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Perspective

Exciting Times for Lipid Nanoparticles: How Canadian Discoveries Are Enabling Gene Therapies

Miffy H. Y. Cheng,*[,‖] Cedric A. Brimacombe,[‖] Rein Verbeke,[‖] and Pieter R. Cullis

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ABSTRACT: In this brief perspective, we describe key events in the history of the lipid-based nanomedicine field, highlight Canadian contributions, and outline areas where lipid nanoparticle technology is poised to have a transformative effect on the future of medicine.

KEYWORDS: *lipid nanoparticles, gene therapy, mRNA, vaccines, CAR-T therapy, CRISPR-Cas9*

1. INTRODUCTION: HISTORY OF LIPID-BASED NANOMEDICINE

A nanomedicine is a nanoscale entity that often consists of a drug carrier loaded with therapeutic agents. The primary role of these nanomedicines is to deliver bioactive agents to disease sites while preventing release in otherwise healthy tissues, thereby improving therapeutic efficacy and reducing toxicity. In search for suitable carriers a large range of nanomaterials have been investigated as potential nanomedicines, ranging from inorganic metals to polymers and lipids. Throughout the past four decades, Canadian researchers have played a central role in nanotechnology development, particularly in the field of lipid-based nanomedicines.

The development of lipid-based nanomedicines began with the discovery of multilamellar liposomes by Bangham et al. in 1965.[1] Subsequently, Gregoriadis et al. demonstrated that drugs and proteins could be encapsulated within liposomes.[2] In the 1980s, Canadian scientists enabled liposomes as a drug-delivery system by developing a high-pressure extrusion technique to manufacture homogeneous unilamellar liposomal systems[3] and others discovered the "remote loading" process to load weak base drugs into liposomes using a pH gradient.[4,5] Additionally, Canadian scientists explored the pharmacokinetics of liposomes and contributed to the "PEGylation" technologies to prolong blood circulation of liposomes (stealth liposomes) and improve the *in vivo* delivery of encapsulated drugs.[6−8] The pharmaceutical potential of these liposomal formulations stimulated the formation of a number of Vancouver-based companies, including Lipex Biomembranes (1985) to produce the Extruder, the Canadian Liposome Company (1986) to produce Abelcet and Myocet, and Inex Pharmaceuticals (1992) to produce Marqibo and Northern Lipids (1992) to solve drug formulation problems for clients using lipid-based approaches. These and other commercialization efforts around the globe resulted in the rapid development of many formulations of anticancer (Doxil, DaunoXome, Depocyt, Myocet, Marqibo), antimicrobial (Abelcet, Ambisome, Amphotec), and macular degeneration drugs (Visudyne) between 1980 and 2000s.

Liposome technology continued to evolve as gene therapy became topical in the mid-1990s. To avoid the toxic effects of permanently positively charged cationic lipids, ionizable cationic lipids were introduced to enable encapsulation of nucleic acids into lipid nanoparticles (LNPs).[9,10] When the ionizable cationic lipid content exceeds the amount needed to neutralize the negative charge on the nucleic acid, these LNPs exhibit a "solid core" hydrophobic interior[11] surrounded by a monolayer of helper lipids such as distearolyphosphatidylcholine (DSPC). These LNPs are distinct from liposomes, as liposomes have an aqueous interior. These and other critical LNP advances were made by Canadian companies Tekmira Pharmaceuticals (previously Inex), Protiva Biotherapeutics (a spin-off from Inex), Precision NanoSystems, and Acuitas Therapeutics (a spin-off from Tekmira) in collaboration with the University of British Columbia (UBC) laboratory of Cullis. For example, in the 2000s, Semple et al. discovered that the potency of LNP−siRNA systems for gene silencing in the liver could be dramatically improved by incorporating ionizable cationic lipids with a pK_a in the region of 6.4.[12] Other advances that enabled commercialization of LNP includes the discovery of microfluidic mixing for scalable formulation of LNP systems containing nucleic acid−based drugs.[13]

