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The Challenge and Prospect of mRNA Therapeutics Landscape

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ABSTRACT

Messenger RNA (mRNA)-based therapeutics hold the potential to cause a major revolution in the pharmaceutical industry because they can be used for precise and individualized therapy, and enable patients to produce therapeutic proteins in their own bodies without struggling with the comprehensive manufacturing issues associated with recombinant proteins. Compared with the current therapeutics, the production of mRNA is much cost-effective, faster and more flexible because it can be easily produced by *in vitro* transcription, and the process is independent of mRNA sequence. Moreover, mRNA vaccines allow people to develop personalized medications based on sequencing results and/or personalized conditions rapidly. Along with the great potential from bench to bedside, technical obstacles facing mRNA pharmaceuticals are also obvious. The stability, immunogenicity, translation efficiency, and delivery are all pivotal issues need to be addressed. In the recently published research results, these issues are gradually being overcome by state-of-the-art development technologies. In this review, we describe the structural properties and modification technologies of mRNA, summarize the latest advances in developing mRNA delivery systems, review the preclinical and clinical applications, and put forward our views on the prospect and challenges of developing mRNA into a new class of drug.

Key Words: mRNA therapeutics; mRNA modification; mRNA delivery; Gene therapy; Nucleic acid therapeutics; *In vitro* transcription

1 INTRODUCTION

Messenger RNA (mRNA) has become an attractive subject of basic and applied research since it was first discovered in 1960s (Brenner et al., 1961). Accordingly, the understanding of mRNA has shifted from a simple link between DNA and protein to a versatile molecule that regulates the functions of genes in all living organisms. Based on this change, numerous types of mRNA-based therapeutics have emerged. In 1990, Wolff et al. firstly reported that intramuscular injection of mRNA into the skeletal muscle of mice led to the expression of encoding proteins (Wolff et al., 1990). Since then, mRNA- based therapeutics have been exploited in a variety of applications, including cancer immunotherapy, infectious disease vaccines, protein substitution and cellular genetic engineering. In 2001, *ex vivo* mRNA transfected dendritic cells entered clinical trial for the first time (Heiser et al., 2002), and hundreds of mRNA-based clinical trials have been launched over the past two decades.

However, mRNA was not considered a new class of drug in the first decades after its discovery. Obstacles such as instability and immunogenicity have hampered its development, making it less pursued than DNA in gene therapy (Burnett and Rossi, 2012; Crooke et al., 2018; Geall et al., 2013; Hajj and Whitehead, 2017; Kallen and Thess, 2014; Kreiter et al., 2011; Reautschnig et al., 2017; Sahin et al., 2014; Van et al., 2015). In recent years, these key problems have been mainly solved by introducing modified nucleosides into mRNA sequences and developing various RNA packaging and delivery systems. A lot of evidences not only proved that mRNA can mediate superior transfection efficiency and longer protein expression time, but also revealed the main advantages of mRNA over DNA. The advantages of mRNA include: (1) mRNA does not need to enter the nucleus to be functional. As soon as it reaches the cytoplasm, the mRNA initiates protein translation. In contrast to mRNA, DNA needs to reach the nucleus first and then be transcribed into mRNA. This process makes DNA less efficient than mRNA, because its function depends on the destruction of nuclear envelope during cell division (Sahin et al., 2014). (2) Unlike DNA and viral vectors, mRNA does not insert into the genome, but only transiently expresses the encoding proteins. Therefore, it provides an excellent safety choice for

researchers and pharmaceutical companies due to its low risk of insertional mutagenesis (Cavazzana et al., 2016; Schlaeger et al., 2015). (3) mRNA can be easily synthesized by an *in vitro* transcription (IVT) process. The process is relatively inexpensive and can be rapidly applied to different therapies (Sahin et al., 2014). (4) Moreover, mRNA is theoretically capable of expressing any protein and may be used to treat almost any disease. Hence, from the view of pharmaceutical industries, mRNA is a very potential drug candidate that will meet the needs of gene therapy, cancer therapy as well as vaccination and so on.

In this review, we summarize the latest progresses in solving a series of key problems around mRNA therapeutics, including avoiding immunogenicity, increasing stability, improving translation efficiency, and enhancing delivery. The current status of preclinical and clinical studies, as well as pipelines of mRNA-based drug candidates for infectious diseases, cancer, and genetic diseases treatment are also overviewed. At last, the concerns and perspectives of mRNA drug industry and biopharmaceutical enterprises are discussed.

2 Structural elements of mRNA

Generally, natural mRNA has a single strand structure, consisting of a 7-methyl-guanosine residue binding at the 5'-end (the 5'-cap) and a poly (A)-tail at the 3'-end. The protein encoding open reading frame (ORF) is marked by a start codon and a stop codon. The untranslated regions (UTRs) locate between the cap/tail and the ORF (Sonenberg and Hinnebusch, 2009). Plasmid DNA, PCR product or synthetic double-stranded oligonucleotide can be used as transcription templates for mRNA synthesis *in vitro*. The transcription process is carried out by T7, T3 or SP6 phage RNA polymerases in the presence of ribonucleoside triphosphates to synthesize a complementary RNA strand (Loomis et al., 2018; Sahin et al., 2014). During this process or after transcription, mRNA is capped by the 7-methylguanosine cap molecules enzymatically at the 5'-end (Li and Kiledjian, 2010; Martin and Moss, 1976). Studies have shown that the 5'-cap plays a crucial role in mRNA maturation, splicing, translation and nonsense-mediated decay (Dwarki et al., 1993; Martin et al.,

1975). The poly (A) tail at the 3'-end is very important for the stability and subsequent translation process of mRNA (Eckmann et al., 2011; Martin and Keller, 1998). Whereas the 3'-UTR region contains α- and β- globin sequences, which can also enhance the stability and translational efficiency of mRNA. Both 3'- and 5'-UTR regions can inhibit the decapping and degradation of mRNA (Ross and Sullivan, 1985; Zinckgraf and Silbart, 2003).

3 Improving the stability and translation of mRNA

One of the major challenges of naked mRNA-based therapy is its short half- life, which is caused by the rapid degradation by abundant extracellular RNases. The half-life of *in vitro* transcribed mRNA (IVT mRNA) and its protein products is a crucial factor affecting the pharmacokinetic (PK) and pharmacodynamics (PD) properties of mRNA-based therapeutics. To optimize the efficiency of mRNA, a variety of chemical modifications to mRNA structures were explored, including modifications to the 5'-cap, poly (A) tail, 5'- and 3'-UTRs, and coding region.

For the modification of the 5'-cap of mRNA, several cap mimics were designed (Figure 1). The mRNA cap consists of 7-methylaguanosine (m^7G), which is connected to the first transcribed RNA nucleotide during transcription *via* a 5', 5'-triphosphate bridge (ppp) (m^7GpppN structure). It not only participates in RNA translation by binding to the translation initiation factor 4E (EIF4E) , but also to the DCP1/DCP2 complex, which regulates mRNA decay (Li and Kiledjian, 2010). The most reported cap analogues are the anti-reverse cap analogues (ARCAs) modified within the ribose moiety of the m^7G (Jemielity et al., 2003; Ziemniak et al., 2013). The ARCA-capped mRNA prevents incorrect cap incorporation during mRNA synthesis, hence exhibits superior translation efficiency. In recent years, another cap analogue, called S analogue that contains a single phosphorothioate (O-to-S) substitution in the triphosphate bridge was developed (Grudzien-Nogalska et al., 2007). It is reported that replacing ARCAs with an S in the β position of the triphosphate bridge (β-S-ARCAs) results in two benefits: high affinity of the cap to EIF4E and low susceptibility to the decapping complex DCP1/DCP2 (Grudziennogalska et al., 2007;

Kowalska et al., 2008). Experiments showed that β-S-ARCAs enhanced the expression of mRNA encoding antigen both *in vitro* and *in vivo*, and were applied in ongoing clinical trial of mRNA vaccine against melanoma (Kuhn et al., 2010). Recently, Jacek Jemielity et al. synthesized a new class of cap analogue, termed 2S analogue, which combines dithiodiphosphate modification, ARCA and extended polyphosphate chain. They found that these 2S analogues elevated the overall translation in human immature dendritic cells and were superior to the previously published phosphate-modified cap analogues applied in clinical trials (Strenkowska et al., 2016).

The poly (A) tail decorates the 3' end of mature mRNA in eukaryotes. It is produced by transcribing its DNA template or by using a recombinant poly (A) polymerase post transcriptionally. The latter is limited because the length of poly (A) tail can vary with each other in the production of mRNA batches, which makes the reproducible batches with a defined poly (A) length very difficult (Gallie, 1991; Körner and Wahle, 1997). Using DNA template to transcribe poly (A) tail *in vitro*, which results in a defined length, is welcomed by the manufacturing industry. It is well known that the poly (A) tail plays a key role in regulating the stability and translation efficiency of mRNA (Chang et al., 2014; Gallie, 1991; Mockey et al., 2006). Longer tails were found to increase the protein expression in various cell types (Elango et al., 2005; Wu and Brewer, 2012). Mockey et al. demonstrated that the protein translation in dendritic cells was improved continuously with a poly (A) tail of 100 nucleotides together with a 5' ARCA cap analogue (Mockey et al., 2006). Holtkamp et al. reported that the long poly (A) tail of 120 nucleotides achieved higher protein expression level than the conventional poly (A) tail of 64 nucleotides (Holtkamp et al., 2006). However, some experts believe that the poly (A) tail is not the longer the better (Choi and Hagedorn, 2003; Jalkanen et al., 2014; Meijer et al., 2007; Yang et al., 2011). They suggested that proper regulation of poly (A) tail length is very important for maintaining specific biological behaviors in cells, but whether the tail needs to be shorter or longer appears to be transcriptional- specific.

The 5'- and 3'-UTRs in mRNA contain specific regulatory sequence elements that

modulate the translation and stability of mRNA. The half-life of mRNA can be improved by introduction of stabilizing elements into UTRs. For example, the 3'-UTRs of α- and β-globin mRNAs are key factors for mRNA half-life of more than 1 day (Holcik and Liebhaber, 1997). In order to increase the stability and translation efficiency, many IVT mRNA conjugating the 3'-UTRs of α- and β- globin mRNAs were designed (Kariko et al., 1999; Waggoner and Liebhaber, 2003; Yu and Russell, 2001). The stabilizing effect can be further improved by incorporating two β- globin 3'-UTRs together in a head to tail orientation (Holtkamp et al., 2006). In addition to the widely applied globin UTRs, various UTRs such as the 3'-UTR of human heat shock protein 70, internal ribosomal entry sites (IRESs) and 3'-UTR of eukaryotic elongation factor 1α (EEF1A1) et al. have been investigated for therapeutic mRNA application (Bergman et al., 2007; Vivinus et al., 2001; Yakubov et al., 2010; Zinckgraf and Silbart, 2003).

For the protein coding region of mRNA, codon optimization leads to controllable translation of the sequence to desired protein. Single synonymous codon substitution may have a significant impact on protein expression, protein folding, and cell function. Because the same amino acid can be translated from a distinct set of codons, there are multiple choices to rewrite an mRNA code to produce exactly the same protein. Recently, researchers of Moderna, Inc. observed that mRNA secondary structure could regulate protein expression by changing the half- life of mRNA translation, and modified nucleotides that stabilize mRNA spatial structure enabled high protein expression level (Mauger et al., 2019). Machine learning is also applied to design the sequence of mRNA to produce more or less desired proteins (Hatzivassiloglou et al., 2001). Until now, this technology has been successfully employed in mRNA- based therapeutics, such as the expression of non-viral proteins and development of infectious disease vaccines (Frelin et al., 2004; Kim et al., 2015).

