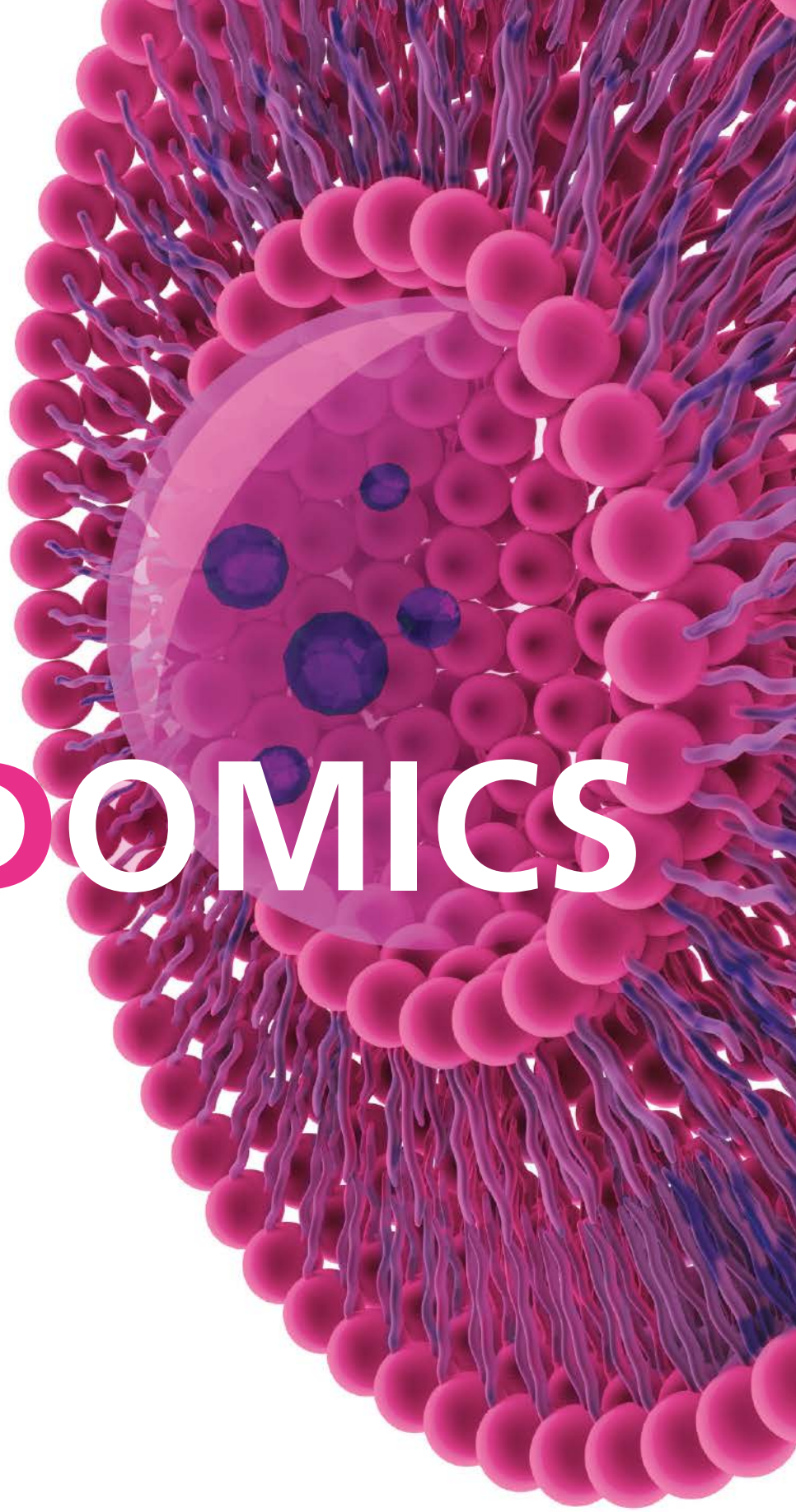

LIPIDOMICS

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Lipid nanoparticles for drug delivery use

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Editorial

Messenger RNA (mRNA), a single-stranded RNA involved in protein synthesis, has emerged as a promising therapeutic strategy in the treatment and prevention of diseases and disorders. mRNA therapies have the potential to transform the standard of care for many different diseases and actualize personalized medicine. During the COVID-19 pandemic, the success of several mRNA vaccines was instrumental in developing immunity against COVID-19 in addition to elucidating a deeper understanding of the disease itself. As a result, the last few years have led to a renewed and vested interest in mRNA therapies in both society and in the greater medical and scientific communities.

This strategy involves the delivery of transcribed mRNA into target cells, where the mRNA is translated into a functional protein. For therapeutic applications, such as gene editing, cellular reprogramming, or protein replacement therapies, the mRNA may encode for an absent or dysfunctional protein to restore normal protein function. As a viral vaccine or cancer immunotherapy, the mRNA encodes for a protein to elicit an immune response.

mRNA therapeutics have a number of advantages over small molecules and therapeutic proteins. They exhibit drug-like behavior, with an ability for repeat dosing and adjusted doses/dosing regimens. Their development from target gene to product candidate is rapid and cost-effective, being relatively simple to manufacture. Furthermore, mRNA therapeutics can act on targets deemed "undruggable" for a small molecule or therapeutic protein. Although mRNA medicines show vast potential, challenges still remain that impede the use of mRNA as a generic therapeutic modality. For example, chronic dosing eventually may activate innate immunity, with attenuated expression of the therapeutic protein of interest. The greatest difficulties, however, relate to delivery and stability. Nucleic acids are hydrophilic and have negative charges, which impede passive diffusion across the plasma membrane. Additionally, nucleic acids can be taken up by phagocytes and degraded by endogenous nucleases, which hinders cellular delivery. Because of the susceptibility to degradation, it is imperative for mRNA therapies to have stable and effective delivery systems that can protect the integrity of the mRNA cargo throughout the transport process, uptake by target organs, tissues, and cells, and subsequent release at the site of action.

Lipid nanoparticles (LNPs) have been the most common and successful nonviral delivery system for mRNA medicines, as exemplified by the approval and clinical use of LNP-mRNA vaccines against COVID-19. LNP delivery systems must be tissue-specific and biodegradable, and they must be able to evade endosomal entrapment and degradation. Additionally, the structure and composition of the delivery system is a crucial consideration during preclinical development of LNP-mRNA formulations, as different chemical compositions can affect the cargo delivery (and delivery efficiency), the safety profile, and immunogenicity. Additional key considerations for LNP-mRNA formulation development include the administration route and manufacturing.

Due to the importance of these elements, this booklet provides an overview of the use of lipid nanoparticles for the delivery of mRNA therapeutics. The studies discussed herein focus on the key aspects of developing an effective drug delivery system: design of LNPs, delivery of LNPs into the body and cells, and subcellular delivery of mRNA cargo. Through these article summaries and expert insights, we hope to educate researchers on the value of LNPs as mRNA drug delivery vehicles as well as important considerations and strategies to enhance the design and development of LNP-mRNA formulations.

