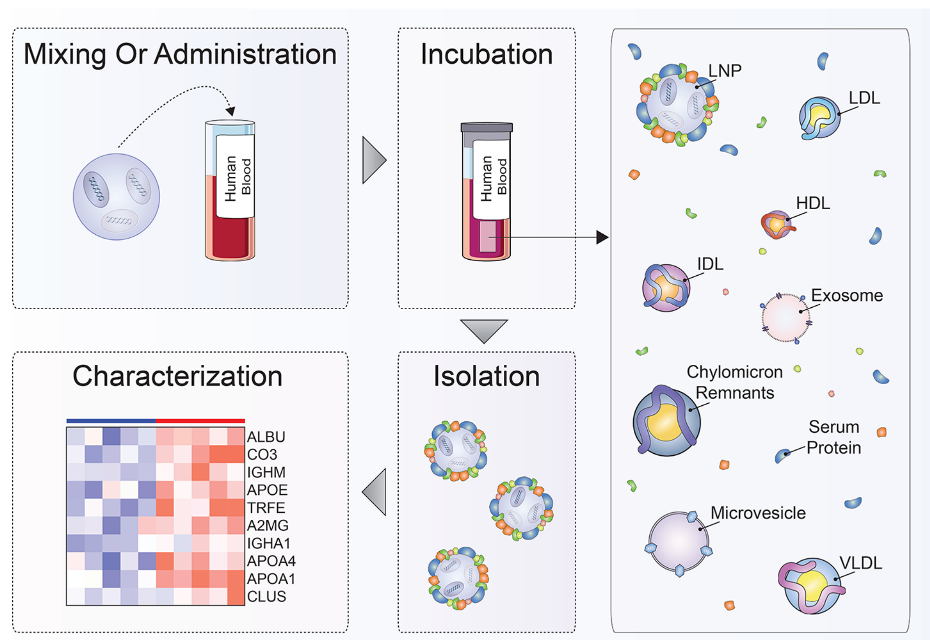

Figure 4. Investigation of the LNP biomolecular corona. The *in vitro* characterization of the LNP corona composition requires the use of appropriate incubation conditions, which should be chosen according to the intended application, disease, and injection site and modality. In this example, LNPs are incubated in human blood to mimic LNP intravenous administration into patients. Following incubation, the LNP−corona complexes must be isolated from unbound plasma proteins, lipoproteins, and extracellular vesicles. The protein and lipid content of the isolatad complexes can then be analyzed and characterized (e.g., by mass spectrometry): LDL (Low-Density Lipoprotein); HDL (High-Density Lipoprotein); IDL (Intermediate-density lipoprotein); VLDL (Very Low-Density Lipoprotein).

be used in clinical practice should be tested in human biological fluids since the corona is species-specific and possibly in fluids derived from patients that will receive the treatment, since the corona is also disease-specific.[44,45,120] NPs for intravenous administration should be tested in full blood, since the corona varies greatly if tested in serum or plasma;[38] moreover, the right temperature (37 °C)[43] and shear stress[121] should be used in order to perfectly mimic the physiological environment.

Once the corona is formed in the correct biological environment, LNP stability, nucleic acid release profile, and the availability of targeting moieties for LNP recognition by cell receptors can be easily assessed *in vitro*. This can predict whether particular lipid compositions will aggregate, release their content prematurely, or be unable to target specific cells due to the "masking" effect of the corona.[28] Characterizing protein corona composition might be required in order to identify which corona component is responsible for (or interferes with) the LNP targeting behavior. Unfortunately, because so far only a few studies have systematically investigated corona composition using appropriate incubation conditions, it remains difficult to correlate protein composition with nanoparticle targeting capability *in vivo*.[119]

After forming the appropriate corona, LNP−corona complexes must be separated from unbound biomolecules and recovered before analysis (Figure 4). This step comes with several challenges. First, the corona composition must be preserved during this separation step. Second, the complexes must be separated from unbound biomolecules with a low molecular weight. Traditionally, several washing steps are performed in order to remove the loosely bound biomolecules that compose the "soft" corona. While this procedure can be relatively straightforward, it is also highly debated, since these weakly bound biomolecules might be relevant for subsequent particle interactions. Third, the complexes must be separated from endogenous nanoparticles, such as lipoproteins and extracellular vesicles. This is particularly challenging for LNPs, which are similar in size and density to these natural nanoparticles (Table 2). When evaluating the top 10 LNP

