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Review

Bioinspired Lipid Nanocarriers for RNA Delivery

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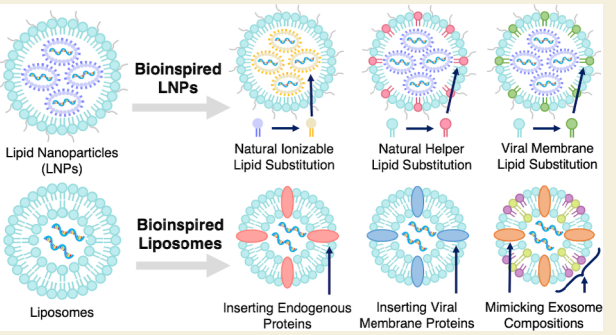
ABSTRACT: RNA therapy is a disruptive technology comprising a rapidly expanding category of drugs. Further translation of RNA therapies to the clinic will improve the treatment of many diseases and help enable personalized medicine. However, in vivo delivery of RNA remains challenging due to the lack of appropriate delivery tools. Current state-of-the-art carriers such as ionizable lipid nanoparticles still face significant challenges, including frequent localization to clearance-associated organs and limited (1−2%) endosomal escape. Thus, delivery vehicles must be improved to further unlock the full potential of RNA therapeutics. An emerging strategy is to modify existing or new lipid nanocarriers by incorporating bioinspired design principles. This method generally aims to improve tissue targeting, cellular uptake, and endosomal escape, addressing some of the critical issues facing the field. In this review, we introduce the different strategies for creating bioinspired lipid-based RNA carriers and discuss the potential implications of each strategy based on reported findings. These strategies include incorporating naturally derived lipids into existing nanocarriers and mimicking bioderived molecules, viruses, and exosomes. We evaluate each strategy based on the critical factors required for delivery vehicles to succeed. Finally, we point to areas of research that should be furthered to enable the more successful rational design of lipid nanocarriers for RNA delivery.



KEYWORDS: *lipid nanoparticles, liposomes, RNA delivery, bioinspired, natural lipids, virus-inspired, exosome-inspired*

1.0. INTRODUCTION

RNA therapeutics leverage RNA-based molecules to regulate gene expression or produce therapeutic proteins to treat or prevent various diseases, including cancer, infectious diseases, immune diseases, and genetic diseases.[1,2] This field has gained more popularity in recent years due to the rapid production and success of the coronavirus 2019 (COVID-19) mRNA-based vaccines;[2−4] however, research has been focused in this area for decades, with several other clinically approved RNA therapies currently on the market.[2,5−8] With advantages including customizability,[2] ease of production,[2,9] and safety,[10] RNA therapeutics have the potential to address several unmet medical needs in the treatment of numerous diseases.

For RNA therapies to function therapeutically, they must reach the cytosol of target cells. This can be challenging as RNA is a large, negatively charged, hydrophilic molecule that cannot pass through the cell membrane independently.[11] Furthermore, RNA lacks stability and is highly susceptible to degradation via exonucleases found within organisms.[11] Hence, appropriate nonviral nanoparticles are required to stabilize, protect, and deliver RNA to the cytosol, among which ionizable lipid nanoparticles (LNPs) are the most clinically viable.[1] The classical formulation of LNPs consists of four components: ionizable lipids, cholesterol, helper lipids, and PEGylated lipids.[12] In 2018, LNPs enabled the first FDA approval of a small-interfering RNA (siRNA) drug called Onpattro;[2] two years later, two COVID-19 vaccines (Comirnaty, Spikevax)[2−4,13] based on LNPs containing mRNA also arrived at the clinic, helping prevent severe infection and saving lives during the COVID-19 pandemic.[14,15] Notably, the success of LNPs highly depends on the ionizable lipid component: once LNPs are internalized by cells via endocytosis, acidification of the endosomal compartment leads to protonation of the ionizable lipid.[14,15] This destabilizes the LNP and endosomal membrane, ultimately enabling the RNA payload to escape from the endosome and enter the cytosol.[14,15] Although current lipid-based nanocarriers have seen some success in translating RNA from bench to bedside, there is still a vast area for improvement in extra-hepatic organ targeting,[16] overcoming biological barriers,[17] and preventing unwanted immune stimulation.[17] To maximize the safety and efficacy of RNA therapy, new RNA delivery systems are highly desired.

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Bioinspired lipid nanocarriers, which combine biological elements with traditional lipid nanocarriers to mimic processes exhibited by biological systems,[18] present a promising opportunity to enable efficient organ-specific RNA delivery while reducing potential toxicity.[16] In this review, we outline and discuss state-of-the-art research on developing bioinspired lipid-based nanocarriers for applications of RNA therapeutics. Nanocarriers inspired by natural materials, endogenous molecules, viruses, and exosomes are specifically discussed.

2.0. INTRODUCTION TO RNA THERAPEUTICS

RNA is a biomolecule involved in the flow of genetic information within an organism. In cells, DNA acts as a template that is transcribed into RNA.[19] A portion of this RNA falls under the category of coding RNA, which is translated into proteins essential for survival.[19,20] In addition, some noncoding RNAs (ncRNAs) are never translated into a protein.[19] The majority of ncRNAs function as regulatory molecules, controlling elements of gene expression and protein function.[20−22] Several genetic and common chronic diseases are linked to mutations in the noncoding and regulatory genomic regions of genes, leading to the dysregulation of noncoding or coding RNAs that are critical for protein synthesis and function.[20] For example, microRNA (miRNA) deregulation is associated with every cancer that has been studied and plays a significant role in metastasis.[22−24] Therefore, the ability to deliver synthetic RNA to cells is a promising therapeutic option to treat and prevent numerous diseases, including certain cancers, infectious diseases, immune diseases, and Mendelian disorders.[1]

RNA therapy involves treating or preventing diseases using RNA-based molecules that can manipulate gene expression or produce therapeutic proteins.[1,2] Compared to traditional treatment methods, RNA therapies offer several advantages. Most conventional treatment strategies rely on small molecules that inhibit or alter protein function by binding to their active sites.[9] A caveat of this method is that only 22% of these proteins can be targeted with small molecule drugs, which limits the types of diseases that can be treated and creates "undruggable targets".[9,25] RNA therapeutics present a solution to this problem as RNA sequences can be designed to target almost any genetic component within a cell.[2] Another critical advantage of RNA therapy is its fast and cost-effective production compared to small molecules or recombinant proteins.[2,9] Once an RNA chemical structure and delivery method are established, RNA sequences can be designed and altered for different applications. This was recently exemplified through the rapid development of mRNA-based vaccines in response to the COVID-19 pandemic.[2−4] The ability to adjust RNA sequences for different targets also presents opportunities for developing vaccines that adapt to evolving pathogens and personalized treatments for rare diseases.[9] RNA is also advantageous for its safety; most notably, RNA is noninfectious, does not integrate into the genome, and is degraded by normal cellular processes after performing its therapeutic task.[10] Despite the several advantages of RNA therapeutics, significant challenges include effectively delivering the RNA to the cytoplasm of target cells and protecting it from exonuclease-mediated degradation.[11] Both obstacles can be addressed by encapsulating RNA in a biocompatible nanocarrier.[1]

2.1. Types of RNA Therapeutics

The applications of RNA therapies depend on if the therapeutic RNA is noncoding or protein-coding. ncRNA therapies regulate gene expression and encompass a broad category of RNAs, including short antisense oligonucleotides, long ncRNA, circular RNA, tRNA, siRNA, miRNA, and several others. The different types of therapeutic noncoding RNA have been extensively reviewed.[22,26] Among ncRNAs, siRNA and miRNA have been widely studied for their use in therapeutics, with several siRNA therapies being approved by the US Food and Drug Administration (FDA) and miRNA therapies in clinical development.[22] Coding RNA therapies often deliver mRNA to the cytoplasm to produce proteins.[9] The applications of protein-coding RNA therapies are highly diverse, including protein replacement therapies, vaccines for infectious diseases, cancer immunotherapy, and gene editing.[9] The following section provides a background on siRNA, miRNA, and mRNA, which are three therapeutically relevant RNA payloads.

2.1.1. Small Interfering RNA (siRNA). siRNA is a short, synthetic, double-stranded RNA (dsRNA) duplex that inhibits gene expression by promoting mRNA degradation.[2,27−29] Traditionally, the siRNA duplex contains two 21−23 nucleotide strands known as the guide and passenger strand, each with a two-nucleotide overhang at the 3′-end.[2,27,28] siRNA therapeutics leverage the endogenous RNA interference (RNAi) pathway to regulate the expression of target mRNAs, which involves interactions with RNA-induced silencing complexes (RISCs) that contain the argonaute 2 (Ago2) protein.[2,27] Once siRNA is delivered to the cytoplasm of cells, it is incorporated into a RISC that retains the siRNA guide strand and discards the complementary passenger strand.[2,27] The guide strand then directs the RISC to the target mRNA via complementary base pairing.[30] After the RISC identifies the target mRNA, the Ago2 protein cleaves the target and induces mRNA degradation.[1,27,29] Through this mechanism, siRNA triggers efficient and specific gene silencing for an individual target.[29] Currently, there are four siRNA-based therapeutics approved by the FDA: Patisiran, Givosiran, Lumasiran, and Inclisiran.

In 2018, Patisiran, designed for treating hereditary transthyretin-mediated (hATTR) amyloidosis, became the first FDA-approved siRNA-based drug.[2] hATTR amyloidosis is caused by mutations in the gene encoding for transthyretin (TTR), which results in a misfolded protein that causes amyloid deposits in the peripheral nerves, heart, kidney, and gastrointestinal tract.[5] Patisiran targets TTR mRNA to inhibit its production and reduce the accumulation of amyloid deposits.[5] Patisiran is formulated in an LNP optimized to ensure delivery to the liver,[31] which is the primary location of circulating TTR.[5] Givosiran is also designed to target the liver but uses siRNA conjugated to a trivalent N-acetylgalactosamine (GalNac) ligand instead of a nanocarrier.[6] This therapy is used to treat hepatic porphyria: a rare disease caused by high plasma levels of aminolevulinic acid (ALA) and porphobilinogen (PBG).[2,6] Next, Lumasiran treats primary hyperoxaluria type 1 (PH1), a rare genetic disease linked to kidney problems due to the overproduction of oxalate in the liver.[7] Lastly, in 2021, Inclisiran was approved by the FDA to treat primary hypercholesterolemia.[2] It lowers cholesterol levels in the bloodstream by targeting mRNA encoding for proprotein convertase subtilisin/Kexin type 9 (PCSK9).[8] Similar to givosiran, this drug uses a GalNac conjugate to target hepatocytes.[8] Based on the existing FDA-approved siRNA therapeutics, the field is limited to therapeutics that target liver diseases. For siRNA therapeutics to become more diverse in their applications, a key challenge is to develop novel nanocarriers that can target different organs in the body.

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Designing new, bioinspired nanocarriers may be a solution to this problem.

2.1.2. MicroRNA (miRNA). miRNA is similar to siRNA in structure, often consisting of a 19−25 nucleotide RNA duplex with two-nucleotide overhangs on the 3′ end.[28] Like siRNA, miRNA also interacts with RISC to cleave a target mRNA through RNAi.[28] However, miRNA differs in function by regulating the expression of several mRNA targets by blocking translation or promoting degradation.[9] The mechanism of target recognition for miRNA is more complex than siRNA, which binds by being entirely complementary to its target. In fact, miRNA relies on imperfect base pairing, meaning it only needs to be partially complementary to its target mRNA.[28] This distinction in target recognition enables miRNA to regulate several mRNAs. miRNA therapeutics are often classified as miRNA mimics (dsRNA molecules that mimic endogenous miRNAs) and miRNA inhibitors (single-stranded RNA (ssRNA) oligos that interfere with miRNA).[9,22] Currently, no miRNA therapeutics are approved by the FDA; however, several companies are working on creating miRNA therapeutics, and many candidates are in clinical trials.[9,32]

Detailed overviews of miRNA-based therapeutics currently in clinical trials have previously been established.[9,32] The following highlights some examples. First, miRagen Therapeutics is developing multiple miRNA therapeutics with applications including the treatment of blood cancers, keloids, and pathologic fibrosis, as well as strategies to accelerate tissue repair.[9] For example, MRG-229 mimics a miRNA called miR-29 to treat pathologic fibrosis.[9,33] miR-29 plays a role in the pathogenesis of pulmonary fibrosis and is significantly reduced in fibrotic lungs, making it a candidate for treating this disease.[34] In addition, InteRNA Technologies has developed a mimic for tumor suppressor miRNA called INT-1B3, which regulates the immunosuppressive tumor microenvironment for treating solid cancers.[9] INT-1B3 is based on miR-193a-3p: a miRNA associated with cancer, metastasis, and therapy resistance when downregulated.[35] Furthermore, SantarisPharma and Regulus Therapeutics are developing antiviral therapies for the Hepatitis C virus (HCV) known as Miravirsen and Rg-101, respectively.[32] Both treatments are inhibitors for miR-122, as this miRNA is essential for HCV replication.[32] Despite the vast number of companies working toward developing miRNA therapeutics, none have been approved for clinical use. A key reason for this is miRNA's ability to regulate multiple genes.[36] This creates undesirable off-target effects, which can lead to toxicity or unwanted adverse effects.[36] While this problem is challenging to eliminate, establishing drug delivery systems that enable miRNA to target specific organs will be critical to improving the safety and clinical translation of these therapeutics.

