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Review

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Nanomedicine-based approaches for mRNA delivery

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Abstract

Messenger RNA (mRNA) has immense potential for developing a wide range of therapies, including immunotherapy and protein replacement. As mRNA presents no risk of integration into the host genome and does not require nuclear entry for transfection, which allows protein production even in non-dividing cells, mRNA-based approaches can be envisioned as safe and practical therapeutic strategies. Nevertheless, mRNA presents unfavorable characteristics, such as large size, immunogenicity, limited cellular uptake and sensitivity to enzymatic degradation, which hinder its use as therapeutic agent. While mRNA stability and immunogenicity have been ameliorated by direct modifications on the mRNA

structure, further improvements in mRNA delivery are still needed for promoting its activity in biological settings. In this regard, nanomedicine has shown the ability for spatiotemporally controlling the function of a myriad of bioactive agents *in vivo*. Direct engineering of nanomedicine structures for loading, protecting and releasing mRNA, and navigating in biological environments, can then be applied for promoting mRNA translation toward the development of effective treatments. Here, we review recent approaches aimed at enhancing mRNA function and its delivery through nanomedicines, with particular emphasis on their applications and eventual clinical translation.

Keywords

mRNA delivery; mRNA engineering; Nanomedicine; mRNA therapeutics; Clinical translation

1. Introduction

Messenger RNA (mRNA) mediates the translation of genetic information from genes into proteins. Delivering exogenous mRNA into cells allows to transiently produce proteins in a precise manner. Such mRNA-mediated transfection offers an attractive alternative to plasmid DNA (pDNA)-based gene therapy by expressing proteins even in non-dividing and hard to transfect cells without the risks of genomic integration.¹ Moreover, while pDNA needs to be delivered inside the nucleus of targeted cells, the access of mRNA to the cytosol and the subsequent engagement with the translation machinery of the cells are sufficient to obtain the proteins of interest. The mRNA delivered inside the cells can also last for several days, which is convenient for developing efficient therapeutic strategies, as well as commercially viable approaches. On the other hand, mRNA presents inherent limitations for being used as a stand-alone drug, including fast degradation by nucleases, limited cellular uptake, and immunogenicity.² While the immunogenic signals triggered by mRNA could be exploited for vaccination or immunotherapy applications,³ major efforts have been dedicated to reduce mRNA immunogenicity

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and improve the stability of the molecule by either chemical modification or by RNA architectonics, aiming at increasing the significance of mRNA as a therapeutic agent. Nevertheless, mRNA is still susceptible to degradation and the cellular uptake of naked mRNA should be improve for eliciting adequate amounts of proteins. Thus, the development of safe carrier systems capable of intracellular delivery of intact mRNA molecules is fundamental for progressing into effective treatments.

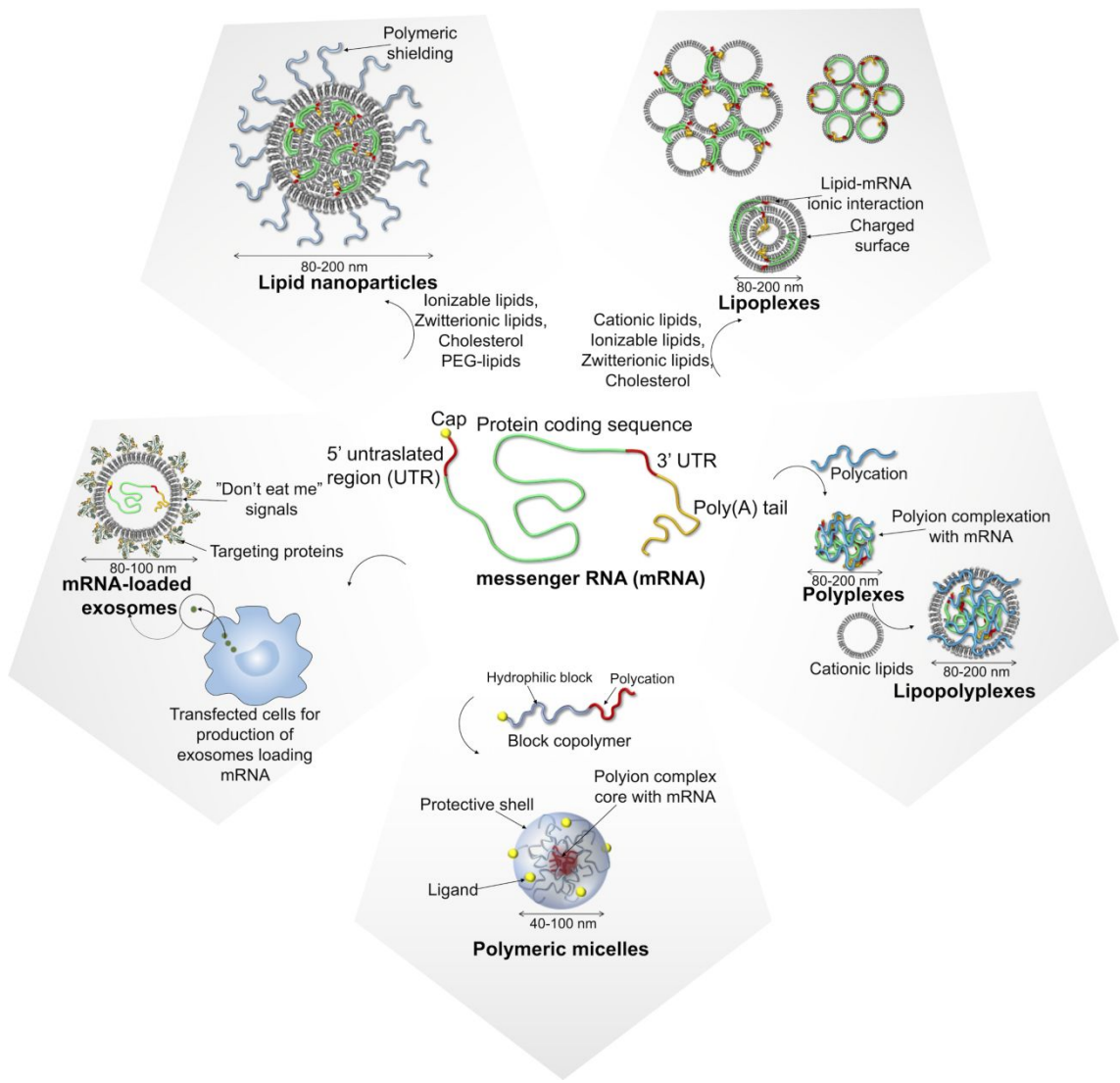


Figure 1. Nanomedicine approaches for mRNA delivery. Various platforms are under intense research and development for the delivery of mRNA based on their unique ability to promote navigation in biological environments, intracellular delivery and engagement with the translation machinery of cells.

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5 2 A wide range of nano-scaled carriers are under intense investigation for developing mRNA
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8 3 delivery systems.¹ Viral vectors, which have been extremely useful for delivering other nucleic acids,
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10 4 have been among the first carriers to be considered for developing mRNA delivery systems.⁴
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12 5 Nevertheless, viral carriers present intrinsic limitations, such as small packing size, immunogenicity,
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14 6 cytotoxicity and complex production processes,⁵ which have spurred the development of safe and
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16 7 effective non-viral vehicles.^{6, 7} These non-viral vehicles can benefit from a myriad of biocompatible
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18 8 synthetic and natural materials for attaining specific physicochemical and functional features directed to
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20 9 develop mRNA-loaded nanomedicines with improved mRNA bioavailability, targeting to specific
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22 10 tissues and cells, and enhanced cellular uptake and intracellular release of mRNA molecules (**Figure 1**).
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24 11 Thus, various non-viral strategies have achieved major breakthroughs in the *in vivo* delivery of mRNA,
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26 12 as well as in the clinical translation of mRNA-based therapies.^{1, 6, 7}
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31 13 Here, we present the recent progress in mRNA-loaded nanomedicine toward innovating
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33 14 vaccination, immunotherapy, treatment of genetic disorders and protein replacement approaches. We
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35 15 have focused on the different non-viral strategies with emphasis on the employed materials and the
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37 16 advantages offered by each approach. Moreover, the mRNA modification methods are also reviewed,
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39 17 highlighting opportunities for synergistically enhancing nanomedicine efficiency. Finally, the trends in
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41 18 the application of mRNA-loaded nanomedicines and their future perspectives are discussed.
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46 20 **2. Engineering mRNA for enhanced function**

47 21 mRNA modification by approaches that enhance the stability and promote its function have
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49 22 been considered for improving mRNA biological activity, as well as reducing immunogenicity issues.
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51 23 Notably, these modification methods do not affect the main structural features of mRNA, *i.e.* being a
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53 24 negatively-charged macromolecule, which provides opportunities for developing orthogonal cooperative
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approaches in combination with nanomedicine with potential for synergistic enhancement of mRNA function. We have categorized these methods as chemical modifications of mRNA, introduction of self-replication functions to mRNA sequences, stabilization through RNA architectonics and circularization of RNA. In the following sections, we review each of these approaches.

2.1. Chemical modifications of mRNA

Chemical modification of mRNA structure is an effective way for promoting mRNAs that are more resistant to enzymatic degradation than their native counterparts, as well as mRNAs with decreased immunogenicity. Major strategies have focused on the introduction of a m7G 5'-Cap structure^{8, 9}, modifications through poly(A) tails^{10, 11}, incorporation of untranslated regions (UTRs)^{12, 13}, and insertion of modified nucleotides¹¹ to the structure of mRNA (**Figure 1**). In the following sections, we summarize the advantages provided by these features.

2.1.1. 5'-Cap structure

During maturation of primary transcripts, a cap is added at the 5' extremity of pre-mRNA.¹⁴ This step occurs very early, before the addition of the first 30 nucleotides. The 5'-cap consists of an inverted 7-methylguanosine connected to the rest of the eukaryotic mRNA via a 5'-5' triphosphate bridge (m7GpppN, Cap 0). Besides Cap 0, the cap can be further modified by methylation at the 2'-O position of the +1 ribonucleotide (m7GpppNm, Cap 1) or the +2 ribonucleotide (m7GpppNmNm, Cap 2), respectively.¹⁵ This methylation is necessary to distinguish self from viral RNA.¹⁶ The presence of the cap is first required for RNA export from the nucleus after binding to the cap binding complex.¹⁷ Once in the cytoplasm, the cap is required for initiation of translation and protection against nuclease degradation.^{18, 19} Accordingly, cap analogs are incorporated in synthetic mRNA by 2 strategies: post-transcriptional capping or co-transcriptional capping.¹⁷ In post-transcriptional capping, capping enzymes

from RNA viruses (mostly *Vaccinia*) are used to produce cap1-capped mRNA using GMP. Although very effective, this strategy requires the expensive production and purification of capping enzymes and is limited to existing natural cap structures.^{17, 20}

In co-transcriptional capping, chemical cap analogs are incorporated during transcription. This allows optimization of chemical analogs, a work well covered by Jemielity's group, as reviewed elsewhere.⁸ During transcription, the m7G cap analog can be incorporated either in correct orientation with the methylated guanosine in 5' (m⁷GpppGpN) or inverted orientation (Gpppm⁷GpN), with a sharp decrease in translation efficiency of mRNA with inverted cap.²¹

The ARCA (Anti Reverse Cap Analog) has been developed for obliged incorporation in the correct orientation.²² In this analog, the hydroxyls of the ribose have been substituted by OCH₃, this stops elongation in the incorrect orientation as it prevents bonds between nucleotides. ARCA-capped mRNAs have 1.8-fold higher translation efficiency over uncapped- or m7G-capped mRNAs.²³

Chemical cap analogs aimed at decreasing cleavage by Dcp1/2 decapping enzymes which cleave the triphosphate bond. Analogues with the second phosphate replaced by an heteroatom have been developed: either carbon²⁴ or nitrogen²⁵. Bridging oxygens have also been substituted with dihalogenmethylenbisphosphonate, and were superior to phosphate substitution both in terms of resistance towards hydrolysis and translation.²⁶

Analogues combining ARCA and phosphorothioated riboses have also been developed (β -S-ARCA analogs).^{9, 27} Such analogues allowed 3-fold higher expression *in vivo* in mice over ARCA-capped mRNA resulting in superior immune response induction.²⁸ Increased translation of β -S-ARCA-capped mRNA was related to increased affinity for the cap-binding translation initiation factor eIF4E and increased resistance to Dcp2 or Dcps degradation over ARCA or m7G caps.^{9, 27, 29}

A further refinement in cap design has been to optimize the first transcribed nucleotide. Indeed, mammalian mRNA can be capped with cap1/cap2 with different first nucleotide (A,G,C,U).³⁰ Sirkoski

et al. compared the translation efficiency of in vitro transcribed and HPLC-purified mRNA harboring caps with different first nucleotides.³¹ They synthesized trinucleotides cap analogs m7GpppNpG where N is the unmethylated or methylated nucleotide and, used them for co-transcriptional capping. They reported that guanine as the first transcribed nucleotide led to the lowest expression in JAWSII murine dendritic cells: 20-fold lower expression of m7GpppGpG cap compared to m7GpppApG cap. This was not due to increased affinity for eIF4E nor increased resistance to decapping enzymes and is in agreement with the abundance of adenosine as first nucleotide in human cell lines.³⁰

One of the drawbacks of co-transcriptional capping is that, due to competition with GTP, not all cap analogs are incorporated alongside transcription and, the efficiency of capping is around 80-90 %.³² To limit antiviral responses, uncapped mRNA is usually removed by Xrn1.^{14, 31, 33}

2.1.2 Poly(A) tail

Another step of pre-mRNA maturation occurs after release from the RNA polymerase II.¹⁴ At the 3' extremity, pre-mRNA is cleaved at 20 nucleotides after a consensus polyadenylation sequence (AAUAAA).³⁴ After cleavage, the polyA polymerase binds to the pre-mRNA and adds a 100 to 250 adenosines polyA tail. The presence of the poly(A) tail is required for translation and for protection against degradation.¹