Emily E. Frieben, Ph.D.
Associate Editor,
Wiley Interdisciplinary Reviews

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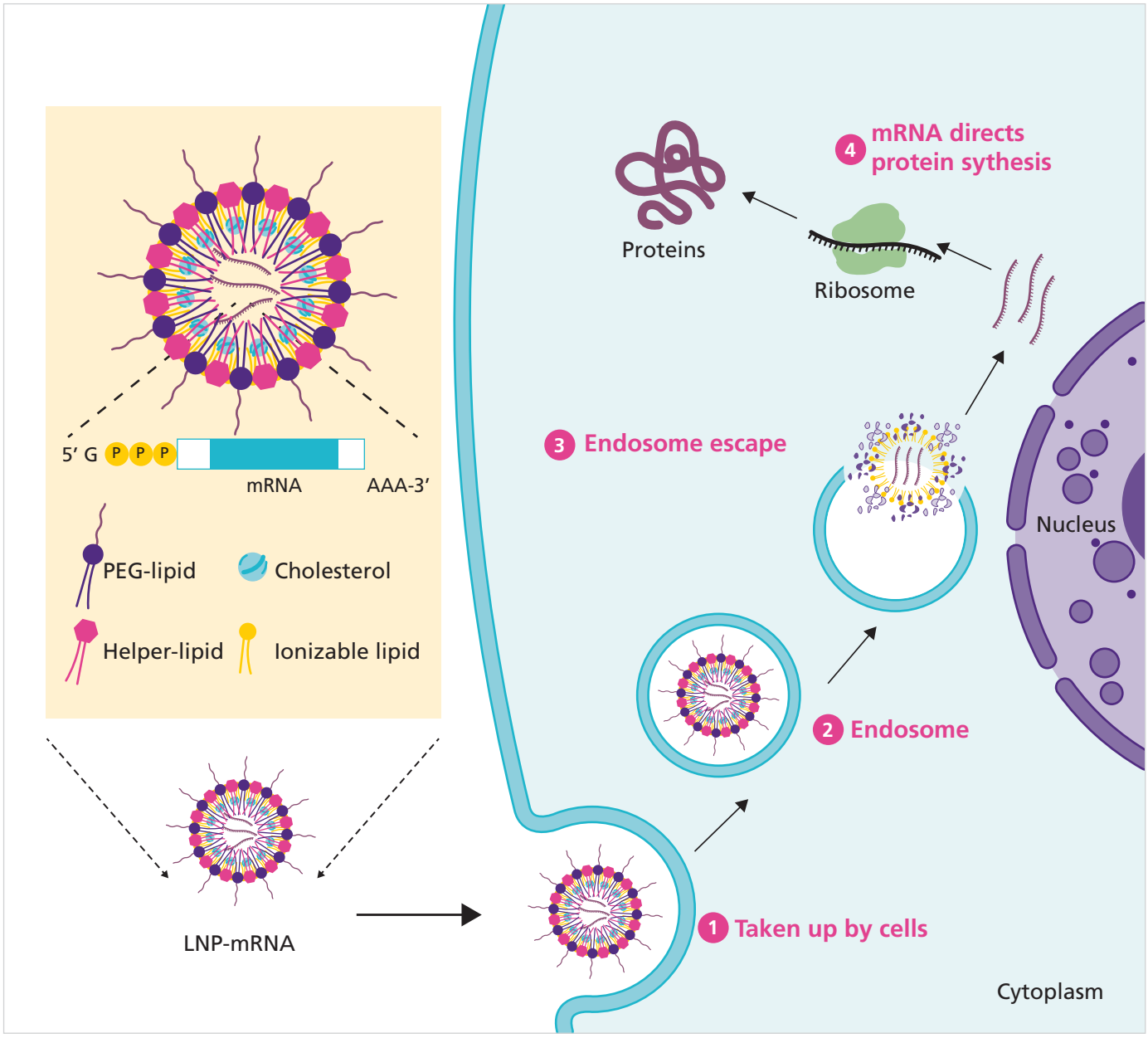
Lipid Nanoparticle Delivery of mRNA Therapies



Delivery of messenger ribonucleic acid (mRNA) by lipid nanoparticles (LNP).

Structural components of mRNA-encapsulated LNPs (left-panel). Under an acidic environment, cationic LNPs form a complex with nucleic acids via electrostatic interactions. Under neutral conditions, the LNPs are neutrally charged and therefore interact less with serum components. Once the mRNA-LNP complex reaches the cell membrane, cationic phospholipids fuse with and destabilize the cell membrane, promoting mRNA-LNP uptake by the cell (1). After being internalized into the cell, the mRNA-LNP complex is engulfed by endosomes (2). The endosomal environment acidifies the ionizable phospholipids, allowing fusion with the primary lysosomal membrane. LNP integrity is disrupted by this interaction, and mRNA is subsequently released, where it can then direct protein synthesis (4). Membrane fusions and LNP structural changes are believed to be the main causes of endosomal membrane destabilization and mRNA escape (3). (Modified from Gu et al., 2022, https://doi.org/10.1002/mco2.167).

Customizable Lipid Nanoparticle Materials for the Delivery of siRNAs and mRNAs

Fenton O.S., Kauffman K.J., McClellan R.L., *et al.*

RNAs, including short interfering RNAs (siRNAs) and messenger RNAs (mRNAs), can regulate protein levels within cells and have potential as therapeutic agents. However, delivering these RNAs to target cells can be a challenge due to several factors. When administered intravenously, for example, circulating nucleases in the bloodstream can cause degradation, pattern recognition receptors can trigger an immune response, and non-specific uptake into off-target tissues can limit their clinical translatability. Additionally, cellular membranes are largely impermeable to naked RNAs due to their size and charge, making it difficult for them to passively diffuse into target cells. To address these issues, researchers are interested in developing delivery materials to improve the therapeutic potential of RNAs. These materials could have a range of potential applications, including diabetes and cancer management, protein replacement therapies, and immune tolerization, among others.

One approach to delivering RNAs is to formulate them with ionizable lipids, which are amphiphilic amine-based small molecules that can reversibly bind RNAs through electrostatic interactions. This complexation can be used to create lipid nanoparticles (LNPs), a type of non-viral nucleic acid delivery vehicle that has demonstrated efficacy in both animals and humans. However, it is not yet fully understood how the chemical structure of the ionizable lipid affects the overall delivery properties of the LNP. This is an important question because the structure of the ionizable lipid could potentially be manipulated to optimize the delivery of RNAs.

To address this question, a team of researchers synthesized a series of degradable diketopiperazine ionizable lipids with varied molecular structure. These ionizable lipids were designed to incorporate three structural motifs: bis-lysine diketopiperazine-based amine cores, ester-based linkages, and a common core approach. The bis-lysine diketopiperazine-based amine cores were chosen because they have previously been used in the synthesis of potent RNA delivery intermediates. The ester-based linkages are degradable, which could improve tolerability, and the common core approach allows for the synthesis of multiple ionizable lipids using identical methodologies and starting materials. The resulting series of eight ionizable lipids included variations in tail lengths, tail geometry, degree of tail unsaturation, and carbon linker lengths.

The researchers then formulated each of these ionizable lipids into LNPs containing either siRNA or mRNA. The LNPs were created using microfluidic mixing approaches, with the identity of the ionizable lipid being the only variable between formulations. The researchers found that all of the ionizable lipids were successfully formulated into LNPs, with the exception of one that did not form stable LNPs with siRNA.

