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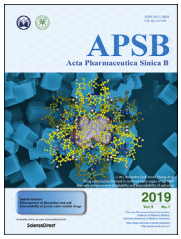

REVIEW

Lipid nanomaterials-based RNA therapy and cancer treatment

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Abstract We summarize the most important advances in RNA delivery and nanomedicine. We describe lipid nanoparticle-based RNA therapeutics and the impacts on the development of novel drugs. The fundamental properties of the key RNA members are described. We introduced recent advances in the nanoparticles to deliver RNA to defined targets, with a focus on lipid nanoparticles (LNPs). We review recent advances in biomedical therapy based on RNA drug delivery and state-of-the-art RNA application platforms, including the treatment of different types of cancer. This review presents an overview of current LNPs based RNA therapies in cancer treatment and provides deep insight into the development of

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future nanomedicines sophisticatedly combining the unparalleled functions of RNA therapeutics and nanotechnology.

1. Introduction

Given the recent developments in scientific and medical technologies, the use of biological molecules as therapeutic agents has become an increasingly popular research topic. Among the identified biological molecules, RNA stands out owing to its unique biological functions and specific characteristics[1]. RNA was originally considered simple, intermediate transmission products that arise during DNA transcription. However, increasing research has reviewed the various roles of RNA and has indicated that they are co-related to most biochemical pathways[2]. One of the most exciting discoveries was using RNA as therapeutic molecules, which led to intense scientific research to develop therapeutic RNA and a constant stream of discoveries and outcomes in this field in recent years[3].

Recently many RNA-based drugs have become the rising stars in pharmacotherapy. However, there are some important challenges. One of the largest obstacles in the clinical use of RNA-based drugs is to deliver RNA *in vitro* or *in vivo*[4]. To develop clinically effective RNA-based drugs, some difficulties must be overcome. The first is the robust defense system of most human cells that keeps exogenous RNA outside cell membranes, as naked RNA are large, negatively charged molecules[5]. RNA intrusion can also trigger the immune system. Different levels of inflammatory responses may occur when introducing certain RNA into the human system[6]. Furthermore, naked RNA is unstable and must be guarded against its tendency to degrade during the delivery process. Thus, most RNA-based drugs require protection to maintain their RNA integrity[7]. Ground-breaking drug delivery nanoplatforms are being rapidly developed, which has led to remarkable progress in RNA-based therapies in the previous five years. For example, several nanostructured vehicles for RNA delivery were designed, manufactured, and tested to guarantee RNA delivery efficacy and protection. Nanotechnology-based systems were also developed to overcome or bypass the barriers in the human body, thus enabling RNA-based drugs to perform their biochemical functions against their intended targets[8]. The merging of nanoscience and bioactive molecule discoveries has clearly led to striking advances in the development of a new era of RNA-based drugs, and this will lead to a revolution in future pharmacological treatments of cancer and other diseases.

2. The introduction of various RNA therapeutics

RNA therapies can enable targeted nucleic acid sequence delivery to edit specific genetic anomalies or mutations (*e.g.*, down-regulation, augmentation, or correction)[9]. Given their reportedly outstanding therapeutic effects, RNA has become a favorable treatment agent for several diseases. The advantages of RNA therapies include the following: (i) the possibility for patient-specific treatments with relatively low cost and high safety levels. (ii) the same type of encoded RNA may perform different cell regulating functions when applied in different therapies. (iii) the RNA sequence design and synthesis are relatively simple. Most importantly, when compared with DNA therapies, RNA therapies keep the host genome intact, as they do not need to enter the nuclear membrane to initiate cytoplasmic protein translation[10]. Prompted by the above characteristics, more customized therapies with improved accuracy have been developed for different diseases (Table 1).

Common RNA therapy approaches can be categorized into coding and noncoding. A major part of the coding RNA approach works on gene stimulation, as it triggers coded-protein antigen synthesis. It affects antibody and cytotoxic lymphocyte production, which consequently induces correlated immunity. In contrast, other small percentages of coding RNA modulate the production and activation of constitutive and functional proteins that can be used for protein supplementation therapy[11]. The noncoding RNA (ncRNA) approach works on gene silencing, as it silences single or multiple related genes to inhibit the production of encoded proteins.

Various types of RNA have been used in RNA therapies, and in the following sections, the primary RNA therapeutics are described and discussed (Fig. 1).

2.1. *mRNA*

Messenger RNA (mRNA) is a type of coding RNA, and each is a single-stranded structured nucleotide sequence produced during complementary DNA transcription. Specifically, three ribonucleotides form one codon, a series of organized codons form one nucleotide sequence, and the nucleotide sequence forms a strand of mRNA, ranging from 300 to 5000 kDa. During the transport of genetic information, mRNA functions as an intermediate agent for protein synthesis between nuclear DNA and the cytoplasm[12]. As mentioned earlier, mRNA can initiate the coding process without crossing the nuclear barrier, and importantly, they do not insert into the host genome. Furthermore, most mRNA naturally degrades in cells after translation and makes them safe to use in patients. Therefore, mRNA applied as a therapeutic agent is superior to other agents in high efficacy and guaranteed safety with each dose. Other advantages of mRNA therapies that make them efficient and desirable for future development include a fast pesticide effect, cost-effectiveness, and the possibility of *in vitro* production[13].

However, the development of mRNA as therapeutics has been limited by their relative size, stability, biological activity, immunogenicity, and translation and delivery efficiency. In order to solve these obstacles limiting the clinical applications of mRNA, their modifications, including nucleotide substitutions and sequence optimizations, are widely investigated. So far, some mRNA, after the processing of architectonic stabilization and chemical treatments, exhibit significantly improved sensitivity to enzymatic degradation and host immunity[14].

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Table 1 Customized RNA therapies developed for different types of chronic diseases.