In general, the structural mRNA elements of 5' -cap, 3'-poly (A) tail, 5'- and 3'-UTRs and coding region are all modification targets. In order to obtain the best mRNA therapeutic efficiency, it is necessary to optimize the combination for specific applications.

4 Avoiding immunogenicity of mRNA

A great issue along with IVT mRNA is its immunogenicity, because exogenous RNA will be recognized as a signal of viral infection. Non-immune cells recognize RNA through the retinoic acid- inducible gene I (RIG-I) receptor and then trigger an innate immune response (Chow et al., 2018; Hornung et al., 2006; Kawai and Akira, 2007; Loo and Gale, 2011). Immune cells can be activated by IVT mRNA and induce inflammation through the Toll-like receptors (Diebold et al., 2004; Heil et al., 2004; Hornung et al., 2008; Kawai and Akira, 2006). The U-rich RNA sequences are known potent activators of Toll-like receptors (Diebold et al., 2004; Hornung et al., 2008). Therefore, it is possible to solve the immunogenicity problem by reducing the U content of mRNA (Thess et al., 2015).

To date, several strategies for nucleotide chemical modification can be selected to reduce the immunogenicity without interfering with the translation properties of mRNA. For example, replace natural adenosine with N^1-methyladenosine (m^1A) or N^6-methyladenosine (m^6A) (Hajj and Whitehead, 2017; Kariko et al., 2005); replace natural cytidine with 5-methylcytidine (m5C); and replace natural uridine with 5-methyluridine (m5U), 2-thiouridine (s2U), 5-methoxyuridine (5moU), pseudouridine (ψ) or N^1-methylpseudouridine ($m^1\psi$) (Anderson et al., 2010; Andries et al., 2015; Kariko et al., 2005; Kariko and Weissman, 2007; Kormann, M.S. et al., 2011) (Figure 2). Among them, m5C and ψ are the most welcomed because they reduce the immunogenicity of mRNA as well as increase the translation efficiency both *in vitro* and *in vivo* (Kariko et al., 2008). It is also proved that increasing the length of poly (A) tail will generate mRNA with low immunogenicity as the U content decreases or is shielded in the sequence (Koski et al., 2004; Weissman and Kariko, 2015).

In addition to modifying nucleotides and adding poly (A) tails, optimizing the codons to render the mRNA GC-rich, minimizing U content is another effective way to eliminate RNA immunogenicity (Mauro, 2018; Thess et al., 2015; Victor et al., 2018). CureVac and Acuitas Therapeutics developed a sequence- engineering method

without any chemical modification of mRNA. They designed the sequence of EPO mRNA by selecting GC-rich codons for each amino acid and delivered the mRNA to pigs using lipid nanoparticles (LNPs) by systemic administration. Results showed that the expression of EPO protein led to meaningful physiological responses without detectable immunogenicity (Thess et al., 2015). However, it should be noted that more GC content is not better, because excessive GC content is not benefit for protein expression (Konu and Li, 2002; Novoa and Ribas de Pouplana, 2012).

After the *in vitro* transcription, a series of purification processes including concentration, precipitation, extraction, and chromatography are needed to produce mRNA. Sophisticated techniques such as anion exchange chromatography, size exclusion columns, high performance liquid chromatography (HPLC) and affinity chromatography are applied to remove dsRNA and truncated transcripts (Henninger et al., 1993; Kariko et al., 2011; McKenna et al., 2007). These purification procedures are reliable methods to eliminate immunogenicity (Batey and Kieft, 2007). It is reported that mRNA with ψ modification is non-immunogenic after purification by HPLC, and the protein translation efficiency is significantly increased (Kariko et al., 2005; Vallazza et al., 2015). In a representative example, Pardi et al. synthesized $m^1\psi$ modified and HPLC purified mRNAs encoding the light and heavy chains of the broadly neutralizing anti-HIV-1 antibody VRC01, and encapsulated the mRNA into LNPs. They found that after systemic administration, the mRNA-LNPs were quickly translated into functional antibodies in mice. A single injection of mRNA-LNPs completely protected mice from challenge of HIV-1 infection (Pardi et al., 2017).

5 mRNA delivery

Efficient and safe delivery of mRNA is one of the biggest challenges in the development of mRNA-based therapeutics, which is more challenging than delivery of small oligonucleotides (Islam et al., 2015; Kowalski et al., 2019; Li et al., 2018). The size of mRNA (300–5,000 kDa, 1–15 kb, Figure 3a) is significantly larger than siRNA and miRNA mimic (13-15 kDa), antisense oligonucleotide (4–10 kDa) and antimiR (4–10 kDa). The N-Acetylgalactosamine (GalNAc)-oligo conjugate exhibited

excellent efficiency and safety of hepatocyte-targeted delivery *in vivo*, but was non-effective for mRNA delivery. Because of their size, charge, and degradability, naked mRNA cannot readily pass through the cell membrane and efficiently leak into the cytoplasm. Researches proved that naked mRNA was taken up by cells *via* the scavenger-receptor mediated endocytosis pathway and accumulated in the endosome (Lorenz et al., 2011; Valadi et al., 2007). Most cells have a low efficiency of mRNA uptake, while the immature dendritic cell is an exception, which can take up mRNA through the macropinocytosis pathway and accumulate mRNA efficiently (Diken et al., 2011; Selmi et al., 2016). However, the broad application of therapeutical mRNAs requires more effective and safer delivery methods, which is key to the realization of potential transformation therapies such as vaccination, protein replacement therapy, and genome editing. Hence, suitable mRNA formulations, e.g., liposomes, polysomes, lipoplexes, and polyplexes, are required and developed to effectively deliver mRNA into most type of cells (Figure 3b). Typically, mRNA-loaded nanoparticles are internalized *via* endocytosis, and then mRNA released from endosomes and lysosomes will initiate translation and produce any types of proteins, including secretory, transmembrane, intracellular and intramitochondrial proteins (Figure 3c).

In recent years, various materials, such as lipids, lipidoids, polymers, peptides, proteins, extracellular vesicles, etc., have been designed and explored for mRNA delivery *in vitro* and *in vivo*. Most of these materials are inspired by siRNA and plasmid DNA delivery technologies. Chemical structures of representative lipid, lipidoid and polymer-based materials is shown in Figure 4. Detailed information regarding to their compositions and ratios, mRNA cargos, routes of administration, indications, sponsors and corresponding references is summarized in Table 1.

Lipids and lipid-derived materials are the main members of delivery systems (Figure 4). By employing lipid or lipid-like materials (lipidoids), various vesicles can be prepared, e.g. liposomes, lipid nanoparticles (LNPs), lipid emulsions, lipid implants, etc. (Pardi et al., 2018) . For example, N-[1-(2, 3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dioleoyloxy-3-trimethylammonium propane chloride (DOTAP),

1,2-dioleoyl-sn-glycero- 3-phosphoethanolamine (DOPE), as classical cationic lipids, were previously used to deliver DNA, siRNA and mRNA (Felgner et al., 1987; Krzyszton et al., 2017; Malone et al., 1989; Manunta et al., 2017). Recently, BioNTech used DOTMA, DOTAP, DOPE and cholesterol to deliver mRNA to dendritic cells, macrophages, lung endothelial cells, chimeric antigen receptor T (CAR-T) cells. Their technology is undergoing clinical trials (Grunwitz et al., 2019; Kranz et al., 2016; Reinhard et al., 2020; Rosigkeit et al., 2018).

A series of lipids and lipidoids have been previously investigated for siRNA delivery, and further thoroughly explored to deliver mRNA *in vivo*. These materials include DlinDMA (Morrissey et al., 2005), Dlin-MC3-DMA (Jayaraman et al., 2012), C12-200 (Love et al., 2010), cKK-E12 (Dong et al., 2014b), 5A2-SC8 (Zhou, K. et al., 2016), 7C1 (Dahlman et al., 2014), and 1,3,5-triazinane-2,4,6-trione (TNT) derivatives (Dong et al., 2014a), etc. (Figure 4). Based on these key lipids or lipidoids, effective mRNA delivery and protein expression can be achieved by adjusting the molar ratio of key lipids to helper lipids, PEG-lipids and cholesterol, changing the helper lipids or PEG-lipids, adding another components (e.g. protamine), or using the same formulations of siRNA or optimized formulations. (Cheng et al., 2018; DeRosa et al., 2016; Geall et al., 2012; Jain et al., 2018a; Kauffman et al., 2015; Kauffman et al., 2016; Li, B. et al., 2016; Nabhan et al., 2016; Oberli et al., 2017; Rybakova et al., 2019; Sago et al., 2018; Sedic et al., 2018; Yanez Arteta et al., 2018; Yin et al., 2016; Yin et al., 2017). Among them, DLin-MC3-DMA is a FDA approved material, which is also used in the first siRNA therapeutic Onpattro (patisiran) approved by FDA and EC (Weng et al., 2019). Several other cKK-E12 derived lipidoids, including OF-02, OF-DegLin and OF-C4-Deg-Lin, have been proved to deliver mRNA to the liver and/or spleen and express protein efficiently through systemic (intravenous) administration (Fenton et al., 2017; Fenton et al., 2016; Fenton et al., 2018).

Additional lipids and lipipoids, including I-DD3/A-DD3/B-DD3, lipid 5 and H, TT3, LP01, C14-113, ZA3-Ep10, MPA-A/MPA-B, C12-(2-3-2), $306O_{i10}$, ssPalm/ssPalmO-Paz4-C2 and ATX-100 (representative lipid of Arcturus) (Figure 4), have been designed and investigated for intravenous or local delivery of mRNA to

targeted tissues and cells (An et al., 2017; Ball et al., 2018; Finn et al., 2018; Jarzebinska et al., 2016; Jiang, L. et al., 2018; Kowalski et al., 2018; Kowalski et al., 2019; Li et al., 2015; Miller et al., 2017; Ramaswamy et al., 2017; Sabnis et al., 2018; Schrom et al., 2017; Tanaka et al., 2018a; Turnbull et al., 2016; Zhang, X. et al., 2017). DOTMA, lipid 5, LP01, C12-(2-3-2) and ATX-100 are representative preclinically and clinically investigated mRNA delivery materials developed by BioNTech (Reinhard et al., 2020), Moderna (Hassett et al., 2019), Intellia Therapeutics (Finn et al., 2018), Ethris (Jarzebinska et al., 2016) and Arcturus Therapeutics (Joseph and Padmanabh, 2016; Ramaswamy et al., 2017), respectively. In a recent study, T cells were bioengineered with DOTMA-lipoplex-encapsulated mRNA vaccine encoding a single-chain variable fragment (scFv) that can specifically bind to CLDN6, a strictly oncofetal cell surface antigen for CAR-T cell targeting. The functionalized CAR-T cells achieved excellent anti-tumor effects in difficult-to-treat mouse models (Reinhard et al., 2020). Moderna recently developed DOTMA LNP-targeted mRNA therapeutic for treatment of arginase deficiency in inherited metabolic liver disorder, an autosomal recessive metabolic disease caused by arginase (ARG1) gene mutation (Truong et al., 2019). The Intellia Therapeutics team achieved clinically relevant levels of *in vivo* genome editing of mouse transthyretin gene in the liver by using the LNP-NT101 delivery system, which contains a biodegradable, ionizable lipid LP01 (Finn et al., 2018).