Polyadenylation of synthetic mRNA can be achieved either by enzymatic polyadenylation of capped mRNA or by cloning a poly(T) sequence in the plasmid template.³⁵ However, enzymatic polyadenylation yields mRNAs with profound differences in poly(A) lengths resulting in a mixture of mRNAs critically affecting reproducibility and purity of mRNA.^{35, 36}

Electroporation of dendritic cells and cancer cells with mRNAs of different poly(A) lengths (0, 16, 42, 51 or 120A) demonstrated the requirement of a poly(A) tail for *in cellulo* translation and a critical impact of poly(A) length on translation efficiency.³⁵ GFP-120A mRNA resulted in a 4-fold higher

transcript level over 51A or 42A mRNA 24 h after electroporation. Increased transcript levels were correlated with higher GFP protein levels. On the contrary, transcript and protein levels were negligible after electroporation of untailed mRNA or mRNA with 16A. Following studies confirmed that the optimal length of the tail is between 120A and 150A, and that it should not be masked by additional bases.³⁷⁻⁴¹

2.1.3. Untranslated regions

5' and 3' UTRs regulate both translation and stability of mRNA.¹⁴ Therefore a strategy to increase translation efficiency and half-life of synthetic mRNA is to include UTRs sequences from stable and highly translated mRNA such as beta-globin, albumin, complement factor 3 (C3) and cytochrome p4502E1 (CYP2E1).^{12, 13} The 5' UTR plays a critical role in the initiation of translation as it controls both mRNA scanning and the selection of the initiation codon.⁴² Because of this role, highly structured 5' UTR regions are avoided and more research has been focused on 3' UTRs. 3' UTRs derived from α - or β -globin, which have long half-lives in erythrocytes (enucleated cells) are the most frequently used for mRNA,^{10, 43} particularly when two sequential β -globin 3' UTRs are fused head to tail.³⁵ Since homologous double UTR (dUTRs) are unstable and prone to recombination, heterologous dUTRs are preferred.^{44, 45} Sahin's group used a SELEX (Systematic Evolution of Ligands by Exponential enrichment) to identify best dUTRs for vaccination and cell reprogramming.⁴⁵ They tested 64 combinations of dUTRs. The two best dUTRs identified were mtRNR1 (mitochondrially encoded 12S rRNA) / β -globin and mtRNR1/ AES (Amino Enhancer of Split) with both 1.89-fold mRNA half-life over double β -globin UTR in human dendritic cells (hDCs). Superior half-life was mirrored with decreased miRNA binding. mtRNR1/AES dUTR also improved mRNA translation in hDCs by 1.6-fold over double β -globin UTR. The intravenous injection of luciferase mRNA lipoplexes in mice revealed a

3-fold higher expression of mtRNR1/AES dUTR over double β -globin UTR, which resulted in superior induction of antigen-specific T cells. For the reprogramming of human foreskin fibroblasts, the AES/mtRNR1 dUTR was the best demonstrating a cell type specificity for optimal dUTR. Recently, Asrani *et al.* evaluated a library of 10 variants of 5' and 3' UTRs in their ability to improve arginase 1 mRNA expression.¹² They reported higher dependence of mRNA translation on 5' UTR sequence relative to 3' UTR variants. Moreover, improved protein production was associated with increased protein translation efficiency, and not to an improved mRNA stability. UTR sequences from C3 and CYP2E1 were found to be the most efficient.

Besides optimization of 5' UTR and 3' UTR regions, sequence engineering of the coding sequence is also required for maximal expression.⁴⁶ Whereas AU-rich motifs are prone to exosome-mediated degradation of mRNA, GC-rich mRNAs have increased expression.⁴⁷⁻⁴⁹ Accordingly, mRNA sequences have been optimized to be GC-rich.⁵⁰⁻⁵²

2.1.4 Modified nucleotides

The first generation of synthetic mRNA lacked the diversity of natural nucleotides present in mammalian mRNA with natural modified nucleotides such as pseudouridine (Ψ), 5-methylcytidine (5meC), N6-Methyladenosine (m6A), 5-methyluridine (m5U), or 2-thiouridine (S^2U).^{11, 53} This led to recognition of mRNA as foreign mRNA by intracellular sensors, such as Toll Like Receptors (TLRs) 7 and 8, resulting in the induction of type I interferon (INF α/β) expression and associated anti-viral responses. These signals resulted in mRNA translation suppression and degradation, and even host cell apoptosis.^{54, 55}

Incorporation of Ψ is the most frequent modification of synthetic mRNA.⁵⁶ Ψ -modified mRNA resists RNase degradation⁵⁷ and lacks TLR activation⁵⁴ resulting in improved translation in cell lysates and cultured cells.^{58, 59} Then, combination of modifications have been preferred to mirror the diversity

1 of natural mRNA. Andries *et al.* reported that mRNA harboring both N methyl Ψ (m1 Ψ) and m5C
2 outperformed mRNA with single Ψ modification or m5C and Ψ modifications in several cell lines and
3 *in vivo*.⁶⁰ Notably, they showed that after intramuscular or intradermal injection of luciferase mRNA
4 lipoplexes, m1 Ψ -mRNA yielded 13-fold higher expression over Ψ -mRNA and 4-fold higher expression
5 over m5C/ Ψ - or m5C/m1 Ψ -modified mRNA suggesting m1 Ψ as a new standard for chemically
6 modified mRNA.

7 Importantly, the incorporation of modified nucleotides is cell-type and mRNA sequence-
8 dependent, requesting application-based screening of modifications.⁶¹⁻⁶³ For instance, Li et al., re-ported
9 that, in THP-1 macrophages, 5meC/ Ψ modified Fluc mRNA resulted in significantly higher Fluc
10 expression, while 5meC/ Ψ modified eGFP mRNA resulted in a decreased GFP expression; m1 Ψ
11 modified Fluc in THP-1 cells generated 8-fold more Fluc than that in hepatocellular carcinoma Hep 3B
12 cells.⁶²

13 14 **2.2. Self-amplifying RNA**

15 Another approach to increase the activity of mRNA comes from mimicry of RNA viruses.
16 Indeed, RNA viruses need to replicate their genome in the host cell for virion production.⁶⁴ Accordingly,
17 researchers have used strategies derived from RNA viruses for vaccination as early as 1999.⁶⁵ These
18 RNA with self-amplification capacity are called self-amplifying RNA (saRNA) or replicons.^{64, 66, 67} In
19 most studies, saRNA are derived from alphaviruses.^{39, 65, 66, 68-72} In alphaviruses, on part of the genome
20 codes for non-structural proteins which assemble to form the RNA-dependent RNA polymerase
21 (replicase), the other part for structural proteins (capsid and envelope proteins).⁷³ After infection,
22 replicase is expressed from the positive strand RNA genome to synthesize the minus RNA strand, which
23 serves as template to amplify the genomic RNA and, for subgenomic RNA synthesis (**Figure 2**). It is

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important to notice that saRNA is amplified after a first round of translation to assemble the replicase, highlighting similar bottlenecks for the early steps of mRNA and saRNA transfection.^{74, 75}

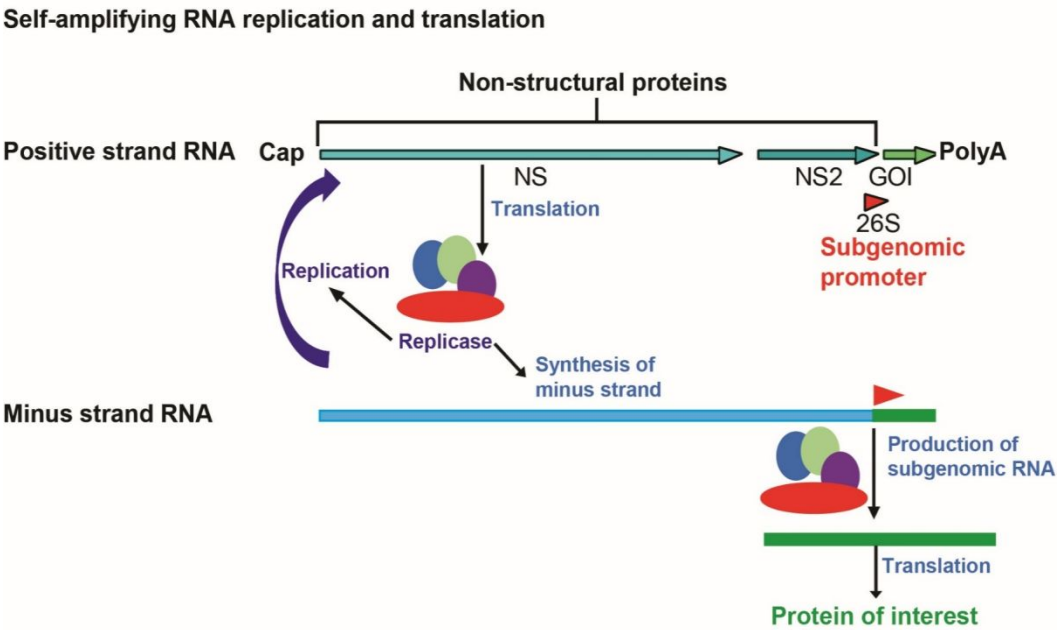


Figure 2. Replication and translation of saRNA. Early after infection the non-structural proteins (NS) are translated and self-assemble to form the replicase. The replicase both amplifies the genome and synthesizes the minus strand RNA. Finally, the replicase translated subgenomic RNA which contains virulence genes allowing production of virions. In synthetic saRNA virulence genes (e.g. capsid) are replaced by the gene of interest. The figure is based on information from a reference ⁷³.

2.3. Circular RNA

Another solution to face the degradation of RNA by exonucleases is the circularization of RNA.⁷⁶⁻⁷⁹ This strategy was inspired by the endogenous circular RNA with covalently linked ends found in viruses ⁸⁰,archaea ⁸¹, all eukaryotes ⁸² among which humans.⁸³ CircRNA is formed by the pre-mRNA splicing machinery from introns ^{84, 85} or from exons ^{71, 86} and its biogenesis is regulated by several factors ⁸⁷. circRNA is generated by back-splicing to join a splice donor to an upstream splice acceptor (**Figure**

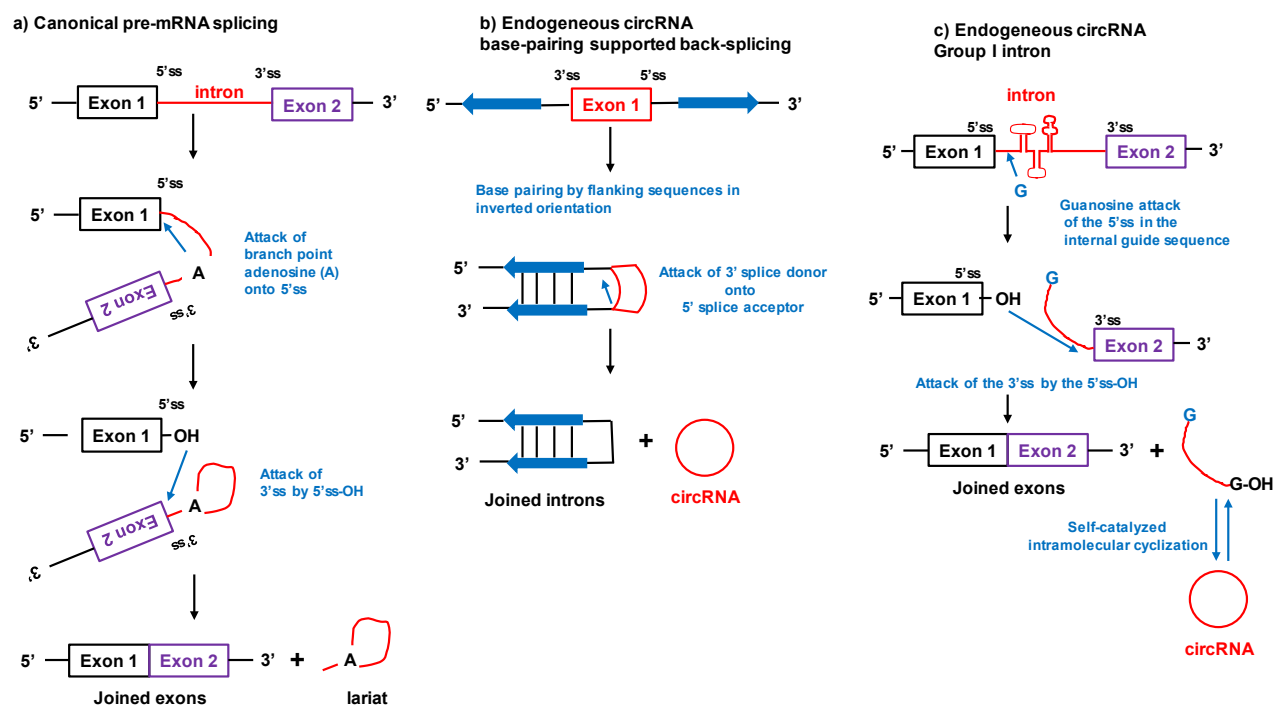


Figure 3. Canonical splicing, back-splicing and self-splicing. **A.** For pre-mRNA, the spliceosome binds to 5' and 3' splice sites (5'ss and 3'ss). The then 2'OH of a 3'ss branchpoint nucleotide performs a nucleophilic attack on the first nucleotide of the 5'ss. This results in the formation of an exon1-3'OH and a lariat intermediate-exon2. Finally, 5'ss-OH attack of exon 2 3'ss joins exons and releases a lariat. **B.** In back-splicing, 5'ss and 3'ss are in reverse orientation. As back-splicing is not sterically favorable, it is supported by complementary base pairing of flanking exons which juxtaposes 5'ss and 3'ss. Then 3'ss attack of the 5'ss splice acceptor joins the two introns and releases circRNA. **C.** An exogenous guanosine (G) attacks the 5'ss separating exon1 5'ss-OH from G-intron 3'ss-exon2. The, 5'ss attack of the 3'ss results in exon joining and release of a linear intron. Group I introns possess a 3' terminal G and this 3'G-OH performs a self-catalyzed nucleophilic attack close to the exogenous G resulting in the formation of circRNA. The figures are based on information from references ⁸⁷⁻⁹¹.