Table 2. Overview of Endogenous Nanoparticles Present in Blood[a]

nanoparticle	size range (nm)	density (g/mL)
Lipoproteins		
Chylomicrons	75−1200	<0.95
Chylomicron remnants	30−80	0.95−1.006
Very low-density Lipoproteins (VLDL)	30−80	0.95−1.006
Intermediate-density Lipoproteins (IDL)	23−27	1.006−1.019
Low-density Lipoproteins (LDL)	18−23	1.019−1.063
High-density Lipoproteins (HDL)	7−13	1.063−1.21
Extracellular Vesicles (EVs)		
Microvesicles	100−1000	1.06−1.1.6
Exosomes	30−150	1.1.3−1.19
Synthetic		
Lipid Nanoparticles (LNPs)	30−200	0.9−1.2

[a]Size and density of lipoproteins and extracellular vesicles are detailed and compared to LNPs.

corona proteins from Table 1 against databases describing the top 100 proteins in extracellular vesicles, all the proteins are also recovered in extracellular vesicle isolates.[122] For lipoproteins, the apolipoproteins and alpha-2-macroglobulin protein family members are especially abundant.[123]

Next, we will describe in brief several techniques that can be used to isolate the LNP−corona complexes, together with the benefits and drawbacks of each approach (Table 3).[37,124−126]

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Table 3. Separation Techniques to Isolate the LNP Corona[a]

recovery method	advantages	disadvantages	refs
Size Exclusion Chromatography (SEC)	Preserves corona integrity	Does not allow separation from lipoproteins Time consuming	121,127,131,132
(Ultra)centrifugation	Fast	Does not allow separation from lipoproteins Might produce aggregates or LNP rupture Might alter corona integrity	131,132
Gradient (Ultra)centrifugation	Fast Gentle on corona integrity	Might not allow separation from lipoproteins Gradient composition might interfere with corona	48
Magnetic Separation	Fast Preserves corona integrity Allows separation from lipoproteins	Requires magnetic LNPs Not suitable for every application	126,128,129,131,132
Cross-linking	Allows separation from lipoproteins Precise	Need to modify nanoparticle formulation Might not retain all corona proteins	47,133

[a]Expert opinion on the most important advantages and disadvantages of different methods.

For a more comparative overview of the separation techniques and parameters currently used in the field of nanotechnology, we refer the reader to the comprehensive review by Böhmert et al.[125]

The most common techniques to isolate an LNP corona are based on separation by size or density. One example is Size Exclusion Chromatography (SEC), which uses a gravity column filled with a porous resin. SEC resolution can be improved by choosing the right resin type and increasing the column length and diameter. The corona is minimally perturbed by this technique: bigger particles and aggregates will be eluted first, while loosely bound proteins with low molecular weight will elute last. However, some studies indicate that this approach cannot separate nanoparticles from certain classes of lipoproteins (Table 2),[127] whose protein content will, therefore, interfere with corona analysis. This is particularly relevant when physiological amounts of blood are used for incubation. Moreover, interactions with the resin might occur. Usually, after running through the column, the sample must be concentrated using ultrafiltration and further washing steps must be applied,[121] making this method too slow for high-throughput studies.

Another widely used technique is centrifugation. Since the sedimentation rate of LNPs (which depends on LNP size and buoyancy) is higher than that of unbound proteins, the LNP−corona complexes will eventually precipitate while the unbound proteins will remain in solution. Therefore, centrifugation time and speed should be optimized according to density of the complexes. The advantage of this technique is its speed and the fact that it requires only small amounts of material. However, centrifuging low-density nanoparticles such as LNPs requires high g-force (ultracentrifugation), which might cause LNP aggregation or disruption and thus inevitably alter corona composition. Moreover, this technique often generates false positives, since many more proteins are isolated in the same fraction as LNPs. Several washing steps should therefore be applied in order to remove the loosely bound proteins. As for SEC, another drawback of centrifugation is that it cannot separate lipoproteins, which have a sedimentation rate very similar to that of LNPs and will probably precipitate as well. In a recent publication,[48] the biomolecular corona of LNPs was characterized using density gradient ultracentrifugation. In this technique, the LNP−corona complexes are first loaded into a medium of graded density and then ultracentrifuged. Fractions with a different density are then collected and analyzed. This method is less aggressive than traditional centrifugation since particles will stay in supension and presumably remain intact. It has not been reported, however, whether or not LNPs will be separated from lipoproteins and EVs or whether the chemical composition of the graded medium might interfere with corona stability and composition.

The LNP−corona complexes of magnetic LNPs can be separated from unbound proteins by loading them into a magnetic separator, washing the loosely bound proteins, and then eluting the LNP−corona complexes.[128,129] This technique is the only one that can easily distinguish between particles and lipoproteins, which are not magnetic and therefore are not retained in the column. However, Bonvin and colleagues recently showed the limitations of this approach and developed a multistep centrifugation method to separate magnetic particles with different characteristics.[129] This technique can supposedly also be applied to LNPs with magnetic nanoparticles encapsulated into their cores.[130] However, encapsulating magnetic iron particles into LNPs might influence the intraparticulate lipid distribution and thus call into question the comparability to LNP−nucleic acid systems.