Disease	RNA	Delivery	Status	Company
Macular degeneration	Aptamer (RNA)	Intravitreal	FDA approval in 2014	Bausch + Lomb
Spinal muscular atrophy	ASO	Intrathecal	FDA approval in 2016	Ionis
Duchenne muscular dystrophy	ASO	Intravenous	FDA approval in 2016	Sarepta
Polyneuropathy	siRNA	Intravenous	FDA approval in 2018	Alnylam
Familial amyloid polyneuropathy	ASO	Subcutaneous	FDA approval in 2018	Ionis
Acute hepatic porphyria	siRNA	Subcutaneous	FDA approval in 2019	Alnylam
Primary hyperoxaluria type 1	siRNA	Subcutaneous	FDA approval in 2020	Alnylam
Familial chylomicronemia syndrome	ASO	Subcutaneous	EU approval in 2019	Ionis
COVID-19	mRNA	Intramuscular	FDA approval	Moderna
Brain cancer	Aptamer (RNA)	Intravenous	Phase I/II	NOXXON
Solid tumors	mRNA	Intravenous	Phase I/II	BioNTech
COVID-19	mRNA	Intramuscular	FDA approved	BioNTech and Pfizer
Advanced melanoma	mRNA	Intratumoral	Phase I/II	BioNTech/Sanofi/ Genmab
Generalized myasthenia gravis	mRNA	Intravenous	Phase I/II	Cartesian
Cystic fibrosis	mRNA	Inhalation	Phase I/II	Translate Bio
Solid tumors/lymphoma/advanced ovarian carcinoma	mRNA	Intratumoral	Phase I/II	Moderna
Solid tumors	mRNA	Intratumoral	Phase I	Moderna
Solid tumors	mRNA	Intratumoral	Phase I	Moderna
Chikungunya infection	mRNA	Intravenous	Phase I	Moderna
Zika	mRNA	Intramuscular	Phase I	Moderna
Cancer	mRNA	Intravenous	Phase I	Moderna
Advanced melanoma	mRNA	Intravenous	Phase I	BioNTech
Solid tumors	mRNA	Intratumoral	Phase I	CureVac
Rabies	mRNA	Intramuscular	Phase I	CureVac
Non-small cell lung cancer	mRNA	Intradermal	Phase I	CureVac
Urea disorder	mRNA	Intravenously	Phase I	Arcturus
Tissue repair	miRNA	Intradermal	Phase I	Mirage (Viridian)
Methylmalonic aciduria	mRNA	Intravenous	Phase I/II	Moderna
Blood cancers	Cobomarsen (MRG-106)	Intravenous/ subcutaneous	Phase II	MiRagen (Viridian)
Keloids	miRNA	Intradermal	Phase II	Mirage (Viridian)
Cytomegalovirus infection	mRNA	Intramuscular	Phase II	Moderna
Cancer	mRNA	Intramuscular	Phase II	Moderna
Ischemic heart disease	mRNA	Epicardial	Phase II	Moderna/AstraZeneca
Diabetic nephropathy	Aptamer (RNA)	Intravenous/ Subcutaneous	Phase II	NOXXON
Lung and pancreatic cancer	mRNA	Intravenous	Phase II	Poseida
COVID-19	mRNA	Intramuscular	Phase III	CureVac

mRNA-based therapies have also been investigated in relation to liver regeneration. Injecting mRNA can trigger the high proliferation of hepatocytes, which can induce restoration of liver function and accelerate tissue regeneration[18]. Furthermore, vascular endothelial growth factor (VEGF) A-encoding mRNA has been explored as a therapeutic for type 2 diabetes mellitus. Research has shown that with upregulated VEGFA expression, skin blood flow subsequently improves, suggesting a potential clinical use regarding angiogenesis[15–19].

2.2. *RNA interference*

ncRNA is a non-protein-coding transcript and is implicated in gene regulation and RNA processing[20]. ncRNA can function in gene silencing, DNA imprinting, and de-methylation and are generally classified into small regulatory ncRNA and long ncRNA. Small ncRNA named small interfering RNA (siRNA) or microRNA (miRNA) is produced during double-strand RNA (dsRNA) cleavage, and these can interfere with targeted mRNA transcription during translation[21,22].

RNA-induced silencing complex (RISC) is a multi-protein complex made by the sequence-specific silencing of cognate genes, and it functions as a core intermediate for mRNA degradation and translational inhibition. RNA interference (RNAi) in the RISC can be triggered by siRNA, shRNA, miRNA, and long ncRNA. Briefly, in the process of mRNA degradation and translational inhibition, an endoribonuclease called dicer breaks long dsRNA and complex hairpin precursors into several shorter du-

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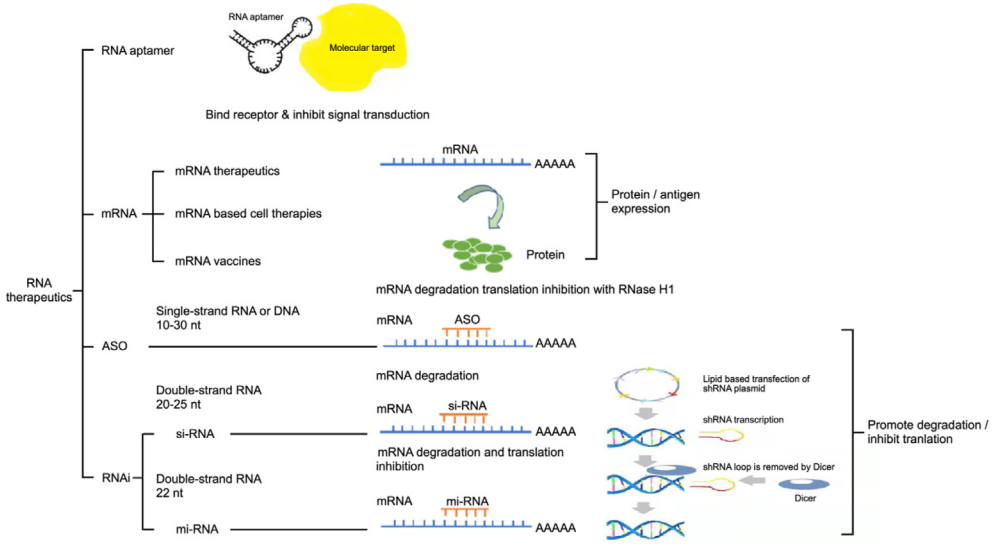

Figure 1 Types of RNA used in RNA therapies: RNA Aptamer, mRNA, ASO, and RNAi.

plexes or siRNA. Then, the siRNA is loaded onto RISC, and once this incorporation has finished, ds-siRNA splits into passenger and guide strands. The RISC then activates and catalyzes the guide strand to bind with the target sequences, whereas former passenger strands are degraded and released. Cellular nucleases then cleave and degrade the bound mRNA. The expression of the target gene is inhibited at the end of this process[23]. Unlike siRNA, most short hairpin RNA (shRNA) applications are viral vector-based and face additional challenges. First, the shRNA sequence naturally forms a tight hairpin structure. shRNA-mediated gene silencing in cells could be impaired under low dicer levels, as they are mostly transcribed by RNA polymerase III or modified polymerase II. shRNA activation is also promoter-dependent, which means its pathway needs the interaction of chromosomal DNA to maintain its function[24].

