

Application of Liposomes in Non-Viral Vectors

Fengyang Wang *

Engineering School, University of Pennsylvania, Philadelphia 19104, USA

* Corresponding Author Email: fengyang@seas.upenn.edu

Abstract. With the development of pathology, the traditional way of drug delivery is gradually unable to meet the needs of increasingly complex cancers, genetic diseases, and other drug delivery needs, so gene delivery was born. Precise, efficient delivery reduces damage to normal cells, improves treatment effect, and reduces the amount of drug used, hence the growing academic interest in gene delivery. To design more efficient and less harmful delivery vectors, non-viral vectors are gradually replacing the traditional viral vectors. As the most advanced non-viral vectors, lipid nanoparticles (LNPs) are widely used, such as Pfizer's COVID-19 vaccine — a vaccine with a prevention efficacy of up to 75% was designed within a short research and development cycle. This article will summarize the existing information on LNPs as non-viral vectors, including delivery processes, target recognition, existing drawbacks, etc., to find vector designs with higher delivery efficiency, lower toxicity, and more generalizability.

Keywords: Gene delivery, non-viral vectors, LNPs, Ionizable lipid.

1. Introduction

Gene delivery therapy is a method of directly transferring exogenous genes into diseased and damaged cells through vectors to treat cells. Compared with traditional drug delivery methods, gene delivery can specifically target specific genes of diseased cells and precisely deliver genetic drugs to specific cells, thereby effectively treating diseases while reducing damage to healthy cells. In addition, because the principle of gene delivery is to fundamentally correct or replace defective genes, it does not treat the symptoms but not the root cause of the disease like traditional treatment methods, and can effectively prevent the recurrence of the disease.

Generally speaking, gene delivery vectors are usually divided into three categories, (i) viral vectors; (ii) non-viral vectors; and (iii) physical methods. A viral vector is usually a modified virus that contains the genes that need to be delivered, but its original genetic sequences that may cause disease have to be removed. One of its main features is the ability to permanently integrate genes into the host cell's genome, thereby enabling long-term gene expression. This means that the effects of the gene can be long-lasting, rather than temporary. Non-viral vectors are usually composed of liposomes, polymers, naked DNA, and nanoparticles. They form stable complex particles by combining with therapeutic DNA, thereby protecting it from degradation and helping it to enter the target cell efficiently. The physical method usually uses artificial means to bypass the vector and directly transplant the gene into the target RNA [1].

In the early days, the scientific community conducted a lot of research on viral vectors. In recent years, as the pursuit of therapeutic effects has become higher and higher, the research on non-viral vectors has also become more and more in-depth. For example, nanoparticles of cyclodextrin polymers were used for the first targeted delivery of small interfering RNA (siRNA). The self-assembly properties of this nanoparticle enable it to effectively deliver siRNA to specific cells to reduce the expression of disease-related genes. This technology shows great potential in treating certain diseases, especially cancer [2]. In addition, there are other different types of non-viral vectors, and this article mainly focuses on elaborating the working principle, advantages, disadvantages, and other properties of the most advanced LNP vectors among non-viral vectors, thereby inspiring the design of more advanced delivery vectors.

2. Composition of LNPs

Usually, the main components of LNPs are ionizable lipids, phospholipids, cholesterol, and PEGylated lipids, as shown in Figure 1 [3]. Ionizable lipids are the most important among the four components. This is because they are key structures for systems with capabilities such as RNA delivery, cell phagocytosis, biocompatibility, degradability, mouldability, and stability and protection. They are generally classified into five major categories, unsaturated ionizable lipids, multi-tail ionizable lipids, ionizable polymer-lipids, biodegradable ionizable lipids, and branched-tail ionizable lipids (Figure 2).