2.1.3. Messenger RNA (mRNA). mRNA is an ssRNA molecule transcribed from DNA and eventually translated into a functional protein.[37] Therapeutic mRNA is synthesized through in vitro transcription and contains key functional regions: the 5′ cap, the 3′ poly(A) tail, the open reading frame (ORF), and the untranslated regions (UTRs).[2,37] The 5′ cap is critical for ribosomes to recognize the mRNA sequence for protein synthesis, while the 3′ poly(A) tail has vital roles in interactions with translation initiation factors.[37] The ORF encodes the protein of interest, beginning with a start codon and ending with a stop codon.[38] The UTRs surround the ORF and do not encode proteins but are essential to regulate mRNA's translation efficiency and stability.[38] Initially, one of the major concerns of using mRNA in therapeutics was its high immunogenicity, as its longer length makes it more likely to induce immune responses when delivered for therapeutic purposes; however, this can be mitigated by modifying mRNA with pseudouridine.[2,37,39] Another challenge is mRNA stability, as it is susceptible to degradation by endogenous exonucleases, but this can be mitigated by encapsulating the mRNA in a nanocarrier.

mRNA therapeutics can be separated into two primary categories. First, mRNA can be used to develop protein-replacement therapies, where the mRNA is used to replace or supplement endogenous proteins.[2,37] The application of mRNA as a protein-replacement strategy has been explored for several diseases, including (but not limited to) cardiac, lung, hematologic, metabolic, orthopedic, and neurogenic diseases, as well as cancer.[37] Most mRNA protein replacement therapies are in preclinical development, with only drugs encoding for a vascular endothelial growth factor (VEGF) and cystic fibrosis transmembrane conductance regulator (CFTR) reaching clinical trials.[37] For example, the clinical trial involving VEGF mRNA (sponsored by AstraZeneca) is based on the drug AZD8601. This drug focuses on enhancing angiogenesis in patients with coronary artery diseases undergoing surgical revascularization.[40] AZD8601 contains naked mRNA and is injected directly into the epicardium in patients undergoing coronary artery bypass grafting surgery.[41] Second, mRNA therapeutics are used in developing prophylactic vaccines for infectious diseases and therapeutic vaccines to fight cancer.[9] In this application, mRNA encodes for antigens and adjuvants, enabling the immune system to protect against or oppose a particular disease.[9] For example, in response to the COVID-19 pandemic, Moderna and Pfizer/BioNTech developed mRNA-based vaccines protecting against disease infection.[2−4] Both formulations adopt LNPs encapsulating the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein.[9] While protein replacement therapy and vaccination are the primary applications of mRNA therapeutics, several other areas are being researched, including cell therapy and gene editing.[9]

Despite the diverse applications of mRNA therapeutics, few drugs are approved for clinical use. As with siRNA and miRNA, significant limitations arise based on the ability to successfully deliver the payload to the correct target cell while protecting the payload and facilitating endosomal escape in the cytoplasm of cells. Next-generation nanocarriers, including bioinspired nanoparticles (NPs), may be critical in accelerating the development of RNA therapeutics to overcome delivery challenges.

3.0. DELIVERY CHALLENGES

For RNA to have a therapeutic benefit when delivered, it must reach the cytosol of target cells to regulate gene expression or produce a functional protein. This is challenging without a delivery vehicle as naked RNA is a large, negatively charged, and hydrophilic macromolecule.[11] This results in electrostatic repulsion between RNA and the anionic cell membrane and prevents diffusion into the cell.[42] Accordingly, the cellular uptake rate of naked mRNA is less than 1 in 10,000 molecules.[43] The existence of ribonucleases (RNases) is another reason administering naked RNA is an inefficient therapy, as these enzymes rapidly degrade RNA and are abundant in blood and tissues.[11]

Due to these challenges, NPs are commonly used as delivery vehicles to stabilize, protect, and deliver RNA into the cytosol. However, even after encapsulating RNA in an NP, many internal barriers work to prevent successful delivery. When intravenously

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(IV) injected or absorbed into the bloodstream, NPs must evade phagocytosis by immune cells and binding to serum proteins.[42,44] Renal clearance by glomerular filtration must also be avoided to prolong the circulation of the NPs.[45] To reach the target tissue, delivery vehicles must cross vascular endothelial cells and diffuse through the dense ECM to the desired cells. The successful traverse of the vascular endothelium by NPs depends on their size and the organ-specific continuity of the endothelium.[46,47] A continuous endothelium is characterized by endothelial cells coupled by tight junctions and anchored to a continuous basal membrane; this is mainly found in the brain, skin, lungs, heart, and muscles. The presence of transcellular pores or a poorly structured basal membrane results in endothelium discontinuity. Some organs, such as the liver, are easier to penetrate due to their more discontinued endothelium, characterized by the presence of 50−200-nm-sized pores.[47−50] Commonly adsorbed proteins also preferentially ferry nanocarriers to the liver.[51] Upon successfully reaching target cells, NPs enter primarily through endocytosis and must escape the endosome before lysosome formation and subsequent enzymatic degradation can occur.[52,53] Endosomal escape may be the most significant bottleneck in the delivery of therapeutics to the cytosol, as it was previously reported that even the most advanced delivery vehicles only escape the endosome 1−2% of the time.[54]

To unlock the full potential of RNA therapeutics, delivery vehicles should be able to deposit RNA in various target organs with minimal off-target effects. RNA therapeutics can potentially treat many diseases across various organs, including the brain, heart, and eyes.[9] However, NPs can only significantly access specific organs when administered systemically. The parenchyma of organs/tissues with discontinuous capillaries, such as the liver, spleen, and bone marrow, is readily accessed by NPs. NPs can also infiltrate inflamed tissues such as solid tumors; however, organs/tissues with continuous capillaries effectively resist penetration.[55] Additionally, the lungs and lymph nodes have been shown to contain moderate quantities of NPs following systemic administration depending on the NP chemistry.[56,57] Notably, inhalation and subcutaneous routes of administration can boost NP levels in the lungs and lymph nodes, respectively, compared to systemic routes.[58−61] Overall, there exists a need for NPs to access organs with continuous capillaries and target specific cells for cancer treatment, gene editing, immunotherapy, or protein replacement therapy.

Based on the mechanisms and delivery barriers of RNA therapy, a nanocarrier should possess key traits to be considered a successful delivery vehicle. First, the carrier should efficiently encapsulate RNA during synthesis to yield a high loading level and minimize material waste, ultimately increasing therapeutic efficacy and lowering cost.[62,63] Nontoxicity, biocompatibility, and biodegradability are essential for nanocarriers, as they can minimize adverse reactions in the human body and increase tolerability to chronic dosing. Moreover, upon serving its therapeutic purpose, a carrier should have a safe and nontoxic degradation and clearance pathway in the body. Additionally, compatible immunogenicity is a crucial property for RNA delivery vehicles, and immunogenicity levels should vary based on the application. High immunogenicity inducing dangerous inflammatory responses should be avoided entirely, while some immunogenicity may be desired for applications such as mRNA vaccines to stimulate proper immune memory.[64] Furthermore, a nanocarrier should be stable and avoid premature degradation in the bloodstream. Two vital functional requirements are high cell transfection efficiency and selective targeting of the desired organ/tissue with minimal off-target effects. A high transfection efficiency indicates the ability to escape the endosome and deliver RNA into the cytosol, one of the significant challenges of RNA delivery. Finally, focusing on translatability, an RNA carrier should have a scalable and economically feasible synthesis process that can be scaled up for clinical trials and commercial use.

4.0. OVERVIEW OF CONVENTIONAL LIPID NANOCARRIERS FOR RNA DELIVERY

There are many nanocarriers for RNA delivery that range in technological maturity. These carriers include polymeric,[1,65,66] lipid-polymeric,[1,67] inorganic,[66] exosome,[68−70] and cell-based[70] systems, which have been reviewed elsewhere. However, lipid-based materials have achieved the most success in the clinic, with the most popular conventional lipid nanocarriers for RNA delivery being liposomes and LNPs. Both consist of an assortment of several lipids arranged into a membrane-core structure.[71,72] Liposomes are formed from the self-assembly of amphiphilic phospholipids into biocompatible bilayer vesicles.[73] Cholesterol and PEG-lipids can be added to the bilayer to increase rigidity and prolong circulation in vivo.[74] Although they can range from 20 to 1000 nm in diameter, liposomes smaller than 100 nm are typically most successful at RNA delivery due to their ability to escape phagocytosis.[72,75] Considered the original lipid-based carrier, liposomes are used in several FDA-approved commercial therapies such as liposomal doxorubicin, Vyxeos, and Lipoplatin.[76] However, no FDA-approved therapies utilize liposomes to deliver RNA.[71,76−78] The critical advantages of liposomes are that they can protect drugs from the external environment, reach target tissues, and release their cargo in a controlled manner.[71] The nanocarriers can carry hydrophobic and hydrophilic therapeutics in their lipid membrane and aqueous core.[72] Long RNA payloads also have ample space inside the large core of liposomes, where they are protected from the external environment until delivery into cells.

In addition to their biocompatibility and efficient encapsulation of RNA, liposomes are easily prepared and modified.[74] Liposomal synthesis techniques include thin-film hydration, solvent injection, and reverse phase evaporation, with emerging methods including freeze-drying and microfluidics.[79] These techniques aim to achieve a narrow polydispersity index (PDI) and effective drug encapsulation.[66] A major drawback specific to liposomes is that organic solvents are frequently used for their synthesis, which can present barriers when scaling up production.[69,80]

LNPs are currently the most effective lipid carriers for mRNA delivery and consist of four major components: ionizable lipids, helper lipids, cholesterol, and PEG-lipids.[12] The ionizable lipids are typically amino lipids that are neutral at pH 7 but become positively charged in the acidic endosomal environment (∼ pH 5−6.5), which helps to destabilize the endosomal membrane and facilitate endosomal escape.[14,15] The positively charged lipids bind to the negatively charged lipids on the endosomal membrane, disrupting the endosomal membrane structure to instead form a nonbilayer, hexagonal (H_{II}) arrangement. This H_{II} structure is believed to promote membrane fusion, endosomal escape of the LNP, and release of the RNA into the cytosol.[14] Based on their geometry, structural helper lipids generally improve the endosomal escape (by favoring the H_{II} phase) or bilayer stability of LNPs.[81] Cholesterol and PEG lipids serve a similar function as they do in liposomes.[81] The four lipid

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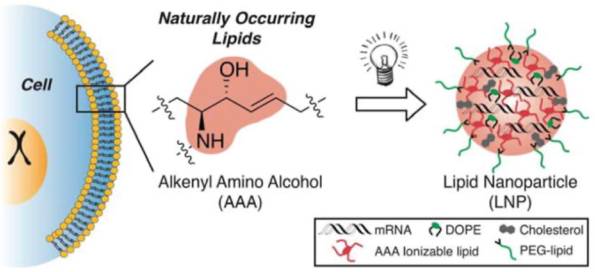
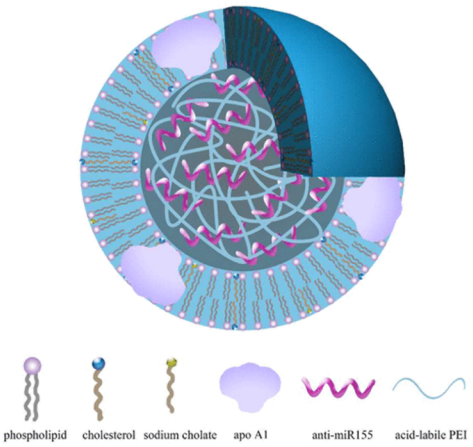
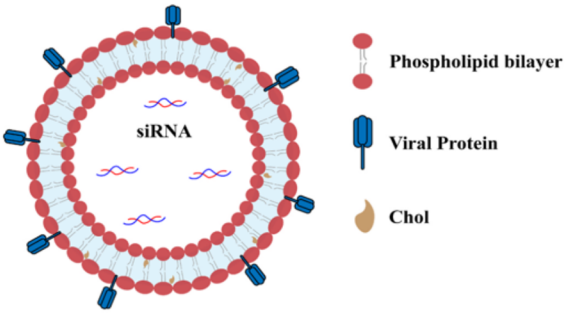
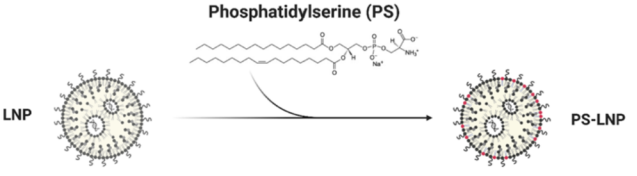
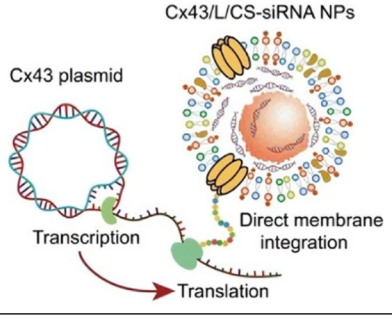
Table 1. Overview of Bioinspired Design Strategies for Lipid-Based RNA Carriers[109−112]