The collaboration between Acuitas, Alnylam Pharmaceuticals (Boston, MA), and the UBC laboratory of Cullis was particularly fruitful and resulted in highly potent LNP−siRNA systems for silencing genes in the liver.[14] This led to the first LNP−siRNA systems for the treatment of the hereditary condition transthyretin-induced amyloidosis (hATTR). Fol-

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lowing a most successful clinical trial,[15] Onpattro was approved by the FDA in 2018 to treat the neuropathies associated with hATTR and became the first systemic RNA interference (RNAi)-based drug to receive regulatory approval.[16] Starting in 2013, Acuitas extended their development of LNP technology to deliver mRNA to the liver, demonstrating that LNP−mRNA systems administered intravenously could result in gene expression in liver.[17] A subsequent collaboration with Professor Weissman (University of Pennsylvania (UPenn), Philadelphia, PA) to investigate the potential of LNP−mRNA systems as vaccines proved particularly successful as demonstrated for the Zika virus.[18] This success led Acuitas to collaborate with BioNTech (Mainz, Germany) to develop an influenza vaccine. When the COVID-19 pandemic hit in early 2020 the LNPs provided by Acuitas was chosen as the delivery system for the development of the Pfizer/BioNTech COVID-19 mRNA vaccine Comirnaty, which has proven to be most effective[19] (Figure 1). Comirnaty is becoming the most successful medicine of all time in terms of doses administered, market size, and global impact on human health.

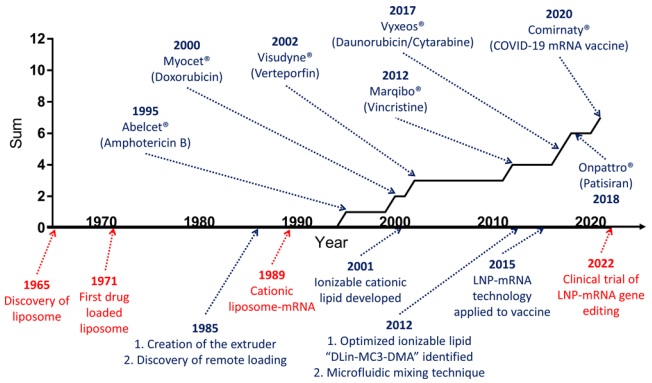

Figure 1. Timeline of regulatory approval of lipid-based nanomedicine and important discoveries contributed by Canadian scientists (in blue). Other key discoveries in liposome and lipid nanoparticle research (in red).

The astonishing commercial success already achieved by drugs enabled by LNP technology developed in Canada is just the beginning. The ability to deliver siRNA or mRNA into cells *in vivo* will enable many forms of gene therapies to treat most diseases. In the rest of this perspective, we highlight several exciting directions for the future of LNP−mRNA therapeutics.

2. LNP−MRNA THERAPEUTICS

The very rapid development of the Pfizer-BioNTech and Moderna mRNA (mRNA) vaccines during the SARS-CoV-2 pandemic provided an extremely effective vaccine, saving countless lives and mitigating the economic and societal damage of the pandemic.[20−22] These dramatic clinical successes have catalyzed an explosion of R&D efforts to exploit LNP-based nucleic acid delivery approaches for a variety of gene therapies. Here we discuss new developments in applications of mRNA-based therapeutics and how they will transform the future of medicine (Figure 2).

2.1. LNP−mRNA Vaccines. While the SARS-CoV-2 pandemic generated significant publicity and momentum for LNP−mRNA vaccine development, the fundamental scientific breakthroughs behind this novel vaccine platform unfolded over decades. The concept behind mRNA vaccines is to trigger immune responses by introducing an mRNA sequence that encodes an antigenic protein within target cells, optimally within specialized antigen-presenting cells (APCs), to produce a robust immune response. The path to safe and effective mRNA vaccines, however, has been extremely challenging, extending over the last 50 years. Fundamental problems included avoiding the immunotoxicity of mRNA, development of nontoxic LNP delivery systems that could encapsulate mRNA efficiently and were also capable of delivering mRNA into target cells *in vivo*, as well as problems such as production and stability. A major breakthrough came when Karikó, Weissman, and their colleagues at the UPenn discovered that the replacement of uridine nucleobases with naturally occurring modified uridines dramatically decreased the immunotoxicity and increased the translation capacity of *in vitro* transcribed mRNA.[23]