In addition, we have developed some lipid or lipid-derived materials and achieved effective gene or siRNA delivery (Deng et al., 2016; Dong et al., 2018; Huang et al., 2017; Li et al., 2014; Liu et al., 2014; Zhang et al., 2016; Zhang, H. et al., 2017; Zhao et al., 2016; Zheng et al., 2018). Whereupon, we continue to develop and investigate a library of lipid-based mRNA delivery systems, and obtain an excellent liver-targeted mRNA delivery system.

The rationales of lipid design for mRNA delivery remain to be further elucidated. However, several aspects have demonstrated to be key determinants of delivery efficiency and safety. Firstly, the component ratio and selected phospholipid have an important influence on delivery efficiency. Although phospholipids are not necessary

for siRNA delivery in some cases, relatively less ionizable cationic lipids and more zwitterionic phospholipids are beneficial to mRNA delivery. mRNA-LNP containing zwitterionic lipid of DOPE was more efficacious than that containing DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), which is more popularly used in siRNA delivery (Cheng et al., 2018; Miller et al., 2017). It is speculated that mRNA is much larger and more flexible than siRNA. A small amount of ionizable cationic lipids are sufficient to load and condense mRNA, form stable nanoparticles, and effectively release the payloads when entering cells. However, a large number of ionizable cationic lipids may bind mRNA too tightly and cannot be released in cells, which may lead to low delivery efficiency. The different performances of DOPE and DSPC may also be attributed to the different impact patterns on the molecular interaction around RNAs in the water pockets of LNPs.

Secondly, biodegradability is of importance for both efficacy and safety. As mentioned before, Dlin-MC3-DMA is an FDA approved excipient. However, in order to further improve the therapeutic index of siRNA-LNP, its biodegradable version L319 was develop by adding ester bonds in the hydrophobic dialkyl chains, which enables rapid elimination of siRNA-LNPs in the liver (Maier et al., 2013). ATX lipids (Arcturus) were also designed to contain an ionizable amino head group and a biodegradable lipid backbone (Figure 4), which can degrade and scavenge much faster in the liver than Dlin-MC3-DMA (Arcturus, 2020), as a result, they were well tolerated in non-human primates (NHPs). Lipid 5, a biodegradable ionizable lipid, was also employed a primary ester on one of the hydrophobic tails to enhance the liver clearance. Furthermore, OF-Deg-Lin is a biodegradable ester version of OF-02, which tends to deliver mRNA to the spleen, whereas the non-biodegradable OF-02 accumulates in the liver and promotes mRNA expression (Fenton et al., 2017) (Figure 4).

Thirdly, lipid saturation significantly influences intracellular mRNA delivery. It was reported that as saturation increases from 2 to 0 double bonds, lamellar (Lα) to reversed hexagonal (H_{II}) phase transition temperature increases, indicating decreasing fusogenicity (Heyes et al., 2005). DLin-DMA had the lowest phase transition

temperature, as the most fusogenic lipid, it showed the most potent siRNA delivery efficiency. Therefore, unsaturated lipids, especially the *cis*-double bonded ones, were designed to promote mRNA delivery. As an example, OF-02 was designed based on cKK-E12 by introducing unsaturated fatty chains to increase mRNA expression *in vivo*. It is hypothesized that unsaturated lipid tails similar to linoleic acid can improve cell membrane fluidity by establishing structural defects, which will promote cellular entry and endosomal escape, two key factors in determining the final efficacy.

Polymers or their derivatives consist another large family of mRNA delivery carrier. Linear or branched polyethylenimine (PEI) is a kind of cationic polymer widely used for nucleic acids delivery *in vitro* and *in vivo* (Boussif et al., 1995; Guo et al., 2010; Guo et al., 2011; Huang et al., 2012; Lin et al., 2011). It is also used to package self-amplifying mRNA (saRNA) encoding influenza virus hemagglutinin and nucleocapsid to protect people from virus infection (Demoulins et al., 2016). In addition to the classical gene delivery polymer PEI, many different polymers have been synthesized to evaluate their capability of mRNA delivery. Polymers of TarN3C10, DD90-C12-122, A1, C1 (PBAE-PCL), amino polyesters (APEs) and hDD90-118 (Figure 4), developed by Daniel G. Anderson and colleagues (Capasso Palmiero et al., 2018; Dong et al., 2016; Kaczmarek et al., 2018; Kaczmarek et al., 2016; Patel et al., 2019), were used alone or together with other lipids (e.g., DSPC, cholesterol, 14/0 PEG2000 PE, or 18:1 PEG2000 PE) to deliver mRNA to hepatocytes or lung epithelium *via* intravenous injection or inhalation, and achieved an ideal potency and safety outcomes. Polymers of N5 (PEH) and polyamine (2-2-2-2) (Figure 4) formed nanoplexes with mRNA and ribonucleoproteins through electrostatic interactions, and elicited high levels of mRNA transfection in different cells by enhancing the mRNA stability and protein synthesis. (Li et al., 2017a; Li et al., 2017b).

In addition to the above materials, there is another kind of biodegradable polymers called charge-altering releasable transporters (CARTs) (Figure 4, CART D_{13}/A_{11} 7), which was designed and synthesized by Robert M. Waymouth and colleagues (Benner et al., 2019; Haabeth et al., 2019; Haabeth et al., 2018; McKinlay et al., 2018;

McKinlay et al., 2017). These materials, specifically oligomers (carbonate-b-α-amino esters), have adopted an unprecedented mRNA delivery mechanism. As oligo (α-amino ester) polycations, they can noncovalently complex, protect, and deliver mRNA and then change physical properties through a degradative, charge-neutralizing intramolecular rearrangement, leading to release of functional mRNA and highly efficient protein translation in cells.

TriMan-lip, a trimannosyl diether lipid, together with Lip1 (O,O-dioleyl-N-[3N-(N-methylimidazolium iodide) propylene] phosphoramidate), Lip2 (O,O-dioleyl-N-histamine phosphoramidate) and PEG-HpK (PEGylated histidinylated polylysine), were used to form lipopolyplexes that efficiently deliver mRNA to dendritic cells and enabled cancer treatment (Le Moignic et al., 2018; Van der Jeught et al., 2018). PAA8k-(2-3-2), an 8000 Da poly(acrylic acid) grafted with (2-3-2) tetramine, was reported to deliver mRNA to lung *via* aerosol administration (Jarzebinska et al., 2016). PE4K-A17-0.33C12, a polyester-based carrier, and pluronic F127 were also used for lung-targeted delivery of mRNA by intravenous injection (Yan et al., 2017). Moreover, CP2k, aPACE, PEI_{10k}-$LinA_{15}$-$PEG_{3.0}$, PEG-PAsp(TEP)-Chol, cRGD-PLG-P(Lys-MP), $PEG[Glu(DET)]_2$, etc. have been reported to deliver mRNA to the lung, liver, or tumor *via* intravenous or subcutaneous administration (Chen et al., 2017; Dunn et al., 2018; Jiang, Y. et al., 2018; Li, M. et al., 2016; Schumann et al., 2018; Uchida et al., 2016) (Figure 4).

Previously, we designed and evaluated plenty of polymers for nucleic acid delivery, and investigated the effects of molecular structure, polymerization form and degree, hydrophobic core, hydrophilic chain, PEG segment, targeting moiety decoration, etc., of polymers on nucleic acid (siRNA) delivery performance (Cheng et al., 2016; Cheng et al., 2013; Du et al., 2017; Guo et al., 2010; Guo et al., 2011; Han et al., 2015; Huang et al., 2012; Lin et al., 2013a; Lin et al., 2011; Lin et al., 2013b; Qi et al., 2012; Wang et al., 2017; Xiao et al., 2017; Zhang, T. et al., 2018; Zhou, J. et al., 2016). Various polymers have been further synthesized and are being evaluated for mRNA delivery. As a representative example, a hybrid polymer composed of cRGD-poly(ethylene glycol) (PEG)-polylysine (PLys) (thiol) and

poly(N-*iso*propylacrylamide) (PNIPAM)-PLys (thiol) successfully delivered mRNA to tumor tissue and mediated potent gene expression (Chen et al., 2017).

Compared with lipids, polymers are relatively less popular in nucleic acid therapeutics development, to a large extent, owing to their molecular complexity and uncontrollable manufacture (Akinc et al., 2019; Crommelin et al., 2019; Leroux, 2017). In this case, simple but effective polymers are most likely to be used in the clinic. Meanwhile, biodegradation and biological response are also key considerations of mRNA delivery we need to pay attention to. The former is beneficial to reduce toxicity and enhance therapeutic index, while the latter may facilitate cellular uptake and bioresponsive endosome escape. PBAEs (Carraso Palmiero et al., 2018; Kaczmarek et al., 2018), CARTs (McKinlay et al., 2017), as well as APEs (Kowalski et al., 2018) are representative biodegradable polymers that exhibit potent mRNA delivery efficiency in animals. Furthermore, targeting moieties can be introduced into polymers to enhance their tissue targeting performance, wherein TriMan-Lip and cRGD-PEG-P(Lys-MP) are examples (Figure 4).

Another commonly used material for mRNA transfection is protamine, an arginine-rich small protein. Protamine can complex with mRNA to form tightly-bound nanoparticles that protect against the influenza virus in mice, ferrets, and pigs (Petsch et al., 2012). Protamine/mRNA complex has also advanced through a number of clinical trials, with several more are ongoing (Sebastian et al., 2014; Weide et al., 2009). However, there is a certain trend in mRNA pharmaceutical companies that protamine is gradually replaced by LNPs as the latter has a better mRNA protection and delivery efficiency. In addition, some other proteins or peptide derived materials e.g., OM-PBAE (Fornaguera et al., 2018), RALA (Udhayakumar et al., 2017), as well as extracellular vesicles (Usman et al., 2018; Wang, J.H. et al., 2018; Yang et al., 2019; Yu et al., 2018), virus-liked particles (Zhitnyuk et al., 2018), chitosan-alginate gel scaffolds (Yan et al., 2018), fluorinated peptoid crystals (Song et al., 2018), DNA-decorated gold nanoparticles (Yeom et al., 2013), and polycation-functionalized zirconium (Zr)-based metal-organic frameworks (MOF) (Sun et al., 2018) have been demonstrated to be used for mRNA transportation *in vitro*

and/or *in vivo*. Several polymers or lipid-based commercial transfection reagents, e.g., *in vivo*-jetPEI™, Lipofectamine™, MegaFectin™, Stemfect™, and TranslT™, are also able to condense and load mRNA, protect their cargo from degradation, and transport them to cells *in vitro* or *in vivo* (Kormann, M.S.D. et al., 2011; Su et al., 2011; Thess et al., 2015).

The nanosized formulations have many advantages, such as easy fabrication, low batch-to-batch variability, good biocompatibility, and scalability compared with many other delivery systems. In addition, some liposomes and polysomes can be easily functionalized with ligands for specific cell or tissue delivery by conjugating with chemical reaction groups. These nanoparticles or nanostructures have been widely used in mRNA-based cancer immunotherapy, antivirus vaccine, and functional protein expression in specific tissues in recent years (Fenton et al., 2017; Oberli et al., 2017; Sedic et al., 2017).

In addition to the delivery system, selecting appropriate administration route for particular tissues or diseases is also important to ensure the successful delivery of mRNA. Electroporation and microinjection are commonly used for mRNA transfection *in vitro* and *in vivo*. Several preclinical and clinical studies have evaluated electroporation with IVT mRNA or patients-derived mRNA for cancer immunotherapy (Table 2). Intravenous injection of naked mRNA can activate the innate immune system, which shows that this technology can be applied to the treatment that requires immune response and relatively small amount of encoded protein. It may not be suitable for other clinical applications such as protein replacement therapy as a relatively large amount of protein is needed. However, when formulated with delivery vehicles, mRNA can be administered *via* various routes, such as intravenous, subcutaneous, intradermal, intramuscular, intratumoral, intranasal, intraperitoneal, intratracheal and retro-orbital injection (Table 1). To date, dozens of preclinical and clinical studies have been conducted to study the therapeutic effects of different mRNA administration routes on infectious disease, cancer and protein deficiency diseases.