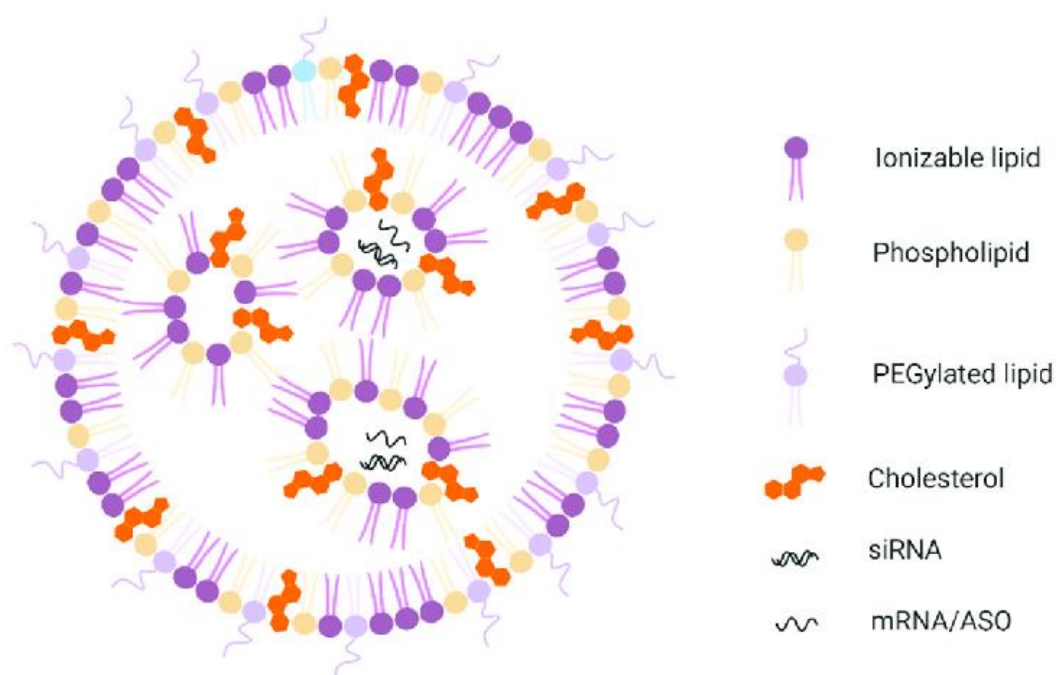


Fig. 1 Typical lipid nanoparticle structure [3].

Ionizable lipids are neutral at physiological pH, have low biological toxicity, and cause almost no harm to the human body. They are positively charged in the acidic environment of the laboratory and thus can combine with negatively charged RNA to form a stable LNP structure [4]. This enables efficient encapsulation and release of RNA molecules. Their ionizable heads have good biocompatibility, which can help the LNPs system interact with the cell membrane of target cells, increase cell phagocytosis, and can be quickly catabolized after releasing the cargo, reducing the risk of long-term accumulation. By changing their structure, the fitness, surface charge distribution, and stability of LNPs can be tuned to meet different therapeutic needs.