The potency of the resulting LNPs was then evaluated by measuring protein knockdown or protein expression in target cells. The researchers found that the structure of the ionizable lipid did have an effect on the potency of the LNP. Specifically, the tail length, tail geometry, and linker spacing of the ionizable lipid all appeared to influence the delivery properties of the LNP. However, more research is needed to fully understand these relationships.

The results of this study suggest that the structure of the ionizable lipid used to formulate LNPs can have a significant impact on the potency of the LNP. Further research is needed to fully understand how the various structural features of the ionizable lipid influence the delivery properties of the LNP. This information could be used to design and synthesize next-generation RNA delivery materials with improved potency and therapeutic potential.

Digest of
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O. S. Fenton, K. J. Kauffman, R. L. McClellan, J. C. Kaczmarek, M. D. Zeng, J. L. Andresen, L. H. Rhym, M. W. Heartlein, F. DeRosa, D. G. Anderson;
Angewandte Chemie International Edition;
2018, 57, 13582

Subcellular Delivery of Lipid Nanoparticles to Endoplasmic Reticulum and Mitochondria

Shi Y., Luo Z., You J.

Delivery of drugs to their site of action holds promise for improving treatment of diseases and disorders. However, the poor bioavailability of many therapeutic agents and the potential for off-target toxicity can limit their clinical use. Endoplasmic reticulum (ER) and mitochondria are subcellular organelles that play important roles in various physiological processes and can be affected in various diseases. Delivery of drugs directly to the ER and mitochondria may be an effective way to improve treatment of these conditions. Subcellular targeting of the ER and mitochondria can be achieved through the use of small molecules or macromolecules with special structural characteristics, but these approaches may have negative impacts on drug structure and activity. Lipid nanoparticles, on the other hand, offer high biosafety, diverse cargo loading, and tunable properties, making them promising candidates for drug delivery to the ER and mitochondria. To understand better subcellular delivery of lipid nanoparticles to endoplasmic reticulum and mitochondria, Shi et al undertook a review to summarize recent advances in the use of lipid nanoparticles for drug delivery to the ER and mitochondria.

Endoplasmic reticulum as the protein and lipid production factory

The endoplasmic reticulum (ER) is a subcellular organelle that plays a role in the synthesis of lipids and sterols, the dynamic equilibrium of intracellular calcium, and the post-translational modification and quality control of proteins. Perturbations in the ER can lead to the accumulation of unfolded or misfolded proteins, activating the unfolded protein response (UPR) to restore proteostasis. However, excessive ER stress can trigger programmed cell death, which is a major contributor to various diseases, including cancer, neurodegenerative disorders, and cardiovascular diseases. The tumor microenvironment (TME) can also induce ER stress in cancer cells, which can be beneficial for their growth, immune evasion, and metastasis. Targeting the ER or manipulating the UPR pathway may be promising strategies for future oncotherapy. In neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, ER stress and the UPR pathway are also involved in the pathogenesis and progression of the diseases. Similarly, ER stress has been linked to chronic liver disease and atherosclerosis. In ocular diseases, ER stress and the UPR pathway have been shown to play a role in the development of retinal degeneration. Delivery of drugs directly to the ER or mitochondria may be an effective way to improve treatment of these conditions. Lipid nanoparticles have potential as drug delivery systems for targeting the ER and mitochondria due to their moderate drug encapsulation, high biosafety, and diverse cargo loading capabilities.

Mechanisms of ER targeting

Lipid nanoparticles (LNPs) are being developed as a delivery vehicle for therapeutic RNAs, such as small interfering RNAs (siRNAs) and messenger RNAs (mRNAs). These RNAs have the ability to regulate protein concentrations within cells at the post-transcriptional level, but their use is limited due to challenges with intracellular delivery. When administered intravenously, for example, RNAs can be degraded by circulating nucleases, trigger an immune response through pattern recognition receptors, and be taken up into off-target tissues, limiting their clinical translatability. Additionally, cellular membranes are largely impermeable to naked RNAs due to their size and charge, limiting their diffusion into the cytoplasm of target cells. To improve the therapeutic potential of RNAs, researchers are developing delivery materials, such as ionizable lipids, which can form LNPs through electrostatic interactions with RNAs. These LNPs have demonstrated efficacy in animals and humans and have the potential to be used in a range of applications including diabetes and cancer management, protein replacement therapies, and immune tolerization. However, more research is needed to understand how the chemical structure of ionizable lipids affects the overall delivery properties of LNPs. Specifically, the influence of factors such as tail length, tail geometry, and linker spacing on LNP potency for degradable diketopiperazine lipids with both siRNA and mRNA cargoes has not yet been explored.

Lipid nanoparticles with ER targeting ability and their applications

Lipid nanoparticles (solid internal structure)

Lipid nanoparticles are a type of drug delivery system that are composed of various types of lipids, including cationic/ionizable, phospholipid, cholesterol, and PEG lipids. These nanoparticles can be further divided into subtypes such as mRNA-encapsulated lipid nanoparticles (mRNA-LNPs), solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), and lipid-polymer hybrid nanoparticles (LPHNs). Lipid nanoparticles have been used for drug delivery and can be modified to target specific subcellular organelles or enhance their biocompatibility and biodistribution. They have been shown to be effective in delivering a variety of therapeutic agents, including nucleic acids, proteins, and small molecules.

Liposomes (hollow internal structure)

Liposomes are a type of lipid nanoparticle that is used as a delivery system for drugs. They are composed of phospholipids and cholesterol and are similar in structure and composition to biological membranes. Liposomes can be used to deliver both water-soluble and fat-soluble drugs and can be targeted to specific subcellular structures. The properties of liposomes, such as their lipid composition, size, polarity, and curvature, can be optimized to improve their targeting to specific structures, such as the endoplasmic reticulum (ER). Inhibitors of N-glycosylation, such as N-butyl deoxynojirimycin (NB-DNJ), can be delivered to the ER using liposomes to enhance their efficacy against viral infections. The post-translational modification of proteins, such as tyrosinase, can also be disrupted by the delivery of NB-DNJ to the ER using liposomes. Liposomes can also be used to deliver other types of drugs, such as anticancer agents, to the ER.

Mitochondria: the powerhouses

Mitochondria dysfunction and associated diseases

Mitochondria are organelles found in eukaryotic cells that produce ATP and regulate cellular apoptosis. They contain their own DNA, which is susceptible to damage by stress-induced oxygen free radicals, leading to mitochondrial dysfunction and various diseases. Mitochondria play a key role in the development of cancer, where they support the rapid proliferation of tumor cells through altered glucose, lipid, and amino acid metabolism and upregulation of anti-apoptotic proteins. Mitochondrial dysfunction is also involved in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease, where it leads to oxidative stress damage and protein aggregation. Mitochondrial dysfunction is also associated with cardiovascular diseases, diabetes, and ageing. Therapeutic agents that target mitochondria are being developed for use in cancer and neurodegenerative diseases.