Inclisiran, a product of Novartis, is a first-in-class siRNA for cholesterol (CHO)-lowering, in which the therapeutic siRNA is chemically linked to triantennary *N*-acetylgalactosamine carbohydrates[25–29]. Recently, Ionis Pharmaceuticals and its subsidiary Akcea Therapeutics jointly announced its antisense oligonucleotide (ASO) drug Vupanorsen (AKCEA-ANGPTL3-LRx). Hypertriglyceridemia, diabetes, and nonalcoholic steatohepatitis are serious risk factors for cardiovascular disease. It has the potential to reduce the risk of diabetes and cardiovascular disease[30].

miRNA is single-stranded RNA with hairpin loop structures that contain a duplex of approximately 22 nucleotides. During miRNA synthesis, the encoded gene is first transcribed into a primary-miRNA by RNA polymerases II and III, which forms a hairpin loop structure and is processed into precursor miRNA (pre-miRNA) by the Drosha-DiGeorge critical region 8 complex. Finally, specific dicers cleave the pre-miRNA to form a mature functional miRNA. The ss-miRNA is then included into the RISC, and thus the guide strand is greatly maintained with the capability to bind the corresponding mRNA. Compared with siRNA, miRNA complementarily binds to the target sequence, which leads to hundreds of possibilities for miRNA and mRNA sequence combinations[31]. With a precise match, the miRNA can regulate target mRNA degradation by inducing the cleavage of endo-nucleo-lytic. However, the imperfect match could adjust translation, resulting in the suppression of mRNA expression.

Among all the types of introduced RNA, most of them are not stable, especially *in vivo*. Besides the package of lipid nanoparticles (LNPs) or other carriers, post-modification of RNA is very important to stabilize the RNA (Fig. 2). The modifications enhance the resistance of the RNA to nuclease digestion and delivery of the RNA to the cell, whether the RNA is delivered alone or in combination with a transfection agent or nanocarriers. The activity of the RNA in the cell is better maintained with modifications.

3. The review of various delivery platforms

Safe delivery is critical for RNA therapy as RNA degradation quickly occurs. An efficient delivery platform is a key to guaranteeing efficient delivery of therapeutic RNA. Ideal delivery platforms should protect RNA from degradation while compensating for their inherent hydrophilicity and electron negativity as they traverse the cell membrane[32]. Additionally, the following features for RNA carriers are essential and must be considered: high loading efficacy, low toxicity, and low immunity[33]. Recent investigations have shown that nanostructured platforms are excellent candidates for RNA delivery[34]. These nanocarriers are stable and can spread easily in organs. Here, we have summarized recent advances in nanotechnology with a primary focus on LNPs (Fig. 3 and Table 2).

3.1. *Fate of nanocarriers*

The size and surface properties of nanocarriers should be the primary considerations during the designing process. The size of nanocarriers can be optimized. On the one hand, they should be small enough to escape the phagocytosis of macrophages, mainly present in the reticuloendothelial systems, such as the liver and spleen. On the other hand, their size is desired to be large enough to prevent themselves from extravasating out of the capillaries. Extensive research has determined that the optimal nanocarrier size is less than 100 nm[35]. In addition, nanocarrier surface modifications can influence the stability and destiny of nanoparticles *in vivo*.

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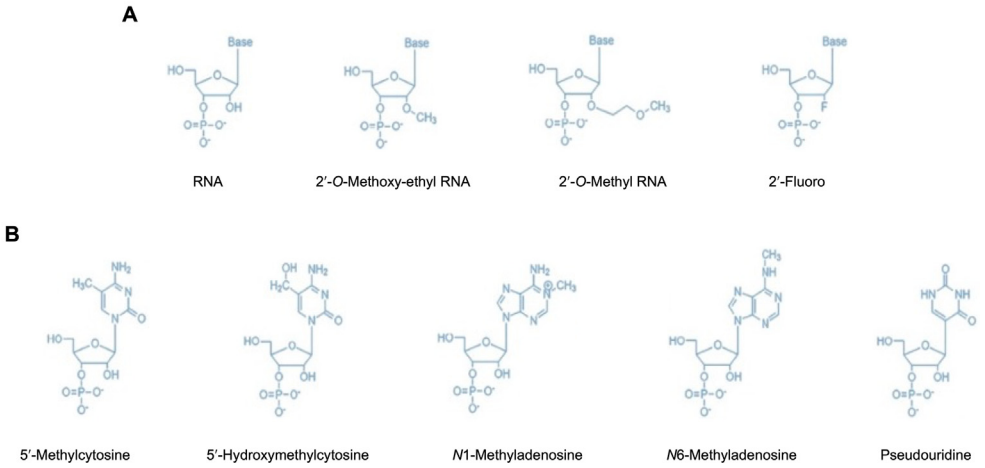

Figure 2 The post-synthetic modifications of RNA for the delivery of the RNA to a mammalian cell. (A) Molecular structure of 2' position of the ribose sugar ring including RNA base, 2'-*O*-methoxy-ethyl RNA base, 2'-*O*-methyl RNA base, and 2'-Fluoro bases. (B) Molecular structure of modification at nitrogen bases, including 5'-methylcytosine, 5'-hydroxymethylcytosine, *N*1-methyladenosine, *N*6-methyladenosine, and pseudouridine.

Modifying hydrophilic polymers [*e.g.*, polyethylene glycol (PEG)] on the surface of nanocarriers can prevent opsonization and subsequent phagocytosis to achieve a long cycle for nanocarriers *in vivo*[36]. In addition, the ability to interact with the target cell can be enhanced by introducing a positively charged or targeted ligand on the surface of the nanocarrier, which enhances its interaction with a negatively charged target cell or a target cell with a specific receptor protein[37].