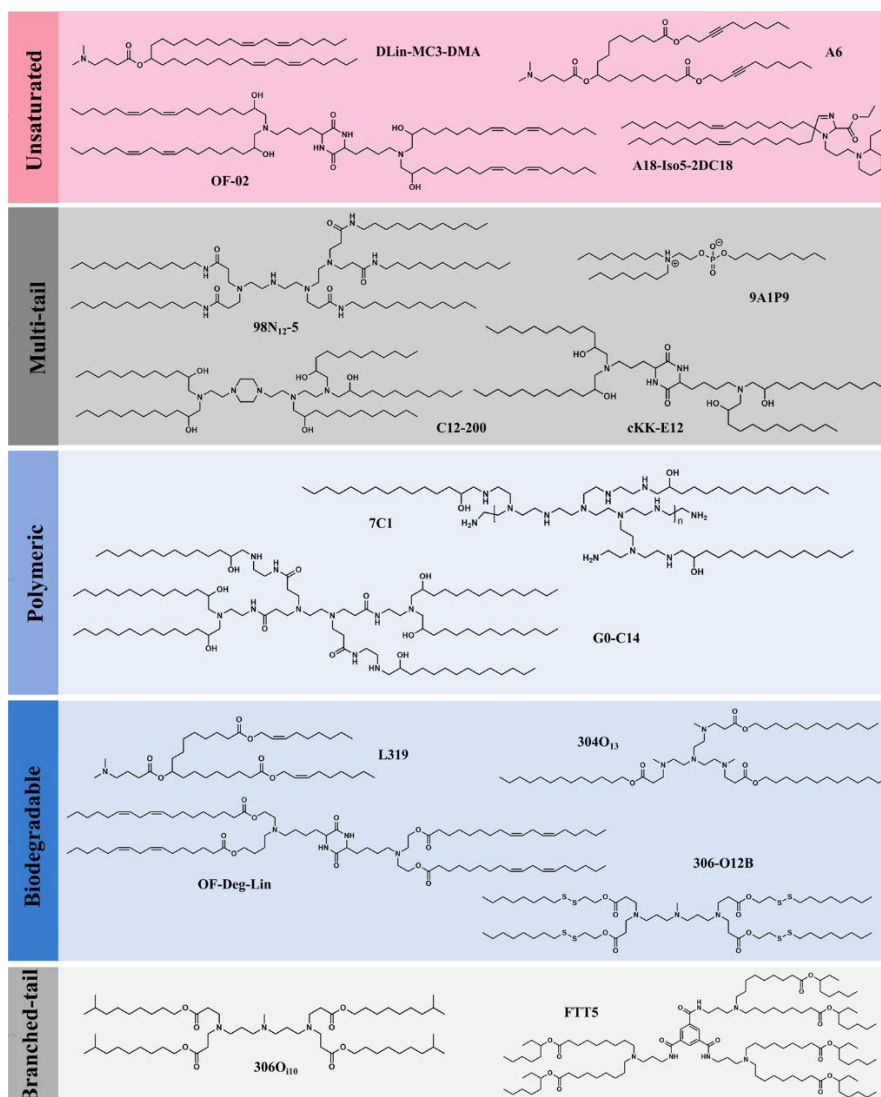


Fig. 2 Some examples of the 5 ionizable lipids categories [4].

3. Delivery Process of LNPs

The entire delivery process consists of three main steps: LNP formation, cellular endocytosis, and RNA release.

First, the synthetic process of LNPs is usually carried out in a laboratory environment. Ionizable lipids are neutral under physiological pH conditions. After artificially lowering the environmental pH value, the head groups of ionizable lipids will be protonated and become positively charged. These positively charged groups attract each other to the phosphate groups of the negatively charged nucleic acids and encapsulate the nucleic acids. At this time, the non-polar tails of these ionizable lipids face outward and combine with the tails of a large number of other non-polar structures, including polyethylene glycol (PEG)-lipid, cholesterol, phospholipid, and other free ionizable lipids, to form a structurally stable vesicle, which is LNPs.

Subsequently, to safely enter the human body environment, the pH of the LNP solution needs to be restored to a level close to the physiological pH. Since the membrane structure of LNPs contains a large number of lipid structures (PEG-lipid, etc.) that are not affected by pH changes, these structures protect the combination of ionizable lipids and RNA from re-dissolving and recovering in a high pH environment [5]. As it is, the stability of LNPs and their efficacy after entering the human body environment are maintained.

After entering the human body circulation penetrating the cell membrane of diseased cells and entering the cytoplasm [6], these LNPs will be engulfed by endosomes. As the endosome matures, the pH inside it will gradually decrease and eventually be lower than the environmental pH when LNPs are formed.

A low pH environment will re-protonate ionizable lipids, and these lipids will interact with the lipids in the endoplasm membrane structure to form a conical inverted hexagonal H_{II} phase structure (Figure 3), resulting in the endoplasm membrane structure being destroyed, thereby releasing the cargo, completing the process of cellular endocytosis and RNA release [7].

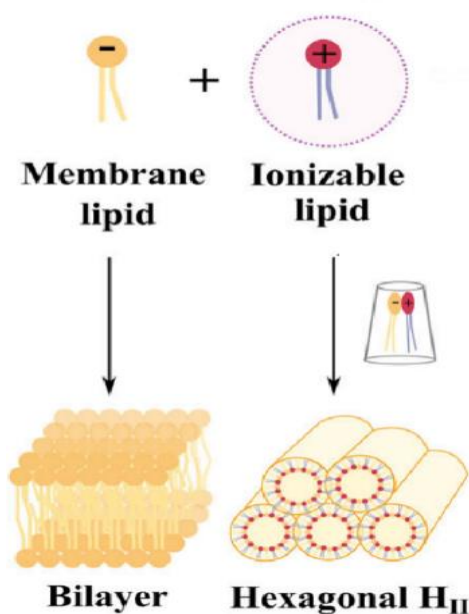


Fig. 3 Bilayer and Hexagonal H_{II} structures [7].