For pre-mRNA canonical splicing, the spliceosome recognizes consensus intron/exon boundary sites: 5' splice site (5'ss) and 3' splice site (3'ss) to remove introns in two steps: 1) formation

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3 1 of a free hydroxyl at the 5'ss, 2) attack of the 3'ss by the 5'ss-OH resulting in exon joining and an excised
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5 2 lariat (**Figure 3A**).⁸⁹
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8 3 Endogenous circRNA is generated when splicing occurs not between 5'ss of exon 1 and 3'ss
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10 4 of exon 2 but between 3'ss of exon 2 and 5'ss of exon 2 that is, back-splicing (**Figure 3B**).^{90, 92} circRNA
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12 5 formation is not frequent due to the low efficiency of the process as upstream 5'ss-3'ss ligation by the
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14 6 spliceosome is sterically unfavorable.⁹³ Thus, circRNA formation is reinforced by repetitive flanking
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16 7 sequences, particularly Alu repetitive sequences in an inverted orientation.^{87, 94}
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18 8 The most frequent method used for *in vitro* production of synthetic circRNA derives from group from
19
20 9 *Tetrahymena* or *Anabena* containing end-to-end fused exons that interrupt half intron sequences.⁹⁰ As
21
22 10 the exon sequences do not participate in the back-splicing they can be replaced by a sequence of interest.^{90,}
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28 12 Group I introns self-splice by recruiting a guanosine (G) which, in the presence of Mg²⁺,
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30 13 performs a nucleophilic attack on the 5'ss phosphate resulting in covalent linkage of G to the end of the
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32 14 intron and generation of a reactive OH on the 5'ss.^{88, 90} The site of G attack is defined by an internal guide
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34 15 sequence present in a duplex formed between the last nt of the 5'ss and the first nt of the intron. The
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36 16 second step involves attack of the 3'ss by the 5'ss-OH. This ligates the 5' and 3' exons and releases a
37
38 17 linear intron. Finally, the linear intron undergoes a self-catalyzed intramolecular cyclization reaction
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40 18 (**Figure 3C**).⁹⁶
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44 19 CircRNA is resistant to exonucleases and highly stable with a median half-life of 24h.⁹⁷ Due
45
46 20 to the low efficiency of back-splicing⁹³, most circRNA have been categorized as side products of pre-
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48 21 mRNA splicing ⁹⁸ or miRNA sponges.⁹⁵ Only few circRNA have been proven to bind and regulate
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50 22 specific miRNA ^{95, 99, 100} or express proteins.^{101, 102}
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54 23 Progression of RNA therapeutics in preclinical and clinical studies ^{103, 104} supported the demand
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56 24 for circRNA to benefit from its enhanced stability.^{76-79, 105} Synthetic circRNA is prepared by
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intramolecular ligation of a linear precursor followed by elimination of linear RNA using RNase R (RNA circularization strategies reviewed in ^{90, 92}). Use of exogenous circRNA for protein expression in mammalian cells is recent (2018).⁷⁸ Authors used a permuted intron-exon method for RNA circularization. This strategy had been applied in 1994 for circRNA production in yeast and bacteria using group I intron of phage T4.¹⁰⁶ Wesselhoeft et al used the group I intron in the thymidylate synthase gene of the T4 phage.⁷⁸ They took advantage of exon-joining by Group I introns and inserted a cassette between 5'ss and 3'ss. Their construct contains 5' and 3' introns. They permuted 5'ss and 3'ss on both sides of the cassette to circularize the insert between splicing sites by group I intron mediated exon joining. The cassette contains an EMCV IRES (encephalomyocarditis virus internal ribosomal entry site) for cap-independent translation followed by Gaussia luciferase with a 3'ss in 5' and a 5'ss in 3' (**Figure 4**). Contrary to group I introns where intron is between two exons, in their construct the 5'ss and 3'ss are separated by the inserted cassette, a distance unfavorable to group I intron self-splicing. Accordingly, authors added homology arms (in green in **Figure 4**) in 5' and 3' to bring introns closer to 5'ss and 3'ss. Using this strategy, they reported circularization of RNA up to 5, 000 nt with close to 100 % circularization efficiency.

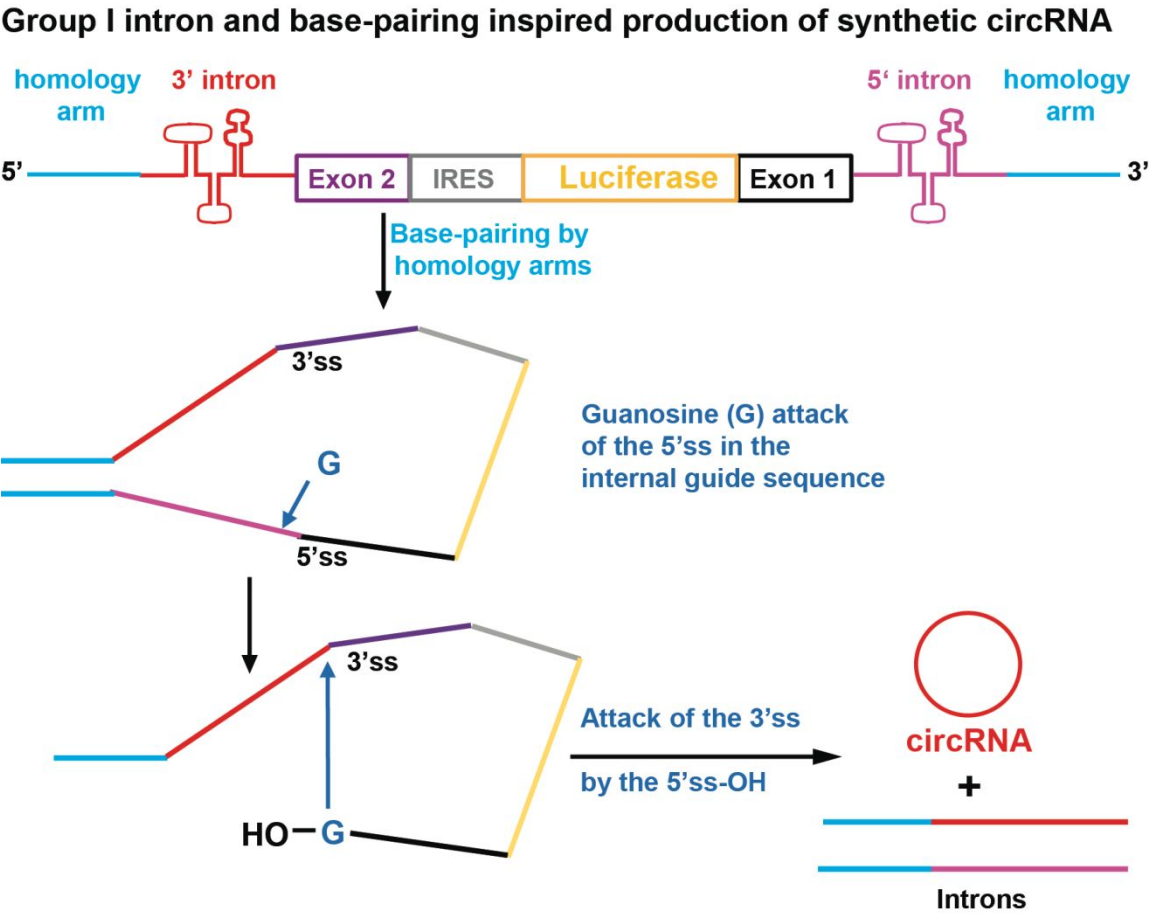


Figure 4. Production of exogenous circRNA by a Group I intron-based strategy. The I intron-based strategy, the 3'ss and 5'ss are too far from each other to allow splicing. Thus, homology arms are added on both sides to bring the two splicing sites closer. Exogenous G attack of the 5'ss releases the 5' intron-homology arm and produces an exon1-OH. This is followed by 5'ss-OH attack of the 3'ss to form a circRNA and release the 3'intron-homology arm. The figures are based on information from a reference ⁷⁸.

Transfection of human cancer cells (HEK293) with circRNA complexed with Lipofectamine Messenger Max (Invitrogen) resulted in 8-fold more luciferase expression over unmodified luciferase mRNA and 50 % more than nucleoside-modified mRNA. This was accompanied with a doubling of luciferase production half-life reaching 80 h compared to 40 h for linear mRNA. Two critical improvements of linear mRNA: HPLC purification and incorporation of modified nucleotides¹⁰⁷ have

also been applied to circRNA and further enhanced its translation and decreased its immunogenicity.⁷⁶,
⁷⁸ Circularization of RNA followed by HPLC purification reduces TLR and RIG-1 sensing.⁷⁸ Concerning
incorporation of modified nucleotides it appears that the circRNA production method needs to be adapted
as replacement of uridine by methyl pseudo uridine or replacement of adenosine by N-methyl adenosine
greatly decreased splicing efficiency.⁷⁹

Along with the stabilization against exonuclease, RNA circularization strategy has a potential
to improve the efficiency of protein production. Among the three protein translational processes,
initiation, elongation and termination, initiation is the rate-limiting phase. In circular RNA without stop
codon, ribosomes continue translation with low frequency of detachment from RNA, thereby skipping
rate-limiting re-initiation step. Indeed, circular RNA without stop codon improved the efficiency of
protein production compared to linear mRNA and circular RNA with stop codon in cell-free translational
system from *E. Coli*, and also in human cells.^{108, 109} For application of this strategy to mRNA therapeutics,
additional mechanism is needed to post-translationally cleave large protein translated from circular RNA
without stop codon for obtaining intended protein.

2.4. mRNA engineering with RNA architectonics

While chemical methods have provided improved translational activity and decreased mRNA
immunogenicity,^{54, 58, 60, 110} only a few types of modified nucleosides allow to preserve mRNA
translational activity, and even widely used modified nucleoside species induce negative effect on
translational processes, depending on their introduction ratio and target cell types (see *Section 2.1.4* for
detail).^{61, 62} Thus, other approaches are needed to expand the options of mRNA engineering.

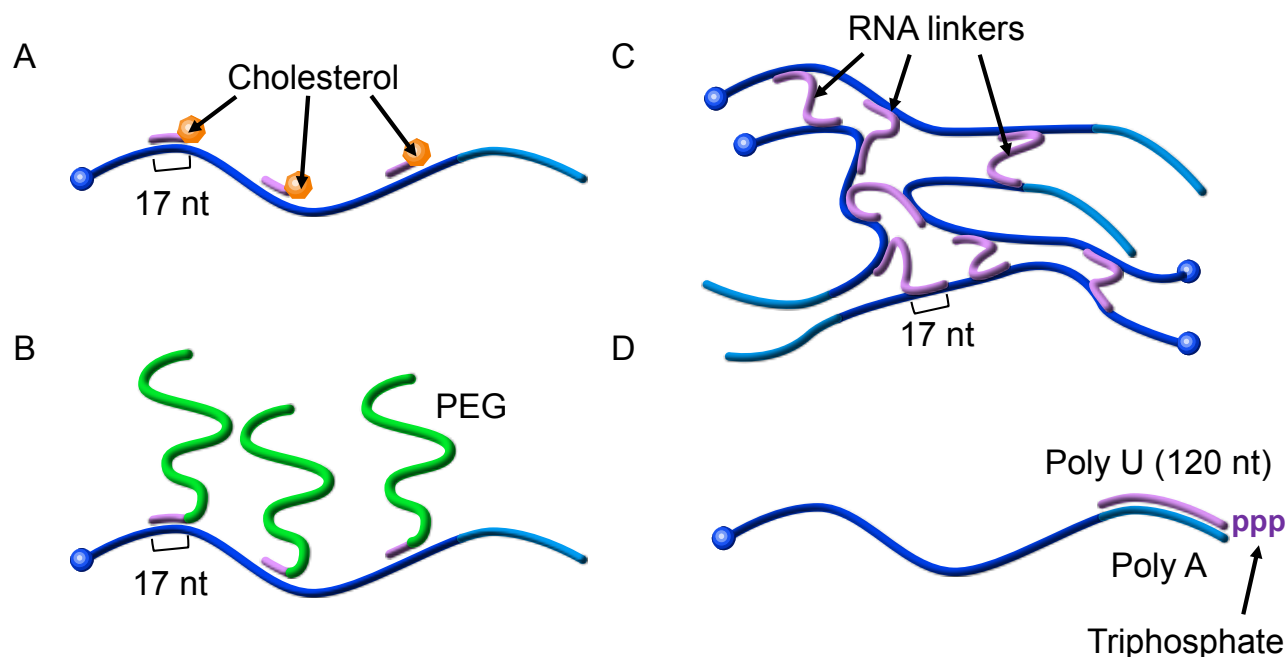


Figure 5. mRNA engineering with RNA architectonics. A, B. Introduction of (A) cholesterol moieties,¹¹¹ and (B) PEG.¹¹² C. mRNA nanoassemblies.¹¹³ D. mRNA hybridized with poly U for mRNA vaccination.¹¹⁴

We proposed an original approach of mRNA architectonics for introducing various functional moieties to mRNA, through hybridization of mRNA with functionalized RNA oligonucleotides (OligoRNAs) (**Figure 5**). Although formation of double stranded RNA structure resulting from OligoRNA hybridization may impair mRNA translational processes and increase mRNA immunogenicity, these unfavorable outcomes were successfully avoided by limiting OligoRNA hybridization length to 17 nt.¹¹¹ This finding motivated us to functionalize mRNA carriers, such as polymeric micelles (PMs) and lipid nanoparticles (LNPs), using OligoRNAs introduced with functional moieties. For structural stabilization of PMs, OligoRNAs introduced with hydrophobic cholesterol (Chol) moiety (Chol-OligoRNAs) was hybridized to mRNA (**Figure 5A**), and then resulting mRNA was mixed with ω -cholesteryl poly(ethylene glycol) (PEG)-polycation block copolymers to prepare PMs (see *Section 3.5* for detail about PMs). Hybridization of just one or a few Chol-OligoRNAs provided drastic

1 effect on tightening mRNA structure in PM core, presumably through stacking between Chol moieties
2 on mRNA and those on the block copolymers. As a result, PMs became stabilized against nuclease
3 attacks and polyion exchange reaction. Eventually, the stabilized PMs showed efficient mRNA
4 introduction in mouse lung with minimal toxicological responses, after intratracheal administration,
5 demonstrating *in vivo* utility of Chol-OligoRNA hybridization strategy.