The reduction of nonspecific uptake should also be considered during designing nano-drug delivery systems (DDSs). It is critical

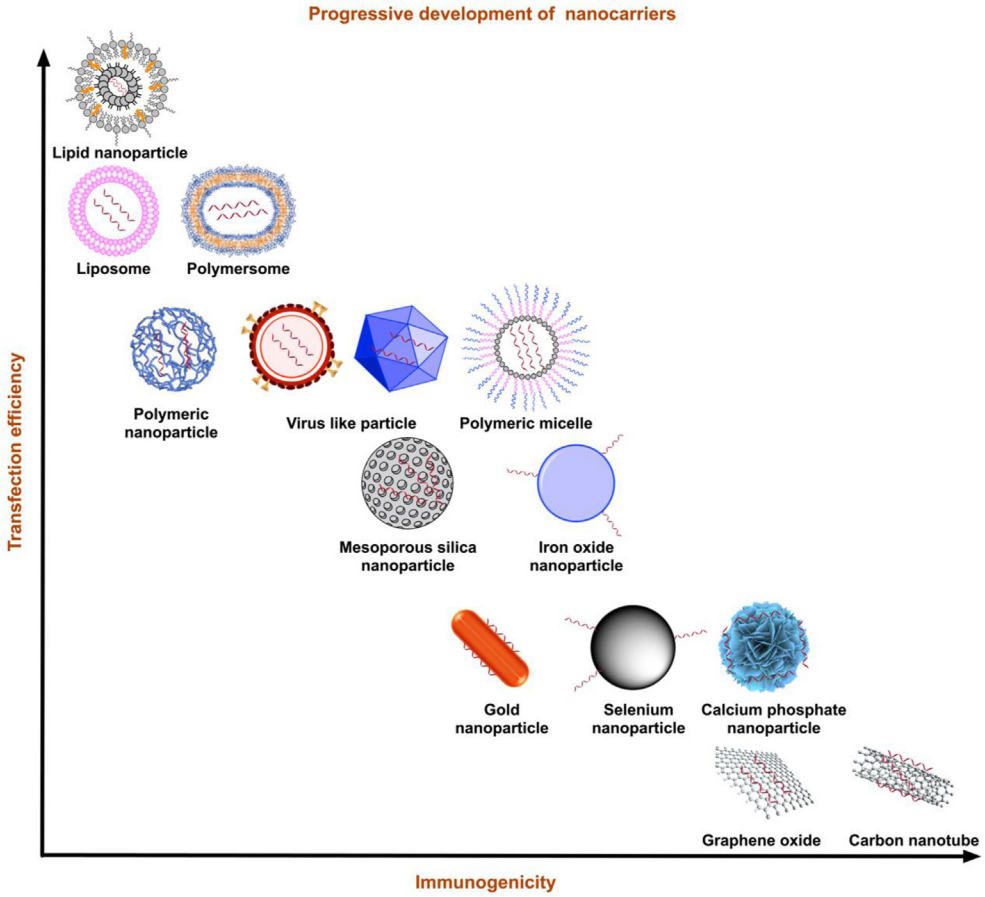

Figure 3 Types of nanocarriers. Summary of nanocarriers developed in the last decade by transfection efficiency and immunogenicity. With balanced transfection efficiency and reduced immunogenicity, LNPs have the potential to evolve into the most promising nanocarriers.

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Table 2 Comparison of different types of carriers for RNAi molecules.

Carrier	Mechanism	Key characteristic	Disadvantage	Advantage
Viral vectors	Bind to the cell receptors High possibility that it can integrated into the genome of host	1) Higher efficiency than LNPs 2) Transfect targeting cells naturally and efficiently	1) High toxicity 2) Genome modification	1) Improved efficiency 2) Biodegradable, low immunogenicity 3) Improve the specificity
LNPs	Conjugate or complexion	1) Good stability 2) Ability to protect the medicine from degradation 3) No immunogenicity 4) Easy to be stored after freeze-dried	1) Low efficiency 2) Stability	4) Low toxicity, good biocompatibility, high security 5) Good production, low cost
Polymer-based nanoparticles	Combined with the skeleton phosphate or connected to the adapter body	1) Higher thermodynamic stability 2) Higher dynamic stability	Low transfection efficiency	
Inorganic nanoparticles	MSNs, carbon nanotubes, QDs, gold nanoparticles	1) High surface area 2) Large pore volume 3) Strong surface absorption	Low efficiency	
Exosome-mimetic nanovesicles		1) High yield 2) Efficient loading 3) Low toxicity	Preparation is not as simple as LNPs	

for the delivery of chemotherapeutics, as specific delivery to the target cells can minimize the side effects. Targeted drug delivery mainly includes passive and active targeting strategies[38]. Passive targeting considers the characteristics of blood vessels in tumor tissues, and it can allow nanocarriers to infiltrate them. It has been demonstrated that liposomes with an average diameter of around 400 nm are favorable for extravasation, and those <200 nm are more likely to be taken up by tumor cells[39]. To date, researchers have reported five types of endocytosis[40].

After interacting with the target cell, the nanocarrier is transferred to early endosomes (EEs)[41]. The internalization of nanoformulations is just the first step in transporting therapeutic drugs. Studies have shown the mechanism of delivering nanocarriers to EEs. Notably, a drug endocytosed through the same pathway can be delivered to different EEs, and nanocarriers taken up *via* different signaling pathways can still be classified into the same EEs[42].

It has been demonstrated that only 1%−2% of liposomes can escape the endosomal pathway[43]. Recent research on the endocytosis pathway indicates that clathrin-mediated endocytosis (CME), fast endophilin-mediated endocytosis (FEME), and clathrin-independent carrier/glycosylphosphatidylinositol-anchored protein-enriched early endocytic compartment endocytosis (CLIC/GEEC) are all involved for nanocarriers with diameters <200 nm, which means that these pathways are unlikely to internalize nanoparticles >200 nm[44]. Notably, the surfaces of nanocarriers are quickly adsorbed by serum proteins (*e.g.*, vitronectin) to form protein crowns after being re-suspended in cell media or injections *in vivo*[45]. To provide data standardization, guidelines fall into 3 categories: (i) experimental methods, (ii) material characterization, and (iii) biological characterization[46]. However, there is very little data available on regulating different uptake pathways in various tissues within the physiological environment and how they improve the transport of nano-DDSs to target tissues.