6 Preclinical and clinical advances of mRNA therapeutics

6.1 mRNA vaccines for cancer immunotherapy

According to the information of ongoing clinic trials registered at http://www.clinicaltrials.gov, mRNA therapies are mainly applied in the field of cancer immunotherapy, specifically for the mRNA-based DC vaccines. The DCs play a crucial role in inducing potent immune responses. They have the ability to direct cytotoxic T lymphocytes and natural killer cells to powerful anti-tumor weapons that are capable of attacking tumor cells (Kirkwood et al., 2012; Palucka and Banchereau, 2012). For mRNA-based DC vaccines, both of IVT mRNA and autologous tumor stem cell-derived mRNA are used to load the DCs with tumor specific antigens.

The DCs can be engineered with mRNA either in an *ex vivo* or *in situ* strategy. For the *ex vivo* strategy, DC precursor cells isolated from patients were activated into mature DCs, loaded with antigen encoding mRNA and re-administrated into the patients. Several methods can be applied to antigen loading of DCs, including nucleofection, lipofection, sonoporation and electroporation, among which electroporation is a frequently used technique (Melhem et al., 2008; Temmerman et al., 2011; Tuyaerts et al., 2002; Van Tendeloo et al., 2001). The most widely used reagents for DC differentiation are granulocyte-macrophage colony stimulating factor (GM-CSF) in combination with IL-4 (Wilgenhof et al., 2013; Wilgenhof, S et al., 2011). GM-CSF is a potent stimulant of the immune system. It recruits immune effectors to the injection site and promotes antigen presentation. DC vaccines pulsed with GM-CSF adjuvant and mRNA, has been used in several clinical trials (NCT03396575, NCT00204516, NCT00204607, NCT00626483, etc.).

The maturation state of DCs is very essential for vaccination, because the mature DCs express high levels of co-stimulatory surface markers, resulting in a better therapeutic efficacy (Benencia et al., 2012; Dalod et al., 2014; Sabado and Bhardwaj, 2010). However, there is also contrary report that mRNA uptake and subsequent antigen expression only occur in immature DCs (Diken et al., 2011). In addition to the maturation state, the ability of DCs to produce IL-12p70, an important T_H1 driving cytokine, was proved to influence the clinical response of DC vaccines (Carreno et al.,

2013; Okada et al., 2011). Production of IL-12p70 can be achieved by stimulating DCs with TLR ligands or proinflammatory cytokines (Napolitani et al., 2005; Warger et al., 2006).

For *in* situ transfection of DCs, direct injection of antigen encoding mRNA into the lymph nodes or co-delivery with TriMix are both welcomed methods. Clinical trials are being conducted in both areas. For example, a phase I clinical trial (NCT01684241) of an intranodally administered naked mRNA vaccine against advanced melanoma has been completed. TriMix is a mixture of three mRNA molecules encoding immunomodulators CD40L, CD70 and truncated, constitutively active TLR4 (Bonehill et al., 2008). It is reported that such so-called TriMix platform showed superior stimulation capacity than other classical stimulatory cytokines cocktail composed of IL-1β, TNF-α, IL-6 and prostaglandin E2 to stimulate DCs, and enhanced the expansion and function of effector T cells (Bonehill et al., 2008; Dewitte et al., 2014; Van Lint et al., 2012; Van Nuffel et al., 2012a).

Although naked mRNA is able to activate TLRs and induce DC activation, this process is insufficient to completely activate the antigen-presenting capacity of DCs (Benteyn et al., 2015; Van et al., 2015). It is an effective way to co-deliver the antigen encoding mRNA with stimuli reagents such as TriMix. The first trial of TriMix-DC vaccine for advanced melanoma therapy was performed in 2010 (NCT01066390) (Van Nuffel et al., 2012b; Wilgenhof, S. et al., 2011). Latter efforts on the combination of TriMix-DC vaccine with checkpoint inhibitor ipilimumab also yielded encouraging results (NCT01302496) (Lebbé et al., 2014).

Boczkowski et al. reported for the first time that DCs induced potent antigen-presenting ability and inhibited tumor growth in mice after they were stimulated by mRNA encoding tumor antigens (Boczkowski et al., 1996). Since then, the availability of tumor-associated antigens such as carcinoembryonic antigen (CEA), human telomerase reverse transcriptase (hTERT), prostate cancer associated antigen (PSA), Wilm's tumor-1 (WT1), gp100, MUC1, tyrosinase and survivin, etc. has increased, and the number of preclinical and clinical studies of mRNA as an off-the-shelf anticancer vaccine has boomed (Table 2). For example, University

Hospital Antwerp initiated several clinical trials to investigate the role of autologous DCs loaded with mRNA coding for WT1 antigen in cancer treatment (NCT02649829, NCT02649582, NCT01291420, NCT01686334, NCT01686334). Patients-derived hTERT and survivin mRNA were loaded into DCs and the trial of vaccine therapy for patients with curative resected prostate cancer was in clinical stage I/II (NCT01197625).

The administration route of DC vaccines has an important impact on DCs' distribution. Only if DCs reach the lymph nodes can they induce immune responses(Van et al., 2012). Several administration routes of DCs have been tested in clinical trials, such as intravenous, subcutaneous, intradermal, intranodal and intratumoral administration (Aarntzen et al., 2012; Connolly et al., 2008; Oshita et al., 2012; Triozzi et al., 2015), among which intradermal administration is the most frequently applied (Van et al., 2015). This is because there are multiple types of immune cells in different layers of skin, including Langerhans cells, T cells, skin DCs and plasma cell-like DCs. Some studies reported that DC vaccines injected intravenously is less effective in lymph node migrations (Eggert et al., 1999; Okada et al., 2001). Other studies showed that, compared with intradermal mRNA administration, internodal application of mRNA exhibits a superior efficacy in inducing antigen-specific T-cell response, which is due to the rapid and efficient engulfment of mRNA by lymph node resident DCs (Johansen et al., 2005; Maloy et al., 2001; Martinez-Gomez et al., 2009; von Beust et al., 2005). However, more and more evidences proved it a matter of debate which DC administration route is superior than the other (Kallen et al., 2013; Lesterhuis et al., 2011). Combined different administration routes may be a good choice to induce more systemic immune response.

In addition to DCs, mRNA is also transfected into other immune cells to generate cancer vaccines, such as Langerhans cells (LCs), cytotoxic T lymphocytes and natural killer (NK) cells. LC is a subset of DC in the skin. Several studies showed that LCs are remarkably efficient in inducing cytotoxic lymphocyte (CTL) responses (Klechevsky et al., 2008). Clinical trials using LC-based cancer vaccines for either

melanoma or myeloma therapy are underway (NCT01995708, NCT01456104). Besides, T cells and NK cells can be transfected with chimeric antigen receptor (CAR) encoding mRNA, leading to antigen binding and cell activation, thus specifically recognizing and killing tumor cells that express these antigens on their cell surface. The CAR strategy was reported to be effective in several animal tumor models and has entered clinical trials (NCT01355965, NCT03415100) (Almasbak et al., 2011; Barrett et al., 2013; Barrett et al., 2011; Zhao et al., 2010).

By studying the ongoing clinical trials of mRNA vaccines for tumor immunotherapy, we found that the combination of DC vaccination with other anti-tumor therapeutics such as chemotherapy, siRNAs, cytokines, and antibodies, etc. is increasing (NCT00672542, NCT02649829, NCT03396575). As reported by Anguille, et al., the combination therapy is based on three principal mechanisms: enhancing the immune response, preserving tumor-associated immunosuppression, reducing tumor burden and increasing immune susceptibility of tumor cells (Anguille et al., 2014). Rational use of DC vaccines in combination with other therapeutics can improve the overall efficacy of the cure rate.

6.2 mRNA vaccines protect against infectious disease

Vaccination for infectious diseases is a widely applied field of mRNA therapeutics. Several mRNA-based vaccines are under investigation for treatment of infectious diseases such as influenza, rabies, HIV, Zika virus infection, etc. Among mRNA technology companies, Moderna, Inc. has the most pipelines of developing vaccines for infectious diseases, mainly based on their LNP platform. Their $H_{10}N_8$ influenza vaccine, a modified mRNA vaccine formulated with LNP, which encodes the viral antigenic protein hemagglutinin (HA), has been investigated in a phase I clinical study in healthy volunteers (NCT03076385). The data presented that 100 μg intramuscular cohort could induce high levels of immunogenicity, and was safe and well tolerated (Bahl et al., 2017; Liang et al., 2017).

Human cytomegalovirus (CMV) is the leading cause of infection in newborns. It can lead to serious complications such as deafness, microcephaly, vision loss and mental deficiencies, etc. There is no approved vaccine for CMV infection. The latest

published data showed that Moderna's mRNA vaccine encoding CMV glycoproteins gB and pentameric complex (PC) produced potent and durable neutralizing antibody titers in immunized mice and NHPs (John et al., 2018). The vaccine is currently undergoing a Phase I clinic trial (NCT03382405).

Another LNP-formulated vaccine, Moderna's Zika mRNA Vaccine encoding viral antigenic protein (Zika virus prM and E) is currently in the Phase I/II clinical study in healthy volunteers (NCT03014089). Children born to mothers infected with Zika virus suffer from microcephaly, a severe disease characterized by abnormally small head and severe neurologic disability. There is no treatment option or approved vaccine for the Zika virus infection or congenital Zika syndrome. Preclinical study of Zika mRNA Vaccine showed that the vaccine prevent the intrauterine transmission of Zika virus in mice and protected the fetuses from Zika-related congenital damage (Richner et al., 2017a; Richner et al., 2017b). This is the first study to establish vaccine protection from the Zika virus during pregnancy. Until now, a few mRNA vaccines for infectious diseases are undergoing clinical trials, mainly rabies, HIV, CMV, influenza and Zika vaccines (Table 2). More preclinical studies are in development and the prospects for success are bright.

6.3 Protein replacement therapy

One of the most frequent applications of mRNA is the introduction of therapeutic antibodies as well as functional proteins, which are missing or malfunctional due to gene mutations. Although these concepts have been proposed for several decades, mRNA molecules were not initially considered as attractive drug candidates. In recent years, advanced technologies such as chemical modification of nucleosides, refined purification process, and novel delivery strategies have largely overcome these shortcomings. mRNA-mediated transcriptional replacement therapy can be applied to produce functional copies of cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is known to be defective in CF patients. In an *in vitro* study, transfection of wild type CFTR mRNA into primary cultured human nasal epithelial (HNE) cells and human bronchial epithelial cells resulted in almost two-fold increase of CFTR expression, and a considerable amount of CFTR were located in the cell

membrane (Bangel-Ruland et al., 2013). Translate Bio's CF mRNA therapy is currently in Phase I clinical study, making it the first company to conduct mRNA therapy testing for rare genetic disease in humans (NCT03375047). Moderna is currently exploring pulmonary mRNA delivery to treat CF.