4. Benefits of LNPs

Since the viral vector itself is part of the virus, even if the disease-causing genes are deleted, there is still no guarantee that it will not be toxic. Studies have shown that viral vectors may cause significant immunogenicity, induce the inflammatory system, and lead to adverse reactions such as degeneration of transduced tissues [8]. At the same time, due to the structure of the virus itself, the size of the nucleic acid that can be delivered is strictly limited [9]. Non-viral vectors can perfectly solve these problems. Since they are composed of lipids and proteins, the safety of non-viral vectors can be fully guaranteed. At the same time, since they can be customized for a specific RNA, the size of the RNA to be delivered can be completely unlimited. In the early stage, due to problems such as the specific binding of non-viral vectors to target cells and cell membrane penetration, viral vectors were more widely used. However, with the deepening of research, these problems have been gradually improved, and non-viral vectors have the potential trend to replace viral vectors.

As the most advanced non-viral vectors so far, LNPs have extremely high delivery efficiency compared with other non-viral vectors. Take Pfizer's COVID-19 mRNA vaccine as an example, according to Pfizer's official data, the BNT162b2 vaccine has a nearly 95% probability of completely preventing COVID-19 infection in Phase III clinical trials. The study included 43,448 participants, 21,720 of whom received the BNT162b2 vaccine. Among vaccinated participants, only eight cases of COVID-19 occurred seven days after the second dose, compared with 162 cases in the group that received a placebo [10]. The vaccine can produce very good results in a short period of time, which reflects the extremely high transport efficiency, precise delivery ability, very stable structure, and excellent mRNA protection of LNPs.

In addition, LNPs generally have lower toxicity and immunogenicity, making them safer and tolerable in clinical applications. And because of its relatively simple structure, it can be structurally adjusted at any time according to different delivery "cargoes" and target cells, which can improve its delivery efficiency and reduce side effects.

5. Targeting of LNPs

LNPs have a phenomenon that they easily accumulate near the liver, that is, liver-enrichment properties. According to existing research, if no intervention is performed, approximately 30%-90% of LNPs will spontaneously accumulate near the liver. Therefore, if the cells that need to be treated are not located in the liver, a very serious off-target phenomenon will occur [11].

To overcome this shortcoming, the reason for the concentration of LNPs in the vicinity of liver cells should first be investigated. The liver has a specialized network of blood vessels called sinusoids. The wider diameter of these blood vessels causes the blood flow rate to slow down at this site, which greatly increases the probability of interactions between substances in the blood and liver cells, ultimately causing LNPs to penetrate the blood vessel walls and enter the liver tissue [12].

Based on this theory, changing the pKa of LNPs is a feasible method to enhance targeting. The surface charge of LNPs will affect the binding ability to the cell membranes of different cells, because the pH microenvironments of different organs are different, so LNPs with different pKa can be more accurately gathered near different tissues. At the same time, changing pKa can also prevent LNPs from gathering together prematurely and being cleared by the immune system. Studies have shown that when pKa is between 2 and 6, LNPs are mainly concentrated in the spleen; when pKa is between 6 and 7, LNPs are mainly concentrated near liver cells; and when pKa rises above 9, LNPs are mainly concentrated in the lungs [13]. The adjustment of Ka is a relatively less difficult and more cost-controllable method for LNPs. Therefore, this method is now commonly used to achieve targeted positioning of LNPs.

6. Existing technology deficiencies in LNPs

Of course, the existing LNPs technology is not perfect. For example, if cholesterol is one of its components, its three-dimensional structure can affect the recognizability of the entire lipid system. Its isomers may have a significant impact on delivery. Some LNPs require the use of plant sterols, but the yield of this substance is very low, and the high cost could restrict the application of such LNPs. These problems are exactly those encountered in the process of industrialization. In addition, for LNPs themselves, the distribution of different lipid components is a concern. The lipid components might not be stable enough during long-term storage. The mechanism of preventing RNA degradation is still unclear, and the spatial structure of LNPs themselves may also play a role. The factors that specifically determine the ability of each LNP to encapsulate the amount of RNA are not yet clear. There are currently no precise answers to these questions. Therefore, LNPs still require more in-depth research to better serve mankind [14].