Design strategy (with description)	Example schematic
Natural lipid substitution (e.g., substituting traditional ionizable lipids in existing LNP formulation with naturally occurring alkenyl amino alcohol lipids) *Image adapted with permission from ref [108]. Copyright 2016, John Wiley & Sons, Inc.*	
Mimicking endogenous molecules (e.g., mimicking HDL by decorating the lipid bilayer membrane of a miRNA carrier with apoA-I, which is highly expressed on the surface of HDLs) *Image adapted from ref [109]. Copyright 2017, American Chemical Society.*	
Mimicking viruses through virosomes (e.g., incorporating viral proteins, such as influenza hemagglutinin (HA), in lipid bilayer membranes) *Image adapted from ref [110]. Copyright 2022, with permission from Elsevier.*	
Mimicking viral membranes (e.g., incorporating phosphatidylserine (PS) in LNPs, a molecule which viruses leverage as an "eat-me" signal for cells) *Image adapted with permission under a Creative Commons CC-BY License https://creativecommons.org/licenses/by/4.0/ from ref [111]. Copyright 2022, Elsevier.*	
Mimicking exosomes (e.g., exosome mimetic liposomes integrating connexin 43 (Cx43) transmembrane proteins using a cell-free protein synthesis mechanism. Cx43 is a protein involved in exosome cell internalization) *Image adapted from ref [112]. Copyright 2019, with permission from Elsevier.*	

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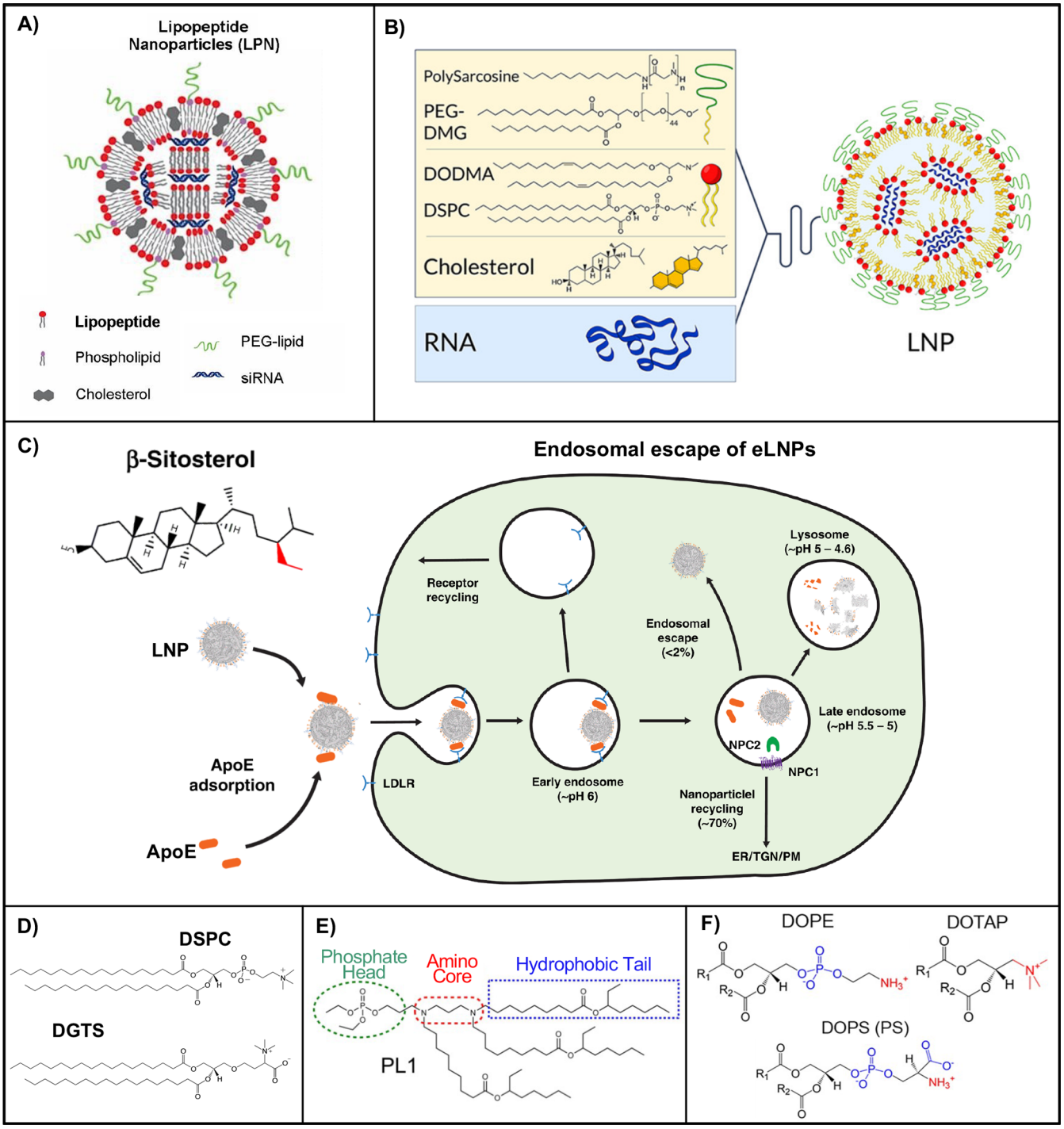

Figure 1. Schematics illustrating current research focused on bioinspired nanocarriers incorporating natural lipids. (A) Schematic of lipopeptide nanoparticles developed by Dong et al.[59] (Adapted with permission from ref 59. Copyright 2014, National Academy of Sciences). (B) Schematic and lipid structures of pSar-LNPs developed by Nogueira et al.[113] (Adapted from ref 113. Copyright 2020, American Chemical Society). (C) Left: The chemical structure of β-sitosterol differs from cholesterol by one ethyl group (highlighted in red). Right: Schematic of endosomal escape of eLNPs developed by Patel et al.[117] Shows desorption of PEG-lipids from LNPs, which allows ApoE binding to occur and causes LDL-mediated cellular uptake in cells. Subsequently, a small amount of RNA escapes the endosome while most is recycled back by lysosomal transporters or directed to degradative endocytic compartments. (Adapted with permission under a Creative Commons CC-BY License https://creativecommons.org/licenses/by/4.0/ from ref 117. Copyright 2020, Nature Portfolio). (D) Chemical structures of two helper lipids, DSPC and DGTS, used by Kim et al. in their LNP formulations.[120] (E) Chemical structure of ionizable phospholipid PL1 developed by Li et al.[123] (Adapted with permission under a Creative Commons CC-BY License https://creativecommons.org/licenses/by/4.0/ from ref 123. Copyright 2021, Nature Portfolio). (F) Chemical structures of three helper lipids used by LoPresti et al. in their LNP formulations.[125] (Adapted with permission under a Creative Commons CC-BY License https://creativecommons.org/licenses/by/4.0/ from ref 125. Copyright 2022, Elsevier).

types combine to form a lipid monolayer, yielding a spherical lipid vesicle with a hydrophobic core. When LNPs are mixed with RNA, ionizable lipids in the core of the particle form shells around the nucleic acids.[82,83] The most successful LNPs follow a

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specific framework: diameter between 80 and 100 nm, neutral zeta potential, PDI less than 0.2, and encapsulation efficiency of at least 80%.[84−90] For example, the DLin-MC3-DMA LNP used in Onpattro (patisiran) was reported to have a diameter of less than 100 nm and neutral zeta potential.[91]

Several LNPs have been approved for commercial use in therapeutics such as Onpattro and, most notably, Pfizer/BioNTech's BNT162b2 and Moderna's mRNA-1273 vaccines for COVID-19.[71] Moreover, LNPs delivering RNA are currently well-represented in clinical trials, given that they are the most advanced delivery vehicle used for the promising application of mRNA therapeutics.[12,71] Originally, methods such as detergent dialysis and ethanol-loading were used to form LNPs.[66] However, novel microfluidic architectures (T-junctions, staggered herringbone micromixers, bifurcating mixers) have helped establish rapid microfluidic mixing as the preferred method to form LNPs loaded with RNA.[87]

As RNA carriers, LNPs possess numerous advantages. When the right ionizable lipid chemistry is incorporated (e.g., no cations in the headgroup, biodegradable bonds present in tails), LNPs are nontoxic and biodegradable carriers that protect RNA from the external environment.[17,89] Additionally, stable and potent LNPs achieving RNA encapsulation efficiencies greater than 95% are frequently reported using microfluidic techniques.[62,63,92] These rapid-mixing microfluidic techniques also enable the high throughput synthesis of smaller and more homogeneous LNPs while requiring less labor.[66,93] Moreover, as mentioned, LNPs allow the endosomal escape of RNA cargos via pH-responsive ionization and efficiently shuttle them into the cytosol. Finally, differential LNP chemistries can lead to selective targeting of the liver, spleen, or lungs when LNPs are administered systemically in mice.[17,56] When specifically comparing them to liposomes, LNPs stand out due to a few key factors. LNPs form micellar structures in their core to further protect RNA, are more stable and rigid, and can be synthesized more homogeneously at a commercial scale.[66]

Despite the strengths and success of lipid-based carriers for RNA delivery, there is still much room for improvement. First, lipid-based carriers struggle to target a variety of organs and generally accumulate in clearance-associated organs such as the liver. One primary reason is that they strongly interact with Apolipoprotein E (ApoE) upon administration, leading to low-density lipoprotein receptor-mediated cellular uptake to hepatocytes.[94] Thus, there exists a need for lipid nanocarriers to reach new organ targets, such as the brain and bone marrow, to enable specific and nontoxic RNA therapies further.[95−100] Although capable of potent RNA delivery, the best lipid nanocarriers still only escape the endosome about 1−2% of the time.[54] Enhancing the cellular uptake and endosomal escape of nanocarriers is crucial for improving the effectiveness of RNA therapies, decreasing dosages, and minimizing material waste.[101] Furthermore, an assortment of challenges stemming from using PEG-lipids can be classified as the "PEG dilemma." PEGylation is used to prolong the circulation of NPs in vivo and help them reach their target destination to perform their intended function.[79,102] However, there are several potential drawbacks to using PEG-lipids, which may stem from developing anti-PEG antibodies in humans after repeated exposure to PEG.[79,102] First, it has been observed that PEGylated liposomes can lose their circulating properties and be rapidly cleared from the blood after repeated doses.[103] Additionally, complement activation-related pseudoallergy can be triggered by PEGylated lipid-based particles due to adverse innate immune responses.[104−106] The presence of PEG in cosmetics, cleaning products, and therapeutics contributes to the increase in anti-PEG antibodies being developed in humans. Given that anti-PEG antibodies can stunt the efficacy of PEGylated lipid-based carriers, there is a need for solutions to the "PEG dilemma".[102]

To help address the targeting limitations of LNPs, Selective Organ Targeting (SORT) was recently developed to deliver LNPs to extrahepatic tissues such as the lung and spleen.[56] However, SORT LNPs rely on using either toxic cationic lipids for lung targeting, which severely limit the dosage and narrow the therapeutic window, or anionic lipids for spleen targeting, which to some extent attenuate the RNA transfection potency.[107] Moreover, only two extra-hepatic tissues, the lung, and spleen, can be targeted by this approach.[56] Hence, there is a need to develop nontoxic and more potent LNPs for the precise and effective delivery of RNA therapeutics across a broader range of different target tissues. Overall, while conventional nanocarriers have demonstrated success in the clinic, there is still ample room for improvement in their safety, efficacy, and extra-hepatic organ targeting ability.

5.0. BIOINSPIRED LIPID-BASED NANOCARRIERS

Bioinspired lipid-based nanocarriers have been investigated to address the challenges associated with lipid nanocarrier RNA delivery. Indeed, traditional lipid nanocarriers are already bioinspired to a certain extent. Their lipid membranes mimic the membranes observed in biology and incorporate naturally occurring lipids such as phospholipids and cholesterol. However, further mimicking biology through increased natural lipid incorporation or replicating endogenous molecule/virus structures has been explored to help address the current challenges of lipid-based RNA delivery. The potential benefits of this method are widespread, ranging from increasing circulation time to improving organ selectivity through targeting endogenous receptor pathways. Crucially, bioinspired nanocarriers aim to improve performance while maintaining the advantages of conventional delivery systems. The current research on lipid nanocarriers that take inspiration from natural materials, endogenous molecules, viruses, and exosomes is reviewed in the following section. An overview of the bioinspired strategies covered in this review is provided in Table 1.

5.1. Substituting LNP Components with Natural Lipids

One of the most common methods of producing bioinspired lipid-based NPs is to incorporate naturally derived lipids into existing synthetic LNPs. This strategy typically aims to improve the circulation time of LNPs, increase their cellular uptake, and improve their endosomal escape. This method generally entails substituting one of the four major lipid types in LNPs with a naturally occurring analog.