An additional challenge was to find a suitable delivery vehicle that could both protect mRNA and facilitate cytoplasmic delivery. Many methods explored the *ex vivo* loading of dendritic cells, "naked" mRNA injections, viral vectors, and polymeric and liposomal nanoparticle formats; of these, LNP delivery systems appear to be the leading technology. While LNP technology was initially focused on systemic delivery of siRNA to hepatocytes, some early reports demonstrated that APCs were also potential targets for LNP−mRNA delivery.[24−26] However, it was not until 2012 that the use of LNPs for mRNA vaccines was first reported.[27] In this study, Geall and co-workers at Novartis built on the success of the LNP formulations of siRNA and formulated a self-amplifying mRNA in LNPs containing an ionizable lipid.[27] This LNP−mRNA formulation, when injected intramuscularly in rodents, was as potent as viral delivery technology with the major advantage of removing the inherent limitations of viral vectors.

Between 2015 and 2017, the potential of LNP technology for delivery of normal (non-self-amplifying) mRNA was also demonstrated. In collaboration with Acuitas Therapeutics, the Weissman Lab at UPenn used LNP−mRNA systems to evaluate the functionality of their nucleoside-modified mRNA constructs, with a first proof-of-concept by Pardi et al. demonstrating successful mRNA expression kinetics by various routes in mice,[28] followed by a series of preclinical studies showing that these LNP−mRNA vaccine systems could induce potent neutralizing immune responses against viral diseases such as Zika[18] and Influenza.[29] CureVac also reported success using Acuitas' LNPs to deliver sequence-engineered mRNA to develop an effective rabies vaccine.[30] Moderna was the first to perform a first-in-human phase I clinical trial for a prophylactic flu vaccine composed of very similar LNPs and *N*1-methyl pseudouridine (m1ψ)-modified mRNA.[17] These pioneering studies laid the foundation for LNP−mRNA vaccines, a revolution in vaccinology, at just the right time.

To date, two m1ψ-modified mRNA vaccines, Comirnaty (BioNTech/Pfizer) and SpikeVax (Moderna), have received FDA approval for use against COVID-19, and several other mRNA vaccines are on the horizon, such as the self-amplifying mRNA vaccine ARCT-154, designed by Arcturus Therapeutics, and CureVac's second-generation vaccine, CV2CoV, developed in collaboration with GSK. These vaccines represent different formats of mRNA vaccines, but they all utilize LNPs as the delivery system.[20] Interestingly, it has recently been shown that LNPs, initially designed as a delivery system, also act as very potent immune adjuvants.[31] This partially explains