6 The hybridization-based mRNA engineering approach is versatile to improve bioavailability
7 of other types of carriers, such as LNPs (see *Section 3.1* for detail about PMs). Although mRNA LNPs
8 have demonstrated their high potential especially in delivery to the liver and the spleen,¹¹⁵⁻¹¹⁷ arduous
9 processes, including the use of microfluidics, are needed for mRNA preparation, especially for
10 preventing LNP aggregation after the mixture of cationic lipids with mRNA.¹¹⁸ To tackle this issue, we
11 developed a simple and robust strategy through hybridization of mRNA with PEGylated OligoRNAs
12 (**Figure 5B**).¹¹⁹ This strategy allowed to prevent aggregation after mixing mRNA with commonly used
13 LNPs, including lipofectamine LTX and DOTAP/Chol liposomes, with minimal influence on mRNA
14 translational activity. The PEGylated LNPs thus prepared exhibited high structural stability even in
15 physiological environment, without forming aggregates in mouse blood circulation, while unPEGylated
16 LNPs formed several micrometer-sized aggregates in blood, and caused lung embolism.

17 Other applications of OligoRNA hybridization strategy include mRNA structuring to improve
18 its nuclease stability. Growing field of RNA nanotechnology revealed that nuclease attack to RNA is
19 weakened by structuring RNA, presumably through steric hindrance, and this strategy was applied to
20 siRNA delivery.^{120, 121} Meanwhile, precise sequence designing is needed to obtain intended base-pairing
21 for structuring RNA. This issue hampers direct application of this technology to the delivery of mRNA,
22 which possesses limited sequence flexibility with fixed structure containing a 5'-cap, 5'- and 3'-
23 untranslated regions, a coding region, and a poly-A tail. To prepare structured mRNA possessing all of
24 these endogenous mRNA components without sequence optimization, we crosslinked mRNA strands

1
2
3 1 using linker OligoRNAs, which possess two complementary arms for hybridization (**Figure 5C**).¹²² Each
4
5 2 mRNA nanoassembly thus prepared was comprised of 7.7 strands of mRNA on average, with average
6
7 3 size below 100 nm, and the nanoassemblies exhibited around 100-fold increase in nuclease stability
8
9
10 4 compared to naïve mRNA, with preserved translational activity. Intriguingly, this result suggests that
11
12 5 nanoassemblies remained accessible to translational factors, although the recognition by nucleases is
13
14 6 effectively weakened presumably through steric hinderance. In mechanistic analysis, nanoassemblies
15
16
17 7 from mRNA with 5' cap were selectively dissociated in the environment mimicking intracellular space,
18
19 8 while those from mRNA without 5' cap failed to dissociate. This result suggests that translational factors
20
21 9 recognize 5' cap in nanoassemblies to trigger translation, and then nanoassemblies dissociate through
22
23
24 10 endogenous mRNA unwinding mechanism of translational complexes, which facilitates further
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26 11 translation. Similar unwinding mechanism might contribute to protein translation from mRNA
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28 12 hybridized with Chol-OligoRNAs or PEG-OligoRNAs. Nanoassemblies exhibited efficient mRNA
29
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31 13 introduction to mouse brain, demonstrating their *in vivo* utility. Rolling circle transcription of plasmid
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33 14 DNA, a widely-used method, can also provide structured RNA with protein expressing capability, and
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35 15 this approach resulted in improvement of RNA nuclease stability,¹²³ which is consistent with our finding
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37
38 16 of mRNA nanoassemblies. However, protein translation from RNA prepared by rolling circle methods
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40 17 relies on an internal ribosomal entry site, which tends to yield a much lower level of translational
41
42 18 efficiency compared to cap-dependent translation.¹²⁴ Other strategies to improve mRNA nuclease
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44 19 stability includes the preparation of long double stranded structure in mRNA by hybridizing long
45
46
47 20 antisense strand.¹²⁵ Although this strategy was effective in facilitating mRNA delivery to the liver after
48
49 21 hydrodynamic injection, its versatility is unclear, because long RNA hybridization to mRNA resulted in
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51 22 impaired translational activity and enhanced mRNA immunogenicity.^{111, 114}

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54 23 Interestingly, in RNA hybridization to poly A region, even 120 nt poly U RNA attachment
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56 24 resulted in only modest decrease in translational activity,¹¹⁴ whereas hybridization of 23 nt or longer

OligoRNA to protein coding region led to the decrease in translational activity.¹¹¹ Meanwhile, mRNA hybridized with 120 nt poly U (mRNA:pU) induced strong innate immune responses, especially when the poly U strand possesses triphosphate structure at its 5' end, which is a substrate of an innate immune receptor, retinoic acid-inducible gene (RIG)-I.^{126, 127} We utilized these unique properties of mRNA:pU, preserved translational activity and enhanced immunogenicity, for mRNA vaccine, to obtain antigen expression and immunostimulatory adjuvant function simultaneously (**Figure 5D**). After introduction to cultured mouse and human dendritic cells, mRNA:pU induced enhanced expression of dendritic activation markers, CD86 and CD40, compared to naïve mRNA. Mechanistic analyses indicate the involvement of RIG-I and Toll-like receptor (TLR)-3 for the immunostimulation by mRNA:pU. Eventually, in mouse vaccination experiment using a model antigen, ovalbumin (OVA), mRNA:pU formulation induced cellular and humoral immunity against OVA more efficiently compared to naïve mRNA. Together, mRNA engineering approach based on OligoRNA hybridization is versatile to improve bioavailability of naked mRNA and various types of mRNA carriers, and also enhance the activity of mRNA vaccine, opening up broad opportunities for expanding the potential of mRNA therapeutics.

3. Nanomedicine-approaches for mRNA delivery

The application of nano-scaled carriers is necessary for allowing mRNA molecules to endure in the harsh *in vivo* environments, as well as promote the intracellular delivery of mRNA. Various materials have been employed for constructing such mRNA-loaded nanomedicines, allowing physicochemical and functional control. By engineering the interaction with mRNA and the carrier components, it is then possible to obtain nanomedicines capable of maximizing the stability of mRNA in biological settings, obtaining optimal translation in targeted cells for therapeutic purposes, and reduce the side effects from mRNA molecules or off-target translation. The choice of the carrier components

1 will depend on the eventual route of administration and therapeutic purpose of the nanomedicines. In the
2
3 following sections, we review major nanomedicine approaches for mRNA delivery categorized by the
4
5 materials used to make the carriers. The delivery systems highlighted in this section are summarized in
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Table 1.

Table 1 Nanomedicine-approaches for mRNA delivery

Category	Key component(s)	Core technologies	Size (nm)	Delivery route(s)	Gene(s)	Ref
Lipid-based nanoparticles	DLin-MC3-DMA ^{a)}	Clinically approved ionizable lipid	75-85	i.v., i.c.v. (mouse)	Luc, frataxin	133
	Lipid 5	Optimized ionizable lipids	86	in vitro, i.v. (mouse, rat, NHP)	Luc, EPO, IgG	135
	TT3 ^{a)}	Orthogonal array optimization	110	in vitro, i.v. (mouse)	Luc, hFIX	136
	A18-Iso5-2DC18 ^{a)}	cyclic head group in ionizable lipid for STING activation	96	in vitro, s.c.	Luc, Cre, OVA, tumor antigens	137
	C12-200 ^{a)}	A large scale <i>in vivo</i> screening of mixture ratio	152	i.v. (mouse)	Luc, EPO	139
	SORT lipid (DLin-MC3-DMA ^{a)} , C12-200 ^{a)} , 5A2-SC8 ^{a)} , DOTAP ^{a)} , 18PA ^{a)} , etc.)	Addition of cationic, anionic and ionizable lipid to change tissue tropism	70-200	i.v. (mouse)	Cre, EPO, IL-10, Cas9	141
	C-24 alkyl phytosterols ^{a)}	Optimized cholesterol	102	in vitro	Luc, GFP, Cas9	143
Polyplexes	5A2-SC8 ^{a)}	Dendrimer LNP	100	in vitro, i.v. (mouse)	Luc, FAH	147
	PEtOx-PEI	Optimization of Mw and charge density	80-450	in vitro	Luc	152
	hyperbranched PBAE	Hyperbranched polycation for nebulization.	150	inhalation (mouse)	Luc, GFP, Cre	153
	Dimannose-PGA/PBAE	Macrophage targeting	100	in vitro, i.p., i.v. (mouse)	IRF5, IKK β	154
	PAsp(EDA), PAsp(TET)	Polyplex stabilization	82, 93	in vitro	Luc	155
	PAsp(TET), PAsp(TEP)	Efficient translation	62, 51	in vitro	Luc	156
Lipopolyplexes	PAsp(DPT), PAsp(TET)	Pre-complexation with eIF4E to facilitate translation	100	in vitro, i.v. (mouse)	Luc	157
	HpK-PEG + HDHE/chol	Protonable histidylated lipids	100	i.v. (mouse)	MART1	38
	PEI + Chol/DOPA/DOPE/DSPE-PEG/Man-lip	Neutral and mannosylated LPP	160-190	i.m., i.v. (mouse)	Luc, HA	39
	HpK-PEG + KLN25/MM27	Mannosylated and histidylated lipids	100-140	i.v. (mouse)	MART1	158
	HpK-PEG +	Histidylated lipids and a tri-	100-	i.v. (mouse)	E7	164

	KLN25/MM27/TriMan-lip	mannosylated diether lipid	150			
	PBAE + EDOPC/DOPE/DSPE-PEG	Biodegradable polymer	n.d.	s.c. (mouse)	OVA	167
	PBAE + PEGylated DD90- C12-122	Alkyl chain installation to PBAE for anchoring PEG-lipid	190	i.v. (mouse)	Luc	168
	TT3 ^{a)} , PLGA	Optimal PLGA formulation for potentiate TT3LNP	100- 250	in vitro	Luc, GFP	169
Polymeric micelles	PEG-PAsp(DET)	Alleviation of mRNA immunogenicity	50	in vitro, i.c., i.n., HTVi (mouse, rat)	Luc, GFP, BDNF, Bcl-2, EPO	40, 175, 176
	PEG-PAsp(TET)-Chol	Micelle stabilization using cholesterol	56	in vitro, i.v. (mouse)	Luc, sFlt-1	41
	PEG-PAsp(DET)-Chol/Chol- mRNA	Introduction of cholesterol to mRNA for stabilization	60	in vitro, i.t. (mouse)	Luc	111
	cRGD-PEG/PNIPAM- PLys(SH)	Tumor targeting; hydrophobic layer and disulfide crosslinking for stabilization	59	in vitro, i.v. (mouse)	Luc, GFP	173
	PEG-PLys(AMP)	Disulfide crosslinking and polycation optimization for stabilization	53	in vitro	Luc	178
	PEG-PGBA	Flexible polycation for stabilization	56	in vitro, i.t., i.v. (mouse)	Luc	181
Exosomes	HEK293T-derived exosome	miRNA-responsive translation system	70- 220	i.v. (mouse)	Luc, PGC1 α	189
	Exosome possessing glioma- targeting peptides	microfluidic cellular nanoporation biochip with electrical pulses to increase exosome production and DNA transfection	30- 170	i.v. (mouse)	PTEN	192

^{a)} See **Figure 6** for chemical structure

Lipid5, ; Heptadecan-9-yl 8-((2-hydroxyethyl)(8-nonyloxy)-8-oxooctyl)amino)octanoate PEtOx, poly(2-ethyl-2-oxazoline); PEI, poly(ethylene imine); PBAE, poly(beta amino esters); PGA, poly(glutamic acid); PAsp(EDA), poly[N-(2-aminoethyl)aspartamide]; PAsp(TET), poly(N-{N'-[N''-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl}aspartamide); PAsp(TEP), poly-[N-(N'-[N''-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl)-2-aminoethyl]aspartamide]; PAsp(DPT), poly{N'-[N-(3-aminopropyl)-3-aminopropyl]aspartamide}; HpK, Histidylated polylysine; HDHE, L-Histidine-(N,N-di-n-hexadecylamine)ethylamide; Chol, Cholesterol; DOPA, 1,2-dioleoyl-sn-glycero-3-phosphate; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidyl-ethanolamine; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; Man-lip, 16:0 1,2-dipalmitoyl-sn-glycero-3-phospho((ethyl-1',20,30-triazole)triethyleneglycolmannose; TriMan-lip, trimannosylated archeal diether lipid; EDOPC, 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine; PLGA, poly(lactic-co-glycolic acid); n.d., not determined; i.v., intravenous injection; i.c.v., intra-cerebroventricular injection; s.c.; subcutaneous injection; i.c., intracisternal injection; i.n., intranasal delivery; HTVi, hydrodynamic tail-vein injection; i.t., intratracheal delivery; NHP, non-human primate; Luc, luciferase; EPO, erythropoietin; OVA, ovalbumin; IL-10, interleukin 10; FAH, fumarylacetoacetate hydrolase; hFIX, human factor IX, IRF5, interferon regulatory factor 5; IKK β inhibitor of nuclear factor kappa- β kinase subunit β ; MART-1, Melanoma antigen recognized by T cells 1; E7, human papillomavirus 16 oncoprotein E7; HA, Hemagglutinin; eIF4E, eukaryotic translation initiation factor 4E; BDNF, brain-derived neurotrophic factor; Bcl-2, B-cell lymphoma-2; sFlt-1, soluble fms-like tyrosine kinase-1; PGC1 α , peroxisome proliferator-activated receptor γ coactivator 1 α ; PTEN: phosphatase and tensin homolog.

3.1. Lipid-based nanoparticles

LNPs are one of the most advanced mRNA delivery systems (reviewed in ^{46, 103}). LNPs are prepared by microfluidic mixing of lipids in ethanol and mRNA in acidic buffer (pH 3.0-4.0).¹²⁸ Lipids include an ionizable lipid ($pK_a < 7$) that will be protonated at acidic pH to condense mRNA and release it inside the cells, cholesterol for stabilization, an helper lipid for endosomal escape (usually DOPE, **Figure 6**) and a PEGylated lipid to prevent aggregation of LNPs.¹²⁹ Using an ionizable cationic lipid for mRNA complexation instead of a permanently charged one limits toxicity *in vivo* and increases half-life of the mRNA.¹³⁰ The rapid mixing leads to the formation of an electron dense core surrounded by a lipid monolayer.¹³¹ Mixing is followed by dialysis against a neutral buffer to remove ethanol.