3.2. LNPs

LNPs, non-viral vectors prepared from multiple lipids, have been applied for the precise delivery of RNA therapeutics. Since 1989, 1,2-di-*O*-octadecenyl-3-trimethylammonium-propane (DOTMA) and 1,2-dioleoyl-3-trimethyl ammonium propane (DOTAP) have become the two most widely used cation lipids. The structure of synthetic lipids is different from that of classical lipids, as the former consist of ester-connected glycerin heads and hydrocarbon tails. For example, DOTMA containing two unsaturated aliphatic hydrocarbon tails that are linked to a quaternary amine through ether groups exhibits the brilliant ability to deliver anionic RNA molecules[47]. This structure makes synthetic lipids more efficient at forming spherical liposomes or lipid complexes to load genetic molecules. In comparison with conventional liposomes consisting of a lipid bilayer, hybrid nanoparticles prepared from lipids and functional polymers exhibit higher stability, effectively avoiding premature leakage of the loaded cargo molecules[48]. If the long-chain molecule PEG is modified on the surface of the LPNs, the nanocarriers can circulate for a longer time *in vivo*. Recently, siRNA-loaded DOTAP/poly(lactic-*co*-glycolic acid) (PLGA) systems were employed to deliver genetic molecules to the lungs for severe lung disease treatment[49]. Generally, typical LNPs consist of four main components: cation lipids, CHO, phospholipids (auxiliary lipids), and PEG-modified lipids, which can increase the loading ability of RNA through positive and negative charges attraction, enhance nanoparticles stability, promote endosome escape, and prevent nanoparticles aggregation, respectively[50]. PEG-modified LNPs can reduce the protein opsonization effect and increase the blood circulation time *in vivo* owing to the PEGylated shell, leading to a stealth effect arising from the hydrophilic steric hindrance. However, the biocompatibility and long-term safety of PEG-modified nanocarriers remained unclear. Reduced transfection efficacy and cellular uptake are two disadvantages of PEG-modified LNPs. Researchers tried to solve these problems by modifying pH-responsive cleavable PEG on the surface of nanoparticles (Fig. 4)[51]. An important feature of this method is sequential targeting, including both passive and active targeting processes. First, the PEG-modified nanocarriers passively target the tumor sites. Then, pH-triggered PEGylation shedding occurs when the nanocarriers are in the tumor acidic microenvironment. Subsequently, the exposed ligands perform active targeting into the tumor cells. This design is sophisticated

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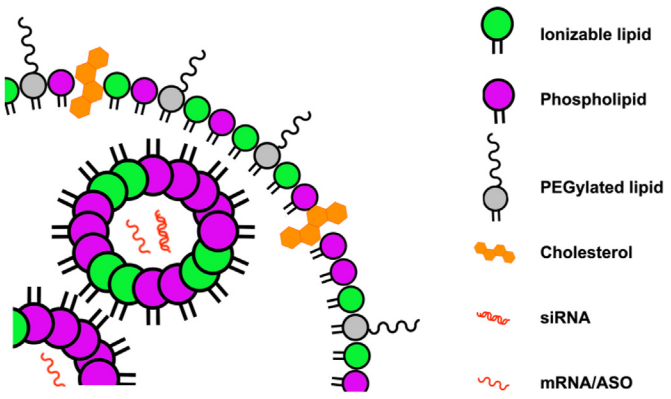

Figure 4 The structure of LNPs. LNPs are comprised of 4 main lipid components, including phospholipids (purple), PEGylated lipids (gray), ionizable lipids (green), and cholesterol (orange) encapsulating nucleic acid cargo including different types of RNA.

for cancer treatment as the microenvironment in tumor sites is always acidic. Many studies have used amino acid-derived peptides instead of PEG to endow nanocarriers with the same properties. For example, Nogueira et al.[52] chose poly-sarcosine as a substitute for PEG, consisting of *N*-methylated glycine as the repeat units. To comprehensive study of the effects of PEG lipid hydrophobic domain on LNP formation, cell internalizations, intracellular translocation, and *in vivo* delivery, researchers have synthesized a majority of PEG lipids with dendritic structures. Nanoformulations 50−100 nm in diameter were prepared to investigate the effects of hydrophobic domain lengths on LNPs. The loading efficiency of siRNA was up to 90%. Only the PEG lipids containing first- and second-generation dendritic structures could be utilized for the effective delivery of siRNA *in vitro* and *in vivo*[53].

Biodegradable LNPs have been fabricated for systematic codelivery of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 to enable effective and precise gene editing. Michael addition reaction was employed to synthesize novel lipids containing biodegradable disulfide bonds in the hydrophobic tails using amines, acrylates, or acrylamides as the reagents[54]. Notably, different linkers, including hydroxylamine, hydrazine, and ethanolamine, were chosen for the synthesis of new ionizable lipids, which exhibited outstanding ability to deliver siRNA into leukocytes. The transfection of leukocytes is reportedly difficult, so an anti-integrin beta 7 monoclonal antibody was added to the LNPs as a leukocyte-specific targeting ligand[55].

Recently, ionizable lipid-like molecules containing branched tails were also reported, which were recognized as the next-generation lipids for the development of LNPs[56]. The ionic lipid-like molecules were prepared through the highly efficient reactions between amine-containing linkers and isodecyl acrylate-containing hydrophobic tails. The obtained ionic lipids worked with CHO, C_{14}-PEG_{2000}, and dioleoyl phosphatidylethanolamine (DOPE) to afford LNPs that were approximately 124 nm before loading mRNA. The loaded DDSs were compared with the LNPs using heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino) butanoate (DLin-MC3-DMA) as the ionizable lipid were prepared, and their loading performances were carefully compared. Unfortunately, for longer RNA (>100 nucleotides), the LNP constitutes had to be revised[57]. Researchers substituted CHO with CHO derivatives in LNPs to achieve their goals. The de-convolution of the morphology, size and internal structures of LNPs are able to be regulated by alternating the type and ratio of ionizable lipids, phospholipids, CHO, and PEG lipids. CHO, a primary component in LNPs, plays a pivotal role in controlling the final morphology of LNPs and affecting the efficiency of genes. Eygeris et al.[58] prepared LNPs using natural phytosterols as the building blocks with varying degrees of crystallinity and rigidity.