Mechanisms of mitochondria targeting

There are several mechanisms for targeting drugs to mitochondria. One method is to use molecules with appropriate physicochemical properties, such as hydrophobicity, to selectively accumulate in the mitochondria. Small molecule compounds, including triphenyl phosphonium (TPP), rhodamine, 1,10-decamethylene bis-4-aminoquinaldinium chloride (DQA), and guanidinium and pyridinium cations, can also be used to target the mitochondria through their electrophoretic interaction with the negatively charged inner membrane. Targeting peptides, such as mitochondria-targeting sequences (MTS) and mitochondria-penetrating peptides (MPP), can also be used to deliver drugs to the mitochondria. Other methods include using nanoparticles, such as liposomes and polymersomes, or conjugating drugs with mitochondria-specific labels.

Lipid nanoparticles with mitochondria targeting ability and their applications

There are several methods for delivering drugs directly to the mitochondria, including using inorganic nanoparticles, albumin nanoparticles, dendrimer polymer-based nanoparticles, PLGA nanoparticles, polydopamine nanoparticles, micelles, and lipid-based nanoparticles. These carrier systems can specifically accumulate in the

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mitochondria of cells and have been used for various applications.

Lipid nanoparticles (solid internal structure)

Mitochondria are organelles within cells that generate adenosine triphosphate (ATP) to support the biochemical activities of cells and mediate signaling pathways that regulate endogenous cellular apoptosis. Dysfunction in mitochondria can lead to various diseases, including cancer and neurodegenerative diseases. There are several mechanisms for targeting mitochondria, including hydropathy, small molecule compounds, and modified drugs or carrier systems. Lipid nanoparticles, in particular, have been studied for their ability to target mitochondria for drug delivery, including for the purpose of overcoming multidrug resistance in cancer treatment. Modifications to lipid nanoparticles, such as the incorporation of docosahexaenoic acid or the use of cationic compounds, can enhance their targeting ability.

Liposomes (hollow internal structure)

Lipid nanoparticles, liposomes, and micelles are three types of nanoparticle-based systems that have been used for mitochondria-targeted drug delivery. Lipid nanoparticles can be modified with small molecules such as triphenyl phosphonium (TPP) or rhodamine to increase their targeting ability to the mitochondria. Liposomes can also be modified with small molecules such as TPP or rhodamine, or with peptides that mimic the targeting signals of mitochondria precursor proteins. Micelles can be formed from amphiphilic block copolymers and can be modified with small molecules such as TPP or with peptides to increase their targeting ability to the mitochondria. These nanoparticle-based systems have been used to deliver drugs such as doxorubicin, lonidamine, and paclitaxel specifically to the mitochondria of cancer cells to improve the therapeutic effects of the drugs while reducing their toxicity.

DQAsomes

Lipid nanoparticles, liposomes, and DQAsomes are all types of carriers that can be used to deliver drugs specifically to mitochondria in cells. Lipid nanoparticles can be modified with specific lipid compositions and surface charges to improve their targeting ability to mitochondria. Liposomes can be modified with small molecules such as TPP, DHA, and Rh123, or with peptides that mimic mitochondrial protein transmembrane signals to target mitochondria. DQAsomes are vesicle-like cationic liposomes that spontaneously form from DQA, a mitochondria-specific targeting ligand, and can be used to deliver plasmid DNA and drugs such as PTX and curcumin to mitochondria. DQAsomes can be modified with HA to improve their stability and targeting ability. These carriers have been used in the delivery of chemotherapy drugs and in the treatment of acute lung injury, and have shown potential in overcoming multidrug resistance in cancer therapy.

Current strategies and future directions

The endoplasmic reticulum (ER) and mitochondria are important membrane-bound organelles in eukaryotic cells that are closely connected and communicate with each other through the mitochondria-associated endoplasmic reticulum membranes (MAMs). MAMs can be targeted for the treatment of diseases, including tumors, neurodegenerative diseases, fatty liver, diabetes, and cardiovascular diseases. While current strategies for targeting the ER and mitochondria have shown promise, there is still a need for more precise, efficient, and safe methods. Further exploration of the mechanisms of cell physiology and the development of improved ER and mitochondria targeting tools may facilitate the diagnosis and treatment of associated diseases and disorders.

Digest of
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Shi Y., Luo Z., You J.;
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2022, 14 (5), e1803;

Branched-Tail Lipid Nanoparticles Potently Deliver mRNA In Vivo due to Enhanced Ionization at Endosomal pH

Hajj K.A., Ball R.L., Deluty S.B., *et al.*

Messenger RNA (mRNA) has many potential therapeutic applications, such as protein replacement, vaccination, cancer immunotherapy, gene editing, and cellular reprogramming. However, the clinical use of mRNA drugs has been limited by a lack of effective and safe delivery systems. mRNA delivery presents unique challenges compared to small interfering RNA (siRNA) delivery because mRNA is much larger, flexible, and single-stranded, and can elicit a stronger immune response. In addition, the effectiveness of a delivery system for mRNA may vary depending on the specific mRNA sequence being delivered. This study aims to investigate the influence of ionizable lipid tail chemistry on the efficacy of lipid nanoparticle (LNP) delivery systems for mRNA. The researchers are interested in how the molecular properties of the LNP, which may be influenced by factors such as lipid tail length and degree of unsaturation, contribute to delivery efficacy.

The researchers used 11 different lipidoids, which were all created from the same amine compound and had different lengths of alkyl acrylate tails. These lipidoids were formulated into lipid nanoparticles (LNPs) with cholesterol and other components, and the LNPs were used to deliver mRNA to mice. The researchers found that LNPs with branched alkyl acrylate tails were more effective at delivering mRNA and had stronger ionization at a pH of 5.0 compared to LNPs with linear tails. They also observed that the biodistribution (the movement and accumulation of the LNPs in the body) of the different LNPs was similar, with the LNPs accumulating in the spleen, kidneys, liver, and lungs.

The researchers then investigated the effect of lipidoid tail chemistry on mRNA delivery using nanoparticles. They used mRNA encoding the firefly luciferase protein as a model and found that lipidoid 306O10, with a carbon tail length of ten, was effective at delivering mRNA to the spleen and liver in mice. However, lipidoid 306Oi10, which has a nearly identical structure to 306O10, was ten times more potent and mostly delivered mRNA to the liver. The researchers also confirmed that the efficacy of these lipidoids was not limited to a specific mRNA cargo and that they compared favorably to other effective mRNA delivery vehicles described in the literature.

The authors then attempted to understand why Oi10 is so effective at delivering mRNA to cells in vivo (inside the body). They first found that there is a weak correlation between the nanoparticle's effectiveness in cell culture and in mice. They also found that size and entrapment of the nanoparticles were not good predictors of efficacy in vivo. The researchers then measured the pKa (a measure of the acidity) of the nanoparticles' surface and found that there was no correlation between pKa and efficacy in vivo. However, they did find that the nanoparticles' ability to become ionized at a pH of 5 (which is present in late endosomes/lysosomes) correlated strongly with efficacy in vivo. They confirmed this finding using a widely used lipid nanoparticle called C12-200.