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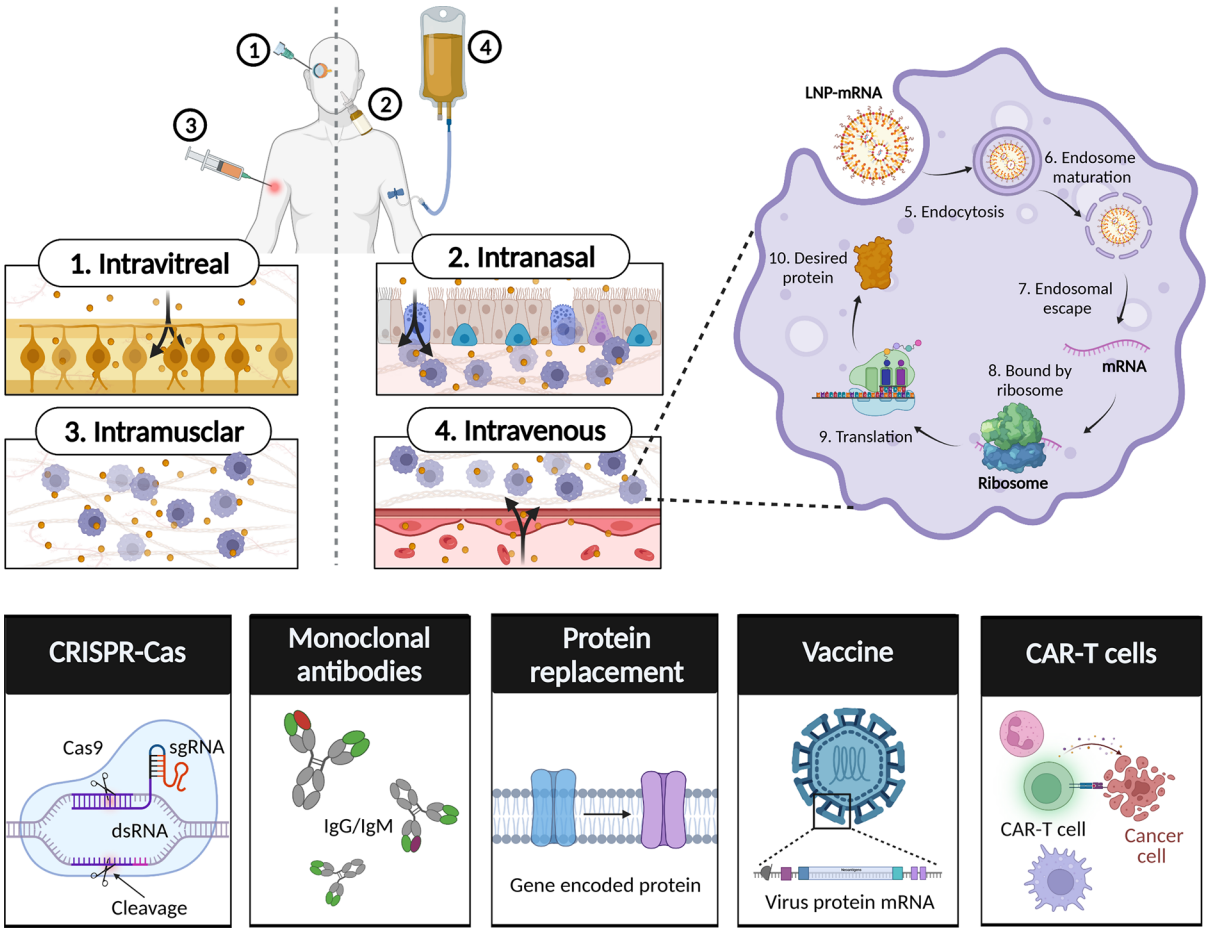

Figure 2. Examples of how the LNP nanotechnology enables the translation of mRNA-therapeutics for a variety of therapeutic applications.

their remarkable potency for mRNA vaccination purposes. However, the underlying molecular mechanisms behind the adjuvant properties of LNPs remain unclear. Furthermore, in-depth investigation is warranted with regard to whether the immune reactions to LNP systems can compromise the benefits of LNP−mRNA therapeutics used for nonimmunogenic purposes.[32] Immunogenicity of LNPs remains a subject of debate; a potential concern that limits the long-term use of LNP−mRNA is the accelerated blood clearance phenomenon, which can result in a dramatic decrease in protein expression upon second and subsequent administrations.[33] However, the latest research from Moderna's phase 1 in human clinical trial against Chikungunya virus showed an unchanged pharmacokinetic profile and a lack of anti-PEG antibodies detected between the first and second dose of LNP−mRNA.[34]

We can expect that LNP−mRNA vaccines will continue to improve in the future; there are ample opportunities. It should be recognized that the LNP−mRNA systems currently employed as vaccines were largely designed to maximize gene expression in the liver following intravenous administration. Studies to develop novel ionizable lipids and new lipid compositions will likely allow the design of safer and more effective LNP−mRNA vaccines. Design of LNP−mRNA systems for different routes of administration such as mucosal delivery is an attractive avenue for further exploration due to its convenient route to induce mucosal immunity. LNPs have played a major role in the success of mRNA vaccines as a result of their very potent delivery efficiency and unique immune adjuvant properties. It is likely that LNP−mRNA vaccines will be the dominant vaccines of the future.