Generally, LNPs with small sizes exhibit better tissue penetration. Thus, the diameter of LNP-based nanoformulations should be rationally controlled for biomedical applications. Microfluidic technology is a versatile and efficient mixing method and makes the preparation of small thermodynamically stable LNPs feasible[59]. However, small-size LNPs can be dissociated in the presence of serum, albumins, and other biological fluids, which means they have poor stability and weak intracellular trafficking[60]. To overcome this problem, Sato et al.[61] investigated the influence of hydrophobic lengths and shapes on the stability of the LNPs. They found that the low stability of small-size LNPs was attributed to the diffusion of lipids within the nanostructures and proteins adsorption on the surfaces. Inspired by the findings, auxiliary lipids were replaced by lecithin, and 22 nm small LNPs were formed. A series of experiments were carried out on the properties of higher molecular-weight scaffolds with 18 or more carbons. To evaluate the networking of amino lipids in the delivery of siRNA in LNPs formulations, Anderluzzi et al.[62] synthesized four lipids that were used to produce LNPs, which displayed similar size and comparable biophysical functions. Notably, *in vitro* antigen expression does not reflect immune response *in vivo*, indicating that *in vitro* assays are insufficient. This highlights the approach to caution and caution required to enter the clinical phase. Researchers should better understand the factors that control the correlations between *in vitro* and *in vivo* assays, as the final therapeutic efficacy of LNP vectors can vary in the two cases. For example, LNPs prepared from ionizable lipids containing saturated hydrocarbon tails were found to improve mRNA delivery[63].

LNPs have also been used to deliver mRNA for cancer and coronavirus therapy[64,65]. After the RNAi drug patisiran was approved by the U.S. Food and Drug Administration (FDA), significant progress was made in the research of LNP carriers, much of which is dedicated to using natural substances in nanocarriers. The experience gained from developing coronavirus disease 2019 (COVID-19) vaccines has led to the reorganization

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and improvement of nanocarriers[66]. Current progress in designing suitable DDSs in academia and industry will ensure the future development of effective RNA therapies.

Advanced LNPs with ionizable lipid components can help to break the mutual restriction between transfection efficiency and cytotoxicity and further produce a series of highly biocompatible LNPs that can be loaded with various RNA[67]. At the same time, the combination of automated high-throughput screening and modern synthesis technology can shorten the evaluation time of the LNPs library, which will make it possible to respond quickly and positively to future crises similar to the COVID-19 pandemic[68]. These positive developments provide strong support for applying LNPs-based RNA delivery vectors (Table 3).

Liposomes have been widely used to deliver both small and macromolecular therapeutic agents[69–71]. High encapsulation efficiency requires a higher amount of cationic lipids, which leads to a rise in the number of positive charges on the surface and subsequent toxicity[72]. To solve these problems, researchers used ionizable lipids containing amino head groups with the acid dissociation constant (pK_a) below 7[73]. Low pK_a makes ionizable lipids neutral at physiological pH (7.4) but positively charged at an acidic pH (<6.0), and as a result, LNPs have a higher nucleic acid encapsulation efficiency under acidic pH. Ionizable and other auxiliary lipids also interact with the negatively charged endosomal membrane, causing membrane disruption and endosomal escape of nucleic acids[74]. Therefore, the ionizable amino lipids and auxiliary lipids constitute the lipid component of LNP preparations (Fig. 2). The lipid composition of the LNPs and liposome preparations and the chemical structures of the recently developed novel ionizable amino lipids are depicted in Fig. 3.

3.3. Limitations of LNPs

The clinical development of LNP technology in chemotherapy and nucleic acid therapy has confirmed the promising potential of lipid carriers in treating a range of diseases[75]. While several concerns faced by LNPs greatly limit their clinic applications. Compared to pre-clinical trials, only a small number of products are successfully approved, which suggest that the application of these nanoparticle-based therapies still faces many challenges when translating from animal model to human clinical trials. Besides, drug leaking is still the bottleneck of LNPs for clinical use, and the stability of nanoparticles is constantly in need of improvement[76].

4. The summary of biomedical applications

4.1. Cancer treatment

According to the data released by the National Cancer Center of China in 2022, cancer is a leading public health challenge and has become one of the primary causes of death in China[77]. The most common and conventional cancer treatments include surgery, chemotherapy, radiotherapy, immunotherapy, and proton therapy, and to date. In recent years, the continuing improvement in conventional cancer treatments has significantly reduced the death rate[78]. Nevertheless, patients face various unsolved healthcare challenges, such as high levels of toxicity, long-term complications, and several other complex factors[79].

Dysregulated gene expression remains a major hallmark of cancer, and consequently, altering the activity of cancer-related genes has been a research focus[80]. RNA plays a crucial role in gene expression. Thus, developing cancer treatments by altering RNA activity could be promising[81]. Nevertheless, nucleases can quickly degrade naked therapeutic RNA in the serum. Moreover, the cell membrane prevents therapeutic RNA from crossing due to the anionic charges on their surfaces. The remarkable progress in nanocarrier development has led to advances in DDSs for *in vivo* delivery of therapeutic RNA[82,83]. Here we introduced RNA therapies in several types of cancers with a focus on LNPs-based nanocarriers.

4.2. Lung cancer

Lung cancer is the most lethal cancer type and causes the number death of cancer worldwide, with an increased rate of 11.4% for new cases annually[84]. Lung cancers can be divided into non-small-cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC)[85]. The heterogeneity and adaptability of lung cancer limit the success of current therapies[86]. Thus, there is great interest in RNA-based therapeutics with the potential to fight cancer.

Nanoparticles are advantageous owing to their high surface area and because the construction of therapeutic RNA nanoparticles can be easily realized. In early-phase research of liposomal DDSs, Zhao et al.[87] fabricated lipid-polycation-hyaluronic acid nanoparticles for VEGF siRNA delivery in a human lung cancer mouse model. These nanoparticles exhibited satisfactory antitumor outcomes through the activation of adenosine

Table 3 LNP vectors in clinical trial.