7. Conclusion

Gene delivery has become a popular treatment method today because it is more efficient, highly targeted, and has fewer side effects than traditional drug delivery methods. Lipid nanoparticles (LNPs), as the most advanced non-viral gene delivery vehicles, have great advantages in treating genetic diseases such as cancer and hereditary diseases. Whether it is far safer than viral vectors or higher loading efficiency than other non-viral vectors, LNPs have established their position in the field of gene delivery.

The delivery process of LNPs includes three steps: encapsulation, endocytosis, and nucleic acid release. Through electrostatic binding to nucleic acids and protection of the lipid structure that is not

affected by pH, nucleic acids can be safely and stably delivered to designated sites, greatly improving exogenous resistance. Therapeutic efficiency of nucleic acids.

Of course, the targeting accuracy of LNPs is also subject to various challenges. Without human intervention, LNPs are easily enriched in liver tissue. By adjusting the pKa of LNPs, its enrichment site can be adjusted, thereby improving the targeting of the delivery system.

Meanwhile, LNPs currently have certain shortcomings, including understanding the distribution and stability of different lipid components, which is critical to the efficacy and shelf life of LNPs. The exact mechanism by which LNPs protect RNA from degradation also remains unclear. Furthermore, the spatial structure of LNPs may greatly affect their functionality, especially in terms of RNA encapsulation ability. These issues indicate that LNPs require more and more in-depth research to adapt to increasingly complex disease treatment theories.

Overall, the application of LNPs in gene delivery is promising but requires continued research and development. Efforts should be focused on perfecting targeting capabilities, minimizing immune responses, and improving release kinetics to fully realize the potential of LNPs as safe, effective, and revolutionary gene delivery systems.

References

- [1] Kamimura K, Suda T, Zhang G, Liu D. Advances in gene delivery systems. *Pharmaceut Med.* 2011, 25(5): 293-306.
- [2] Yin H., Kanasty, R., Eltoukhy A. et al. Non-viral vectors for gene-based therapy. *Nature Reviews Genetics*, 2014, 15: 541-555.
- [3] Wahane A., Waghmode A., Kapphahn A., et al. Role of lipid-based and polymer-based non-viral vectors in nucleic acid delivery for next-generation gene therapy. *Molecules*, 2020, 25: 2866.
- [4] Han X., Zhang H., Butowska K., et al. An ionizable lipid toolbox for RNA delivery. *Nature Communications*, 2021, 12: 7233.
- [5] Suzuki T., Suzuki Y., Hihara T., et al. PEG shedding-rate-dependent blood clearance of PEGylated lipid nanoparticles in mice: faster PEG shedding attenuates anti-PEG IgM production. *International Journal of Pharmaceutics*, 2020, 588: 119792.
- [6] Lin J.C., Tam Y.Y.C., Hafez I., et al. Influence of cationic lipid composition on the uptake and intracellular processing of lipid nanoparticle formulations of siRNA. *Nanomedicine: Nanotechnology, Biology and Medicine*, 2013, 9(2): 233-246.
- [7] Semple S.C., Akinc A., Chen J., et al. Rational design of cationic lipids for siRNA delivery. *Nature Biotechnology*, 2010, 28(2): 172-176.
- [8] Gardlík R., Pálffy R., Hodosy J., et al. Vectors and delivery systems in gene therapy. *Medical Science Monitor*, 2005, 11(4): 110-121.
- [9] Katare D.P., Aeri V. Progress in gene therapy: a review. *IJTPR*, 2010, 1(33): e41.
- [10] Polack FP, Thomas SJ, Kitchin N, et al. C4591001 Clinical Trial Group. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *New England Journal of Medicine*, 2020, 383(27): 2603-2615.
- [11] Soutschek J., Akinc A., Bramlage B., et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature*, 2004, 432: 173.
- [12] Gyanani V., Goswami R. Key design features of lipid nanoparticles and electrostatic charge-based lipid nanoparticle targeting. *Pharmaceutics*, 2023, 15(4): 1184.
- [13] Dilliard S.A., Cheng Q., Siegwart D.J. On the mechanism of tissue-specific mRNA delivery by selective organ targeting nanoparticles [Medical Sciences]. *Proceedings of the National Academy of Sciences of the United States of America*, 2021, 118: e2109256118.
- [14] Eygeris Y., Gupta M., Kim J., et al. Chemistry of lipid nanoparticles for RNA delivery. *Accounts of Chemical Research*, 2021, 55(1): 2-12.