At present, most mRNA-based gene therapies are carried out in preclinical studies. In 2016, AstraZeneca and Moderna initiated a phase I clinical trial of AZD8601, an investigational mRNA-based therapy that encodes vascular endothelial growth factor-A (VEGF-A). Previously, scientists injected the VEGFA encoding mRNA directly into the hearts of mice. They found that this mRNA can produce enough proteins to improve the survival and health of animals after a heart attack (Zangi et al., 2013). On May 1, 2018, a randomized, double-blind phase II clinical trial was launched to investigate the safety and tolerability of AZD8601 following epicardial injection in patients with moderately impaired systolic function undergoing Coronary Artery Bypass Grafting (CABG) surgery (NCT03370887). This is by far the most advanced program of Moderna.

The proteins translated by mRNA can be converted into therapeutic proteins through a series of processes including folding, post-translational modification, aggregation into secretory granules, and transport to the outside of the cell. Multiple factors may affect the final physiological effect of proteins during these processes. For example, the signal peptide takes great role in directing protein secretion and is applied widely to improve the expression of protein in cells. Extracellular mRNA should be transfected ideally into cells that the encoded protein is naturally secreted. Otherwise the signal sequence should be optimized (Roberts et al., 2011; Sahin et al., 2014; Weissman, 2015). Another major factor should be considered is cell- or tissue-specific delivery of IVT mRNA. The differences of post-translational modifications among various cells is varied from each other. For example, glycosylation, proteolysis, cofactor-dependent folding and clearance of misfolded proteins are cell dependent in heterologous tissues (Weiss et al., 2010). Besides, compared with long-term gene therapies such as plasmid DNA transfection, mRNA delivery results in protein expression with a shorter duration. Sometimes this is

recognized as a limitation of mRNA therapy. However, in the case of repeated administration, many pathological defects can be treated with transient expression of therapeutic proteins. Further understanding of the pharmacokinetic and pharmacodynamic properties of mRNA is certainly required to direct the dosage of mRNA.

6.4 Modulating cell fate and differentiation

Another promising direction of mRNA-based therapy is to use mRNA to program cells and redirect their fates. The discovery of iPSCs provides potential cell sources for building disease models, regenerative medicine, or tissue bioengineering. Both DNA and RNA-based technologies have been successfully used to transfect somatic cells to generate iPS cells, while employment of IVT mRNA has shown faster gene expression and is able to generate integration-free clinical relevant iPSCs (Preskey et al., 2016; Rohani et al., 2016; Yoshioka et al., 2013).

Until now, RNA-based technologies applied in cell reprogramming and lineage-conversion are still in the stage of laboratory research. Many attempts have been made around the main considerations of mRNA, such as the cytotoxicity, immunogenicity, and transfection efficacy etc. Warren et al. reprogrammed multiple human somatic cells to pluripotency cells efficiently and safely (Warren et al., 2010) by transfecting four chemically modified mRNAs encoding *KLF4*, *c-MYC*, *OCT4*, and *SOX2* factors into somatic cells. Yoshioka et al. reported another approach based on Venezuelan equine encephalitis-reprogramming factor (VEE-RF) RNA. The VEE-RF RNA replicon expressed four reprogramming factors. iPS cells were generated efficiently by a single VEE-RF RNA transfection into human fibroblasts, (Yoshioka et al., 2013). These mRNA-based iPS cell reprogramming techniques may have a great chance to be transferred into clinical application.

The IVT mRNA is also applied to direct the differentiation of iPSCs into terminally differentiated cells. It was also demonstrated by Warren et al. that the iPSCs would trans-differentiate into terminally differentiated myogenic cells after repeated introduction of human MYOD1 mRNA (Warren et al., 2010). Besides, using therapeutic mRNA to program undesired, diseased cells to synthesize toxic

intracellular proteins, thus inducing cells self-destruct, is attracting the attention of researchers and biotechnology companies. Moderna, Inc. designed an mRNA encoding a toxin protein, which contains two different miRNA binding sites (miRts) in the 3' UTR. Upon LNP encapsulation and intratumoral injection of the mRNA, sufficient toxic proteins were expressed in hepatocellular carcinoma cells (HCCs) and induced cell apoptosis, but the expression of toxic protein in healthy hepatocytes was limited (Jain et al., 2018b). This is because different miRNAs are recruited into HCCs and healthy hepatocytes by mRNA, and the abundant miRNA 122 in healthy hepatocytes degrades mRNA through the mechanism of small interfering RNA. The other recruited miRNA142 is abundant in hematopoietic cells, resulting in the suppression of protein expression in many antigen presenting cells. Such miRNA-mediated Trojan horse mRNA allowed for a solution to mitigate off-target expression and immune response in mRNA therapy.

6.5 Gene editing using mRNA recombinant techniques

Gene editing technologies hold great promise in treatment of genetic maladies by using engineered nucleases to knock in or knock out the defective gene precisely. These nucleases include zinc-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs), and the RNA guided clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) endonucleases. To date, successful editing has been mediated by DNA plasmid vectors. But the persistent expression of nucleases mediated by plasmids is a double-edged sword. On the down side, the continuous expression of nucleases increases the chance of off-target genome editing. Consequently, the delivery of mRNA encoding ZFNs, TALENs, and Cas endonucleases seems to be an attractive alternative, as their expression is transient.

In recent years, mRNA encoding genome editing tools have been widely used in the construction of human original cells and transgenic animals. The CRISPR/Cas9 system is one of the most common and simple systems used for generating modified genome carrying animals. For example, Cas9 mRNA and guide RNA (gRNA) were introduced into the zygote by microinjection or electroporation, and successfully

produced a large scale of mutant mice (Hashimoto and Takemoto, 2015; Mashiko et al., 2013; Yasue et al., 2014). Other Cas nucleases, such as Cpf1, have also been applied in generation of knockout mice (Kim et al., 2016). In a proof-of-concept study, Cas9 mRNA (≈4500 nt) and sgRNA (≈100nt) were intravenously administered with a novel zwitterionic amino lipids (ZALs) delivery system to induce targeted DNA editing in mice (Miller et al., 2017). Such non-viral RNA delivery system provides a powerful tool for *in vivo* gene editing. Genome editing in human hematopoietic stem cells, progenitor cells (HSPCs) and T cells were also achieved efficiently by electroporating ZFN mRNA and adeno-associated virus (AAV) serotype 6 vectors into these cells (Wang et al., 2016; Wang et al., 2015). However, the safety and efficiency of gene editing technology in clinical translation should be paid much attention to, because a study on genome editing of hematopoietic stem cells and progenitor cells based on CRISPR/Cas9-AAV6 found that Cas9 mRNA invoked transcriptional changes, elicited viral response and overall transcription down-regulation (Cromer et al., 2018).

7 Concerns over the mRNA medicinal industry

Based on the prospect that mRNA can be turned into a powerful therapeutic for treatment of genetic diseases, cancer, infectious diseases, and other diseases, more and more well-funded biotech companies have been established, such as Moderna, CureVac, BioNTech, Argos Therapeutics, RaNA, Translate Bio, Ethris, Arcturus, Acuitas, etc. Although these companies have made breakthroughs in technology, they still have not completely solved the key problems of delivery, off-target effects and immunogenicity of mRNA. Turning mRNA into a drug still has a long way to go. For protein replacement therapy, the dosage needs to be carefully addressed, as the amount of protein produced by the same dose of mRNA may vary greatly in different populations. It is reasonable to screen mRNA candidates encoding proteins that are effective at low doses with broad therapeutic windows (Sahin et al., 2014). That's why vaccines for cancer immunotherapy and infectious diseases are the first choice of mRNA industry. For cancer immunotherapy, a big problem may be the selection of

mRNA antigens. For example, mRNA-4157 (Moderna) consists of 20 mRNAs, which are screened by sequencing the genes of patients-derived tumor and blood. Techniques are still being developed to screen new antigens and predict their potency to produce sufficient immune responses. Important new antigens may be missed, while inefficient and off targeted antigens may be selected, leading to safety problems. It is difficult to figure out how many antigens are needed to produce sufficient immune response because mutant clones in tumor tissues can vary widely.

Deliver mRNA into cells and prompt it escaping from endosome are additional challenges. mRNA spans hundreds to thousands of nucleotides and is much larger than other kinds of RNA drugs such as siRNA. So newly designed delivery systems are required. An even bigger long-term challenge will be the tissue selectivity of mRNA. The commonly used LNPs tend to aggregate in the liver, making mRNA useful for liver targeted therapy. However, the delivery of mRNA into other organs requires an appropriate administration route, such as AstraZeneca's clinical trial of heart attack in which VEGF mRNA is administered by epicardial injection, or a new smart deliver system.

Another noteworthy issue is the transparency of mRNA companies, including technological advances and disclosure of patents. These companies continued to raise funds from private investors but largely kept details of their science. It is possible that investors may see the scientific data, but outsiders can only guess.

8 Summary and perspective

Over the past two decades, mRNA has been one of the least explored frontiers of drug discovery. It has attracted billions of dollars. Compared with traditional protein pharmaceuticals, mRNA has a shorter production cycle, lower cost and easier pollution control. RNA vaccines also avoid several issues associated with DNA vaccines. Moreover, two of the most concerned issues of mRNA, immunogenicity and stability, are under control to some extent upon chemical modification of selected nucleotides. With the approval of other RNA drugs ASO and RNAi, mRNA research field will be hotter if more positive data are released. In the short-term mRNA

technology may have various problems, but it is definitely worth exploring in the long run.

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AUTHOR CONTRIBUTION

Y. W. and Y. H. wrote the paper. T. Y., C. L., B. H., M. Z. and S. G. involved in information collection and discussion. H. X. and X.J. L. provided insightful discussions and suggestions. Y. H. supervised the project.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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Figure Legends

Figure 1. 5'-cap analogues that have been used to express proteins.

Figure 2. Representative base modifications used for *in vitro* transcription of mRNA.

Figure 3. mRNA delivery and protein expression. (a) Scheme of the structure of *in vitro* transcribed (IVT) mRNA. (b) Representative mRNA delivery formulations. (c) mRNA cellular uptake and protein expression process.

Figure 4. Representative chemical structures of lipids, lipidoids and polymers developed for mRNA delivery. The organs or cells that current delivery materials can transport to are shown in the middle. The key considerations of developing novel lipids, polymers, as well as other materials were also included in the figure. mRNA-NP, mRNA-loaded nanoparticle.