Disease	Type of RNA	Target	Vector	Clinicaltrial	Status
Elevated LDL-cholesterol	siRNA	PCSK9	LNPs	NCT01437059	Phase I
Solid tumors	siRNA	KSP and VEGF	LNPs	NCT01158079	Phase I
Hepatitis B	siRNA	HBV antigen	LNPs	NCT02631096	Phase II
Solid tumors	siRNA	PLK1	LNPs	NCT01437007	Phase III
Hepatic fibrosis	siRNA	HSP47	LNPs	NCT02227459	Phase II
Hepatocellular carcinoma	siRNA	MYC	LNPs	NCT02314052	Phase II
Hematological and solid tumors	siRNA	MYC	LNPs	NCT02110563	Phase I
CMV infection	mRNA	Pentamer and T cell antigen	LNPs	NCT03382405	Phase I
Zika	mRNA	prM and E	LNPs	NCT04064905	Phase I
OTC deficiency	mRNA	OTC	LNPs	NCT03375047	Phase I/II
COVID-19	mRNA	S-protein	LNPs	NCT04283461	Phase I
Solid tumors, lymphomas and ovarian cancer	mRNA	OX40L	LNPs	NCT03323398	Phase I/II
Solid tumors and lymphomas	mRNA	OX40L	LNPs	NCT03739931	Phase I

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monophosphate-activated protein kinase and inhibition of rapamycin, and these functions were equivalent to those of the anticancer drug metformin. Moreover, mesoporous silica nanoparticles (MSNPs) are considered another powerful DDSs. Dilnawaz et al.[88] developed DDSs for lung cancer therapy that co-delivered anticancer drugs (*e.g.,* etoposide or docetaxel) with survivin siRNA by the MSNPs. They suggested that this system has a significant apoptotic effect when using high-dose co-deliver drugs *in vitro*. Nascimento et al.[89] reported polyethylenimine (PEI) functionalized siRNA/MSNPs immobilized on electro-spun nanofibers. Another cancer treatment strategy is to disrupt cancer cell proliferation. In recent decades, although researchers have identified several siRNA that can suppress the growth of cancer cells, most of them are often associated with adverse side effects. Consequently, developing siRNA DDSs with low levels of systemic side effects is a field that requires further research.

4.3. Glioblastoma

Glioblastoma is one of the most common and aggressive brain tumors and shows poor treatment response, with a <2-year average survival rate. Glioblastoma pathogenesis is mainly associated with the BBB, as it creates a formidable obstacle when treating glioblastoma[90].

To increase the efficiency and safety, Kong et al.[91] utilized PEI-modified gold nanoparticles as carriers to deliver siRNA to glioblastoma, the delivery efficiency and therapeutic results were further promoted by conjugated Arg-Gly-Asp peptides as the targeting agent on the surface. In another study, Zheng et al.[92] employed a polymeric vehicle to encapsulate siRNA through the combination of hydrophobic interactions, hydrogen bonds, and electrostatic interactions. Heterogeneous inheritance and epigenetic aberrations in glioblastomas make it exceptionally difficult to use conventional drugs. RNAi, however, is a promising strategy for glioblastoma treatment. A combination of RNAi using nanoparticles could inhibit tumor growth and increase the survival rate of patients. To treat glioblastoma specifically, Sukumar et al.[93] chose a direct nose-to-brain transport pathway for the delivery of miRNA using hybrid polymeric nanoparticles, which exhibited dramatically different results from the commonly used subcutaneous administration. New therapies and challenges specific to glioblastoma require further investigation for practical applications.

4.4. Pancreatic cancer

Pancreatic cancer is one of the most aggressive cancer. Previous reports have demonstrated that oncogenic Kirsten ras (KRAS) mutations are involved in the pathogenesis of most pancreatic cancers[94]. Consequently, KRAS mutations have become a primary research target for treating pancreatic cancers[95].

There are recent reports about the application of RNA therapies for pancreatic cancer. For example, Zeng et al.[96] prepared DDSs consisting of siRNA and PEG-*b*-PLL on account of the extraordinary sequence specificity of RNAi to direct arsenic-induced apoptosis and KRAS silencing. Uchida et al.[97] developed a novel nano-micelle composed of PEG-polycation block copolymer-CHO as an mRNA vehicle for treating pancreatic cancer. The mRNA nano-micelle efficiently expressed proteins in the tumor sites. The liposome-based RNA DDSs offered advantages over viral-based RNA DDSs, which were characterized by rapid blood circulation clearance and low efficiency. Kamerkar et al.[98] engineered exosomes that could recognize oncogenic KRAS specifically as carriers for siRNA or shRNA. They found a significant increase in the overall survival in mouse models, suggesting that this newly developed exosome therapy could suppress pancreatic cancers. Han et al.[99] targeted activated pancreatic stellate cells to construct a tumor microenvironment-responsive nanosystem.

4.5. Liver cancer

In 2020, liver cancer became the second most lethal cancer type worldwide. Patients with 5-year survival only ∼18%[84]. Primary liver cancer has been categorized into two main forms: hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma, which constitute 75%−85% and 10%−15% of all cases[100]. It is usually caused by chronic liver damage, and the common inducing factors include alcohol abuse, nonalcoholic fatty liver disease, and hepatitis virus infection. Several small-molecule drugs for the treatment of HCC have failed, and RNA-based drug development has shown promising results for liver cancer therapy[101].

Dendrimers are an excellent example of a powerful vector application. Driven by electrostatic interactions, Khan et al.[102] used dendritic molecules to efficiently complex with negatively charged siRNA under an acidic microenvironment. They utilized an alkyl to generate amine-rich ionizable dendrimer cores and demonstrated the siRNA could be specifically delivered to target cells, such as endothelial cells and hepatocytes. The main challenge when applying dendrimers in clinical use is overcoming tissue damage during delivery by lowering their toxicity and improving potency.

4.6. Prostate cancer

Prostate cancer is the second most common cause of death in males globally, and its case numbers are similar to those for lung cancer[84]. Conventional prostate cancer therapies include surgical prostate removal, hormone therapy, and radiotherapy. These therapies have exhibited remarkable clinical efficacy. However, the life quality of patients receiving these traditional therapies is impacted seriously by multiple side effects, such as those from surgical or chemical castration[103]. While prostate cancer can be induced by various risk factors, such as race, age, family history, and geneticity. The clinical management of prostate cancer is difficult owing to our relatively limited understanding of related genes. However, as RNA therapeutics can be used to silence the target genes, RNAi becomes an unparalleled therapeutic modality in the treatment of prostate cancer[104].