2.2. LNP−mRNA for Reprogramming Immune Cells. LNP−mRNA systems have many other biomedical applications beyond prophylactic vaccines. One of the most exciting new areas of therapy in development is that of LNP−mRNA-based chimeric antigen receptor T cell (CAR-T) immunotherapies. CAR-T is an immensely powerful immunotherapy platform where patient T cells are harvested and reprogrammed *ex vivo* to express a CAR, which enables them to bind specific epitopes on virtually any cell to target them for T cell mediated destruction.[35] CAR-T therapies have had remarkable success in inducing long-term remission in some B cell leukemias;[36] however, this approach is greatly hindered from broad-scale applicability due to the requirement for patient leukapheresis, GMP facilities, and the use of viral vectors for CAR gene delivery. To be truly transformative, the development of an off-the-shelf CAR-T therapy will be necessary, and LNPs offer an avenue for delivery of CAR encoding mRNA via LNPs directly *in vivo*.

A particularly exciting proof-of-concept demonstration of this approach was published by Rurik et al.[37] In this study, mRNA encoding a CAR which targeted fibroblast activation protein (FAP), a marker of activated fibroblasts that secrete excessive extracellular matrix in many chronic heart conditions, was encapsulated into LNPs decorated with a T cell targeting anti-CD5 targeting antibody. After infusion, transient FAP CAR expression was observed in 17.5−24.7% of murine T cells after 48 h; notably, multiple T cell subtypes were transfected.

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In a model of cardiac-injury induced fibrosis, injection of anti-CD5−LNP−FAP CAR mRNA resulted in improved cardiac function after injury due to reduced fibrosis mediated by *in vivo* generated CAR T cell targeting of overactive cardiac fibroblasts. Impressively, these LNP-derived CAR T cells functioned similarly to equivalent *ex vivo* generated cells. This study also highlights how CAR-T therapies have treatment applicability beyond B cell leukemias and other cancers (in this case, cardiac fibrosis) and many other novel applications are conceivably possible.

Key challenges which need to be overcome to successfully develop an off-the-shelf CAR-T therapy based on this technology are as follows: (1) identifying similar CAR-mRNA−LNP formulations that do not require targeting ligands for T cell transfection, which are costly and inhibitory for large-scale application, (2) developing CAR-based treatments for both solid tumors and noncancer diseases, and (3) engineering LNP−mRNA-based systems for generating other CAR-expressing immune cell types such as macrophages, natural killer cells, and neutrophils, all of which have demonstrated activity in preclinical studies, including some for solid tumuors.[38−40] We anticipate that the spectrum of druggable conditions using an off-the-shelf CAR-mRNA−LNP formulation will be enormous. We also note that reprogramming of immune cells in this fashion is not limited to the expression of CAR molecules, and most conceivable protein(s) can be artificially expressed for reprogramming purposes.

2.3. LNP−mRNA Gene Editing. Another LNP-based technology that has received considerable attention is an LNP−mRNA system encoding gene-editing machinery, which deliver proteins such as CRISPR−Cas9 and CRISPR−Cas9−base editors to correct specific mutations in genetic diseases when there is a single causative defective gene. A particularly attractive aspect of using CRISPR-based gene editing approaches is the potential for "curative" levels of gene editing for diseases, removing the necessity for repeated treatment infusions, and as such, this area of investigation has garnered significant interest and investment. The progress has been sufficiently rapid that there are now at least three clinical trials ongoing using LNP-based delivery of CRISPR mRNA (NCT04601051, NCT05120830, and NCT04560790). One such trial is building on the genetic knockout of TTR, the same target gene as the Onpattro RNAi-based LNP drug; in preclinical studies, authors demonstrated that a single administration of coencapsulated Cas9 mRNA and an optimized guide sequence achieved permanent gene editing (a gene knockout via nonhomologous end joining) in up to 60% of mouse hepatocytes, which resulted in >97% reduction of TTR levels in the blood.[41] In phase 1 clinical trial, TTR knockout was achieved in participants receiving an mRNA dose of 0.3 mg/kg, resulting in an 80%−96% reduction in serum TTR level,[42] demonstrating a rapid translation into clinical settings.