Table 1 Representative mRNA delivery systems

Delivery materials	Component ratio (mol% if not specified)	Ratio of material to mRNA	Payload mRNA	Administration route	Targeted cell or tissue	Disease m indication(
Lipid						
DOTMA or DOTAP, DOPE or cholesterol	NA	1.3/2 (charge)	eGFP, Luc, HA, OVA, gp70	i.v.	DC, macrophage	Cancer (me
DOTMA/DOPE, or DOTMA/cholesterol	2/1 or 1/1 (mol)	4/1 or 1.3/2 (charge)	Luc, Cr	i.v.	Lung endothelial and macrophage	NA
Liposome (DOTAP, cholesterol, 1/1(mol)), protamine	1/2 (lipids/protamine, w/w)	3/1 (w/w)	IL-22BP	i.p.	Tumor	Colon canc
DLinDMA, DSPC, cholesterol, DMG-PEG2000	40/10/48/2	3/1 (N/P)	Luc, saRNA encoding RSV-F	i.m.	Vascular endothelia or myocytes.	RSV infect
DLin-MC3-DMA, DSPC, cholesterol, DMG-PEG2000	50/10/38.5/ 1.5	10/1 (w/w)	EPO	i.v.	Hepatocyte	Anemia
DLin-MC3-DMA, DSPC, cholesterol, DMG-PEG2000	55/10/32.5/ 2.5	30/1 (w/w)	Luc, Frataxin	intrathecal	Dorsal root ganglia	Friedreich's
DLin-MC3-DMA, DSPC, cholesterol, DMG-PEG2000	50/10/38.5/ 1.5	NA	CFTR	nasal pumping	Airway epithelia	Cystic fibro
DLin-MC3-DMA, DSPC, cholesterol, DMG-PEG2000	50/7/40/3	NA	FIX, Luc, GFP	i.v.	hepatocyte	hemophilia
DLin-MC3-DMA, DSPC, Cholesterol, DMG-PEG2000, DSPE-PEG2000	50/10.5/38/ 1.4/0.1	16/1 (w/w)	Luc, IL10	i.v.	Leukocyte	Inflammato disease
DOTAP, DOPE, Chol, MP_{1000}-LPX	50/10/35/5	5/1 (N/P)	GFP	In vitro	DC	NA
Antibody-modified liposome (ionizable cationic	50/10/38.5/ 1.5	20/1 (w/w)	Luc, eGFP	i.v.	Lung endothelial	NA

lipid/phosphatidylcholine/cholesterol/PEG-lipid)						
Lipidoid						
C12-200, DOPE, cholesterol, DMG-PEG2000	35/16/46.5/2.5	10/1 (w/w)	EPO, FIX, Cas9 mRNA and anti-FAH sgRNA	i.v.	Hepatocyte	Anemia (E hemophilia hereditary t (anti-FAH
cKK-E12, DOPE, cholesterol, C14-PEG2000, Sodium Lauryl Sulfate (additive)	15/26/40.5/2.5/16	10/1 (w/w)	Luc, TRP2, gp100, OVA, β-gal, Cas9 mRNA and sgRNA	s.c., i.v.	DC, macrophages, neutrophils, and B cells	Cancer; hypercholes (anti-pcsk9
cKK-E12, DOPE, 20α-OH, C18-PEG2000	NA (extrapolated from figure 3M: 50/12.5/35/2.5)	NA	Cre	i.v.	Endothelial cells, hepatocytes, Kupffer cells, immune cells	NA
cKK-E12, DOPE, cholesterol,, 14:0 PEG2000 PE	35/16/46.5/2.5	NA	Anti-HER2 Antibody	i.v.	Hepatocyte	HER2-posit cancer
OF-02, DOPE, cholesterol, DMG-PEG2000	35/16/46.5/2.5	10/1 (w/w)	EPO	i.v.	Hepatocyte	Anemia
OF-Deg-Lin, DOPE, cholesterol, DMG-PEG2000	35/16/46.5/2.5	10/1 (w/w)	Luc	i.v.	Hepatocyte, B cell	NA
OF-C4-Deg-Lin, DOPE, cholesterol, DMG-PEG2000	35/16/46.5/2.5	10/1 (w/w)	Luc	i.v.	Liver, spleen	NA
Lipid 5, DSPC, cholesterol, DMG-PEG2000	50/10/38.5/1.5	5.67/1 (N/P)	Luc, hPBGD	i.v.	Hepatocyte	Acute inter porphyria
Lipid 5, DSPC, cholesterol, DMG-PEG2000	50/10/38.5/1.5	5.67/1 (N/P)	hEPO, mCherry	i.v.	Hepatocyte	Anemia
Lipid 5, DSPC, cholesterol,	50/10/38.5/	5.67/1	hMUT	i.v.	Hepatocyte	Methylmalo

DMG-PEG2000	1.5	(N/P)				acidemia
Lipid H, DSPC, cholesterol, DMG-PEG2000	50/10/38.5/ 1.5	5.67/1 (N/P)	influenza HA, prM-E from Zika	i.m.	Muscle	Influenza o Infection
ATX-100, DSPC, cholesterol, DMG-PEG2000	50/7/40/3	NA	GFP, FIX	i.v.	Hepatocyte	Hemophilia
TT3, DOPE, cholesterol, DMG-PEG2000	20/30/40/0. 75	NA	Luc, FIX	i.v.	Hepatocyte	Hemophilia
TT3, DOPE, Gd-DTPA-BSA, cholesterol, DMG-PEG2000	20/12/18/40 /0.75	NA	Luc, eGFP	i.v.	Hepatocyte	NA
TT3, DOPE, cholesterol, DMG-PEG2000	15/25/45/0. 75	NA	Cas9 mRNA, anti-HBV or pscr9 sgRNA	i.v.	Hepatocyte	HBV or hypercholes
LNP-INT01, DSPC, cholesterol, DMG-PEG2000	45/44/9/2	4.5/1 (N/P)	Cas9 mRNA and anti-TTR sgRNA	i.v.	Hepatocyte	TTR amylo
TNT-b_{10}, DOPE, cholesterol, DMG-PEG2000	30/40/35/0. 75	NA	Luc	i.v. i.p. s.c.	Spleen, liver (hepatocyte)	NA
C14-113, DSPC, cholesterol, DMG-PEG2000	50/10/38.5/ 1.5	10/1 (w/w)	eGFP	Intramyo cardial	cardiomyocyte	Heart disea
ZA3-Ep10, cholesterol, PEG-lipid	100/77/1 (mol)	7.5/1 (w/w)	Cas9 mRNA and anti-LoxP sgRNA	i.v.	Hepatocyte	NA
5A2-SC8, DOPE, cholesterol, DMG-PEG2000	23.8/23.8/4 7.6/4.8	20/1 (w/w)	Luc, mCherry, FAH	i.v.	Hepatocyte	Hepatorena tyrosinemia
MPA-A (or MPA-Ab), DOPE, cholesterol, DMG-PEG2000	20/30/40/0. 75	NA	Luc, Cas9	Intratum oral, i.v.	Cancer cell	NA
C12-(2-3-2), DOPE, cholesterol, DMPE-PEG2000	8/5.29/4.41/ 0.88 (mol)	17/1 (N/P)	Luc	i.v.	Liver, lung	NA
C12-(2-3-2), DPPC, cholesterol, DMPE-PEG2000	NA (projected: 8/5.29/4.41/ 0.88, mol)	8/1 (N/P)	Luc, ACE2	i.v.	Liver, lung	Liver and l fibrosis
C12-(2-3-2), DPPC, cholesterol, DMG-PEG2000, and collagen, sucrose	8/5.29/4.41/ 0.88 (mol for lipids)	8/1 (N/P)	Luc, BMP-2	local implanta tion	Osteoblast	Bone defec
7C2	50/23.5/6.5/	10/1	Cre, Cas9	i.v.	Splenic	Inflammato

(7C1/cholesterol/C14-PEG2000/18:1 Lyso PC) or 7C3 (7C1/cholesterol/C14-PEG2000/DOPE)	20 or 60/10/25/5	(w/w)	mRNA and anti ICAM2 sgRNA		endothelial cells, hepatocyte	
306O$_{i10}$, DSPC, DOPE, cholesterol, 14/0 PEG2000-PE	38.8/3.6/10.9/44.5/2.25	8.75/1 (w/w)	Luc	i.v.	Hepatocyte	NA
306O$_{i10}$, DOPE, cholesterol, C14-PEG2000	35/46.5/16/2.5	NA	Luc	i.v.	Hepatocyte	NA
LNP$_{ssPalm}$, ((ssPalm, DOPE, cholesterol) + DMG-PEG2000)	(3/3/4) +3% or 1% mol	NA	Luc, eGFP	Intracerebroventricular	Brain neuronal cells and astrocytes	NA
LNP$_{ssPalm}$, ((ssPalmO-Paz4-C2, DOPC, cholesterol) + DSG-PEG5000)	(60/10/30) + 3% mol	NA	Luc	i.v.	Inflammatory lesions	NA
Lipid and polymer hybrid						
TarN3C10, DSPC, cholesterol, DMG-PEG2000	5/2/2/1 (w/w)	5/1 (w/w)	EPO	i.v.	Hepatocyte	Anemia
DD90-C12-122, 14/0 PEG2000 PE	100/7 (mol%)	57/1 (N/P)	Luc	i.v.	Lung	NA
A1, 18/0 PEG2000 PE	100/5 (mol%)	50/1 (N/P)	Luc	i.v.	Lung	NA
C1 (PBAE-PCL), 14/0 PEG2000 PE	100/15 (wt%)	50:1 (w/w)	Luc	i.v.	Lung	NA
Amino-polyester (I-DD3, A-TD3, B-DD3), DOPE, cholesterol, DMG-PEG2000	50/25/23.5/1.5	8/1 (N/P)	Luc, Cre	i.v.	Lung endothelium, hepatocyte, splenic APC	NA
Liposome (DOTAP, CHEMS, cholesterol, PEG 2000-lipid) and and micelle (GalNAc-C5-PEG12-ECT (CTA))	NA	NA	Luc, OTC	i.v.	Hepatocyte	Ornithine transcarbamylase deficiency
DOTMA, PLGA	13/60 (w/w)	30/1 (w/w)	mCherry	In vitro	DC	NA
Lip1, Lip2, TriMan-Lip, and PEG-HpK	47.5/47.5/5 (for lipids)	3/1 (w/w)	OVA, E7, MART1	i.v.	DC	Cancer

PLGA4-7 LPNs ((TT3, DOPE, cholesterol, DMG-PEG2000) + PLGA4)	25/25/45/0.75 for lipids (mol), PLGA/mRNA = 9/1 (w/w)	NA	Luc, eGFP	In vitro	Hep3B	NA
CLAN (PEG_{5K}-$PLGA_{11K}$, $PLGA_{11K}$, BHEM-cholesterol)	21.875/1.925/2 (w/w)	25.8/0.1 (w/w)	OVA	i.v.	DC	Lymphoma
Polymer						
CP 2k	N/A	16/1 (N/P)	Luc, HIV gp120	i.n.	Nasal associated lymphoid tissue	HIV
hDD90-118	N/A	50/1 (w/w)	Luc	inhalation	Lung epithelium	NA
PAA8k-(2-3-2)	N/A	2/1 (N/P)	Luc	aerosol	Lung	NA
PE4K-A17-0.33C12, Pluronic F127	95/5 (w/w)	30/1 (w/w)	Luc	i.v.	Lung	NA
CART D_{13}/A_{11} 7 (oligo(carbonate-b-α-amino ester))	N/A	10/1 (cation/anion)	eGFP, Luc	i.m. i.v.	Myocyte (i.m.), spleen and liver (i.v.)	NA
PEI_{10k}-$LinA_{15}$-$PEG_{3.0}$	N/A	4.5/1 (w/w)	eGFP	i.v.	Pulmonary microvascular endothelium	NA
PEG-PAsp(TEP)-Chol	N/A	8/1 (N/P)	sFlt-1, Luc	i.v.	Tumor	Pancreatic
cRGD-PEG-P(Lys-MP), PNIPAM-PLys(SH)	N/A	1.5/1 (N/P)	Luc, GFP	i.v.	Tumor	Neuroglio
$PEG[Glu(DET)]_2$	N/A	2/1 (N/P)	follistatin	s.c.	Hepatocyte	Muscle wa disorder