The researchers then tested the surface ionization of LNPs from a synthesized library of 22 branched-tail at pH 5. They found that those made with the Oi10 tail had the highest surface ionization within the group. They also found that LNPs made with branched-tail lipidoids were more effective at delivering mRNA in vivo than those made with straight-tail lipidoids. The researchers theorized that the branch in the Oi10 tail increases the distance between the lipidoid molecules in the LNP bilayer, which may facilitate protonation of the amines and lead to higher surface ionization. They also found that differences in LNP surface ionization were not caused by differences in the ability of the individual lipidoid molecules to protonate, but by differences in the molecular packing of the LNP components. The authors conclude that further studies are needed to fully understand the role of molecular organization on LNP efficacy.

In summary, the authors report that a family of lipid materials was found to be effective for delivering mRNA to cells in the body. The LNP 306Oi10 was particularly effective and had an in vivo mRNA delivery efficacy that was comparable to the most effective materials previously studied. The potency of lipidoids containing the branched tail Oi10 was found to be related to strong surface ionization at the late endosomal pH of 5.0, which is thought to facilitate better membrane disruption and endosomal escape. The effectiveness of these branched-tail lipidoids in mRNA delivery makes them a promising material for use in various therapeutic applications.

Digest of
10.1002/smll.201805097; Hajj, K. A., Ball, R. L., Deluty, S. B., Singh, S. R., Strelkova, D., Knapp, C. M., Whitehead, K. A.; Small; 2019, 15 (6), e1805097;

Nanoscale Platforms for Messenger RNA Delivery

Li B., Zhang X., Dong Y.

mRNA is a single-stranded RNA that plays a crucial role in the expression of proteins in cells. Abnormal levels of mRNA can lead to various diseases and disorders. mRNA can be used as a therapeutic agent to treat various diseases and conditions because it has several advantages, including the ability to be delivered in a controlled manner and the ability to produce a transient protein expression. However, mRNA has historically faced challenges such as instability, poor cell penetration, and immunogenicity. In recent years, advances in nucleic acid chemistry and the decline in production costs of mRNA have led to its increased use as a therapeutic agent. mRNA-based therapeutics have also benefited from the development of new delivery methods. However, there are still challenges to overcome in the development of mRNA-based therapies, including the need for better delivery systems and the need to address immune responses to the mRNA.

The path from exogenous mRNA to functional proteins

Exogenous mRNA (genetic material that originates outside an organism) can be delivered to cells using nanoparticles, which protect the mRNA from degradation and facilitate its uptake and release into the cytosol (the fluid-filled space inside cells). The pharmacokinetics (how a drug is absorbed, distributed, metabolized, and eliminated by the body) and cellular trafficking of mRNA-encapsulating nanoparticles can vary based on the route of administration, the properties of the nanoparticles, and the type of cells. Researchers have investigated the use of lipid nanoparticles (LNPs) for mRNA delivery, and have found that LNPs can effectively deliver mRNA to cells and result in the production of functional proteins, although the process can be complex and may involve multiple pathways. Further research is needed to optimize the delivery of mRNA using nanoparticles, including identifying the most effective administration routes and improving endosome escape.

Nanoscale delivery platforms

Lipid and lipid-derived nanoparticles are a class of delivery systems that have been used to encapsulate mRNA since the late 1970s. These nanoparticles have been shown to effectively deliver mRNA into various types of cells, including human, mouse, and rat cells. Liposomes have also been used in clinical trials for the treatment of advanced melanoma. Cationic liposomes, which are formulated using molecules such as DOTMA and DOPE, have been particularly effective at delivering mRNA and other nucleic acids into cells. The size and surface charge of the liposomes can affect their ability to deliver mRNA to specific organs or tissues. Different storage conditions may also impact the effectiveness of lipid nanoparticles for mRNA delivery.

Polymer-based nanoparticles for mRNA delivery

Polymeric nanoparticles are a type of nanoscale platform that can be used to deliver mRNA (a type of genetic material). One example of a polymer that has been used for this purpose is polyethylenimine (PEI), which has a strong affinity for nucleic acids and can facilitate the escape of the nanoparticle from endosomes (small, membrane-bound structures within cells). PEI has been used to deliver small interfering RNA (siRNA) and to induce immune responses in mice. Other polymers that have been used for mRNA delivery include poly(glycoamidoamine) brushes, poly(beta-amino ester), and polyethylene glycol-polylactide (PEG-PLA). In addition to chemical conjugation, some researchers have used graphene oxide or jetPEI (a linear PEI derivative) to deliver mRNA. These polymeric nanoparticles have shown promise in various preclinical studies, although more research is needed to determine their safety and effectiveness in humans.

Protein derivatives mRNA complexes

Protamine is a protein that has been used in the development of mRNA-based vaccines. In 2000, it was shown to be effective in inducing an immune response in mice when used to condense mRNA encoding for beta-galactosidase. Protamine has also been used in clinical trials as a vaccine for melanoma and rabies, and has been coated with lipids and polymers to improve stability and tumor accumulation. Coat proteins derived from viruses have also been used to deliver mRNA, with prom-

ising results in animal models. These approaches show potential as a means of delivering mRNA for the purpose of inducing an immune response, but further clinical investigation is needed.

Other types of nanoparticles for mRNA delivery

Researchers have studied various types of nanoparticles for mRNA delivery, including gold nanoparticles, polymer-lipid hybrid nanoparticles, and peptide complexes. These nanoparticles have been found to be effective in delivering mRNA to cells and inducing protein expression, and have been tested in both in vitro and in vivo settings. Some approaches involve pre-assembling mRNA with translation components or incorporating poly(A) binding proteins into the nanoparticles. mRNA has also been self-assembled into nanoscale particles, which can be delivered to cells using transfection agents.

Future directions

mRNA-based therapies, which involve the use of mRNA to treat a variety of conditions including infectious diseases and cancers, have made significant progress in the past decade but still face a number of challenges. These challenges include a lack of understanding of mRNA's translation efficiency, half-life, and immunogenicity, as well as the need for new approaches to engineering mRNA sequences and structures. Additionally, mRNA purity and impurities produced during the in vitro transcription process must be carefully characterized and analyzed. A number of delivery platforms for mRNA are being applied in clinical trials, and the features of these platforms, such as targeting specific organs and cells, high delivery efficiency, biodegradability and biocompatibility, and appropriate duration of pharmacological effects, must be taken into account when designing new platforms. It is expected that the combination of optimal therapeutically relevant mRNA and proper delivery systems will lead to significant contributions to human health in the near future.

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Anionic Lipid Nanoparticles Preferentially Deliver mRNA to the Hepatic Reticuloendothelial System

Pattipeiluhu R., Arias-Alpizar G., Basha G., *et al.*

RNA therapy involves the delivery of RNA molecules to target cells in order to control gene expression. Lipid nanoparticles (LNPs) are commonly used for this purpose, but so far have mainly been effective at targeting hepatocytes (liver cells). Researchers are interested in expanding the use of LNPs to target other cell types, but have not yet fully understood the biological mechanisms underlying their transport and uptake in the body. One potential target for LNP-based therapies is liver sinusoidal endothelial cells (LSECs), which are specialized scavenger cells involved in maintaining blood homeostasis and clearing waste and pathogens from the body. LSECs express a variety of scavenger receptors on their surface, which could potentially be exploited for LNP targeting. However, LSECs also have a number of unique features, such as a fenestrated endothelial monolayer, a lack of tight junctions, and an inhomogeneous distribution of scavenger receptors, that may complicate LNP targeting. The authors discuss current efforts to understand and overcome these challenges in order to develop LNP-based therapies that can specifically target LSECs.