Other efforts include using CRISPR−Cas9−base editors, which are attractive as they can make precise alterations without double-stranded DNA breaks, which also allows them to be used in nondividing or slowly dividing cells. These fusion proteins consist of a catalytically impaired Cas9 linked to a cytidine or adenine deaminase, which can convert C/G to T/A or A/T to G/C without requiring homologous recombination.[43,44] An exciting application of base editors is the development of LDL cholesterol reducing treatments by inactivation of the PCSK9 gene. PCSK9 was identified as a candidate because the natural loss of function mutations (2−3% in some populations) resulted in lowered levels of low-density lipoprotein (LDL) cholesterol in the blood, with no other notable health consequences;[45−47] most PCSK9 is produced in the liver, where LNP-based RNA delivery is highly effective.[48] In a recent landmark study performed in nonhuman primates, it was shown that a single administration of LNP CRISPR base editor mRNA with a precisely designed guide resulted in very high levels of PCSK9 knockout in the liver (∼90%) and a resultant reduction of LDL cholesterol of ∼60%. These improvements were stable for up to 8 months after a single dose, demonstrating the powerful potential for single treatments for cholesterol reduction and prevention of atherosclerotic heart disease, which is the leading cause of death worldwide.[49] The potential for single-dose curative therapies by gene editing is incredible, and these developments are only the tip of the iceberg with regard to applications and new treatments.

Major challenges for CRISPR-based gene editing are to minimize off-target editing while selecting for on-target mutagenesis; therefore, it is vital for the LNP delivery system to both selectively and specifically deliver editing machinery to the targeted tissues and cells. Successful on-target editing also depends on the administration route of LNP−mRNA, which dramatically affects LNP−mRNA biodistribution, and subsequently, Cas9 protein expression and therapeutic outcome.[50] It is important not only to understand the functionality of the LNP−mRNA platform but also to identify whether the disease state is playing a role in LNP organ/cell tropism. Despite these ongoing challenges for nanomedicine, new high-throughput screening methodologies for identifying organ- or cell-specific transfection-competent LNP formulations are being rapidly developed and implemented,[51−54] and these limitations are likely to be overcome as LNP technology further matures.

3. CONCLUSION

LNP−mRNA therapeutics are driving an explosion of advances for the treatment of acute and chronic disease as well as vaccines. The sheer speed at which these new therapies can be developed is revolutionizing medicine. None of these dramatic advances in therapeutics would be possible without the lipid nanoparticle delivery systems described in this perspective, highlighting the transformational role of this Canadian-developed nanotechnology.

AUTHOR INFORMATION

Corresponding Author

Miffy H. Y. Cheng − *Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada;* orcid.org/0000-0002-0261-4642; Email: miffy.cheng@ubc.ca

Authors

Cedric A. Brimacombe − *Polymorphic BioSciences, Vancouver, British Columbia V6T 1Z3, Canada*

Rein Verbeke − *Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada; Ghent Research Group on Nanomedicines, Faculty of Pharmacy, Ghent University, 9000 Ghent, Belgium;* orcid.org/0000-0003-1849-5411

Pieter R. Cullis − *Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British*

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Columbia V6T 1Z3, Canada; orcid.org/0000-0001-9586-2508

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.molpharmaceut.2c00365

Author Contributions

‖M.H.Y.C., C.A.B., and R.V. contributed equally to this work.

Notes

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