There have been extensive efforts to develop RNA delivery systems for prostate cancer therapy. For example, Hasan et al.[105] prepared siRNA/PLGA nanocarriers with a siRNA encapsulation efficiency of up to 32%−46% through the soft lithography method. Furthermore, Chen et al.[106] developed nanoplexes with the functional group thiol−ene. These complexes showed a remarkable ability for hydrolytic degradation, which allowed the click functionalization of thiol−ene releasing interleukin-8 siRNA. In addition, researchers have now designed novel carriers with abundant stimuli-responsive functions, which are widely used for the construction of RNA-based nanomedicines. Xu et al.[107] proposed a multifunctional envelope-type siRNA nanoparticle. Owing to these and previous efforts, future research may be able to focus on specific genes to promote the efficacy of targeted therapies.

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4.7. LNPs-based therapy targeting T cells

LNPs-based therapy targeting T cells have been developed for combination with surgery, chemotherapy, radiotherapy, and targeted pathway inhibition, as these are known to fight aggressive diseases by triggering the immune reactions of patients[108]. Various forms of cancer immunotherapy have emerged recently, and research into tumor-infecting viruses, cell therapy, cancer vaccines, immunogenic cell death, immune checkpoints, targeted antibodies, cytokines, and immune factor adjuvants has seen great progress. Recent work showed that by injecting T cell-targeted LNPs containing the mRNA needed to reprogram T lymphocytes, the researchers could target T cells to treat heart disease[109]. The efficacy of many RNAi drugs has been proven, and they have now entered clinical trials, such as Patisiran and ENVISION in phase III trials[110]. The vast strides in RNA-based therapies for cancer have elevated the interest in RNA-based immunotherapies as a typical immunotherapy method and for their ability to elucidate the RNA in anticancer immune functions for different cancer types[111]. Therefore, this section further discusses and summarizes current RNA-based immunotherapies for cancers.

Vaccines are an effective therapeutic option widely used to protect and treat infectious diseases like measles, polio, and even COVID-19[112]. Current vaccines are usually designed to trigger antigen-specific cells by presenting tumor-associated antigens to antigen-presenting cells. In 1999, the first effective self-replicating RNA vector was constructed by Ying et al.[113] to improve the immunogenicity of nucleic acid vaccines. They found that the treated mice had a positive survival rate after receiving the RNA vector-enhanced nucleic acid vaccine, which indicated that RNA could be an excellent candidate for novel cancer vaccines. After the first successful attempt, developments in the field of RNA-based vaccines have become extremely rapid, especially in recent years. One of the notable applications was reported by Sahin et al.[114], who implemented an RNA-based poly-neoepitope to mobilize immunity against a spectrum of cancer-specific mutations through individualized mutanome vaccines. Clinical evidence indicated that this personalized RNA vaccine had positive effects against melanoma.

5. The prospects, challenges, and opportunities

Developmental landmarks have been achieved in drug delivery, cancer therapy, and vaccine in new RNA-based technologies. The current state-of-the-art research direction is translating from experimental use to clinical application of RNA therapeutics. Novel nanocarriers as drug delivery tools have unique advantages when applying nanoplatforms for RNA-based technologies. Structural stability of nanoplatforms during administration can effectively prevent bioactive molecules from degradation. Most importantly, Therapeutic RNA combing with delivering nanocarriers have high biocompatibility, allowing effective constituent of drugs to overcome biological barriers and targeting specific cells and tissues. In addition, because of its biocompatibility and stability, patients under treatment can alleviate undesired inflammation in tissue or organs, thus achieving a better therapeutic effect. These unique advantages have demonstrated the bright future of nanotechnology-assisted therapies. Different fields (*e.g.*, biologists, chemists, material engineers, or physicians) have devoted enormous efforts to better rationally design, development, and experiment of RNA targeting nanoplatforms with special characteristics. We believe mRNA therapeutics will be continuously explored for the development of new vaccines, whereas miRNA-based therapeutics show brilliant potential in wound healing. However, great opportunities also come with enormous challenges, especially in the development of ideal therapeutic agents for the optimization of these nanocarriers and in the achievement of the intended goal. These unsolved challenges are the biggest obstacle to stop the broad applications of these vectors as genetic molecules carriers. Finding proper methods to reduce the bioaccumulation and stop the aggregation in the body fluids for nanocarriers becomes important since the structural stability of nanocarriers makes it more easily to accumulate in the circulation system. Besides, nonspecific adsorption of drugs in circulation makes the balance of stability and activity fragile and challenging to achieve. Take the development and application of carbonaceous nanomaterials, inorganic nanoparticles, and LNPs as examples, even though they already stand out in biocompatibility and bio-stability, especially for LNPs, which have enhanced biocompatibility and represent the most advanced and promising nanocarriers, their potential toxicity and carcinogenicity have remained unsolved. Other than the above holdback, more research is needed to clear the doubt between active dosage and DNA damage.

Despite the challenges, new trends of continuously exploring the application of RNA-based healthcare materials will never stop the steps. Artificial intelligence (AI) approaches applying to investigating potential RNA therapeutic agents in future research directions might bring new opportunities for biomedical applications and revolute the current pharmacological therapies. AI approaches are outstanding in several aspects. First, AI approaches are made by large, organized, structured datasets. The designed algorithms with high sensitivity allowed AI tools to process, integrate, interpret, and find patterns of datasets. Thus, they have the potential to level up medical diagnosis, detection of genetic disorders and mutations, infectious disease characteristics, and treatment accuracy, all cost-effectively and significantly. Additionally, AI tool shows complexities and variability due to the application of multiple algorithms, further enhances the problem solute capability and results in reliability, which could also contribute to analyzing RNA-related mechanisms such as RNA structure, predicting alterations such as RNA folding, and performing fast and accurate in complex gene expression patterns recognition. The above advantages gave the AI tool particular suitability to design RNA therapeutics for silencing and correcting to develop patient-specific gene mutation inhibitors and perform genome editing.

Recently, with enormous growth in cancer-related RNA therapeutics research, AI tools have been proven useful in RNA-based cancer treatments. Thus, we believe the upcoming request and interest in RNA-based research will ensure the continuous development of RNA therapeutics and the improvement of nanotechnology-assisted RNA delivery, such as LNPs, in the coming decades. We hope this review will offer new views and motivate scientists in all fields during the ongoing revolution in patient pharmacotherapy and personalized medicine.

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Author contributions

Xingcai Zhang and Yingli Sun conceived the idea. Xingcai Zhang, Yingli Sun, and Luo Hai performed the major literature search and wrote the original draft. Xingcai Zhang, Yingli Sun, Guocan Yu, and Yibo Gao revised and edited the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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