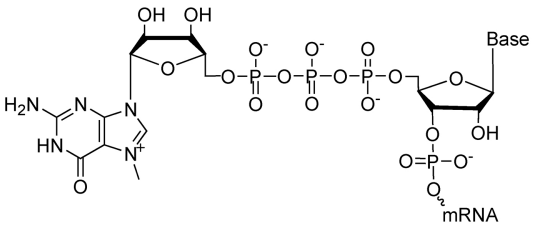
aPACE	N/A	100/1 (w/w)	EPO	i.v.	Hepatocyte	Anemia
P(Asp-AED-ICA)−PEG	N/A	20/1 (N/P)	GFP	in vitro	Cell line	N/A
PEG–PLys(AMP-26)	N/A	2/1 (N/P)	Luc	In vitro	Huh-7	NA
N5 (PEH), recombinant, human eIF4E	N/A	eIF4E to mRNA: 1/1(w/w) ; N5 (PEH) to mRNA: 50/1(N/ P)	ovalbumin, SIINFEKL peptide, Luc	i.v.	Lung	NA
Polyamine (2-2-2-2), PABP	N/A	PABP to mRNA: 1/1(w/w) , Polyamine to mRNA: 50/1 (N/P)	Luc	i.v.	Lung	NA
Polymer and peptide						
OM-PBAE	N/A	25/1 (w/w)	Luc, eGFP	i.v.	Spleen, liver	NA
PPx-GALA	N/A	4/1 (N/P)	eGFP, OVA	In vitro	Cell line, dc	NA
poly(lactic acid), CPP (LAH4-L1, LAH4, RALA)	NA	NA	eGFP	In vitro	DC	NA
RALA	N/A	10/1 (N/P)	eGFP, OVA	i.d.	DC	NA
Biologics						
Exosome	N/A	NA	MGMT	intratumoral	Glioma cell	Glioma
RBC EV	N/A	NA	Cas9 mRNA and anti-	In vitro	Leukemia cell	NA

			miR-125b-2 locus sgRNA			
EV	NA	NA	HchrR6	i.p.	Tumor	Breast canc
EV	NA	NA	EGFP, CD-UPRT	intratum oral	Tumor	Schwannor oma
VSVG-L7Ae virus like particle	NA	NA	eGFP	In vitro	iPSC and monocyte	NA
Lipid hybrid						
Chitosan-alginate gel scaffold, Stemfect™	NA	NA	Luc, OVA	s.c.	DC	NA
CombiMag (neodymium, NdFeB), Stemfect™ (lipid)	NA	NA	LMX1A, FOXA2 and PITX3	In vitro	hNP1	Generate dopaminerg
Squalene, DOTAP, sorbitan trioleate, polysorbate 80	4.3/0.4/0.5/ 0.5 (wt%)	7.1 (N/P)	Clade C envelope glycoprotein	i.m.	Myocytes, DC, macrophage, neutrophil	HIV infecti
Others						
Nhex2NcF4Nce6 (fluorinated peptoid crystal)	N/A	30/1 (mol%)	eGFP	In vitro	H1299	NA
AuNP-DNA	NA	NA	BAX	intratum oral	Tumor	Cancer
MOF-PGMA(EA)	N/A	1.5/1 (N/P)	Luc	In vitro	Cell line	NA

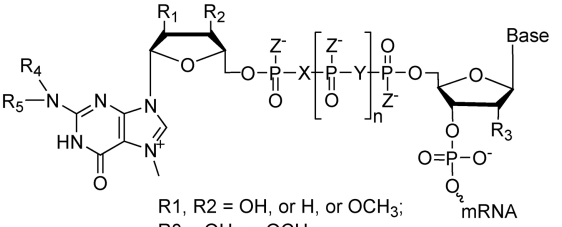
Abbreviation: N/A, not applicable; NA, not available; i.d., intradermal; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; i.v., intravenous; s.c., subcutaneous; r.o., retro-orbital injection; ACE2, angiotensin converting enzyme 2; APC, antigen presenting cell; ASSET, anchored secondary scFv enabling targeting; AuNP, gold nanoparticle; BAX, BCL-2 (B-cell lymphoma 2)-associated X-protein; Cas9, clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9; CD-UPRT, cytosine deaminase (CD) fused to uracil phosphoribosyltransferase (UPRT); CFTR, cystic fibrosis transmembrane conductance regulator; CPP, cell-penetrating peptide; Cre, cyclization recombination enzyme; DC, dendritic cell; DLinDMA , 1,2-dilinoleyloxy-3-dimethylaminopropane; DLin-MC3-DMA, (6Z,9Z,28Z,31Z)- Heptatriaconta- 6,9,28,31-tetraen-19-yl 4-(dimethylamino) butanoate; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyloxy-3-trimethylammonium propane chloride; DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; E7, human papillomavirus 16 (HPV16)

oncoprotein E7; eGFP, enhanced green fluorescence protein; eIF4E, eukaryotic initiation factor 4E; EPO, erythropoietin; EV, extracellular vesicle; FAH, fumarylacetoacetate hydrolase; FIX, factor IX; FOXA2, forkhead box protein A2; gp100, glycoprotein 100; gp120, glycoprotein 120; gp70, glycoprotein 70; HA, influenza virus hemagglutinin; hBMP-2, human bone morphogenetic protein 2; HBV, Hepatitis B virus; hMUT, human methylmalonyl-CoA mutase; hNP1, H9-derived human neural progenitor cell line; hPBGD, human porphobilinogen deaminase; ICAM2, intercellular adhesion molecule 2; IL-22BP, IL-22 binding protein; iPSC, induced pluripotent stem cell; LMX1A, LIM homeobox transcription factor 1 alpha; Luc, luciferase; MGMT, O-6-methylguanine-DNA methyltransferase; MART1, melanoma antigen recognized by T-cells 1; MIT, Massachusetts Institute of Technology; N/P, molar ratio of the amino group (N) to the phosphate group (P); OTC, ornithine transcarbamylase; OVA, ovalbumin; PAA, poly(acrylic acid); PABP, poly(A) binding proteins; PEG, polyethylene glycol; PEI, polyethylenimine; PITX3, pituitary homeobox 3; PLGA, poly(lactic-co-glycolic acid); RBC, red blood cell; RSV-F, respiratory syncytial virus fusion glycoprotein; saRNA, self-amplifying mRNA; sFlt-1, anti-angiogenic protein; ssPalm, SS-cleavable proton-activated lipid-like material; TRP2, tyrosinase-related protein 2; TTR, Transthyretin; β-gal, β-galactosidase; 20α-OH, 20α-hydroxycholesterol.

m7GpppN (native)

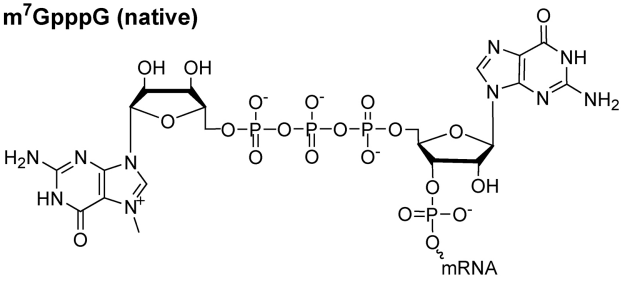


5'-Cap Analogs

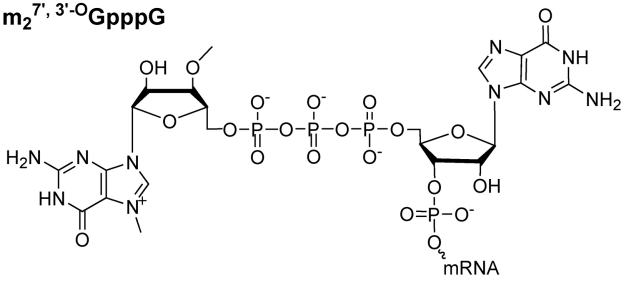


R1, R2 = OH, or H, or OCH_3;
R3 = OH, or OCH_3;
R4, R5 = H, or CH_3;
X, Y = O, or CH_2; Z = O, or S;
n = 1, or 2, or 3

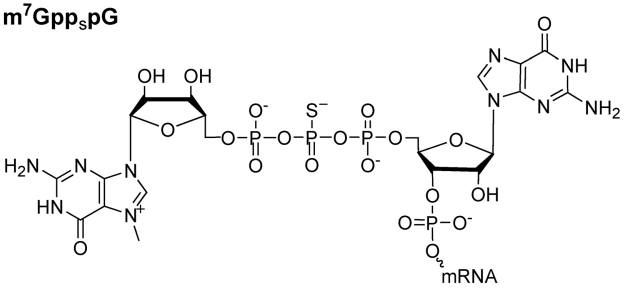
m^7GpppG (native)



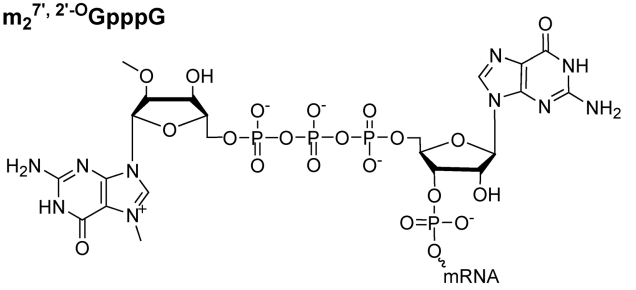
$m_2^{7',3'\text{-}O}GpppG$



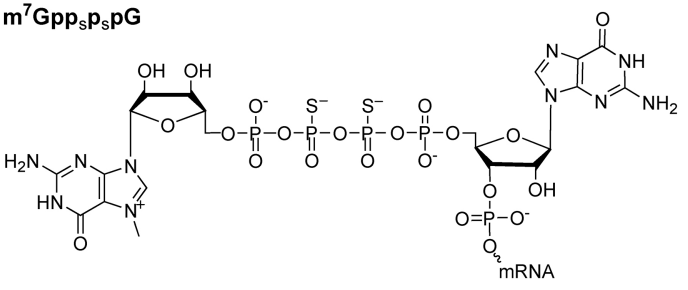
m^7Gpp_spG



$m_2^{7',2'\text{-}O}GpppG$



$m^7Gpp_sp_spG$



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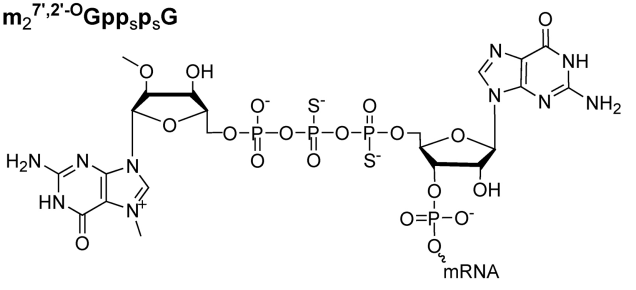
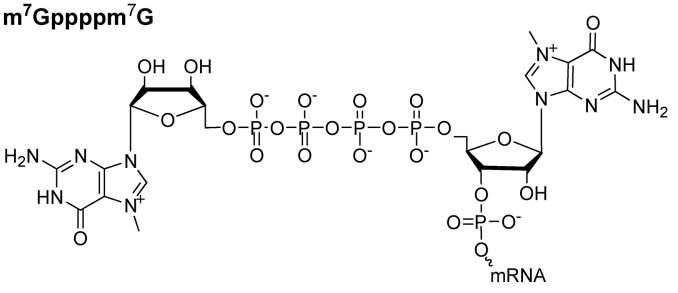