LNPs were first developed for siRNA delivery, mostly to the liver. The system has produced an FDA-approved siRNA LNP called Onpattro, which includes the DLin-MC3-DMA (MC3, **Figure 6**) ionizable lipid.¹³² Accordingly, earlier studies on mRNA LNPs used ionizable lipids with a structure similar to DLin-MC3-DMA.^{46, 133} LNPs are internalized by clathrin-mediated endocytosis, resulting in their accumulation in endosomes and possible clearance by exocytosis.^{134, 135} Protonation of the ionizable lipid at endosomal pH (5.5-6.5) and presence of fusogenic lipids such as DOPE disrupts the endosomal membrane allowing intracellular delivery of mRNA and its translation.^{46, 135}

Biodegradability is an important parameter for the clinical translation of mRNA LNPs. Sabnis *et al.* screened a library of different amino lipid-based LNPs and compared their safety and expression levels in rodents and non-human primates to MC3-LNPs.¹³⁵ They identified ethanolamine as the best headgroup and their library included ethanolamine with different lipid tails. Intravenous administration of all the variants led to expression in the liver of mice. By measuring lipid levels in the liver along time, they reported that ester linkages accelerated liver clearance. Whereas primary esters increased pK_a and decreased mRNA expression, secondary esters improved mRNA expression. The best lipid, lipid 5 (Heptadecan-9-yl 8-((2-hydroxyethyl)(8-(nonyloxy)-8-oxooctyl)amino)octanoate), contains one lipid chain with a primary ester and one lipid chain with a secondary ester and provided 3-fold higher

expression over MC3-LNP. Higher expression was correlated with enhanced endosomal escape with 6-fold more cytosolic mRNA after transfection of HeLa cells with lipid 5-LNPs over MC3-LNPs. Lipid 5-LNPs were more rapidly cleared from liver, spleen and plasma than MC3-LNPs and, contrary to MC3-LNPs did not accumulate in the heart, arguing for better biosafety. Evaluation of the LNPs then moved to Cynomolgus monkeys. Intravenous injection of 0.01 mg/kg of mRNA encoding either human erythropoietin or anti-human IgG influenza antibody resulted in 5-fold higher protein levels of lipid 5-LNPs over MC3-LNPs, with minimal activation of immune markers.

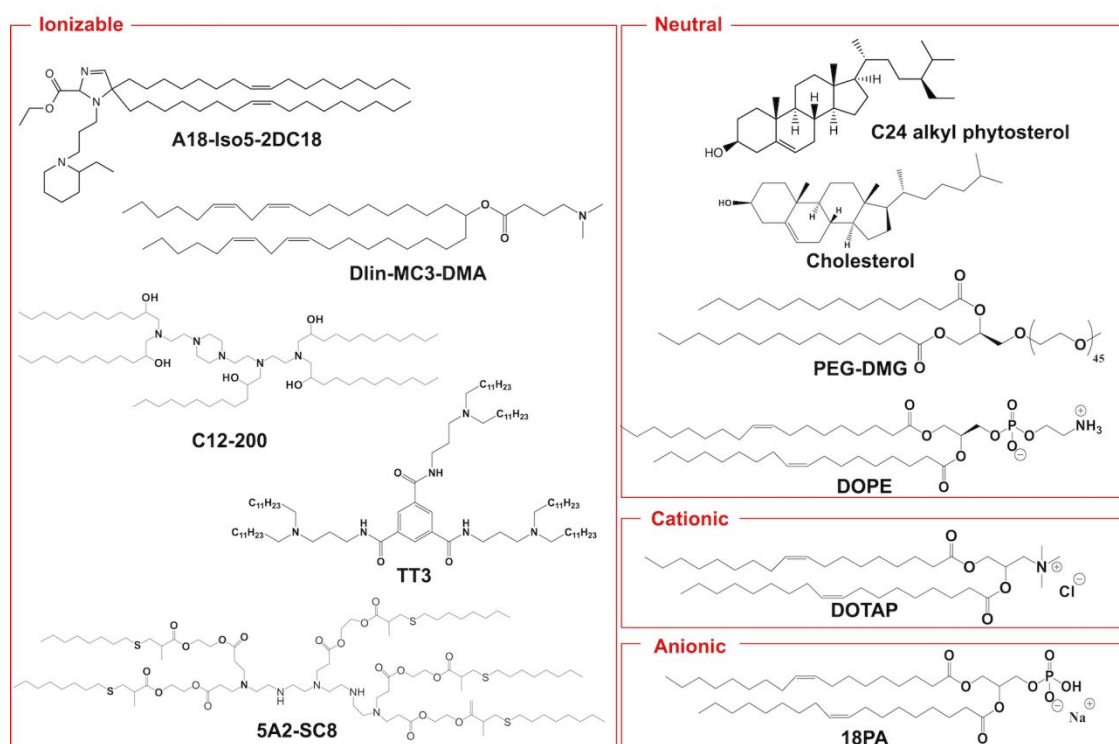


Figure 6. Common lipid and small surfactant structures for preparing LNPs.

Dong *et al.* introduced LNPs where the ionizable lipid is based on TT (N^1, N^3, N^5 -tris(2-aminoethyl)benzene-1,3,5-tricarboxamide, **Figure 6**), a group consisting of a phenyl ring, three amide linkers and three amino lipid chains.¹³⁶ LNPs were prepared with TT3 (T3, DOPE, Cholesterol, DMG-

PEG2000 at molar ratios of 20/30/40/0.75). Intravenous injection of TT3-LNPs resulted in 6-fold higher bioluminescence in mice spleen over C12-200 LNPs (**Figure 6**). They also prepared LNPs using mRNA coding human factor IX (hFIX), a blood clotting factor deficient in hemophilia. Intravenous injection of hFIX mRNA TT3-LNPs at a dose of 1.1 mg/kg was able to restore FIX levels to normal physiological values in FIX knockout mice, suggesting a broad potential for protein replacement therapies.

A further improvement over MC3-LNPs was to use a cyclic lipid headgroup instead of a linear one.¹³⁷ The Anderson group screened a library of LNPs formed with headgroups containing linear or heterocyclic amines (dimethylamino, dimethylaminopropyl, dibutylaminopropyl, dipropylaminopropyl) which were benchmarked against MC3-LNPs. Irrespective of the number of carbon bonds, subcutaneous injection of OVA mRNA LNPs with heterocyclic polar heads showed superior activation of murine splenocytes compared to linear polar heads. Piperazin and azepanyl polar heads induced the highest levels of specific lymphocytes. LNPs with the A18 polar head increased IFN- γ secretion by 10-fold over MC3-LNPs and 75-fold over LNPs with a linear tertiary amine polar head (**Figure 6**). This was explained by enhanced affinity of cyclic lipids towards the binding pocket of the STING protein, resulting in superior expression of the dendritic cell activation markers CD40, CD86 and HLA-DR (Human Leukocyte Antigen DR isotype). A18-LNPs achieved superior protection against tumor growth and extended survival over MC3-LNPs in two murine cancer models.

Helper lipids such as DOPE and cholesterol are critical components of LNPs (**Figure 6**).^{46, 103} DOPE is preferred to DSPC as helper lipid as presence of DSPC leads to the formation of protrusions on the surface of LNPs and mRNA LNPs enriched in DSPC have low transfection efficiency *in cellulo* and lower expression *in vivo*.^{131, 137-139} mRNA LNPs containing DSPC were also reported to induce inflammation at the injection site.¹⁴⁰

All mRNA LNPs to date include cholesterol as a stabilizer and to promote membrane fusion.^{135, 137, 139-144} Substitution of cholesterol by natural analogues has been proposed to increase the potency of

mRNA LNPs.¹⁴³ Authors prepared LNPs with MC3:sterol:DSPC:DMG-PEG at 50:38.5:10:1.5 molar ratios using three groups of cholesterol analogs. Group I included Vitamin D analogs (e.g. Vitamin D3), group II analogs included phytosterols (e.g. β -Sitosterol, **Figure 6**), group III included pentacyclic terpenoids (e.g. botulin). LNPs containing group I analogs had similar size and mRNA encapsulation efficiency as cholesterol LNPs but low transfection efficiency. Inclusion of group III analogs decreased encapsulation efficiency by ≥ 50 % and increased the size of LNPs resulting in poor transfection efficiency. The group II analog β -Sitosterol did not alter LNPs size nor encapsulation efficiency but improved translation efficiency by 48-fold in cancer cells. This enhancement in transfection efficiency was even observed in primary cells, with a 14-fold enhancement in *ex vivo* transfected human peripheral blood macrophages over MC3-LNPs. Improved transfection was due to differences in both microstructure of LNPs and intracellular trafficking. Whereas cholesterol-LNPs presented a smooth surface, β -Sitosterol-LNPs' surface was highly faceted suggesting phase separation of lipid domains¹⁴⁵, packing defects suggested to improve fusion with membranes and thus intracellular delivery.¹⁴⁶ Confocal microscopy imaging of transfected cells highlighted a higher rate of cellular uptake and retention of β -Sitosterol-LNPs, with a no significant enhancement in endosomal escape but higher cytoplasmic mobility of β -Sitosterol-LNPs.

Cheng *et al.* developed LNPs for mRNA delivery and gene editing in lungs, spleen or liver after intravenous injection.¹⁴¹ The tissue tropism of these particles is based on modulation of the internal charge of the LNPs by addition of an additional sorting lipid. LNPs consisted of ionizable lipid (11.9-50%), DOPE as co-lipid (5-23.8 %), cholesterol (19.3-47.6 %), DMG-PEG (1,2-dimyristoyl-rac-glycerol-methoxy(poly(ethylene glycol, 0.8-4.8 %) (**Figure 6**) and an increasing percentage of sorting lipid (0-50%). They compared three different ionizable lipids: the 5A2-SC8 dendrimer (**Figure 6**) which allowed FAH (fumarylacetoacetate hydrolase) mRNA delivery in the hepatocytes of FAH knockout mice and extended their survival¹⁴⁷, the MC3 lipid and the same lipid composition as the FDA-approved

siRNA LNP Onpattro¹³², the C12-200 lipid (**Figure 6**) which allowed mRNA delivery in the liver.¹³⁹ For LNP prepared with these 3 different ionizable lipids, expression of mRNA in organs was controlled by the content of DOTAP (1,2-dioleoyl-3-trimethylammonium-propane, **Figure 6**) permanently charged cationic lipid. LNPs without DOTAP were expressed in the liver, LNPs with intermediate DOTAP content (10-15%) were expressed in the spleen and LNPs with 50 % DOTAP were expressed in the lungs. Measurements of surface charge showed that DOTAP is in the core of LNPs together with the mRNA as zeta potentials were close to 0. Interestingly, the type of lipid dictated organ specificity. Using an ionizable lipid as sorting lipid reinforced the liver tropism of LNPs. Adding an extra 20 % of 5A2-SC8 increased mRNA expression in the liver by 3-fold. Using a zwitterionic sorting lipid favored spleen targeting with exclusive distribution to the spleen of LNPs containing 80% DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) or 50% DOPC (2-((2,3-bis(oleoyloxy)propyl)dimethylammonio)ethyl ethyl phosphate). Negatively charged sorting lipids also favored spleen targeting with exclusive expression in the spleen of LNPs with 10-40% 18PA (1,2-dioleoyl-sn-glycero-3-phosphate) (**Figure 6**). Selective organ targeting (SORT) LNPs achieved high expression of erythropoietin and interleukin 10 after intravenous injection and nearly 100 % gene editing in murine hepatocytes after a single intravenous injection of 0.3 mg/kg of mRNA.

3.2. Polyplexes

Polymer-mRNA complexes (polyplexes), particularly using PEI (polyethylenimine) is one of the earliest strategies used for nucleic acid delivery.¹⁴⁸ Whereas the high cationic density of PEI allows RNA complexation and endosomal escape by “proton-sponge” effect,¹⁴⁹ this also induces cytotoxicity¹⁵⁰ and alters cellular homeostasis.¹⁵¹ Formation of conjugates of low molecular weight PEI with lipids decreases the cytotoxicity while preserving transfection efficiency.¹⁴⁹ Using a library of poly(2-ethyl-2-oxazoline) with varying molar mass and charge densities identified the optimal M_w of PEI for pDNA or

mRNA delivery, the EI content being controlled by hydrolysis kinetics.¹⁵² Whereas large and densely charged polymer (83 kDa and 100 % hydrolysis) was optimal for pDNA transfection of HEK 293T cells, the optimal polymer for mRNA was smaller and with lower charge density (45 kDa and 80 % hydrolysis). This polymer has not been used *in vivo* yet.

Poly(β -amino) esters (PBAEs) are biodegradable polymers that are less toxic than PEI and showed efficient mRNA delivery *in vivo*.^{153, 154} An hyperbranched PBAEs (hPBAEs) formulation has been designed for nebulized mRNA delivery to the lungs.¹⁵³ To evaluate the influence of PBAEs structure on mRNA inhalation, Authors compared linear PBAEs (lPBAEs) and hPBAEs. Whereas lPBAEs and hPBAEs showed similar transfection of A549 lung cells in culture, only hPBAEs were suited for nebulization. Indeed, size of lPBAEs increased from 150- to 1200-nm after nebulization with high polydispersity. Oppositely, hPBAEs size remained stable under 200 nm with low polydispersity. Nebulization of luciferase mRNA polyplexes prepared with hPBAEs resulted in 16-fold higher luciferase protein levels in mice lungs compared to 25 kDa branched PEI polyplexes. Notably, hPBAEs allowed mRNA delivery to all lung lobes and the transfection of 24.6 % of lung epithelial cells with no transfection of lung immune cells nor alveolar damage. This technology holds potential for future clinical application of mRNA polyplexes.