Inspired by endogenous lipoproteins, Dong et al. screened several lipopeptides for their effectiveness as ionizable lipids in an LNP formulation containing distearoylphosphatidylcholine (DSPC), cholesterol, and PEG-DMG (a commonly used PEG-lipid).[59] A schematic of the lipopeptide nanoparticles is shown in Figure 1A. The group found that lysine-derived lipopeptides were most effective at delivering FVII-silencing siRNA and identified a lead material, cKK-E12, which incorporated a lipopeptide with a dilysine-derived diketopiperazine core and four amino alcohol-based lipid tails. cKK-E12 effectively targeted hepatocytes in vivo and achieved a remarkably low efficacious dose at the time (ED50−0.002 mg/kg in mice).[59] More recently, Fenton et al. formulated a group of alkenyl amino

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alcohol (AAA) ionizable lipids, a moiety found in sphingosine and other bioactive molecules, with cholesterol, 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE), and C14-PEG-2000 to form LNPs.[108] A schematic of the LNPs is depicted in Table 1. The group compared the performance of the LNPs with cKK-E12 when delivering mRNA encoding for human erythropoietin (EPO) in vivo to C57BL/6 mice. Their most potent LNP, OF-02, increased EPO production 2-fold compared to cKK-E12 across a broad linear dose−response window, establishing itself as the most potent LNP at the time in 2016. Additionally, OF-02 had nearly identical biodistribution to cKK-E12 and limited batch-to-batch variability, indicating its scale-up potential.[108]

Attempting to solve the "PEG dilemma," Nogueira et al. replaced PEG-DMG with polysarcosinated lipids in an LNP formulation, as illustrated in Figure 1B.[113] Polysarcosine (pSar) is a polypeptoid composed of repeating units of the endogenous amino acid sarcosine (*N*-methylated glycine). pSar has been found to exhibit stealth-like properties similar to PEG and have low immunogenicity.[114−116] The researchers found that pSar and PEG-DMG LNPs delivering mRNA encoding for Firefly luciferase (Fluc) had comparable performance when administered intravenously to Balb/C mice. However, pSar LNPs promoted longer EPO secretion in mice with significant EPO levels still present at 48h postadministration. Another finding of the study was that pSar nanocarriers induced lower proinflammatory cytokine secretion and reduced complement activation in an in vitro human whole blood model compared to PEGylated LNPs. Nogueira et al. suggested that the lower toxicity of pSar may positively affect cellular uptake and processing of the pSar-LNPs, leading to prolonged expression of the target protein.[113]

Helper lipids and cholesterol are other components of LNPs that have been replaced by naturally derived molecules in studies. For example, Patel et al. investigated the replacement of cholesterol with C-24 alkyl derivatives.[117] C-24 alkyl derivatives are phytosterols that play a critical part in constituting the membrane composition and dynamics of plant cells and are consumed in our diet.[118] The group incorporated these natural cholesterol analogs into LNPs and named them enhanced LNPs (eLNPs). Remarkably, the eLNPs were comparable in size and encapsulation efficiency to traditional LNPs but exhibited an 11- to 211-fold improvement in transfection in vitro when delivering mRNA, depending on the alkyl derivative and mRNA dosage used. Cryo-TEM revealed that eLNPs incorporating the alkyl derivative β-sitosterol differed in structure from LNPs mainly due to their highly faceted surface, which showed more defects. Interestingly, β-sitosterol differs from cholesterol by only one ethyl group, as shown in Figure 1C.

Further experiments exhibited the higher uptake and retention of eLNPs in HeLa cells and the superior transfection of eLNPs in NPC1-deficient fibroblasts compared to LNPs. This led Patel et al. to postulate that the different surface morphology of eLNPs enabled them to fuse more easily with membranes and possibly take more favorable trafficking pathways for enhanced transfection .Given that NPC1 is a transmembrane protein located on lysosomes, the researchers also stated that eLNPs probably interact less with lysosomal transporters, thus increasing their residence time and enhancing their endosomal escape.[117] This proposed phenomenon is illustrated schematically in Figure 1C.

Another group, Kim et al., also incorporated β-sitosterol into their LNPs and found similar effects on the structure and transfection efficiency of their LNPs.[119] Combining the use of β-sitosterol with a denser PEG-lipid layer, Kim et al. developed LNPs that achieved effective mRNA transfection in mouse epithelial cells upon nebulization and inhalation.[119] In a separate study, Kim et al. substituted the helper lipid DSPC with a series of naturally occurring membrane lipids originating from the cell membrane of plants and microorganisms.[120] They primarily studied diacylglyceryltrimethylhomo-Ser (DGTS), a lipid found in algae associated with lipid metabolism and cell survival in stress conditions.[121] The structures of DSPC and DGTS are provided in Figure 1D. DGTS LNPs had worse transfection in vitro (HeLa and A549 cells) than DSPC but better performance delivering mRNA in vivo (IV administration, Balb/C mice). Upon repeating in vitro testing after nebulizing the LNPs, DGTS only performed 12-fold worse than DSPC as opposed to 50-fold prenebulization. Thus, the authors concluded that DGTS may possess advantages over DSPC for in vivo delivery and tolerability for nebulization.[120]

Recently, novel biomimetic phospholipids have been employed to improve LNP formulations, taking inspiration from the molecules that are natural components of the cell membrane and are already used in many formulations as helper lipids.[122] For example, Li et al. tested the effectiveness of phospholipid and glycolipid-mimetic lipids as ionizable components in LNP formulations.[123] After screening numerous lipids, Li et al. isolated the phospholipid PL1, pictured in Figure 1E, as the optimal ionizable lipid for their LNP formulation. PL1 contained a biomimetic phosphate head, an ionizable amino core, and three hydrophobic tails. Upon encapsulating mRNA encoding for costimulatory T-cell receptors (CD137 or OX40), PL1 LNPs effectively delivered the cargo to a T-cell line in vitro and in vivo to T-cells within tumors resulting in a significant therapeutic effect.[123]

Previously, the development of SORT LNPs revealed that adding distinctly charged lipids to existing LNP formulations could affect serum protein adsorption by controlling the global/apparent pK_a of LNPs; subsequent interactions between surface-bound proteins and cognate receptors highly expressed in specific tissues enabled RNA delivery beyond the liver.[56,124] LoPresti et al. performed a similar investigation, instead entirely substituting DOPE with distinctly charged lipids rather than adding a fifth lipid component.[125] Seven endogenous lipids and a synthetic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were tested.[125] The neutral lipids were DOPC, sphingomyelin (SM), and a ceramide; the anionic lipids were phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidic acid (PA); the cationic lipids were ethyl phosphatidylcholine (EPC) and DOTAP. The structures of DOPE, DOTAP, and PS are pictured in Figure 1F. The researchers found that neutral lipids promoted protein expression in the liver, and anionic and cationic lipids shifted expression to the spleen and lungs, respectively. These results were obtained from administering LNPs containing 40 mol % of the new helper lipid to mice and were consistent across formulations containing three different ionizable lipids. Several possible mechanisms for the difference in organ targeting were suggested, including that the different surface charges affected the protein corona that coated the LNPs and thus led to differential trafficking pathways, as observed with SORT LNPs.[124] Importantly, SM, PA, DOPC, and EPC LNPs had very low efficacy in vivo; thus, those helper lipids were determined to be unlikely components of potent LNP formulations. However, the authors stated that those lipids may perform better when a standard helper lipid such as DOPE

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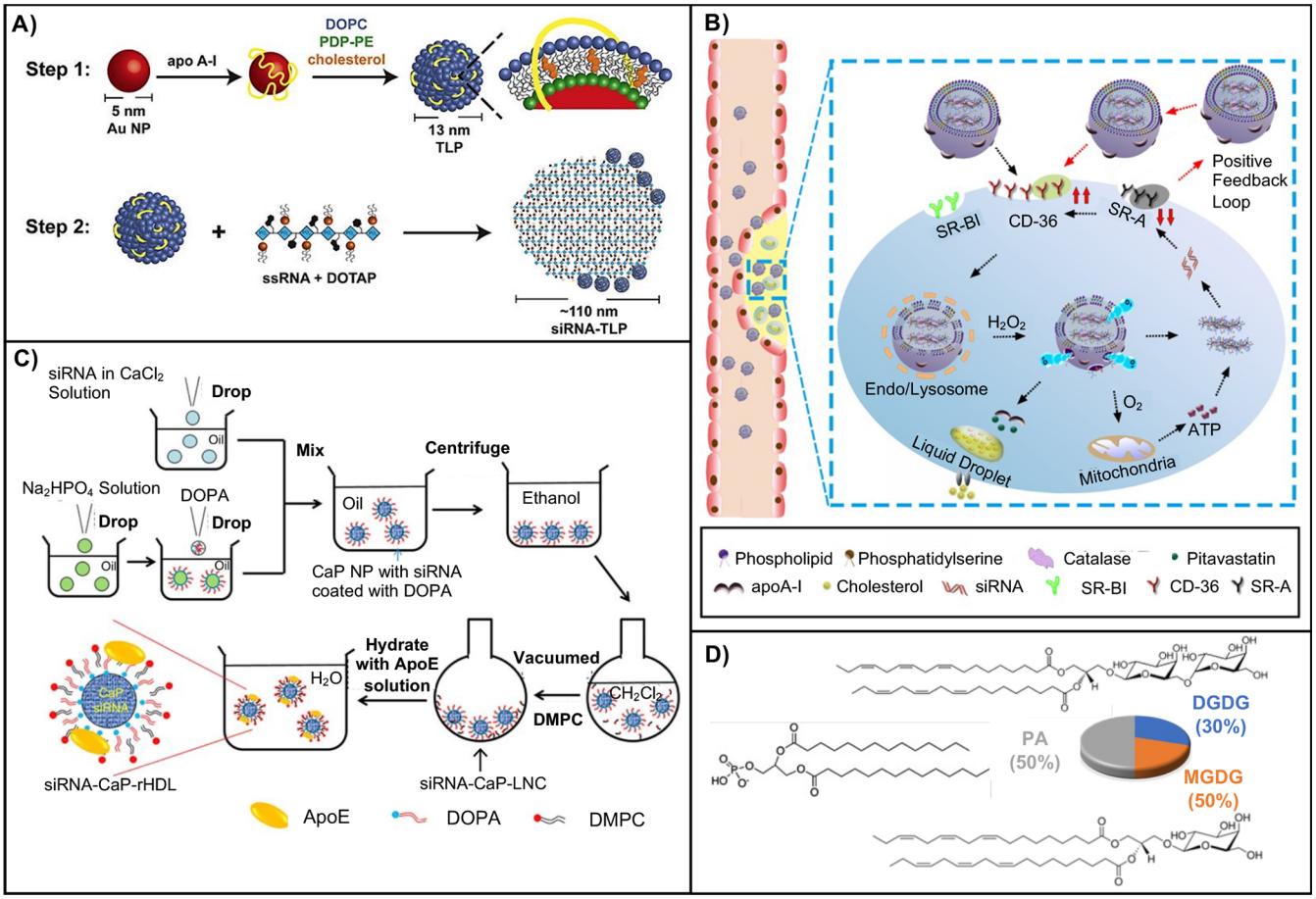

Figure 2. Schematics illustrating current research focused on bioinspired nanocarriers mimicking endogenous molecules. (A) Schematic of synthesis procedure and components of siRNA-TLPs developed by McMahon et al.[132] In Step 1, TLPs are synthesized. In Step 2, TLPs are mixed with single-stranded RNA (ssRNA), complement strands of a siRNA duplex, complexed with DOTAP. (Adapted from ref 132. Copyright 2016, with permission from John Wiley & Sons, Inc.). (B) Schematic illustration of dual-targeting HDL-mimics developed by Jiang et al., showing how they dynamically enhance plaque targeting via a positive feedback loop and lower intracellular lipid disposition.[139] (Adapted from ref 139. Copyright 2019, with permission from Elsevier). (C) The components and outline for the preparation of siRNA-CaP-rHDLs developed by Huang et al.131. (Adapted with permission under a Creative Commons CC-BY License http://creativecommons.org/licenses/by/4.0/ from ref 131. Copyright 2017, Nature Portfolio). (D) Chemical structure and the ratio of lipids used in GDNP-mimicking nLNPs developed by Sung et al.[142] (Adapted from ref 142. Copyright 2022, with permission from Elsevier).

is used alongside them, as PA was one of the more successful lipids tested in SORT LNPs.[56] In contrast, DOTAP and PS maintained high efficacy while shifting specificity to the lungs and spleen, respectively. Remarkably, the PS LNPs developed by LoPresti et al. had higher efficacy than the PS SORT LNPs, driven by greater spleen expression.[125] Finally, the group reported that anionic lipids were the best at transfecting immune cell lines RAW 264.7 and Raji B cells.[125] As with SORT, it is essential to note that the use of cationic lipids promotes toxicity, while the inclusion of anionic lipids limits transfection potential.[107]

To summarize, bioderived ionizable lipids are first worth screening to achieve increased LNP potency, while replacing PEG-lipids with pSar-lipids can lead to comparable efficacy with the benefit of lower immune activation. Remarkably, substituting cholesterol with β-sitosterol can significantly improve LNP uptake, retention, and transfection efficiency potentially due to changes in LNP surface morphology. Regarding helper lipids, DGTS may possess advantages over DSPC for administration routes requiring nebulization, such as in highly sought-after nasal and lung vaccines.[126−129] Incorporating differently charged endogenous helper lipids can shift protein expression to the liver, lung, or spleen, and PS stands out as one of the most efficacious helper phospholipids with similar performance to the cationic DOTAP. Additionally, the poor performance of DGTS compared to DSPC in vitro (HeLa and A549 cells) but better performance in vivo (IV administration, Balb/C mice) indicates that in vitro models are not always accurate predictors of in vivo performance. Overall, substituting one of the four lipid components with natural lipids is a valuable strategy to improve the efficacy of LNPs without sacrificing their core properties and scale-up potential.