$m^7Gppppm^7G$



$m_2^{7',2'\text{-}O}Gppp_sp_sG$

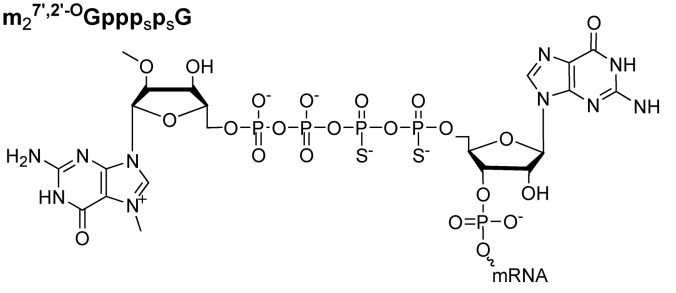


Figure 1

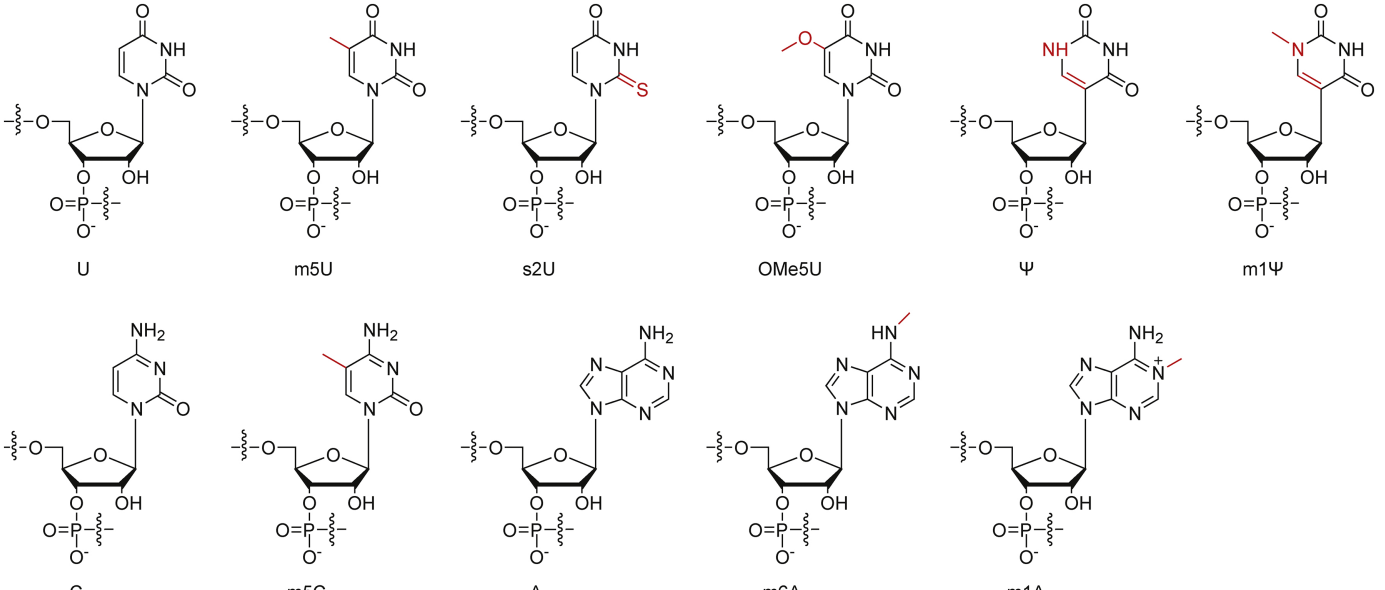


Figure 2

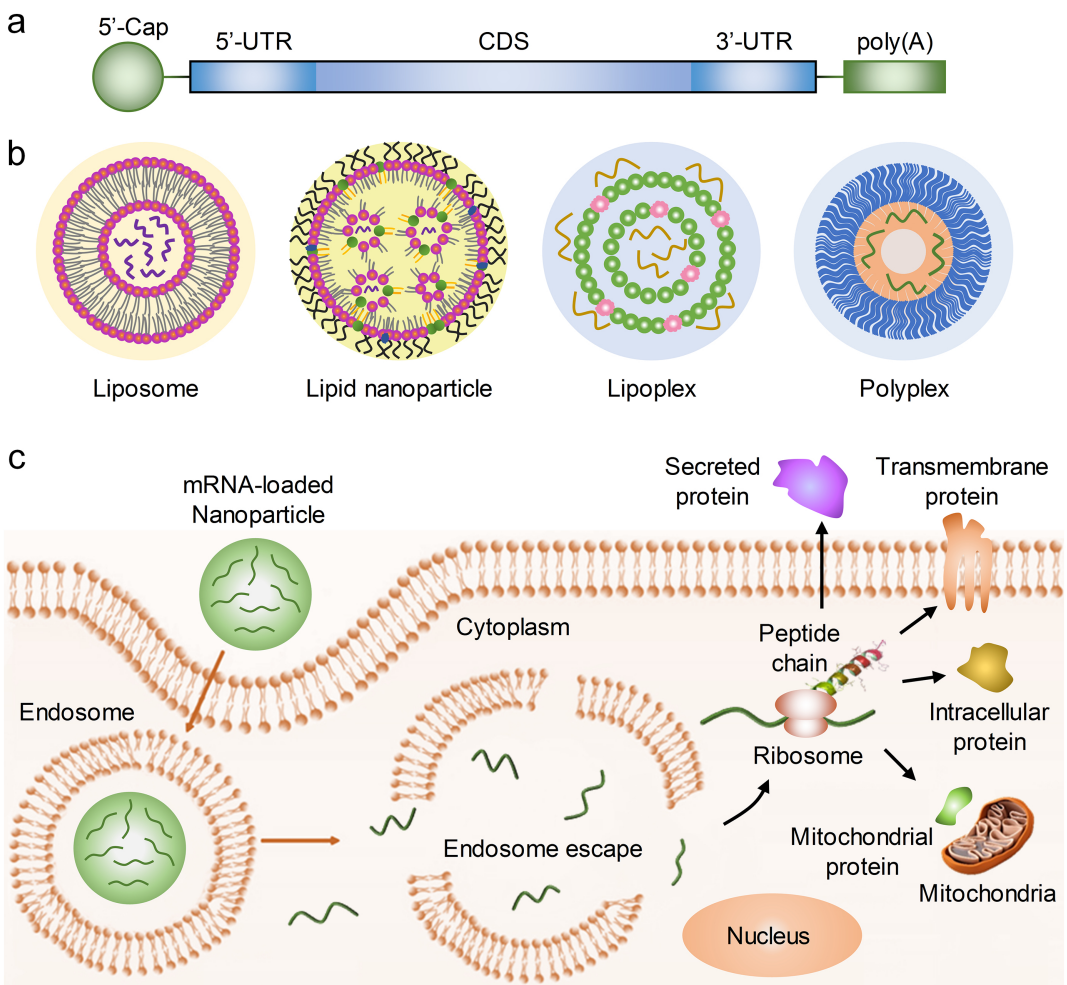

a
5'-Cap
5'-UTR
CDS
3'-UTR
poly(A)
b
Liposome
Lipid nanoparticle
Lipoplex
Polyplex
c
mRNA-loaded
Nanoparticle
Secreted
protein
Transmembrane
protein
Cytoplasm
Peptide
chain
Endosome
Intracellular
protein
Ribosome
Endosome escape
Mitochondrial
protein
Mitochondria
Nucleus

Figure 3

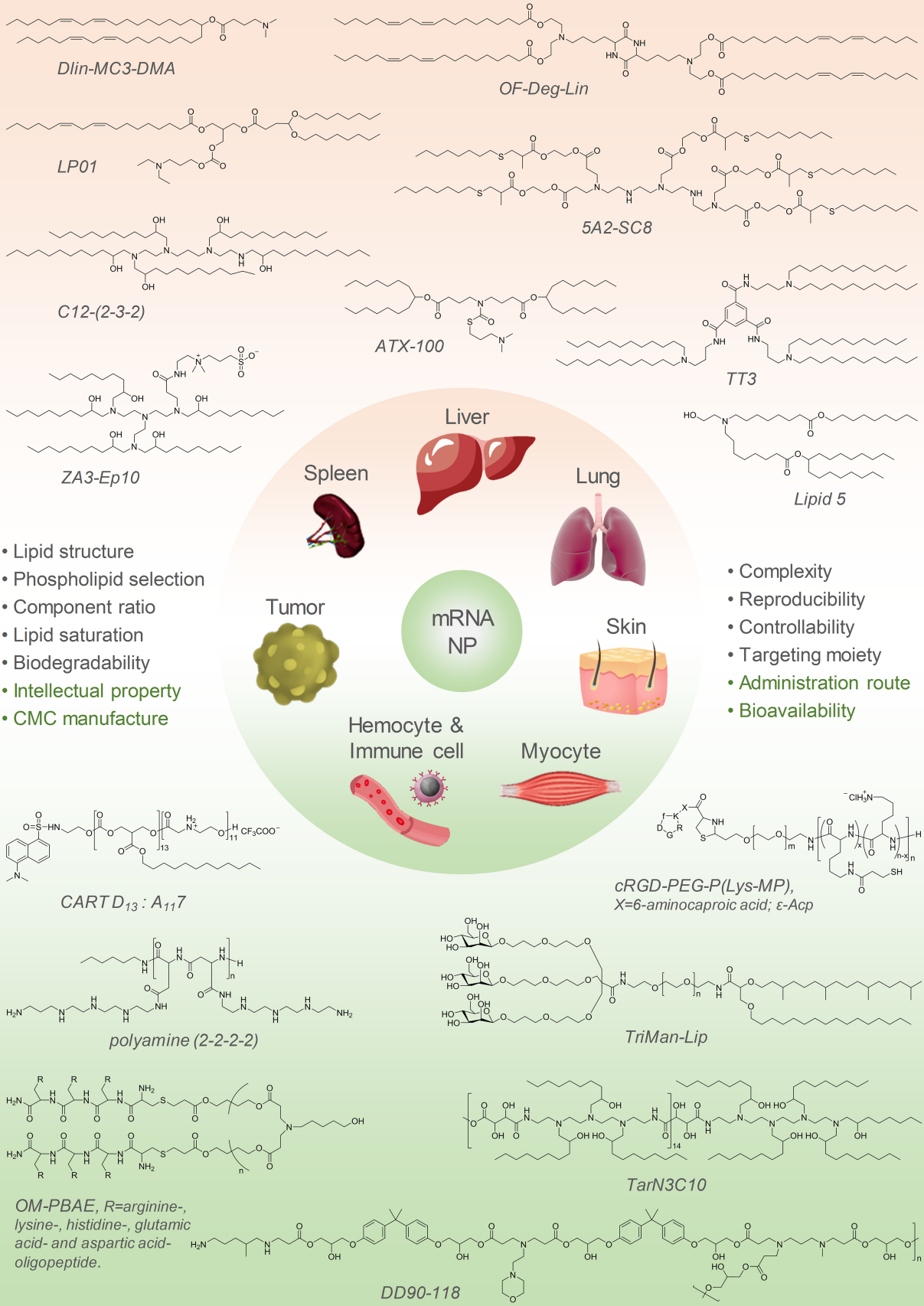

Dlin-MC3-DMA
OF-Deg-Lin
LP01
5A2-SC8
C12-(2-3-2)
ATX-100
TT3
ZA3-Ep10
Lipid 5
Liver
Spleen
Lung
Tumor
mRNA NP
Skin
Hemocyte & Immune cell
Myocyte
• Lipid structure
• Phospholipid selection
• Component ratio
• Lipid saturation
• Biodegradability
• Intellectual property
• CMC manufacture
• Complexity
• Reproducibility
• Controllability
• Targeting moiety
• Administration route
• Bioavailability
CART D13 : A117
cRGD-PEG-P(Lys-MP),
X=6-aminocaproic acid; ε-Acp
polyamine (2-2-2-2)
TriMan-Lip
TarN3C10
OM-PBAE, R=arginine-, lysine-, histidine-, glutamic acid- and aspartic acid-oligopeptide.
DD90-118

Figure 4