A few recent studies directly compared the techniques mentioned above, with conflicting results.[126,131,132] This highlights, once more, the need for alternative, reliable methods to isolate and characterize the corona. One attempt by Zhou and colleagues applied the principle of protein cross-linking to retain proteins on the surface of cysteine functionalized gold nanoparticles.[133] NP−corona complexes were fixed with formaldehyde, centrifuged, washed with SDS, and sonicated in order to retain the hard corona proteins only. Interestingly, the group compared the corona formed with this new method to the corona formed by standard or sucrose cushion centrifugation and found differences in protein composition. Similarly, Pattipeiluhu and colleagues used a photoaffinity labeling approach to capture and purify corona biomolecules from three clinically relevant liposomal formulations.[47] The formulations were modified by substituting phosphatidylcholine with a structurally similar photoaffinity lipid probe able to covalently cross-link, upon sample irradiation, to any biomolecule in close proximity. To allow its isolation, the probe also contained an azide group on the fatty acid chain terminus. After serum incubation, samples were irradiated for 15 min and then liposomes were solubilized. By using standard bioorthogonal click chemistry protocols, the azide present on the fatty acid chain terminus was then coupled to either a fluorescent alkyne-Cy5 probe or an alkyne-

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biotin label for, respectively, direct visualization or pull-down and isolation of the proteins bound to the phosphatidylcholine probe. This approach can identify proteins directly in contact with the LNP surface without interference from lipoproteins or loosely bound corona proteins (soft corona). However, these methods require modification of the formulation and might interfere with corona formation as well. Further experiments are needed to validate this technique, since there is no guarantee that every protein present on the surface will be isolated and identified.

After incubation and separation of NPs and their coronas, the protein content is typically analyzed by liquid chromatography mass spectrometry (LS-MS), which has become the gold standard for qualitatively and quantitatively identifying the single proteins that form the corona.[134] Other available techniques can identify the macroscopic effects that the corona exerts on nanoparticles, such as dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), electrophoretic light scattering (ELS, ζ-potential), and (cryogenic) transmission electron microscopy (TEM), or elucidate more subtle protein changes, such as nuclear magnetic resonance spectroscopy (NMR), fluorescence correlation spectroscopy (FCS), ultracentrifugation, and surface plasmon resonance (SPR). These methods have been extensively reviewed elsewhere.[119]

Importantly, the approaches described here can only provide a partial insight into the complex corona composition of LNPs. A combination of different techniques and the use of standardized controls is necessary to overcome some challenges and to gain an accurate and deep understanding of the LNP biomolecular corona. Comprehensive studies and sophisticated methods are urgently needed to correlate the corona compositions of LNP with their clinical outcomes.

■ CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Gene therapy retains great appeal for treating almost any pathology by silencing genes, expressing proteins, or correcting genetic defects in almost any tissue. In order to avoid degradation and the onset of unwanted immune reactions, genetic materials can be encapsulated into delivery vectors. Due to the recent advances in LNP technology[2,3,87] and particularly after the approval of Onpattro, the first LNP-based siRNA therapeutic for treating hereditary transthyretin-mediated amyloidosis, LNPs for nucleic acid delivery have gained a great deal of attention.[12,13] Currently, LNP systems are being investigated as a platform technology for a broad range of conditions and diseases, from delivering siRNA to reducing cholesterol,[135] or using the liver as a bioreactor to produce therapeutic proteins,[136−138] to developing mRNA-vaccines against cancer[139] or infections,[137,140] including the novel coronavirus SARS-CoV-2.[141−144]

Onpattro and many other systemically administered LNPs efficiently target the liver,[3,135] thus allowing the treatment of a broad spectrum of disorders originating in hepatocytes. This liver tropism is not only based on the presence of hepatic fenestrae that allow circulating LNPs to access the sinusoidal surface of hepatocytes. In fact, it is the presence of ApoE in the LNP corona that confers Onpattro liver tropism, mediated by the recognition of ApoE by the LDL receptor on hepatocytes. Without ApoE, LNPs such as Onpattro lose their transfection potency,[10] indicating that target cell recognition of specific corona components is necessary for efficient delivery. Therefore, the long-standing (but highly controversial) dogmatic focus on the preferential accumulation of nanoparticles within tumors owing to their leaky vasculature, i.e., enhanced permeability and retention (EPR) effect, might not provide the full picture.[145] Probably not by chance, it has been shown that targeting solid tumors by PLGA particles also depends on the LDL receptor recognition of ApoE.[146] A recent study even demonstrated that nanoparticles do not diffuse passively through the leaky tumor endothelium, but rather are recognized directly by endothelial cells.[147]