The control of the structure of the polycations may also provide opportunity for intracellular navigation. For example, polyplexes made from *N*-substituted polyaspartamides having odd number of repeats, *i.e.* polyaspartamides modified with ethylene diamine (P(Asp(EDA))) or triethylamine (P(Asp(TET))), showed higher and prolonged expression *in vitro* compared to polyaspartamides having even number of repeats, *i.e.* polyaspartamides modified with diethylenetriamine P(Asp(DET)) or tetraethylenepentamine (P(Asp(TEP))).¹⁵⁵ This effect was associated with the protonation behaviors of the aminoethylene groups in response to changes in pH during endosomal uptake, which direct the endosomal escape and the stability of the polyplexes. Thus, despite the polyaspartamides with even

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2
3 1 number of repeats promoted endosomal escape, they showed lower stability of mRNA in the cytosol of
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5 2 cells, which may lead to mRNA degradation and less protein translation. On the other hand,
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7 3 polyaspartamides having odd number of repeats facilitated a rapid escape from endosomes, though their
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9 4 higher stability in the cytosol resulted in prolonged protein translation. Thus, the stability of these
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11 5 polyplexes in the cytosol appears to correlate with the protonation of the primary amino groups at the
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13 6 side chains. These primary amines are almost fully protonated for polyaspartamides having odd number
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15 7 of repeats, while for polyaspartamides having even number of repeats, the protonation is lower.
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19 8 The polycation structure may also affect the initiation of mRNA translation. For example, by
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21 9 evaluating the translation of polyplexes from a series of polyaspartamides in cell free conditions, it was
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23 10 found that the translation efficiency of polyplexes formed from polymers having higher number of
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25 11 aminoethylene repeating units, *i.e.* (P(Asp(TET))) and (P(Asp(TEP))), was comparable to that of naked
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27 12 mRNA. On the other hand, the efficiency of polyplexes from polyaspartamides having less repeating
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29 13 units, *i.e.* P(Asp(EDA)) and P(Asp(DET)) was significantly decreased.¹⁵⁶ This behavior was explained
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31 14 by the high negative charge of the triphosphate of the m⁷G group in the cap of mRNA. Thus,
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33 15 polyaspartamides presenting higher number of aminoethylene repeating units may bind to the
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35 16 triphosphates in the caps more strongly, and promote binding to eIF4E. Such binding profile was
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37 17 confirmed in a separate study by using FRET imaging studies of Cy5-mRNA/Cy3-eIF4E complexes
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39 18 electrostatically stabilized by polyaspartamides,¹⁵⁷ showing that P(Asp(DPT)) and P(Asp(TET)) keep the
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41 19 co-localization of mRNA and eIF4E inside cells. These findings indicate the relevance of the polycations
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43 20 design for regulating intracellular delivery and targeting to the translational machinery of cells.
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51 22 **3.3. Lipopolyplexes**

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53 23 Lipopolyplexes (LPP) are ternary Lipid-Polycation-nucleic acid complexes.^{39, 158, 159} These
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55 24 ternary complexes were introduced by Leaf's group by complexation of DNA by protamine or polylysine
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1 into polyplexes before interaction with DOTAP cationic liposomes.^{160, 161} Lipopolyplexes allowed a
2 better protection against DNases and a superior gene expression *in vivo* over DOTAP liposomes / DNA
3 lipoplexes. This technology was later applied to RNA delivery by Pichon's group.¹⁶²

4 We used LPP where mRNA is first complexed with PEGylated and histidylated polylysine
5 (PEGHpK) before interaction with cationic liposomes made of cholesterol/HDHE (L-Histidine-(N,N-di-
6 nhexadecylamine)ethylamide).³⁸ The interesting feature of this formulation is the spleen targeting
7 obtained following intravenous administration. Vaccination of mice with LPP prepared with mRNA
8 (termed LPR) coding a melanoma antigen resulted in superior inhibition of melanoma tumor growth
9 compared to mRNA polyplexes or mRNA lipoplexes. To better target dendritic cells harboring mannose
10 receptors on their surface, liposomes harboring mannose moieties have been synthesized. In this second
11 set of study, liposomes were made with bio-inspired lipids: N-methyl imidazolium lipophosphoramidate
12 (cationic lipid, KLN25) and histamine lipophosphoramidate (protonable lipid, MM27), and a
13 mannosylated lipid. These cationic lipopolyplexes made of PEGHpK polymer and KLN25/MM27
14 mannosylated liposomes were designated LPR. Following IV injection, they allowed a better *in vivo*
15 transfection of DCs over unmannosylated LPR resulting in superior tumor growth inhibition and
16 extended survival of mice bearing B16F10 melanoma.¹⁵⁸ To further increase the transfection of DCs *in*
17 *vivo*, a second generation of mannosylated LPR were also formed with the PEG-HpK polymer and
18 KLN25/MM27 liposomes but included a glycolipid containing a tri-antenna of α-D-mannopyranoside
19 instead of a monovalent mannose for improved endocytosis by DCs.¹⁶³ LPR prepared with the tri-antenna
20 targeting motif allowed better transfection of DCs *in vitro* and a stronger tumor growth inhibition in mice
21 compared to untargeted LPRs or LPRs with a mannosylated lipid harboring only one mannose.^{164, 165}
22 Indeed, knowing the structure of oligosaccharides on the ligands recognized by mannose receptors on
23 dendritic cells, having more than one moiety leads to an increase of the receptor avidity and an improved
24 targeting.¹⁶⁶ One of the interesting feature of this formulation is the fact that mRNA lipopolyplexes e did

not rely on type I IFN for effective T-cell immunity by contrast to lipoplexes (Figure 7A). This property led to a similar protection against melanoma growth after vaccination with unmodified or m1Ψ-modified mRNA encoding a tumor antigen (Figure 7B)

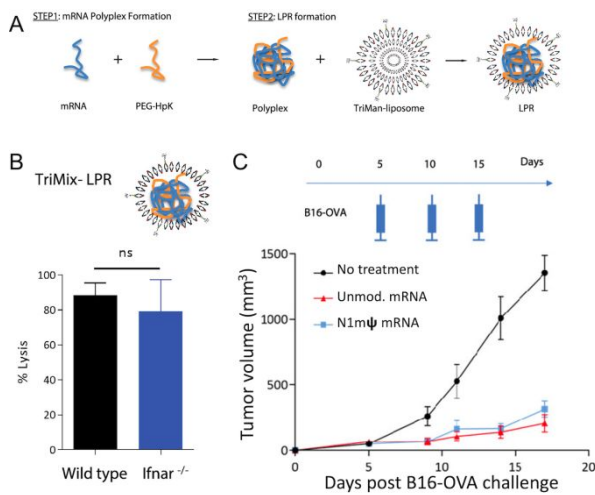


Figure 7. Cationic and trimannosylated lipopolyplexes induce protection against melanoma. A. Preparation procedure of hybrid lipid-shell polymer core mRNA nanoparticles (LPR). **B.** Percent of target cell lysis as measurement of cytolytic T cell response following a single intravenous immunization with LPR containing Trimix RNA in wild-type mice and *Ifnar*^{-/-} mice. **C.** Tumor growth curves of mice vaccinated with unmodified or modified OVA mRNA trimannosylated LPR bearing B16-OVA melanoma. Adapted with permission from reference ¹⁶⁴. Copyright 2018 American Chemical Society.

LPP have also been prepared using other polymers such as PBAE and PEI.^{39, 167} 4 kDa PBAE/mRNA polyplexes were encapsulated in (1,2-dioleoyl-sn-glycero-3-ethylphosphocholine/1,2-dioleoyl-sn-glycero-3-phosphatidyl-ethanolamine/1,2-distearoyl-sn-glycero-3-hosphoethanolamine-N-[amino(polyethyleneglycol)-2000, EDOPC/DOPE/DSPE-PEG) liposomes.¹⁶⁷ Subcutaneous injection of these LPP resulted in stronger stimulation of DCs and better antigen presentation over lipoplexes or polyplexes confirming the superiority of the ternary platform. Importantly, LPP formulation has a potential to improve the functionalities of existing polyplexes and LNPs. For example, PBAE-based polymer was installed with alkyl tail to anchor PEG-lipids, which allowed efficient mRNA introduction to the lung after systemic delivery.¹⁶⁸ Incorporation of poly(lactic-co-glycolic acid) (PLGA) to TT3-

based LNP (**Figure 6**) was effective in increasing mRNA introduction efficiency to several human cell lines.¹⁶⁹

3.5. Polymeric Micelles

Polymeric micelles (PMs) based on polyion complexation have also shown high potential for delivering mRNA.^{170, 171} The PMs are self-assembled in aqueous conditions by ionic and hydrogen bonding between the negatively charged mRNA and block cationomers, having a neutral segment and polycation block. Thus, PMs form a nano-compartmentalized structure with a hydrophilic protective shell surrounding the mRNA payload in the polyion complex core.¹⁷² The mRNA inside the PMs is packaged in globular shape,^{40, 173} probably due to the flexibility of single stranded mRNA. The cationomers can then be engineered to elicit effective protection of the loaded mRNA against enzymatic degradation, as well as promote the cellular uptake by endocytosis and the eventual endosomal escape and access to the cytosol. PMs also allowed to suppress immune responses of mRNA, probably by inhibiting the interaction of mRNA with TLRs and/or hindering TLR mediated signals by endosomal disruption.^{40, 174} These features indicate PMs as promising carriers for dealing with major challenges of mRNA therapy, *i.e.* instability, immunogenicity and translation efficiency.

The potential of PMs for effective mRNA delivery has been demonstrated *in vivo* after local or systemic administration. mRNA-loaded micelles based on the block cationomer poly(ethylene glycol)-*b*-poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-P(Asp(DET))) showed successful transfection in the nasal epithelium,¹⁷⁵ the central nervous system,⁴⁰ and the liver.¹⁷⁶ The PEG-P(Asp(DET)) block copolymer was designed to protect mRNA from rapid degradation and improve the intracellular delivery of mRNA by polyion complexation and shielding with the PEG blocks. Moreover, the polyaspartamide block, *i.e.* P(Asp(DET)), presents 2 pKas, *i.e.* 6.2 and 8.9, which allow for effective polyion complexation at pH 7.4 and enhance endosomal escape after protonation at endosomal pH (pH

5-6.5).¹⁷¹

While mRNA-loaded PMs are stable under physiological salt conditions, polyelectrolytes in biological environments can disrupt them by interfering with the polyion complex between polymers and mRNA.¹⁷⁷ Thus, efforts directed to stabilize mRNA-loaded PMs have focused on maintaining the micelle structure by cross-linking the core with stimuli-sensitive covalent bonds,¹⁷⁸ or by introducing hydrophobic moieties in polycation segments or nucleic acids.^{41, 111} For example, PMs prepared by block copolymers having cholesteryl group at the end (**Figure 8A**), as well as a relatively large PEG segment of 23 kDa and polycation blocks for endosomal escape, showed improved blood circulation upon intravenous injections, achieving 3-orders of magnitude higher concentration in blood compared to naked mRNA. The high stability and extended bioavailability of these micelles promoted their tumor targeting, which resulted in high translation of a mRNA encoding luciferase (**Figure 8B**). The ability of these micelles to elicit antitumor effects was tested by using mRNAs producing the anti-angiogenic sFlt-1 in a model of pancreatic cancer. The enhanced delivery by the cholesterol modified micelles significantly improved the efficacy compared to the micelles without cholesterol (**Figure 8C**).⁴¹ PMs having their core crosslinked *via* reduction-sensitive disulfide bonds by using cyclic-RGD peptide-modified PEG-poly(L-Lysine-thiol) copolymers (cRGD-PEG-P(Lys(SH))) showed improved stability compared to PMs prepared from PEG-P(Lys) block copolymers. Moreover, by adding a hydrophobic layer of thermoresponsive blocks into these micelles just by mixing with poly(N-isopropylacrylamide)-poly(lysine-thiol) (PNIPAM-P(Lys(SH))) copolymers at a temperature below the lower critical solution temperature (LCST) of PNIPAM and then incubating the micelles at physiological temperature, it was possible to further improve the bioavailability of polyion complex micelles 3-orders of magnitude higher compared to PMs prepared from PEG-P(Lys) block copolymers.¹⁷⁹ The enhanced stability of the PMs from cRGD-PEG-P(Lys(SH))/PNIPAM-P(Lys(SH)), as well as the tumor targeting by the cRGD ligand on the surface of the PMs improved the mRNA accumulation in a mouse model of glioblastoma.¹⁷³

Besides crosslinking and increasing the hydrophobicity of the core, the stability of PMs could be enhanced by improving the affinity of the polymers to the mRNA. In this regard, controlling the rigidity of the main chain of the cationomers could provide a straightforward approach for promoting the stability of mRNA-loaded PMs. In fact, molecular dynamics simulations have shown that polycations with higher flexibility may have stronger binding to DNA and double stranded RNAs (dsRNAs) by increasing the entropic gain through water release.¹⁸⁰ We have recently demonstrated this possibility by using 2 cationomers having similar structure, except for their polycation backbone, *i.e.* the standard PEG-P(Lys) bearing a relatively rigid polycation backbone composed of peptide bonds and PEG-poly(glycidyl butylamine) (PEG-PGBA) block copolymer, which has a relatively flexible polycation block comprised of a polyether backbone (**Figure 8D**).¹⁸¹ While both copolymers spontaneously formed PMs after mixing with mRNA in aqueous conditions, PMs made from PEG-PGBA required less polymers to stabilize the micelle structure. By isothermal titration calorimetry measurements (**Figure 8D**), PEG-PGBA was found to have 50-fold higher binding to mRNA than PEG-P(Lys) due to the decreased in the binding enthalpy and the increase in the binding entropy, which leads to lower Gibbs free energy and enhanced the binding affinity to mRNA (**Figure 8D**). The stronger binding of PEG-PGBA resulted in PMs with significantly enhanced the protection against enzymatic attack and disassembly by polyanions. The PMs from PEG-PGBA showed higher intracellular delivery of mRNA, resulting in higher protein translation *in vitro* and *in vivo*. When systemically injected, the PMs from PEG-PGBA were able to circulate in the bloodstream longer time than the PMs from PEG-P(Lys).