Study results

Design and characterization of anionic srLNPs

The authors have previously shown that anionic nanoparticles are efficiently cleared from circulation in zebrafish embryos by specialized scavenger cells (SECs) expressing stabilin-1 and -2 receptors. They have now developed anionic lipid nanoparticles (LNPs) with the goal of targeting these same receptors in liver sinusoidal endothelial cells (LSECs) in mammals. By replacing the zwitterionic helper phospholipid in their LNPs with an anionic analog, they were able to create an anionic surface charge on the LNPs, which they hypothesized would redirect LNP targeting and functional RNA delivery from hepatocytes to LSECs through the stabilin pathway, while simultaneously inhibiting LNP uptake by hepatocytes. They compared the ability of the anionic LNPs to target LSECs and hepatocytes in vitro and in vivo, and found that the anionic LNPs preferentially targeted LSECs over hepatocytes. Additionally, they found that the anionic LNPs were more effective at gene knockdown in LSECs than in hepatocytes. These results suggest that anionic LNPs may be a promising tool for targeted gene manipulation in LSECs.

Biodistribution of LNPs in embryonic zebrafish

In this study, two types of lipid nanoparticles (LNPs) containing a fluorescent probe and fluorescently tagged mRNA were injected into wild-type zebrafish embryos. One type of LNP (DSPC–LNPs) remained mostly in circulation, while the other type (srLNPs) accumulated within certain cells in the vasculature (blood vessels) of the embryos. This selective accumulation of srLNPs within certain cells was confirmed to be mediated by stabilin scavenger receptors. Injections of both types of LNPs into mutant zebrafish embryos without these receptors showed that srLNPs remained in circulation while DSPC–LNPs did not significantly change in distribution. Both the lipid and mRNA probes remained stable within the LNPs during circulation and during uptake by cells.

LNP-mediated mRNA delivery and expression in embryonic zebrafish

The researchers investigated the use of lipid nanoparticles (LNPs) for delivering functional mRNA to zebrafish embryos by initially attempting to use Cy5-labeled eGFP mRNA payloads. However, they observed low levels of mRNA expression. Therefore, they switched to unlabeled eGFP mRNA. This alteration did not significantly alter the structure, surface charge, or mRNA encapsulation efficiency of the LNPs. When the LNPs were injected into the zebrafish embryos at 1.5 hours post-injection (hpi), they were found to associate with certain cells within the vascular system of the embryo. These cells included sinusoidal endothelial cells (SECs) and blood-resident macrophages. At 24 hpi, intense eGFP fluorescence was observed specifically within SECs and macrophages, indicating successful delivery and expression of the mRNA.

To further test the specificity of LNP-mediated mRNA delivery, the researchers also used mutant zebrafish lacking certain receptors. They found that, in these mutants, the LNPs were taken up by macrophages but not SECs, suggesting that the uptake of LNPs by macrophages is not dependent on these receptors.

The researchers also investigated the ability of LNPs to deliver mRNA to functional hepatocytes. They found that the LNPs were taken up by these

cells, leading to mRNA expression and protein production. However, the LNPs were less efficient at delivering mRNA to hepatocytes compared to SECs and macrophages.

Overall, the results suggest that LNPs are effective at delivering functional mRNA to specific cells within the zebrafish embryo, and have potential for use in RNA-based therapies.

Hepatocyte targeting and mRNA expression in older zebrafish embryos

The liver of the zebrafish undergoes significant growth and development during the first few days of its life. This includes the formation of new blood vessels, the maturation of functional hepatocytes (liver cells), and the emergence of features necessary for the liver to process lipid nanoparticles. The zebrafish also has a range of proteins involved in lipid transport and metabolism that are similar to those found in humans. As a result, the zebrafish has been used as a model to study various aspects of endogenous lipid transport and metabolism, including the clearance and metabolism of lipid-based nanomaterials. In this study, researchers used zebrafish embryos to investigate the potential for using apolipoprotein E (apoE)-mediated pathways to target lipid-based nanomedicines to the liver. They administered apoE-targeted liposomes (tiny bubbles made of fats) to zebrafish embryos and found that they were taken up by the liver and associated with, but not yet taken up by, hepatocytes (liver cells). These results suggest that apoE-mediated mechanisms of targeting lipid nanoparticles to hepatocytes are present, functional, and exploitable in the zebrafish.

LNP-mediated mRNA delivery and expression in mice

The authors examined the distribution and mRNA expression patterns of lipid nanoparticles (LNPs) in mice, with a focus on the liver. LNPs were injected into mice and their distribution was tracked using a fluorescent probe. The LNPs were found to accumulate in the liver, with one type of LNP showing significantly enhanced uptake in all liver cell types. The LNPs were also found to be able to deliver functional mRNA to hepatic cells, with one type of LNP showing significantly enhanced mRNA delivery to hepatic cells compared to the other type. The results demonstrated the potential for LNPs to be used as a delivery system for mRNA therapies, particularly in the liver, and highlighted the importance of considering both LNP targeting and mRNA expression in the evaluation of such systems.

Discussion and future directions

This research has designed a new platform called LNP-mRNA that is capable of targeting the hepatic reticuloendothelial system (RES) in the liver to enhance mRNA expression within these cells. This approach allows for well-reasoned predictions of the platform's behavior in the body without the need for testing on animals. The platform is based on anionic LNP formulations, which have a measured surface charge of less than -15 mV and are between 20 and 100 nm in size, and it has been shown to be effective in targeting the hepatic RES through the stabilin-mediated recognition and uptake within sinusoidal endothelial cells (SECs). This platform has the potential to be used as a gene therapy for liver-specific and systemic diseases, including autoimmune diseases, in which the hepatic RES plays a central role. The platform can be optimized through the use of ionizable lipids and sterol components, chemically modified RNA, and miRNA suppression to improve target specificity. The research also suggests that the use of zebrafish embryos can be a valuable tool in the discovery and optimization of LNP formulations due to the ability to visualize the LNP in real-time and at cellular resolution.

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Lipid-Based Nucleic Acid Therapeutics with *In Vivo* Efficacy

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Introduction and overview of lipid-based nucleic acid therapeutics

Therapeutic interventions using nucleic acid (NA) delivery have the potential to treat various diseases, including cancer, infectious diseases, and inherited genetic disorders. However, the delivery of naked NA is often ineffective due to delivery barriers. To improve efficacy, various viral and non-viral delivery systems and physical delivery methods have been explored, including lipids, polymers, and lipid-based nanocarriers (LNCs). LNCs consist of various components, including ionizable lipids or lipid-like materials, phospholipids, cholesterol or cholesterol analogs, and PEGylated lipids, and may also include targeting amphiphiles. Lipid-based nanocarriers have shown promise in clinical trials, but their effectiveness can be influenced by the structural properties of the payload and component lipids, as well as formulation and manufacturing factors.