5.2. Mimicking Endogenous Molecules

Another method of producing bioinspired lipid nanocarriers is to go beyond substituting one lipid type and instead model the whole particle architecture after an endogenous molecule. This method exploits endogenous pathways to reach and transfect target cells with higher efficacy.

High-density lipoproteins (HDLs) are the smallest lipoproteins and natural carriers of RNA in vivo, ferrying and delivering miRNA to cells.[130,131] Scavenger receptor type B-1 (SR-B1) is a high-affinity receptor for spherical HDLs that exhibit surface expression of apolipoprotein A-I (apoA-I), and it

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is expressed in a wide variety of cell and tissue types.[130,132−134] Thus, HDLs deliver miRNA with high selectivity to cells expressing SR-B1, motivating the synthesis and evaluation of HDL-mimicking particles for RNA delivery. McMahon et al. pursued this concept by developing templated lipoprotein particles (TLPs) to mimic spherical HDLs.[132] The TLPs were created by mixing the lipids DOPC, cholesterol, and a phospholipid with 5 nm gold NPs decorated with Human apoA-I. The apoA-I served to mimic HDL, while the gold NPs were used to aid synthesis and could theoretically be removed in future iterations. When incubated with a mixture of single-stranded siRNA and DOTAP (used to neutralize the negative charge of siRNA), 110 nm-sized siRNA-TLP complexes were formed. A schematic of the structure and synthesis of the siRNA-TLPs is illustrated in Figure 2A; the particles were anionic, uniform in size, and successfully protected siRNA from degradation. During testing in multiple cancer cell lines, the siRNA-TLPs successfully targeted SR-B1 and potently reduced two established protein targets in prostate and other cancers, androgen receptor and enhancer of zeste homologue.[135−137]

Furthermore, 13 doses of 0.7 mg siRNA/kg over 26 days significantly reduced tumor volume in a mice prostate cancer xenograft model. Crucially, no off-target toxicity was observed during this treatment. Along with successfully targeting SR-B1 by mimicking HDL, the TLPs validated that delivering ssRNA complements of a siRNA duplex is a viable method for efficaciously delivering siRNA.[132] Showing the versatility of the TLPs, Wang et al. used the system in a follow-up study to successfully deliver miRNA to corneal and limbal epithelial cells as well as stromal keratocytes through topical application to the ocular surface.[138] Corneal re-epithelialization was significantly improved in diabetic mice who suffered alkali burn-induced inflammation after treatment with miRNA-TLPs.[138]

Another group that targeted SR-B1 with HDL-mimics containing apoA-I was Lu et al.[109] In a similar fashion to McMahon et al., they condensed anionic anti-miR155 miRNA with cationic acid-labile polyethyleneimine (PEI) and surrounded it with a lipid bilayer coat comprised of cholesterol, phospholipids, sodium cholate, and apoA-I. The final particles were 180 nm in size with a −37 mV zeta potential; a schematic of the particles is shown in Table 1. Through several in vitro tests, the group validated the miRNA protection, lack of cytotoxicity, and macrophage SR-B1 targeting of the system. Lu et al. reported a 22% reduction of relative miRNA level in Raw 264.7 cells after treatment with their approach compared to regular liposomes or an 80% reduction of miRNA compared to untreated cells.[109] These were promising results despite the lack of in vivo testing. A final SR-B1-honing structure was reported by Jiang et al., who targeted not only SR-B1 but the scavenger receptor CD36 commonly expressed on macrophages.[139] By complexing SR-A siRNA and catalase with ATP-responsive ternary polyplexes and housing them in a lipid bilayer containing PS, Jiang et al. created an HDL-mimic for treating atherosclerotic plaque. The mimic and its dual-targeting mechanism are depicted in Figure 2B. The final particles were 200 nm in diameter with a zeta potential of −27 mV. A 3-month dosage regimen of the NPs reduced plaque areas by 65.8% and decreased macrophages by 57.3% in ApoE$^{-/-}$ mice.[139]

A hyperactive Ras pathway is characteristic of many cancers, with around 30% of all human cancers exhibiting at least one Ras gene mutation.[131] The activation of Ras promotes cancer cells to engulf extracellular proteins through macropinocytosis to gain nutrients.[140,141] Huang et al. decided to exploit this pathway to infiltrate cancer cells more efficiently with a lipoprotein-biomimetic nanostructure.[131] The group designed a nanosized calcium phosphate-siRNA complex surrounded by a layer of two phospholipids, which they subsequently incubated with apolipoprotein E3 (ApoE3) to create HDL-mimicking NPs named siRNA-CaP-rHDLs. The synthesis outline and schematic of the siRNA-CaP-rHDLs are shown in Figure 2C. ApoE3 was employed to help target low-density lipoprotein receptors in the blood−brain barrier (BBB) and glioblastoma cells. ApoE3 was key to the formulation as the group aimed to target activating transcription factor-5 (ATF5), an overexpressed antiapoptotic transcription factor in glioblastoma, with their siRNA treatment. The siRNA-CaP-rHDLs were 20−40 nm in diameter with a PDI of 0.2−0.4, indicating a moderately variable synthesis process.

Further characterization studies confirmed that Ras-activated cell lines exhibited elevated uptake of CaP-rHDL and that compared to previous work on HDL mimics, the CaP-rHDL could load more siRNA and better protect against nuclease degradation.[143] ATF5 siRNA delivered by CaP-rHDLs significantly suppressed ATF5 mRNA and protein expression and induced apoptosis in glioblastoma cells. In a xenograft tumor mice model, a 0.36 mg/kg siRNA dose significantly extended the survival time of treated mice with minimal side effects. In addition, due to the common Ras pathway the CaP-rHDL exploits, its siRNA cargo and targeting protein could be modified to treat many different types of cancer.[131]

A final method of mimicking lipid-based endogenous molecules for RNA delivery is to create LNPs based on NPs naturally found in edible ginger (GDNPs). The significant advantages of this strategy are hypothesized to be the lack of toxicity and increased production scale compared to traditional synthetic LNPs (due to an abundant natural source).[144] Zhang et al. initially isolated GDNPs from ginger which contained many lipids, a few proteins, and around 125 miRNAs.[144] The group successfully used the particles to reduce acute colitis, enhance intestinal repair, and prevent chronic effects in mouse colitis models.[144] In a follow-up study, Zhang et al. loaded the GDNPs with siRNA-CD98 and reduced CD98 expression in colon tissues after oral administration.[145] Most recently, Sung et al. reverse-engineered the GDNPs and found that they were mostly comprised of the lipids PA, monogalactosyldiacylglycerol (MGDG), and digalactosyldiacylglycerol (DGDG).[146] The galactolipids MGDG and DGDG form the stable lipid bilayer of chloroplast in plant leaves. The authors noted that many plant-derived NPs do not contain cholesterol but are stable enough to target the lower GI tract. Thus, they mixed PA, MGDG, and DGDG at the ratio found in GDNPs (5:2:3) and observed that the lipids self-assembled into new LNPs (nLNPs). The structure of the three lipids is provided in Figure 2D.

Interleukin-22 (IL-22) has anti-inflammatory effects against ulcerative colitis in the colon, so Sung et al. complexed IL-22 mRNA with the cationic polymer turbofectamine and encapsulated the system inside nLNPs. The polymer served to reconcile the negative charge of the RNA such that it could combine with the negatively charged nLNPs. The final diameter and zeta potential of the mRNA-nLNPs were 200 nm and −18 mV, respectively. Oral delivery of the mRNA-nLNPs promoted IL-22 expression in the colonic mucosa of mice, coinciding with an accelerated healing process indicated by more body weight and colon length recovery and a reduction in histological index and pro-inflammatory cytokines. Consistent with the fact that synthetic LNPs offer a more consistent product than naturally derived counterparts, Sung et al. noted that the nLNPs had a

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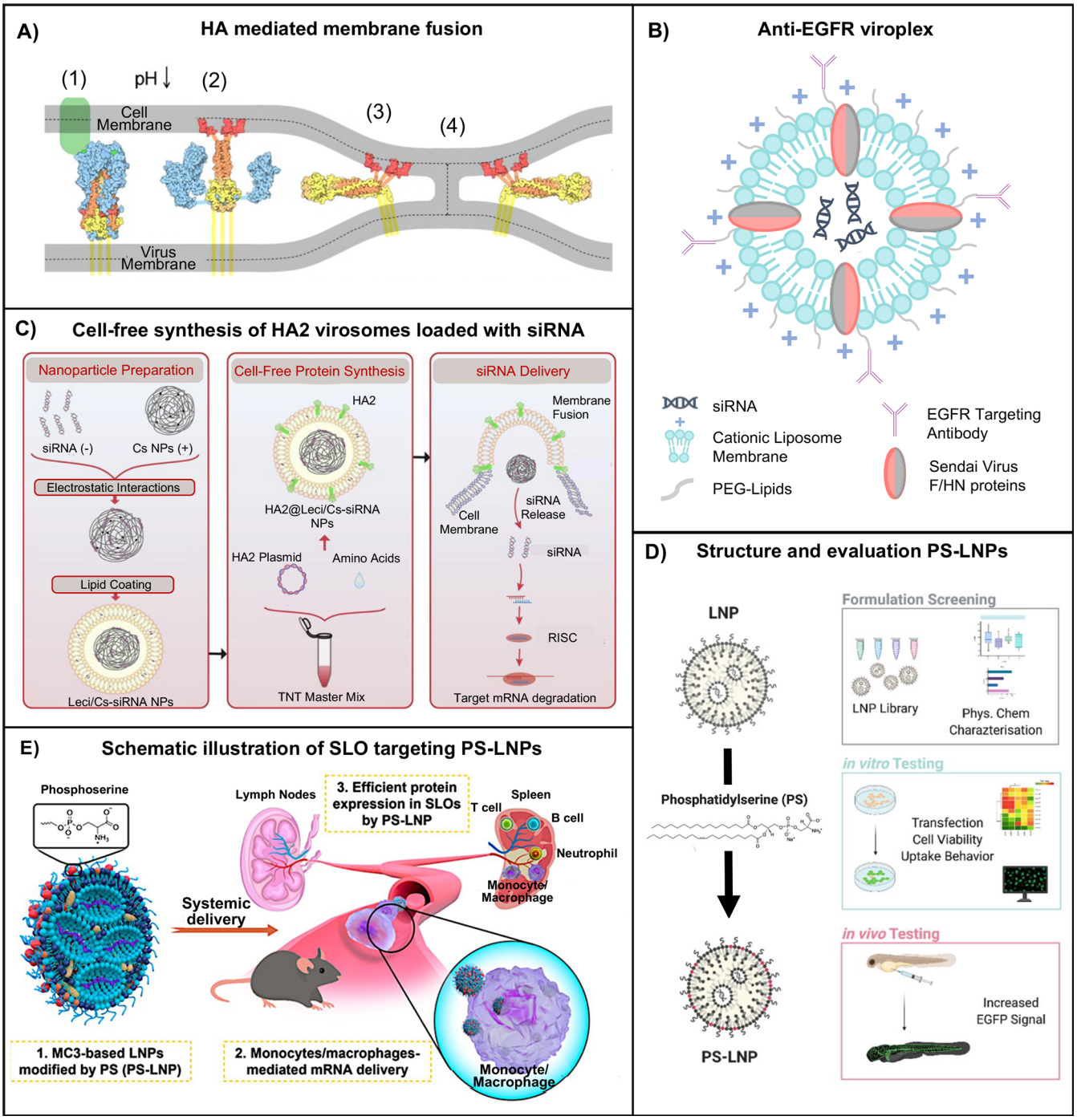

Figure 3. Schematics illustrating current research focused on bioinspired nanocarriers mimicking viruses. (A) Shows the critical steps involved in HA-mediated membrane fusion. (1) HA binds to the cell membrane through sialic acid groups (green); (2) pH reduction in endosome causes a conformation change that causes fusion peptides (red) to interact with the cell membrane; (3) Another conformation change fuses the membranes to form a "stalk"; (4) multiple HA proteins work to accomplish this, and eventually the stalk collapses to form a pore. (Adapted from ref 110. Copyright 2022, with permission from Elsevier). (B) Outlines the modifications to cationic liposomes made by Kim et al.[156] to form EGFR-targeting Viroplexes that deliver siRNA. (C) Schematic showing the cell-free synthesis of HA2 virosomes and the mechanism of siRNA delivery (Adapted from ref 154. Copyright 2022, with permission from Elsevier). (D) Illustrates the design of PS-LNPs inspired by viral membrane lipids and provides an overview of methods to evaluate their ability to deliver RNA. (Adapted with permission under a Creative Commons CC-BY License https://creativecommons.org/licenses/by/4.0/ from ref 111. Copyright 2022, Elsevier.) (E) Schematic showing PS-LNP nanoparticles and their ability to target SLOs through macrophage/monocyte mediated delivery, enabling mRNA delivery to the spleen and lymph nodes (Adapted from ref 171. Copyright 2022, American Chemical Society).

more consistent composition and reduced batch-to-batch variability while inheriting many core properties of the original GDNPs.[142,146]

In conclusion, mimicking HDL is an effective strategy to deliver RNA to the various cells expressing SR-B1 and to target the commonly hyperactive Ras pathway in cancers. This method has proven to be efficacious in vivo and may unlock RNA

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delivery to historically difficult-to-reach targets; however, it requires the synthesis of complex multilayered nanostructures which have unclear scale-up potential. In addition, modeling the lipid composition and ratios of LNPs after GDNPs may be a potent strategy for RNA delivery to the colon to treat bowel diseases such as colitis. However, synthesis complexity and toxicity concerns are increased due to the complexation of RNA with a cationic polymer to achieve charge reconciliation. Mimicking endogenous molecules is thus an intriguing strategy to exploit new tissue-targeting pathways. However, there is uncertainty about its scalability in the current state.