These observations indicate the possibility to construct stable and functional mRNA-loaded PMs for systemic applications through precise control of the chemical structure of the cationomers. Such nanomedicine platforms could allow guiding mRNA molecules into hard to reach tissues and cells for eliciting unprecedented therapies. The development of such advanced PM formulations will be associated with systems capable of enduring harsh *in vivo* settings, promoting extravasation in through the blood

vessels of the target organ and effectively engaging with the targeted cells for efficient protein translation.

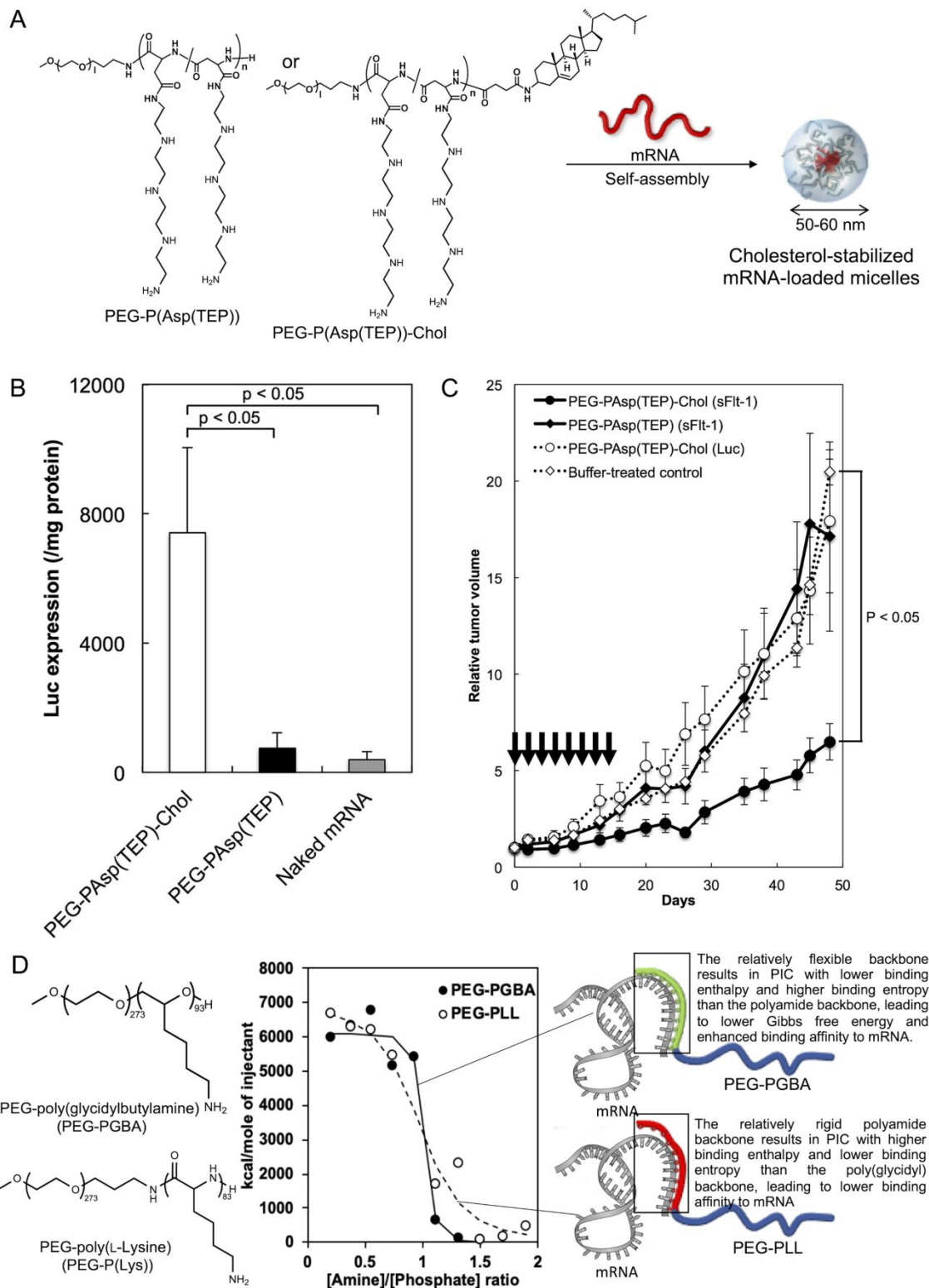


Figure 8. Control of the cationic structure enhance mRNA-loaded micelles performance. A. Self-

assembly of mRNA-loaded micelles by PEG-P(Asp(TEP)) or cholesterol-conjugated PEG-P(Asp(TEP)) (PEG-P(Asp(TEP))-Chol). The block copolymers contain TEP units for endosomal escape and cholesterol for hydrophobic stabilization. **B.** Expression of luciferase in pancreatic BxPC3 tumors after systemic mRNA delivery by PEG-P(Asp(TEP))-Chol or PEG-P(Asp(TEP)) based micelles. Data expressed as the mean \pm S.E.M (n = 4). Statistical significance determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. **C.** Efficacy of systemically injected micelles loading sFlt-1-encoding mRNA against BxPC3. Data presented as the mean \pm S.E.M. (n = 8). Statistical significance determined by ANOVA followed by Tukey's multiple comparison test. Arrows indicate injection points. Reprinted in part with permission from reference ⁴¹. Copyright 2016 Elsevier.

D. The flexibility of the polycation segment affect the binding affinity to mRNA. Structures of PEG-P(Lys) bearing a relatively rigid polycation backbone composed of peptide bonds and PEG-poly(glycidyl butylamine) (PEG-PGBA) copolymer with a backbone having flexible ether bonds (left panel). Isothermal titration calorimetry curves of the formation of mRNA-loaded micelles from PEG-P(Lys) or PEG-PGBA (right panel). Adapted with permission from reference ¹⁸¹. Copyright 2020 John Wiley and Sons.

3.6. Exosomes

In the search for biocompatible mRNA delivery systems, exosomes secreted by almost cell types have been proposed as biological nanocarriers (Reviewed in ^{182, 183}). Exosomes are 50-150 nm vesicles originating from the fusion of multivesicular bodies with the plasma membrane.¹⁸⁴ These vesicles are a means of exchanging proteins, lipids and nucleic acids between producing and receiving cell types¹⁸² with as much as 1,300 different mRNAs in mast cell-derived exosomes.¹⁸⁵ Thanks to their cellular origin, the surface of exosomes presents membrane proteins, transmembrane proteins and immunoglobulins which are “don’t eat me” signals decreasing their clearance from the circulation.^{186, 187} In addition, these

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3 1 endogenous membrane proteins promote their fusion with cells allowing intracellular delivery of
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5 2 cargo.^{182, 188} Nucleic acids can be loaded in exosomes either indirectly or directly. For indirect loading,
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7 3 cells are transfected with nucleic acids before isolation of exosomes: transfection using pDNA to obtain
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9 4 mRNA-loaded exosomes. For direct loading, purified exosomes in suspension are electroporated with
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11 5 nucleic acids, this method is called bulk electroporation (BEP). Cell-derived exosomes are isolated and
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13 6 purified from conditioned media of cells transfected with pDNA coding the desired mRNA or from
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15 7 biological (e.g. blood) by ultracentrifugation or sucrose gradient ultracentrifugation and then stored at -
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17 8 80 °C until use.¹⁸²

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21 9 To decrease the expression of delivered mRNA in undesired tissues, Sun *et al.* applied tissue-
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23 10 specific miRNA-controlled mRNA expression to exosome borne mRNA as an alternative to tissue
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25 11 targeting.¹⁸⁹ In their system, the mRNA IRES contains two specific miRNA binding sites and binding of
26
27 12 the miRNA induces a conformational change resolving tertiary structures inhibiting translation. Using
28
29 13 luciferase mRNA harboring miR-21 binding sites they reported a 6-fold increase in luciferase expression
30
31 14 in the presence of miR-21. They later moved to adipocyte-targeted expression. They produced exosomes
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33 15 by transfection of HEK293T packaging cells with a plasmid coding PGC1 α (Peroxisome proliferator-
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35 16 activated receptor Gamma Coactivator 1 α) harboring two miR-148 binding sites. PGC1 α is an essential
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37 17 transcription factor for fat browning¹⁹⁰ and miR-148 an adipose tissue specific miRNA.¹⁹¹ Exosomes
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39 18 isolated from cell culture media contained 50-70 mRNA copies per 100 exosomes. Exosomes were
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41 19 intravenously injected to fat diet-induced obesity mice and they proved that PGC1 α expression was
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43 20 increased in adipose tissue but not in liver and lungs, common off-target accumulation organs of
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45 21 exosomes after intravenous injection. Moreover, overexpression of PGC1 α in adipocytes promoted fat
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47 22 browning suggesting promising applications for obesity therapy.

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49 23 Using microfluidics and multiple transfections, Yang *et al.* were able increase the production
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51 24 of mRNA-loaded exosomes and decorate them with a targeting peptide for anti-glioma therapy.¹⁹² They
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developed a microfluidic cellular nanoporation biochip (CNP) where pDNA enters cells through 500 nm channels at the bottom and secreted exosomes are collected from the top, followed by isolation using sequential ultracentrifugation. As secretion of exosomes is a stress response, they compared exosome production after stress by starvation, hypoxia, heat or electrical pulses. Electrical pulses generated 10-fold more exosomes than all the other stress signals with a maximal secretion of 150 exosomes per 10⁴ mouse embryonic fibroblasts (MEFs) at 200V. Using a plasmid coding the tumor suppressor PTEN (Phosphatase and TENsin homolog) and RT-qPCR quantitation, they demonstrated that exosomes produced by CNP and electrical pulses stimulation contained 1,000-fold more intact mRNA over BEP cells or Lipofectamine 2000[®]-transfected cells. To target glioma cells, they used a CDX-CD47 plasmid coding the glioma targeting peptide CDX¹⁹³ fused to the N terminus of the exosome-enriched transmembrane protein CD47 to produce targeted exosomes (Exo-T). Adding the CDX-CD47 plasmid resulted in the production of PTEN mRNA-loaded Exo-T harboring the CDX peptide on their external surface. Presence of the targeting peptide increased cellular uptake and PTEN expression in murine U87 glioma cells over untargeted exosomes. Finally, intravenously injected Exo-T were able to cross the blood-brain barrier and accumulate in mice brain leading to superior tumor growth inhibition and extended survival of mice bearing orthotopic gliomas compared to untargeted exosomes. As CNP improve the yield of mRNA loading in exosomes using a microfluidic platform and versatile targeting can be implemented using a CD47 fusion plasmid, this technology shows high translational potential for exosomal mRNA targeted delivery.

4. Applications

4.1. Vaccination

Early applications of mRNA have been focused to the development of vaccines. This is because mRNA presents several advantages for eliciting potent immunization, as well as the potential to fast and cheap

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3 1 processing and easy handling.³ Moreover, as variety of antigens can be encoded in the mRNA sequence,
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5 2 it is possible to utilize the same carrier platform for eliciting effective immunization against a wide-range
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7 3 of diseases. The carriers used for mRNA vaccination are designed to deliver the antigen-encoding mRNA
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9 4 molecules to antigen presenting cells (APCs). Inside the APCs the mRNA molecules produce proteins
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11 5 that are processed into peptide epitopes for presentation through the major histocompatibility complex
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13 6 (MHC) class I. The efficiency of the immunization will depend on several aspects, including the route
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15 7 of vaccination, the cellular targeting, and the translation efficiency and the toxicity of the carrier.
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22 9 **4.1.1. Carrier development for mRNA vaccines**

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24 10 Among various mRNA carriers for mRNA vaccines, LNPs are most vigorously studied. In the
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26 11 researches introduced in *Section 3.1*, LNP development was mainly directed to maximizing its protein
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28 12 expression potential. Meanwhile, some researches optimized LNP formulations based on its vaccination
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30 13 capability. Oberli *et al.* ranked LNPs on their ability to induce ovalbumin (OVA)-specific T cells 7 days
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32 14 after subcutaneous injection.¹⁴⁰ They compared several ionizable lipids (C12-200, cKK-E12 and
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34 15 503O13) and positively charged lipids combined with a lipid-anchored PEG, cholesterol and a helper
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36 16 lipid (DSPC or DOPE). The best formulation, B11, was composed of cKK-E12/DOPE/Chol/C14-
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38 17 PEG2000 (10/15/26/40.5/2.5 mol %) and induced 4.2 % OVA-specific T cells. Therapeutic vaccination
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40 18 of mice harboring established B16-F10 melanoma with B11 LNPs prepared with TRP2 (Tyrosinase
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42 19 Related Protein 2) and gp100 (glycoprotein of 100 kDa) tumor antigen mRNAs extended their survival
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44 20 compared to LNPs prepared with irrelevant mRNAs.
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49 21 Lutz *et al.* compared the vaccinal potency of LNPs to licensed influenza vaccines in non-human
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51 22 primates.¹⁹⁴ They prepared LNPs using the ionizable lipid L319, DSPC, cholesterol and PEG-DMG at a
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53 23 molar ratio of 555:10:32.5:2.5. They showed that intramuscular vaccination of cynomolgus monkeys
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55 24 with LNPs formed with mRNA encoding the hemagglutinin antigen from the pandemic influenza strain
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H1N1pdm09 induced functional antibody titers above protective levels for 5 months after vaccination. Antibody titers were comparable to those obtained after vaccination of monkeys with the licensed Flud vaccine. In addition, the LNP vaccine induced antigen-specific CD4 and CD8 T cells which were absent in the Flud[®] group.

To advance the application of mRNA-LNP vaccines, the Coalition for Epidemic Preparedness innovations initiated a partnership agreement worth up to US\$ 34 million with the German mRNA vaccine company Curevac to develop a downscaled and transportable facility called RNA Printer for the production of several grams of mRNA LNPs in a few weeks. Altogether, these data show that the LNP technology is a powerful and biocompatible mRNA delivery platform and warrants clinical trials in the near future. As of now few mRNA LNPs are under phase 1 clinical trial for several applications: SARS-CoV-2 Infection (NCT04283461 in USA and BNT162 in Europe), a phase 1 melanoma trial (mRNA-4157) and a phase 1 trial in several cancers (NCI-4650), phase 1 against cystic fibrosis (NCT03375047).