Main classes of therapeutic nucleic acids in use and their mechanisms of action

Therapeutic nucleic acids are a class of medications that target genetic material to alter gene expression or function. There are seven main types: plasmid DNAs, which provide functional copies of mutated or defective genes; antisense oligonucleotides, which bind to and alter mRNA; small interfering RNAs (siRNAs), which bind to and degrade mRNA; microRNAs (miRNAs), which regulate protein-coding genes through mRNA binding; small activating RNAs (saRNAs), which activate gene expression through mRNA binding; CRISPR-associated protein and clustered regularly interspaced short palindromic repeats (CRISPR/Cas), which can edit genomic DNA; and in vitro transcribed messenger RNAs (IVT mRNA), which provide functional copies of genes. These nucleic acids can be delivered through synthetic or viral vectors and may work through various mechanisms, including mRNA degradation, gene activation or repression, and genomic editing.

There are several challenges to delivering nucleic acids (NAs) to the body. After intravenous injection, unmodified NAs can interact with proteins in the blood, be degraded by enzymes, and accumulate in the liver. They can also trigger an immune response and be eliminated by the kidneys. Modification of the NAs can help to address some of these issues, but can also make them less effective. NAs must also be able to enter target cells and reach their intended location within the cell, which can be difficult due to their size and charge. One way to improve the delivery of NAs is to encapsulate them in nanoparticles called lipid nanocapsules (LNCs). However, LNCs can also be cleared from the body quickly and may not effectively reach their target cells due to their size and surface properties.

Components of lipid-based nanocarriers with nucleic acid payload and their structural properties

Payload: Chemical modification of nucleic acids to reduce toxicity, change their PK/PD properties, and improve delivery

Examples of nucleic acids that can be modified include plasmid DNA (pDNA), antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), miRNAs, saRNAs, and in vitro transcribed (IVT) mRNAs. Modification methods include altering the sugar backbone, phosphodiester bond, nucleobase, and/or ORF and poly A tail. Modifications can improve circulation half-life, reduce immunogenicity, and increase stability and translation efficiency. However, care must be taken to avoid off-target effects and unintended consequences.

Delivery system components – Cationic/ionizable lipids or lipid-like materials

LNCs (lipid-nucleic acid complexes) are composed of cationic or ionizable lipids and nucleic acids (NAs) and are used to deliver NAs to target cells. The cationic lipid component helps the complex enter the cell and escape endosomes, while the NA's charge ratio and co-lipids (such as phospholipids and cholesterol) can be adjusted to reduce non-specific binding and improve stability in circulation. The acidic environment inside the target cell causes ionizable lipids to become positively charged and release the NA payload into the cytosol. Surfactants, including simple surfactants, gemini surfactants, and lipophilic polycations, have been studied for use in nucleic acid delivery systems and compared for transfection efficiency. Lipidoids, a type of ionizable surfactant with dendrimer-like structures, have increased the

diversity of lipid delivery materials and have shown in vivo efficacy at smaller doses. LNCs using these materials, like Smarticles™ with pH-tunable properties, have potential for drug delivery and treating various diseases.

Co-lipids

Co-lipids, such as phospholipids, cholesterol and other sterols, PEG lipids, and targeting moieties, play a role in the formulation and targeting of lipid nanoparticles (LNPs) for in vivo applications. Phospholipids, such as DOPE and DSPC, can improve the stability and effectiveness of LNPs, but their unsaturation and presence in certain amounts is important for optimal performance. Cholesterol is found in the bilayer of conventional liposomes and in the outer monolayer and core of solid core LNPs (scLNPs), where it helps with stable encapsulation and particle stability. PEG lipids, which coat the surface of LNPs, can influence their size, shelf life, and pharmacokinetics. Targeting moieties, either endogenous or exogenous, can be used to direct scLNPs to specific cells in the body.

Formulation

There are several key factors that need to be taken into consideration when formulating LNCs (lipid-based nanoparticles) with nucleic acid payloads. These factors include the N/P ratio (nitrogen to phosphate ratio), the size of the payload, the molar ratio of the lipid components, the initial nucleic acid to lipid ratio, the pH of the formulation buffer, the ionic strength of the formulation buffer, and the dialysis buffer. These factors can influence the physical and chemical properties of the nanoparticles and their performance in vivo (in living organisms).

Nitrogen to phosphate ratio

The N/P ratio (the ratio of nitrogen to phosphorus atoms in a molecule) is important in the design of lipid nanoparticles (LNCs) used for gene silencing and transfection. A higher N/P ratio is associated with improved transfection efficiency and decreased toxicity, but can also result in larger particle size and increased surface charge. The N/P ratio can be adjusted by altering the lipid molar ratio, initial nucleic acid to lipid ratio, and flow rate ratio during mixing. The N/P ratio of the clinically approved drug Patisiran is 3, resulting in a particle size of about 50 nm and a close to neutral surface charge at physiological pH.

Payload size

Larger payloads, such as mRNA, mcDNA, or pDNA, may affect particle size, morphology, and encapsulation efficiency of scLNPs. These scLNPs with clinical efficiency are typically generated at a N/P ratio of 3-6 and exhibit an amorphous solid core coated with a lamellar structure. LNCs made with rapid mixing techniques to entrap mRNA, mcDNA, or pDNA at a N/P ratio of 1.5 resulted in a heterogeneous mixture of small liposomal structures and larger amorphous solid core particles. scLNPs with a N/P ratio of 4 and composed of DLin-KC2-DMA/DSPC/Chol/PEG-c-DMA (50/10/38.5/1.5 mol%) showed >80% encapsulation efficiency for siRNA, mRNA, and pDNA payloads.

Lipid composition

The composition of lipids in a SNALP (small, nucleic acid-lipid particle) can greatly impact its ability to effectively silence genes in the liver. A 20/25/45/10 mol% ratio of ionizable lipid, phospholipid, cholesterol, and PEG-lipid, respectively, was found to be a poor hepatic gene silencing agent, but increasing the ionizable lipid to 40 mol% improved the activity to a level that could be clinically translated. A further 6-fold improvement in potency was achieved with a 50/10/38.5/1.5 mol% ratio. The lipid composition can also affect payload entrapment, size, and morphology of the particles. Increasing the ratio of one lipid at the expense of others can alter the morphology of the particles.

Initial nucleic acid to lipid ratio

The encapsulation efficiency of a lipid composition, used to encapsulate a nucleic acid payload, can vary based on the formulation method used. Using the preformed vesicle (PFV) method, a lipid composition of DLinDMA/DSPC/Cholesterol/PEG-S-DMG (40:10:40:10 mol%) was able to achieve a maximum final encapsulation ratio of 0.05 (wt/wt) corresponding to 80% encapsulation with an initial nucleic acid to lipid ratio of 0.061 (wt/wt). However, using microfluidic mixing techniques, a different lipid-based nucleic acid therapeutic was able to achieve a final encapsulation ratio of 0.08 (wt/wt), corresponding to over 95% encapsulation. The initial nucleic acid to lipid ratio can be controlled by adjusting the concentrations of the

total lipid and nucleic acid stock solutions and the flow rate ratio during mixing.