5.3. Mimicking Viruses

A promising approach for developing nanocarriers with better organ targeting and enhanced endosomal escape for RNA therapies involves taking inspiration from viruses. In nature, most viruses consist of a 20−500 nm protein capsid containing genetic material in either DNA or RNA.[147] Viruses have evolved to bypass the immune system, prolong their blood circulation time, and overcome physiological barriers to infect hosts with their genetic material.[148] Essentially, viruses act as natural gene delivery systems that promote genomic transfer by enhancing cell recognition, cellular binding, endocytosis, penetration, and nuclear import.[149] Most importantly, viruses have efficient cellular uptake and endosomal escape attributed to their structure and ability to respond to changes in their microenvironment.[148,149] Because these properties overlap with essential traits of drug delivery systems in RNA therapeutics, viruses have inspired the development of multiple nanocarriers. One strategy is to directly use a viral vector to deliver RNA, which involves removing the genetic material of a virus and replacing it with a therapeutic gene; however, safety concerns arise due to potential immunogenicity, toxicity, inflammation, and insertional mutagenesis.[147] This has motivated the development of lipid-based carriers that incorporate lipids and proteins found in viruses, resulting in a safer alternative to viral vectors and more efficacious delivery than conventional nonviral vectors.[150] Current research focuses on endowing lipid-based nanocarriers with the fusogenic properties of viruses through the incorporation of fusion proteins or specific lipids found within viral membranes. These molecules enable viruses to insert their genetic material into cells by initiating fusion between the viral and host membrane.[151] Traditionally, fusion proteins are incorporated into liposomes to form virosomes, while membrane lipids that enhance cell uptake are added as components to LNPs.

5.3.1. Virosomes. Virosomes contain viral and nonviral components, forming a liposomal phospholipid bilayer embedded with viral fusion glycoproteins.[110] Because of this, virosomes can enhance the RNA delivery of nonviral systems while reducing cytotoxicity compared to viral systems.[150] Examples of membrane fusion proteins used in virosomes include influenza virus hemagglutinin (HA),[152−154] vesicular stomatitis virus G (VSV-g) protein,[155] and Sendai virus proteins.[156−159] Currently, most virosome systems are designed for applications delivering siRNA. For example, Jonge et al. developed an influenza-based virosome for siRNA delivery, which relied on HA for membrane fusion activity.[153] HA can enhance RNA delivery in virosomes by binding with sialic acid receptors on cells to induce endocytosis and by undergoing conformational changes in the acidic endosomal pH to cause endosomal escape.[110]

The process of HA-mediated fusion is shown in Figure 3A. Jonge et al. synthesized the virosomes by solubilizing the influenza virus in a phosphatidylcholine (PC) detergent and extracting the viral protein and membrane components using centrifugation.[153] From here, siRNA was complexed with a cationic lipid, *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC), and added to the solubilized viral components where virosome self-assembly occurred. To evaluate gene silencing, the authors delivered virosomes containing green fluorescent protein (GFP)-silencing siRNA to cells expressing GFP. The virosome performance was compared to lipofectamine, a commercial transfection agent used only for *in vitro* transfection of nucleic acids.[160] This resulted in decreased fluorescence comparable to the lipofectamine control, indicating that functional siRNA was successfully delivered to the cell cytoplasm. To determine if HA-mediated membrane fusion was responsible for the siRNA delivery, the authors inactivated the HA protein on the virosomes by exposing them to low pH before cell incubation. Pre-exposure to low pH causes HA to undergo a conformational change before entering the endosome, disabling its ability to mediate endosomal escape.[153] When virosomes with inactivated HA were delivered, cell GFP expression was unaffected. This result indicated that the siRNA could not reach the cytoplasm without the HA undergoing a conformational change in the endosome and highlighted that the functional integrity of the fusion protein is critical for siRNA delivery. In addition to their ability to enhance siRNA delivery in vitro, the authors found that the virosomes had minimal cytotoxicity, with cell viability remaining between 80 and 100%. However, the optimized virosomes only achieved a siRNA encapsulation efficiency of ∼37%.

More recently, Kim et al. developed cancer-targeting antibody-conjugated cationic virosomes (viroplexes) incorporating Sendai virus proteins for use in siRNA-based cancer therapies.[156] The structure of these viroplexes is shown in Figure 3B. Sendai virus contains fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins that enhance virosome uptake by binding to sialylated glycans on the cell surface, initiating fusion of the virus envelope with the host-cell membrane.[161] Kim et al. extracted and purified the F/HN viral proteins using a detergent-based method. Cationic liposomes complexed with siRNA were composed of *O,O*-dimyristyl-*N*-lysyl glutamate (DMKE) as positively charged lipids, cholesterol, and PEG lipids.

The purified F/HN proteins were added to the cationic liposome solution to form the viroplexes, and an antibody targeting epidermal growth factor receptor (EGFR) was conjugated to the particle surface. EGFR is overexpressed in tumors, making it an ideal target for developing cancer-specific delivery vehicles.[162] Compared to noncationic virosomes and cationic liposomes with no viral proteins, the viroplexes exhibited the highest siRNA transfection during in vitro tumor cell studies. Moreover, when used to deliver antitumoral siRNA to mice, significant tumor growth inhibition was observed for both cationic liposome and viroplex delivery systems. Most notably, when the viroplexes were delivered in combination with doxorubicin, significantly enhanced tumor inhibition was observed compared to all other controls. Ultimately, Kim et al. concluded that the viroplexes resulted in the most effective siRNA delivery to target the cancer cells, as indicated by high transfection in vitro and tumor suppression in vivo. While these cationic virosomes showed great promise, the cytotoxic nature

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of positively charged nanoparticles in healthy cells[163] must be considered when analyzing the safety of these delivery systems.

Currently, a significant limitation of virosomes is the traditional preparation method. As described by Jonge et al.[153] and Kim et al.,[156] preparation typically involves solubilizing an inactivated virus with a detergent, using ultracentrifugation to extract the desired protein and membrane components, and relying on the hydrophobic effect for viral protein insertion in a lipid-bilayer particle.[110] This method is problematic as it requires multiple steps, the detergents can destroy the structural and functional integrity of the membrane protein, and the lipid assembly process often yields poor encapsulation efficiency.[154]

Recently, Wang et al. developed a one-step virosome preparation method using cell-free protein expression technology.[154] Cell-free protein expression systems rely on transcription and translation machinery found in cells to synthesize proteins in vitro using synthetic DNA templates.[164] The details of these expression systems have been reviewed previously.[110,164] Using this method, the authors prepared HA virosomes with a lecithin-based liposomal membrane and a chitosan-siRNA core for better RNA encapsulation. The steps for producing these virosomes are outlined in Figure 3C. This system displayed minimal cytotoxicity when delivered to A549 and NCI-N87 cells, with cell viability higher than 70% even at a lipid concentration of 200 ug/mL. Additionally, the HA virosomes exhibited superior cell uptake compared to liposomes and the independent chitosan-siRNA complexes. The virosomes were also tested for silencing VEGF in cells using siRNA, and the HA virosomes showed more efficient gene silencing (19%) compared to liposomes (6%). This demonstrated that the cell-free synthesis method enables the formation of functional HA-virosomes that enhance cell uptake and siRNA delivery. While this method is still in development, further optimization of the formulation and protein expression may enable the creation of entirely synthetic virosomes.[110]

With respect to commercial development, Entos Pharmaceuticals has established a proteo-lipid delivery system similar to virosomes. The platform relies on neutral lipids to form the lipid-bilayer structure and integrates proprietary fusion-associated small transmembrane (FAST) proteins derived from reoviruses.[67,165] FAST proteins are small, nonstructural viral proteins that are not involved in viral entry into cells.[166] Instead, these proteins are expressed by reovirus-infected cells and mediate fusion of infected cells with uninfected cells.[166] It is reported that the use of FAST proteins in Entos Pharmaceuticals' platform enables rapid fusion of the lipid nanocarrier to the cell membrane, thus enabling cargo delivery directly to the cytoplasm while bypassing conventional endocytic pathways.[67,167] There are limited studies available that assess the performance of this platform in nucleic acid therapeutics; however, the company reports applications in the delivery of RNAi, miRNA, mRNA, and DNA.[167] Currently, Entos is working on clinical trials assessing the performance of a COVID-19 DNA vaccine using their established platform.[168] The company also has several other nucleic acid therapies under preclinical and clinical development.[168]

Overall, virosomes present a promising option for improving the delivery of RNA through fusion-mediated cell uptake. With advantages including better cell uptake and enhanced endosomal escape, virosomes can provide solutions to several challenges associated with RNA delivery. The research focused on virosome-mediated siRNA therapeutics demonstrates better gene silencing and cell uptake than traditional liposomes.[153,156] Entos Pharmaceuticals exemplifies successful commercialization of a virosome-like proteo-lipid vehicle platform; however, there are limited studies assessing the performance compared to conventional delivery systems. In the future, direct comparisons of virosomes with other clinically relevant delivery systems, such as LNPs, will be critical to evaluate the efficacy of virosomal delivery systems. Despite the advantages, virosomes lack efficient preparation methods, with established procedures relying on cumbersome processes involving using detergents to extract viral proteins. With advancements in cell-free protein synthesis, this problem can be mitigated; however, integrating this strategy in virosome preparation still requires further optimization. Moreover, high siRNA encapsulation efficiency is difficult to achieve using conventional preparation strategies. Therefore, for virosomes to become a clinically relevant delivery system in RNA therapeutics, research should focus on developing more efficient and scalable preparation techniques.

5.3.2. Mimicking Viral Membranes. In addition to virosome development, recent work has investigated the delivery of LNPs incorporating phospholipids found on viral membranes. In particular, the phospholipid phosphatidylserine (PS) has been explored.[111] PS is a component in several viral membranes and functions by camouflaging the virus as a dead cell via apoptotic cell mimicry.[111,169] In mammalian cells, PS is naturally contained within the cell membrane; however, once these cells undergo apoptosis or necrosis, PS is exposed outside the cell membrane.[111] This process induces PS recognition, causing apoptotic clearance. In viruses, PS found on the outer viral membrane disguises it as an apoptotic body and causes cells (including macrophages and tissue cells) to engulf virions through cell clearance mechanisms.[169] Moreover, cells contain PS-mediated virus entry-enhancing receptors (PVEERs). These receptors enable viruses to evade the immune system through both anti-inflammatory signaling and facilitating cell internalization without requiring extracellular exposure of receptor binding domains or fusion proteins.[169] For these reasons, incorporating PS in LNP compositions can theoretically create an "eat-me" signal for cells, increasing LNP uptake. For example, Lotter et al. investigated the partial substitution of the helper lipid 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) with PS. A summary of the experiments run by Lotter et al. is shown in Figure 3D. By varying the PS content of their LNPs from 0 to 10%, the group found that 1−5% of PS, corresponding to 10−50% substitution of DOPC, was optimal for transfection efficiency and protein translation in vitro in HuH-7 cells.