Several examples[10,146,147] show that the corona plays a fundamental role in tissue-selective targeting and that the particle chemical identity is not sufficient to describe the complex transformations and interactions happening *in vivo*. By the time they reach target cells, LNPs have been in contact with hundreds of biomolecules and barriers, which inevitably alter their chemical nature. Thus, at this point the biologically relevant entity is no longer the bare particle, but rather a nano-object with a distinct size, charge, shape, and biomolecular corona. The relevance given to the biological identity of LNPs comes from the observation that the corona alters the physicochemical properties of LNPs[18,20,26] and thus their stability,[49,50] integrity, release profile,[61,62] biodistribution,[115,117] and targeting capability.[28] As a consequence, LNP development must not disregard the effect of the biological environment during LNP delivery.

Despite its important implications, the biomolecular corona has been a neglected component of the nanomedicine field for a long time, particularly regarding LNPs for gene therapy. In fact, *in vitro* testing of LNPs, including particle characterization (particle size, charge, and stability), is often performed using bovine serum, usually at non-physiological concentrations (10% FBS). In addition, release profiles are rarely tested in relevant biological media, in contrast with the extensive literature on this topic.[37−46] Not surprisingly, *in vitro* testing has revealed weak correlations with LNP behavior *in vivo*.[148,149] Of note, the ionizable cationic lipid DLin-MC3-DMA, a key component of Onpattro, has been selected in an *in vivo* screening aimed at measuring its silencing efficacy, since *in vitro* experiments had a low predictability.[106]

Understanding how to engineer LNPs to selectively target tissues beyond the liver will be key to develop next-generation gene therapies. High-throughput screenings are powerful tools that could reveal the correlation between LNP composition and biodistribution.[103−105] A critical question is whether the observed biodistribution can be traced back to a particular corona composition. We now know that any LNP modification might potentially affect the composition of the biomolecular corona, since the nature and dynamics of the corona depend heavily on the physicochemical characteristics of LNPs.[34−36] Advanced methods to isolate LNPs from biological media will be crucial to overcome technical challenges hindering the high-throughput elucidation of the LNP corona composition. Studying the corona fingerprint, which represents all the corona components that confer targeting capabilities to a particle, can help us understand how these proteins affect LNP recognition by cells.[115,117] Finding a particular corona composition that can direct LNPs toward a specific target organ, cell type, or receptor,[114−116] as in the case of Onpattro, will be critical to controlling the LNP pharmacodynamic profile *in vivo*.

To conclude, this review highlights that the biomolecular corona defines the biological identity of LNPs for gene therapy. On one hand, the physicochemical characteristics of

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LNPs and the biological environment strongly affect corona composition. On the other hand, the physicochemical characteristics of LNPs change upon corona formation, which is specific to the biological environment in which particles are administered. As a consequence of this specificity, the same LNP will possess a different corona for each individual and each disease.[44,45,120] This emphasizes, once more, that the "one size fits all" nanomedicine approach should be replaced with a more personalized framework.[120,150,151] Instead of thinking about this specificity as an obstacle to LNP delivery, we should use it as a way to predict which treatment will be more suitable for each patient. Consequently, our field needs to consider the inevitable formation of the biomolecular corona while designing, characterizing, and testing LNPs *in vitro*. The future challenge in developing next-generation LNP-based gene delivery systems will be finding a way to use the corona to our advantage.

■ AUTHOR INFORMATION

Corresponding Authors

Valentina Francia − *Department of Biochemistry and Molecular Biology, University of British Columbia, V6T 1Z3 Vancouver, British Columbia, Canada; Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, 3584 CX Utrecht, Netherlands;* orcid.org/0000-0003-4911-6832; Email: v.francia@umcutrecht.nl

Dominik Witzigmann − *Department of Biochemistry and Molecular Biology and NanoMedicines Innovation Network (NMIN), University of British Columbia, V6T 1Z3 Vancouver, British Columbia, Canada;* orcid.org/0000-0002-8197-8558; Email: dominik.witzigmann@ubc.ca

Authors

Raymond M. Schiffelers − *Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, 3584 CX Utrecht, Netherlands;* orcid.org/0000-0002-1012-9815

Pieter R. Cullis − *Department of Biochemistry and Molecular Biology and NanoMedicines Innovation Network (NMIN), University of British Columbia, V6T 1Z3 Vancouver, British Columbia, Canada;* orcid.org/0000-0001-9586-2508

Complete contact information is available at:
https://pubs.acs.org/10.1021/acs.bioconjchem.0c00366

Notes

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