Buffer solutions

The pH of the formulation buffer is important when using ionizable lipids or lipidoids to encapsulate nucleic acids (NAs), as it affects the charge on the lipids and therefore their interactions with the NA. Efficient encapsulation is usually achieved at pH values lower than the pKa of the ionizable lipids, with pH 3 or 4 being optimal for optimized ionizable lipids. Buffers containing metal ions such as sodium can lead to larger, more heterogeneous particles, while buffers without metal ions such as HEPES are less likely to produce these particles. After the initial particle formation, the suspension is often dialyzed against various buffers to remove residual ethanol and adjust the pH, with the final structure of the particles being formed at this step. Particle size can increase during dialysis, and high ionic strength of the formulation buffer can negatively impact particle formation by reducing the charge on the cationic or ionizable lipids.

Manufacturing and manufacturing aspects

Manufacturing

Cationic lipoplexes are particles that are used for the delivery of nucleic acids (NAs) into cells. They are typically made by mixing preformed liposomes with NAs. Liposomes can be made using a variety of techniques, such as thin film hydration followed by size reduction, extrusion, sonication, microfluidization, or the ethanol injection method. These methods can produce particles in the size range of 200-300 nm with an encapsulation efficiency of around 50%. However, they have several drawbacks, including poor control over the physicochemical properties of the particles, lack of scalability, and reproducibility issues. In addition, these methods can produce particles that are toxic to cells. Therefore, there is a need to develop new methods for the production of cationic lipoplexes that address these issues.

Manufacturing/mixing aspects in rapid mixing techniques

Rapid mixing techniques such as microfluidic hetero-coacervation (MHF) and spiral hemostasis mixer (SHM) involve several manufacturing and mixing factors that can impact the size and size distribution of particles produced. These factors include flow rate, flow rate ratio, chip geometry, and mixing time. In MHF, higher flow rates and flow rate ratios lead to smaller particles with a narrower size distribution, while lower flow rates and flow rate ratios lead to larger particles with a wider size distribution. Chip geometry can also affect particle size, with smaller channel diameters and specific microfluidic chip designs resulting in smaller particles. Mixing time can impact particle size and size distribution, with higher Reynolds numbers increasing mixing times but potentially reducing mass-transfer and leading to particle aggregation. In SHM, higher flow rate ratios lead to smaller "limit-size" particles based on the lipid constituents, and chip geometry can decrease particle size through increased chaotic advection. However, commercial instruments like the NanoAssemblr have predetermined chip geometry.

Preclinical applications

Lipid nanoparticles have been used to deliver therapeutic agents such as small interfering RNA or mRNA to treat various liver diseases and cancer, improve chloride efflux in cystic fibrosis, reduce HBV DNA in a mouse model of infectious disease, and provide immune protection against viral challenges in mice, ferrets, and non-human primates. mRNA vaccines have also shown promising results in clinical studies for COVID-19 and are in a phase I trial for another infectious disease.

Clinical status of lipid-based nucleic acid therapeutics

Lipid-based nucleic acid therapeutics are a type of treatment that involves the use of lipids (fats) to deliver therapeutic DNA, RNA, or oligonucleotides (small molecules) to specific parts of the body. These therapies have been studied in various diseases, including lung diseases, cancer, and infectious diseases. Lipid-based vectors such as lipoplexes and liposomes have been tested as delivery vehicles for DNA gene therapies and RNA therapies such as small interfering RNA (siRNA) and antisense oligonucleotides (ASOs). Some examples of lipid-based nucleic acid therapeutics that have advanced to clinical trials include DC-Chol-based lipoplexes and DNAbilize technology for cancer,

Onpattro (patisiran) and GalNacsiRNA conjugate (Vitusiran) for TTR amyloidosis, and Alnylam's Inclisiran, a GalNac-siRNA conjugate targeting PCSK9 for cholesterol reduction. The effectiveness of these therapies is still being evaluated.

Conclusions and future perspectives

The use of lipid-based nucleic acid (NA) therapeutics, including small interfering RNA (siRNA) and messenger RNA (mRNA) vaccines, has advanced significantly in recent years through research into formulation parameters and manufacturing techniques. This has led to improved stability and reduced toxicity of the therapies, as well as the ability to target specific organs with the use of specialized lipids. However, extrahepatic targeting of these therapies following systemic delivery remains a challenge, and further research is needed to better understand the properties of these nanosystems and improve their efficacy. Despite these challenges, lipid-based NA therapeutics have already had several successful products in the market and show promise for future development.

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Rational Design of Anti-Inflammatory Lipid Nanoparticles for mRNA Delivery

Zhang H., Han X., Alameh M.G., *et al.*

Ionizable lipid nanoparticles (LNPs) are a type of non-viral delivery platform used to deliver RNA therapeutics, including the COVID-19 mRNA vaccines. They are composed of ionizable lipids, cholesterol, polyethylene glycol (PEG)-conjugated lipids, and phospholipids. LNPs can protect and deliver mRNA therapeutics to target cells and tissues, but they can also trigger immune responses and reduce the translation efficiency of mRNA. To improve the safety and effectiveness of LNPs, researchers are developing LNPs that include anti-inflammatory agents such as corticosteroids. In this study, the researchers explored the use of a specific corticosteroid called dexamethasone (Dex) in LNPs to reduce pro-inflammatory cytokines and improve the tolerability and effectiveness of LNPs for protein replacement and gene editing therapies. They found that LNPs incorporating Dex effectively reduced the production of pro-inflammatory cytokines and increased protein expression in cells.

The purpose of this study was to investigate the use of lipid nanoparticles (LNPs) as a delivery system for mRNA in vitro. The LNPs were synthesized by mixing an ethanol phase containing certain lipids and an aqueous phase containing the mRNA. The LNPs were then filtered and their polydispersity index and average diameter were measured. The mRNA concentration and encapsulation efficiency in the LNPs were also measured. The LNPs were then used to treat human HepG2 cells and murine RAW264.7 cells at various doses for 24 hours, after which the cells were tested for luciferase expression and viability. The results showed that the LNPs were able to efficiently deliver mRNA to the cells and that the C:D ratio of cholesterol to dexamethasone in the LNPs influenced the transfection efficiency and cytotoxicity in the cells.

Lipid nanoparticles (LNPs) were created by mixing an aqueous phase containing mRNA and an organic phase containing several components in a microfluidic device. The LNPs were tested for transfection efficiency and cytotoxicity in HepG2 cells, and the anti-inflammatory effects were evaluated on murine macrophages. In mice, the LNPs were found to induce an inflammatory response, but the incorporation of dexamethasone into the LNPs reduced this response. In vivo transfection of the LNPs encoding for luciferase in the liver was successful and the LNPs were found to be effective at reducing inflammation in a mouse model of acute lung injury. The LNPs with dexamethasone showed potential as a non-viral vector for mRNA delivery, particularly for anti-inflammatory purposes.

This study investigated the use of dexamethasone-incorporating lipoprotein nanoparticles (C9D1 LNPs) as a potential anti-inflammatory therapy. The C9D1 LNPs were found to significantly reduce levels of the pro-inflammatory cytokine TNF-alpha in vitro and in vivo compared to non-modified lipoprotein nanoparticles (C10D0 LNPs). In addition, the C9D1 LNPs improved mRNA transfection by 1.5-fold in mice, suggesting that the incorporation of dexamethasone into LNPs may be a promising strategy for reducing inflammation and enhancing the therapeutic effects of mRNA-based therapies.

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