The 2.5% PS LNPs increased the EGFP signal 3-fold compared to the DOPC LNPs with no impact on cell viability. Further studies displayed that 2.5% of PS LNPs had a 13.5% increase in cellular uptake compared to DOPC LNPs, with the authors suggesting the activation of Annexin V (a cellular protein that binds strongly to PS) as a significant factor contributing to this result.[170] Furthermore, Lotter et al. stated that PS also increases clathrin-mediated endocytosis by enhancing interactions with scavenger receptors of target cells. In a final in vivo study, the potency of PS was confirmed as PS LNPs had a 3.7-fold increase in transfection potency compared to DOPC LNPs in the tail region of a zebrafish embryo after IV injection.[111]

Luozhong et al. also investigated the impact of adding PS to LNPs, explicitly focusing on leveraging PS to target secondary lymphoid organs (SLOs), including lymph nodes (LNs) and the spleen.[171] As discussed previously, PS causes apoptotic mimicry in a similar manner to viruses, enabling cell entry by signaling cell

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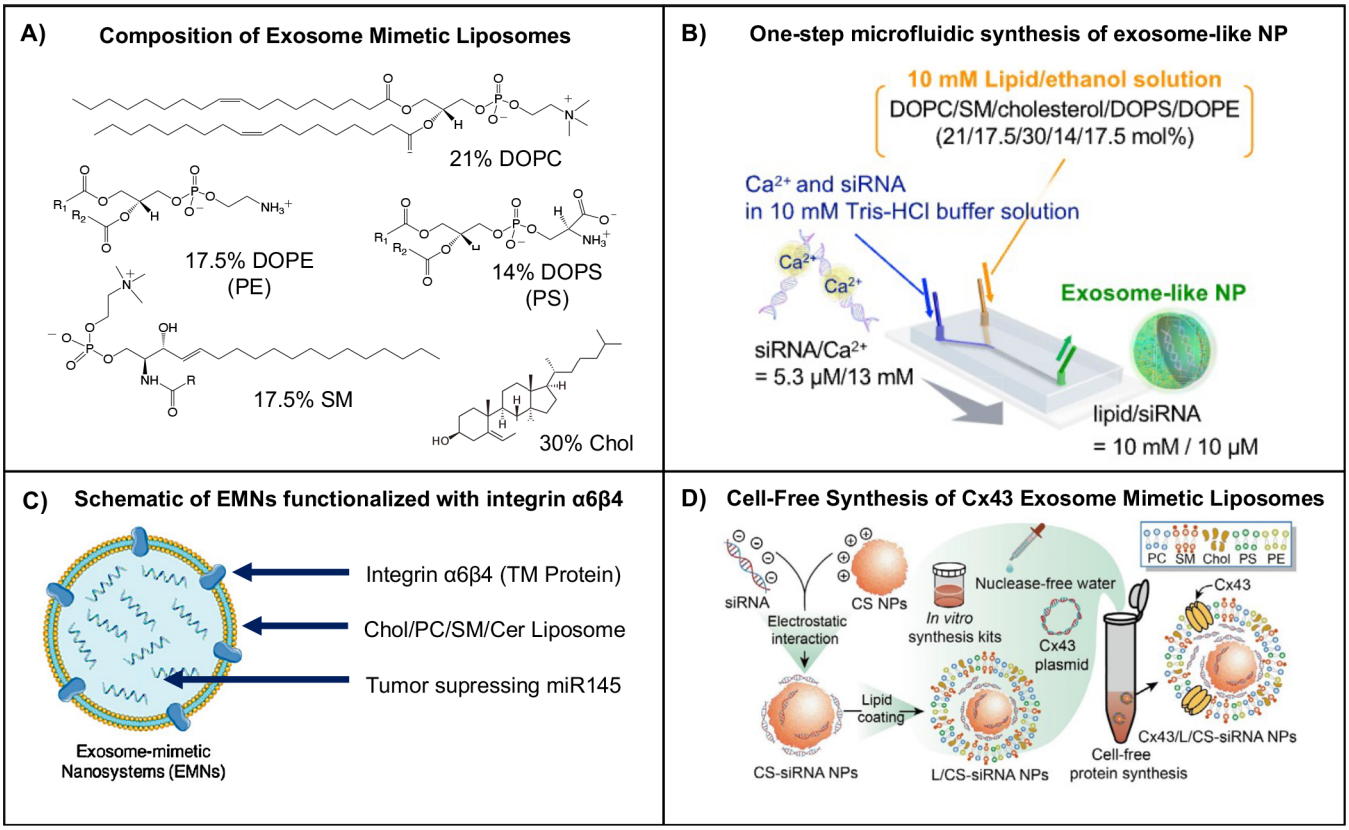

Figure 4. Schematics illustrating current research focused on bioinspired nanocarriers mimicking exosomes. (A) Illustrates the chemical structures of the lipids used in exosome mimetic liposomes, reported by Lu et al.[179] (B) Visual representation of a one-step microfluidic synthesis method to prepare exosome-like NPs (Adapted from ref 180. Copyright 2021, American Chemical Society). (C) Structure of tumor-suppressing exosome mimetic nanosystem functionalized with tumor-targeting integrin *α6β4* reported. (Adapted with permission under a Creative Commons CC-BY License license http://creativecommons.org/licenses/by/4.0/ from ref 177. Copyright 2019, BioMed Central Ltd.). (D) Schematic for the preparation of exosome-mimetic Cx43/L/CS-siRNA NPs. siRNA was complexed with CS and subsequently coated with an exosome-like lipid bilayer. Cell-free synthesis was then used to incorporate Cx43 proteins in the lipid bilayer. (Adapted from ref 112. Copyright 2019, with permission from Elsevier).

clearance pathways. These signals primarily communicate with phagocytes, like macrophages, which are abundant in SLOs.[171−173] Because of this, Luozhong et al. hypothesized that incorporating PS in LNPs could redirect LNP delivery from the liver to SLOs. A summary of the SLO targeting mediated by PS-LNPs is illustrated in Figure 3E. To test their theory, a series of experiments compared PS-LNP formulations with traditional MC3-LNP formulations (containing MC3, DSPC, cholesterol, and DMG-PEG2000). The PS-LNPs contained 5% 1,2-dioleoyl-sn-glycerol-3-phosphate-L-serine (DOPS), with the phosphoserine functional group required for targeting. An additional formulation containing 1,2- dioleoyl-sn-glycerol-3-phosphate (PA) (an anionic lipid with no phosphoserine targeting group) was used as a reference.

In an initial in vivo study, mice were IV injected with LNPs containing Fluc mRNA to evaluate luciferase expression across different organs. The MC3-LNPs showed maximum protein expression in the liver, while both PS- and PA-LNPs showed higher expression in the spleen. Notably, compared to PA-LNPs, the signal in the spleen for PS-LNPs was 45-fold stronger. In addition, the PS-LNPs accumulated in superficial cervical lymph nodes (SCLNs), while the MC3-LNPs and PA-LNPs did not. The total luminescence signal for PS- and MC3-LNPs was comparable, indicating that the PS LNPs achieved spleen targeting while maintaining high transfection efficiency. Biodistribution studies after the systemic injection of LNPs containing Cy-5 labeled RNA showed that MC3- and PA-LNPs exhibited almost no fluorescence in lymph nodes. Conversely, PS-LNPs showed Cy-5 signals in all lymph nodes, with SCLNS being the strongest (90% of the signal). To better understand the PS-targeting mechanism, in vitro tests were performed using HepG2 cells (hepatocyte cell line) and RAW264.7 cells (a monocyte/macrophage cell line). MC3-LNPs had ten times higher expression in HepG2, while PS had higher transfection in RAW264.7. PS-LNPs also exhibited higher transfection in primary murine splenocytes. Ultimately, this result suggested that PS-lipids increase LNP transfection in immune cells, especially macrophages. Additional in vivo tests confirmed that PS-lipids recruit blood or tissue-derived monocytes, suggesting that monocytes and macrophages mediate PS LNP delivery. To ensure the role of macrophages in SLO targeting using PS, Fluc mRNA LNPs were delivered to a macrophage-depleted mouse model. In this model, the PS-LNPs were no longer delivered to SLOs, and FLuc expression occurred in the liver.

In summary, incorporating PS in LNP compositions creates a biomimetic nanoparticle with enhanced cell uptake and SLO targeting. Because PS acts as a natural "eat-me" signal for macrophages and other tissue cells, adding small amounts (2.5%) to conventional LNP formulations has resulted in different enhancements in cell uptake and protein translation both in vitro and in vivo. Furthermore, because PS triggers cell clearance pathways associated with macrophages, it can efficiently mediate LNP delivery to SLOs, which is essential for applications requiring mRNA delivery to immune cells.

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Another key advantage is that PS can be seamlessly incorporated into current LNP self-assembly mechanisms to enhance cell uptake and organ targeting. For these reasons, developing virus-mimicking LNPs using PS is an ideal strategy to create bioinspired nanocarriers with enhanced cellular uptake, protein translation, and organ targeting properties.

5.4. Mimicking Exosomes

Exosomes are a subset of extracellular vesicles (EVs) secreted by various cell types, including cancer cells, specific immune cells, and epithelial cells.[68,69,174] Typically, exosomes consist of 30−120 nm diameter lipid membrane vesicles enriched with various proteins, lipids, and nucleic acids from their parental cell.[68,80,175] The primary function of exosomes is to facilitate the transport of molecules between cells, which directly affects intercellular communication and other biological processes.[69,80] Exosomes have vital advantages over conventional nanocarriers such as LNPs. These advantages include their heightened ability to evade phagocytosis in the bloodstream and circulate for longer in bodily fluids, cross biological barriers, and efficiently transfect target cells using their surface proteins.[68,69] Due to their endogenous source, exosomes can be more biocompatible and less immunogenic than synthetic carriers. However, exosomes possess some major disadvantages related to their production. There lacks standardized methods for the large-scale production of exosomes with consistent properties due to the many variable factors in the biological extraction of exosomes.[68] Once extracted, it is challenging to purify exosomes without sacrificing yield and incurring high costs.

Moreover, passive loading techniques result in low amounts of RNA in exosomes, especially for longer RNAs such as mRNA.[68,176] Finally, the complex composition and undefined biological functions of exosomes impede their transition to the clinic, as these characteristics may promote unwanted side effects.[112,177,178] To circumvent these challenges, recent literature has focused on developing exosome-mimetic nanoparticles for drug delivery applications. This strategy leverages the lipid-bilayer structure of liposomes, which closely resembles that of exosomes.[175] To achieve a more biomimetic nanoparticle structure, specific exosome-like lipid compositions and surface proteins can be integrated into the liposomes.[112,175,177,179] Traditionally, these systems require bottom-up preparation strategies, which enable more control of the final product composition and result in more clinically translatable structures.[175] Current work has investigated using exosome-mimetic particles for siRNA and miRNA delivery.[112,177,179,180]

5.4.1. Mimicking Exosome Lipid Compositions. One strategy to create exosome-mimicking nanocarriers entails developing liposomal formulations that contain lipid compositions found in exosomes. Exosomes are rich in sphingomyelin (SM) and cholesterol (Chol), with reduced levels of phosphatidylcholine (PC).[181] Moreover, both PS and phosphatidylethanolamine (PE) are also traditionally found in exosomes, with PS playing a vital role as an "eat-me" signal.[181] The distribution of lipids found in exosome membranes is hypothesized to contribute to the release and delivery of exosome cargos to receptor cells,[181] making this composition desirable to mimic for RNA delivery. This strategy was implemented by Lu et al., where they developed exosome-mimetic liposomes with a lipid composition of DOPC/SM/Chol/DOPS/DOPE at a 21/17.5/30/14/17.5 molar ratio (as shown in Figure 4A).[179] These exosome-mimics were prepared using a thin-film hydration method, with siRNA loading occurring during the hydration step. For comparison, cationic DOTAP liposomes and conventional PC Chol liposomes were prepared. The synthesized exosome-mimics were ∼119 nm in size with a zeta potential of −24 mV, comparable to exosomes reported in the literature. However, the encapsulation efficiency was only 31%, which was lower than the DOTAP liposomes (70%) and PC Chol liposomes (37%). This result is expected, given the electrostatic repulsion between the anionic siRNA and the anionic lipids within the exosome mimic. Through in vitro studies, the exosome-mimics demonstrated cell viability 4-fold higher than the DOTAP liposomes, highlighting the safety of the noncationic delivery systems. Cell uptake in A549 and HUVEC cells displayed that exosome-mimics had better uptake than PC Chol liposomes but significantly worse uptake than DOTAP liposomes. Notably, the cell uptake and gene silencing ability of the exosome-mimics was >3-fold higher than the PC Chol liposomes. When investigating gene silencing effects through the delivery of siVEGF to cells, the exosome-mimics demonstrated >3-fold higher gene silencing than PC Chol liposomes. Despite this, the gene silencing was much lower than the cationic DOTAP liposome formulation, showing room for improvement in future research. This study also exemplifies the challenges to achieving high encapsulation efficiency when formulating exosome-mimics.

Kimura et al. attempted to solve issues with poor RNA encapsulation in exosome mimics by developing a one-step microfluidic synthesis process for exosome-mimicking NPs, outlined in Figure 4B.[180] This process involved solubilizing siRNA in a buffer solution containing Ca^{2+}, which counteracted the anionic charge of the siRNA to reduce electrostatic repulsion. The lipids used in the exosome-mimics (DOPC/SM/Chol/DOPS/DOPE at 21/17.5/30/14/17.5 molar ratio) were solubilized in ethanol. These aqueous and organic solutions were then combined in a microfluidic mixer to form the exosome-mimics, with an optimal flow rate of 500 uL/min at an aqueous: organic ratio of 2. Using this method, the authors achieved an encapsulation efficiency of over 50%, while alternative methods yielded encapsulation efficiencies below 20%. Moreover, when delivering luciferase siRNA in vitro to HeLa cells with the new exosome-mimics, 50% luciferase knockdown and minimal cytotoxicity were achieved. Compared to cationic LNPs, the luciferase expression was significantly lower (80% knockdown in cationic LNPs), but cell viability was improved.

Overall, by mimicking the lipid composition of exosome membranes, Lu et al. demonstrated enhanced in vitro gene silencing and cell uptake performance compared to conventional liposome formulations.[179] However, siRNA encapsulation was still significantly limited as no cationic lipid component was used during preparation, thus leading to electrostatic repulsion. While Kimura et al. developed a microfluidic method to enhance encapsulation and liposomal size control, encapsulation peaked at ∼50%.[180] New strategies to improve the encapsulation efficiency of siRNA should be investigated in future research, such as complexing the siRNA in cationic particles before microfluidic mixing or incorporating an ionizable component during formulation. In addition, these exosome-mimics showed poor results compared to traditional cationic formulations, demonstrating that the lipid composition alone may be insufficient for effective RNA delivery when progressing to in vivo studies. However, these studies also emphasize that exosome mimics can overcome safety challenges related to cationic liposomes. In conclusion, further lipid formulation

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optimization may be required to develop high-performing exosome mimics, given the complexity of exosome structures.

5.4.2. Mimicking Exosome Transmembrane Proteins. To overcome challenges related to cell uptake and organ targeting, recent research has focused on developing exosome-mimetic liposomes that incorporate transmembrane (TM) proteins found in endogenous exosomes. Exosomes secreted from different cells have different compositions and TM proteins, which can generate a cell "homing" effect,[182−184] enabling exosomes to specifically target their origin or "home" cell type for delivery of their cargo. Thus, functionalizing exosome-like liposome formulations with TM proteins presents a strategy to achieve enhanced cell targeting, cell uptake, and RNA delivery. For example, Vázquez-Ríos et al. proposed exosome-mimetic nanoplatforms for targeted cancer delivery by mimicking the structure of tumor-derived exosomes, as illustrated in Figure 4C.[177] Tumor-derived exosomes have tumor-homing properties that enable cancer and metastasis targeting.[177,185] Transmembrane proteins (TMs) found in tumor-derived exosomes, such as integrin $\alpha 6\beta 4$, contribute to this tumor-homing ability.[177] More specifically, integrin $\alpha 6\beta 4$ is involved in lung targeting.[185] For this reason, Vázquez-Ríos et al. used an ethanol-injection methodology to create liposome-based exosome-mimics functionalized with integrin $\alpha 6\beta 4$ for cancer-targeted delivery of miR-145, a well-known tumor-suppressing miRNA.[186] The liposome composition consisted of Chol/PC/SM/Ceramide (Cer) at a 0.9/1/0.4/0.03 w/w ratio. In vitro testing in A549 cancer cells showed that the functionalized exosome-mimetic nanosystems (F-EMNs) resulted in a 5-fold increase in Cy-5 labeled miR-145 expression compared to nonfunctionalized EMNs. This increase in miR-145 delivery was associated with reduced cell proliferation and reduced expression of N-cadherin (a protein that promotes tumor cell survival, migration, and invasion).[187] Overall, this result showed that the F-EMNs resulted in enhanced delivery of functional miR145 to cancer cells. In vivo tests focused on comparing the F-EMNs to tumor-derived exosomes after intraperitoneal injection in mice with lung cancer. Both groups generated similar levels of miRNA in the lung and tumor. Notably, F-EMN-treated mice displayed lower fluorescent signals in their kidneys compared to the exosome-treated group, suggesting that the mimetic system can better target tumors and reduce systemic toxicity.

In addition, Lu et al. developed exosome-mimetic NPs incorporating the TM protein Connexin 43 (Cx43) for siRNA delivery.[112] Cx43 is found in exosomes and is thought to enhance cytosolic delivery.[188] Evidence suggests that Cx43 leads to the docking of exosomes on target cells through a process that forms gap-junction (GJ)-like structures, which transfer exosome payloads into cells through the channel pore.[188] Incorporating proteins like Cx43 in lipid-bilayer NPs typically occurs via cell-based protein preparation methods; however, this can cause protein precipitation, insufficient membrane insertion, and cytotoxicity.[189] Thus, Lu et al. proposed a cell-free synthesis method to generate their Cx43 exosome-mimetic particles, which leverages the lipid bilayer NPs to prevent protein aggregation and facilitate the oligomerization, refolding, and membrane integration of Cx43. Figure 4D shows a schematic of this preparation method. Thin-film hydration was used to prepare exosome-mimetic liposomes composed of DOPC/SM/Chol/DOPS/DOPE (21/17.5/30/14/17.5 molar ratio), which encapsulated a cationic chitosan-siRNA complex. This structure was combined with a Cx43 plasmid and transcription and translation reagents, resulting in the synthesis and membrane integration of Cx43. When delivering the Cx43 liposomes encapsulating a membrane-impermeable dye in vitro, 58% of the cells were dye-positive, while only 5% of cells treated with liposomes lacking Cx43 showed a positive signal. Moreover, when adding a GJ inhibitor, the dye-positive cells decreased by 43% in the Cx43 liposome-treated group, suggesting that the TM protein facilitates cell uptake through GJ mechanisms. Lu et al. also looked at VEGF silencing in U87 MG cells. Cx43 siVEGF liposomes significantly decreased VEGF protein expression (∼30% downregulation) relative to controls, including the liposomes lacking Cx43. When combined with endocytosis and GJ inhibitors, VEGF downregulation was reduced. This result suggested that gene silencing and siRNA delivery was facilitated through clathrin and caveolin-mediated endocytosis, as well as gap junction mechanisms. Lastly, the Cx43 exosome-mimetic NPs exhibited cell viability above 85%, showing minimal cytotoxicity. Despite these positive results, the authors acknowledged that the exosome-mimetic NPs still have inadequate in vitro RNA transfection compared to the lipofectamine commercial transfection agent, indicating that further optimization is required.

To conclude, incorporating TM proteins into exosome-mimetic liposomes resulted in better performance than liposomes lacking TM proteins. By selecting proteins expressed on cell-specific exosomes, effective cell targeting can be achieved, as shown by Vázquez-Ríos et al.[177] Moreover, Lu et al. showed that incorporating TM proteins can enhance cell uptake while being minimally cytotoxic.[112] With the advancements in cell-free synthesis methods, protein-functionalized exosome-mimetic particles can be synthesized by a safer, more efficient, and clinically translatable method. However, as mentioned by Lu et al., the RNA delivery was inferior lipofectamine controls, which suggests that an ionizable lipid element may be required for more RNA to reach the cytosol through enhanced endosomal escape. Nevertheless, adding TM proteins expressed in endogenous exosomes presents a novel method to generate functional exosome-mimetic liposomes with cell-targeting abilities while maintaining biocompatibility and minimal toxicity.

6.0. CONCLUSIONS AND FUTURE OUTLOOK

There are many design strategies to create bioinspired lipid nanocarriers for RNA delivery, with the primary goal being to achieve greater potency than established synthetic liposomes and LNPs. Bioinspired design can enhance several RNA carrier properties, including tissue targeting, cellular uptake, and endosomal escape.

First, substituting LNP components with natural lipids can increase LNP efficacy while maintaining scale-up potential. For example, replacing cholesterol with β-sitosterol modifies LNP surface morphology and increases transfection efficiency. Also, substituting traditional helper lipids with bioderived counterparts shifts the primary organ targets of RNA therapies and can potentially stabilize LNPs during nebulization for effective intranasal and lung vaccines. Finally, incorporating pSar-lipids instead of PEG-lipids decreases unwanted immune activation while maintaining the efficacy of LNPs.

Additionally, mimicking endogenous molecule structures utilizes existing endogenous pathways to target hard-to-reach tissues. Mimicking HDL helps target cells expressing SR-B1 and the Ras pathway in cancers, showing positive outcomes in vivo. Meanwhile, RNA delivery to the colon may be unlocked by

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mirroring GDNPs. However, these strategies are marred by the requirement to synthesize multilayered nanostructures, which can be complicated to scale up.

Taking inspiration from viruses is another method of bioinspired design, given that viruses naturally transfer nucleic acids from cell to cell. Virosomes improve cellular uptake and endosomal escape through their enhanced fusogenicity and have outperformed liposomes for siRNA delivery. However, virosome preparation requires viral protein extraction and results in inefficient siRNA encapsulation. A more feasible methodology is to incorporate PS into LNP membranes to mimic viral membranes. This method achieves enhanced cellular uptake and protein translation in vitro and in vivo due to PS acting as a natural "eat-me" signal. Moreover, RNA delivery to immune cells can be improved using PS due to its macrophage and SLO targeting. Importantly, the seamless incorporation of PS into existing LNPs does not compromise synthesis simplicity or scalability.

Mimicking exosomes by developing liposomal formulations with similar lipid compositions to the natural molecules is a final pathway to developing bioinspired lipid nanocarriers. Exosome-mimetic liposomes have demonstrated enhanced cellular uptake and gene silencing in vitro compared to specific conventional liposomes. Furthermore, incorporating TM proteins into exosome-mimics further increases their performance and enables more specific cell targeting. However, exosome-mimics struggle to achieve suitable encapsulation efficiencies and are outperformed in vitro by traditional cationic liposomal systems (e.g., Lipofectamine). Thus, ionizable lipid components in exosome-mimics should be used to help reconcile the negative charge of RNA during formulation, thereby increasing encapsulation efficiency while maintaining biocompatibility.

To conclude, the current research findings first point to incorporating natural lipids into LNPs as the most advanced and clinically translatable bioinspired design strategy for lipid-based nanocarriers. This method enhances the properties of existing LNPs without sacrificing their current advantages, such as ease of synthesis and scalability. Natural lipid substitution is a good strategy to help overcome RNA delivery challenges, such as the lack of cellular uptake and endosomal escape, to improve LNP potency. However, this method only provides slight modifications to current LNP formulations and thus may be limited in what it can achieve. Overall, further research should be undertaken to optimize the incorporation of natural lipids such as β-sitosterol and PS into state-of-the-art LNP formulations and compare the performance to their unmodified counterparts. Current research also reveals the potential benefits of mimicking endogenous molecules, viruses, or exosomes. Doing so can exploit endogenous pathways to target SR-B1-expressing cells, the Ras pathway in cancer, and other hard-to-reach tissues and cells. Nevertheless, these methods are still immature and have unclear translational potential in their current state. They require multiple components to efficiently encapsulate RNA, thereby increasing synthesis complexity. Thus, exploring ionizable lipids in these systems is suggested to help reconcile the positive charge of RNA while maintaining biocompatibility.

In addition to optimizing RNA carrier systems, several other areas of research in the RNA delivery field should be pursued to fill knowledge gaps and enable better carrier design. First, creating in vitro models that are more predictive of in vivo performance is vital to better screen and develop potent RNA carriers. Moreover, a more profound elucidation of cellular uptake and endosomal escape pathways taken by RNA delivery vehicles is required to improve rational design strategies for the particles. The correlation between cellular uptake and endosomal escape in different cells and organs should also be further investigated. Finally, cell-free protein synthesis methods should be optimized to enable simpler and more efficient virosome and exosome-mimic production.

The further exploration of bioinspired lipid nanocarriers and the other areas above will help overcome the major bottleneck of RNA therapy: delivering RNA safely and efficaciously to target cells. Given the potential of RNA therapeutics, these advancements could substantially impact global health and the lives of those struggling with disease.

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Notes

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ABBREVIATIONS

RNA	ribonucleic acid
COVID-19	coronavirus 2019
LNP	lipid nanoparticle
FDA	food and drug administration
PEG	polyethylene glycol
siRNA	small interfering RNA
mRNA	messenger RNA
DNA	deoxyribonucleic acid
ncRNAs	noncoding RNAs
miRNA	microRNA
dsRNA	double stranded RNA
RNAi	RNA interference
RISC	RNA-induced silencing complex
Ago2	argonaute 2

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hATTR	hereditary transthyretin-mediated
TTR	transthyretin
GalNac	N-acetylgalactosamine
ALA	aminolevulinic acid
PBG	porphobilinogen
PH1	primary hyperoxaluria type 1
PCSK9	proprotein convertase subtilisin/Kexin type 9
ssRNA	single stranded RNA
HCV	Hepatitis C virus
ORF	open reading frame
UTR	untranslated region
VEGF	vascular endothelial growth factor
CFTR	cystic fibrosis transmembrane conductance regulator
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
RNases	ribonucleases
NP	nanoparticles
IV	intravenously
PDI	polydispersity index
HII	hexagonal
ApoE	apolipoprotein E
SORT	selective organ targeting
DSPC	distearoyl phosphatidylcholine
AAA	alkenyl amino alcohol
DOPE	1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine
EPO	erythropoietin
pSar	polysarcosine
Fluc	firefly luciferase
eLNP	enhanced LNP
DGTS	diacylglyceryltrimethylhomo-Ser
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
SM	sphingomyelin
PS	phosphatidylserine
PG	phosphatidylglycerol
PA	phosphatidic acid
EPC	ethyl phosphatidylcholine
HDL	high-density lipoproteins
SR-B1	scavenger receptor type B-1
apoA-I	apolipoprotein A-I
TLP	templated lipoprotein particles
ApoE3	apolipoprotein E3
BBB	blood−brain barrier
ATF5	activating transcription factor-5
GDNP	ginger derived nanoparticle
MGDG	monogalactosyldiacylglycerol
DGDG	digalactosyldiacylglycerol
nLNP	new LNP
IL-22	interleukin-22
HA	hemagglutinin
VSV-g	vesicular stomatitis virus G
PC	phosphatidylcholine
DODAC	*N,N*-dioleoyl-*N,N*-dimethylammonium chloride
GFP	green fluorescent protein
F	fusion
HN	hemagglutinin-neuraminidase
DMKE	*O,O*-dimyristyl-*N*-lysyl glutamate
EGFR	epidermal growth factor receptor
FAST	fusion-associated small transmembrane
PVEERs	PS-mediated virus entry-enhancing receptors
DOPC	1,2-dioleoyl-sn-glycerol-3-phosphocholine
SLOs	secondary lymphoid organs
LNs	lymph nodes
PA	1,2- dioleoyl-sn-glycerol-3-phosphate
SCLNs	superficial cervical lymph nodes
EVs	extracellular vesicles
PE	phosphatidylethanolamine
Chol	cholesterol
TMs	transmembrane
Cer	Ceramide
F-EMNs	functionalized exosome-mimetic nanosystems
Cx43	Connexin 43
GJ	gap-junction.

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