Besides LNPs, mRNA complexation with protamine, a small arginine-rich protein, provides a promising platform.¹⁹⁵ Protamine/mRNA complex functions as an immunostimulatory adjuvant, by stimulating TLR7 signaling. While tight complexation with protamine resulted in drastic reduction of protein translational capability of mRNA, translational efficiency was recovered by decreasing protamine/mRNA ratio to prepare mixture of naked mRNA and protamine/mRNA complexes.¹⁹⁶ The optimal formulation that balanced the properties of immunostimulation and protein translation was used in clinical trials of vaccination against infectious diseases (NCT02241135),¹⁹⁷ and cancer (NCT01915524).¹⁹⁸ While carriers were used in most researches of mRNA vaccine, even naked mRNA provided vaccination effects as described in the next section.

4.1.2. Route of vaccination

mRNA-based vaccines are usually administered through local or systemic injection depending

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3 1 on the antigen expression localization requirements. Direct subcutaneous, intramuscular, or intradermal
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5 2 injection of mRNA have been the main routes for vaccines directed to infectious diseases, while
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7 3 intravenous injections are used when the systemic expression of antigens is required.
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12 5 *Intradermal*
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14 6 After intradermal delivery, mRNA-transfected resident DC need to migrate to lymph nodes to
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16 7 present antigens to T cells and induce an adaptive immune response.¹⁹⁹ Intradermal injection of naked
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18 8 mRNA resulted in the transfection of dermal DC with subsequent priming of antigen-specific CD8⁺ T
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20 9 cells.²⁰⁰ Using fluorescently-labeled mRNA, authors could show mRNA expression occurred in the
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22 10 dermis not the epidermis and, that dermal DCs and dermal macrophages internalized the mRNA.
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24 11 Although mRNA was delivered to dermal DCs and macrophages to the same extent (5% mRNA positive
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26 12 cells for both), using transgenic mice expressing the DC-specific integrin cD11c fused to the diphtheria
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28 13 toxin receptor they could demonstrate that dermal DCs are the main contributor to mRNA expression in
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30 14 the dermis. Internalization occurred *via* macropinocytosis as pre-conditioning the injection site with the
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32 15 micropinocytosis inhibitor rottlerin dramatically decreased the expression of luciferase mRNA in the
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34 16 dermis. Finally, T cell priming was reduced in rottlerin-treated animals confirming that intradermal
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36 17 mRNA vaccination proceeds by macropinocytosis in dermal DCs. Feasibility of this administration route
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38 18 for mRNA vaccine has already been demonstrated even in large animals, with mRNA LNP inducing the
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40 19 production of antibodies against human immunodeficiency virus type 1 (HIV-1) in rhesus macaques.²⁰¹
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49 21 *Intranodal*
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51 22 Because ultimately, the naked mRNA requires to reach a lymph node, some groups directly
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53 23 inject the mRNA in the lymph nodes: by intranodal injection.⁴⁶ As the lymph nodes are rich in immune
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55 24 cells, intranodal injection results in mRNA accumulation in both macrophages and dendritic cells,
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antigen-presenting cells capable of inducing an adaptive immune response.²⁰² However, in injections need to be performed by highly-trained personnel: by surgically exposing the inguinal lymph node followed by injection and wound closure in mice,²⁰³ by a radiologist guided by echography in inguinal lymph nodes in humans.²⁰⁴

Kreiter et al. compared the vaccine activity of naked mRNA administered by intranodal, subcutaneous or intradermal routes in mice.²⁰⁵ Using luciferase mRNA they showed highest bioluminescence after intranodal injection, 3-fold higher than intradermal injection at 16h whereas no signal was detected after subcutaneous injection. By injecting mRNA coding the influenza antigen hemagglutinin they reported that only intranodal injection led to the expansion of antigen-specific T cells both in lymph node, blood and spleen. Therapeutic evaluation as a preventive cancer vaccine demonstrated superior survival of intranodally vaccinated mice over s.c. or i.d. vaccinated mice with s.c. route offering no protection at all.

The same authors then evidenced that naked mRNA selectively accumulated in lymph node DCs after intranodal injection *via* macropinocytosis highlighting the importance of this endocytic pathway for naked mRNA vaccination both by intradermal and intranodal routes.²⁰²

Joe et al. compared intranodal mRNA vaccination to intramuscular pDNA vaccination against influenza.²⁰³ Intramuscular pDNA vaccination was used as a benchmark. mRNA vaccination induced more antigen-specific cytotoxic T lymphocytes both in the lymph nodes and the spleen over pDNA. Intranodal mRNA also partially protected mice from viral challenge.

Intranodal mRNA vaccination recently reached Phase 1 clinical trial in HIV patients.²⁰⁴ HIV patients received three intranodal doses of mRNA coding HIV immunogen sequences. This vaccine was well tolerated in patients and induced a moderate HIV-specific T cell response supporting the ongoing Phase 2 clinical trial.

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Intravenous

Successful outcomes obtained by intranodal administration provided proof of concept to the strategy of targeting lymphoid organs for effective vaccination. Intravenous administration allows for targeting multiple lymphoid organs throughout the body, with an easier and less invasive procedure, compared to intranodal administration. While uptake of mRNA carriers by reticuloendothelial system typically induced negative influence on their application to disease treatment, this process provides beneficial outcomes in mRNA vaccine. After intravenous administration of cationic mRNA LNPs based on DOTAP, efficient protein expression was obtained in CD11⁺ antigen presenting cell in the spleen.²⁰⁶ In addition, this formulation activated APCs in several organs, including the spleen, liver, bone marrow and lymph nodes. As a result, intravenous administration of the LNPs loading model antigen mRNA induced enhanced cellular immunity compared to their intradermal and subcutaneous administration. Another group also demonstrated the advantage of intravenous administration over intramuscular and subcutaneous administration, using negatively charged mRNA LNPs based on lipofectamine RNAi Max.²⁰⁷ Several reports attempted to improve the efficacy of intravenous mRNA vaccination by introducing (tri-)mannose ligands to LNPs for targeting dendritic cell,^{208, 209} or by incorporating lipid-based immunostimulatory adjuvant to LNPs.²¹⁰ In another report, α -galactosylceramide was co-encapsulated to LNPs with mRNA encoding tumor antigens to activate natural killer T cells, leading to improved efficiency of cancer vaccines.²¹¹

Intravenous mRNA vaccination was tested also in patients with advanced malignant melanoma, in a phase I clinical trial. In the study, LNPs with various surface charges were prepared by tuning LNP/mRNA mixture ratio, for optimization in animal experiments. As a result, LNPs with slightly negative charge induced efficient protein expression from mRNA in the spleen, bone marrow and lymph nodes, with histological observation showing efficient protein expression in dendritic cells. This formulation showed efficient anti-tumor activity in several mouse models, and successfully induced

antigen-specific T cell responses in a clinical trial.¹¹⁶ A subsequent study indicated successful immune activation in the spleen of patients, using [¹⁸F]Fluoro-2-deoxy-2-D-glucose positron emission tomography (FDG-PET).²¹² The same group recently used this LNP formulation to potentiate chimeric antigen receptor (CAR)-T cell therapy against solid tumor in an animal study.²¹³ In many cases of CAR-T cell therapy against solid tumors, CAR-T cells are inactivated rapidly after transplantation due to low accessibility to target cells, leading to reduced proliferative signal in CAR T cells. To solve this problem, mRNA expressing cancer antigen that CAR-T cells target was administered using LNPs to obtain its expression in APCs throughout lymphoid tissues in the body. Through interaction with APCs expressing the cancer antigen, CAR-T cells proliferated with preserved activity, thereby inducing efficient anti-cancer effect in mouse models.

4.1.3. saRNA vaccines

saRNA vaccines were first used in the form of Virus RNA Particles (VRPs).^{214, 215} In VRPs the structural proteins have been replaced by the antigen, they are provided *in trans* in packaging cells to produce replication-defective VRPs.^{215, 216} As reviewed previously,²¹⁶ alphavirus-based VRPs showed promising results, among which protection against Meta pneumonia virus, respiratory syncytial virus and dengue in primates.^{217, 218} However, VRPs face the same biosafety and production limits as viral vectors.²¹⁹

Synthetic saRNA has been developed as an alternative to VRPs and, since synthetic saRNA do not use packaging cells delivery systems are required. Delivery of saRNA faces the size challenge of saRNA: as saRNA code for the replicase and the antigen(s) they are longer than mRNA: 10, 000 nt vs 1,000 nt for mRNA. Despite this increased length saRNA are produced by *in vitro* transcription from a DNA template with yields similar to mRNA.³⁹

Because synthetic saRNA are more recent than mRNA, fewer formulations have been

developed for their delivery.^{56, 104, 220} saRNA has been used for vaccination both by intradermal injection of naked or complexed saRNA followed by electroporation⁷⁴, as intramuscular injection of saRNA lipopolyplexes³⁹, intramuscular injection of saRNA complexes with dendrimers.⁶⁹ In these studies, saRNA demonstrated higher expression and extended duration of expression over mRNA.^{66, 67, 71} A few examples will be detailed.

Vogel et al. used PEI polyplexes to deliver saRNA coding an influenza antigen.⁶⁶ Using luciferase reporter gene they evidenced extended saRNA expression after intramuscular injection of polyplexes over mRNA polyplexes with an expression delay of saRNA attributable to the necessary production of replicase, minus strand RNA and translation of minus strand RNA. saRNA gave equivalent protection as mRNA at 64-fold lower dose. Moreover, combining saRNA coding hemagglutinin from three strains of influenza protected against sequential viral challenges. They later reported the induction of CD4 and CD8 specific T cells against conserved HIV-1 regions after vaccination with saRNA polyplexes.²²¹ These results validate the versatility and therapeutic potential of saRNA polyplexes.

A dendrimer platform has been used for multipurpose intramuscular saRNA vaccination.^{69, 222} VEEV-derived saRNA was complexed using Modified Dendrimer Nanoparticles (MDNPs) into c.a. 100 nm stable in human serum. Intramuscular injection of MDNPs resulted in the induction of antigen-specific T cells *in vivo*. Interestingly, when authors injected MDNPs prepared with luciferase saRNA, they reported an increase in luciferase activity between 36 h and 60 h post injection suggesting *in situ* replication of the saRNA. MDNPs formed with antigen-coding saRNA protected against lethal influenza challenge, ebola virus, Zika virus and *Toxoplasma gondii*.^{69, 222}

We introduced neutral targeted lipopolyplexes (LPP) for the delivery of VEEV-derived saRNA to dendritic cells.³⁹ Here, saRNA was first complexed in positively-charged polyplexes using PEI (polyethylenimine) before interaction with anionic PEGylated liposomes to form neutral LPP (**Figure 9A**). A mannosylated lipid (16:0 PA-PEG3-mannose 1,2-dipalmitoyl-sn-glycero-3-phospho((ethyl-

1',2',3'-triazole)triethyleneglycolmannose) was included in liposomes to engage the mannose receptor on dendritic cells.^{158, 159} LPP were capable of transfecting a murine dendritic cell line *in vitro*. Intramuscular injection of LPP prepared with luciferase saRNA resulted in durable expression in the quadriceps (**Figure 9B**). Vaccination with LPP formed with saRNA coding influenza antigens led to the induction of antigen-specific CD4 and CD8 T cells (**Figure 9C**). Another benefit of using saRNA, particularly saRNA derived from Venezuelan equine encephalitis virus has another advantage as the non-structural proteins also inhibit type I IFN signaling.^{74, 223}

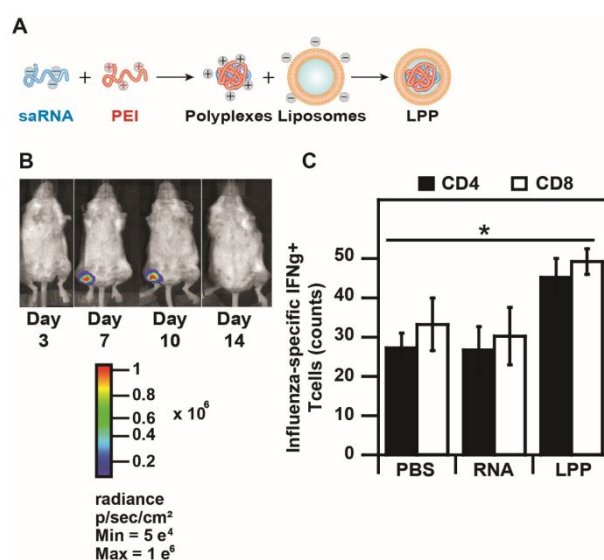


Figure 9. Formation of LPP-saRNA and activity *in vivo*. **A.** To form neutral Lipopolyplexes, saRNA is first complexed with branched PEI to form positively charged polyplexes. Then, positively charged polyplexes are mixed with anionic liposomes resulting in the formation of neutral LPP. **B.** Intramuscular injection of LPP- luciferase saRNA complexes allowed sustained expression *in vivo*. **C.** Vaccination of mice by intramuscular injection of LPP- hemagglutinin saRNA induced antigen-specific IFN- γ secreting cells with superior immunogenicity over naked RNA or unvaccinated mice. Adapted with permission from reference ³⁹. Copyright 2019 Elsevier.

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4.2 Protein replacement therapy

Alongside with vaccine, mRNA delivery was widely used for supplying therapeutic peptides or proteins, which is called as protein replacement therapy. As early as 1992, *in vivo* protein replacement therapy was reported, in which vasopressin mRNA was injected to hypothalamus of model rats that lacks vasopressin expression, to treat diabetes insipidus.²²⁴ However, over 20 years after the report, therapeutic application of mRNA was focused on vaccine and immunotherapy, with very few reports addressing the potential of mRNA for protein replacement. A major challenge for mRNA-based protein replacement is the control of mRNA immunogenicity, as inflammatory responses after mRNA delivery would hamper the therapeutic processes and pose safety concerns. This is in sharp contrast to the cases of vaccine and immunotherapy, in which induction of innate immune responses is essential to obtain preventive or therapeutic outcomes. To control mRNA immunogenicity, various techniques have been developed. Among them, replacement of mRNA nucleosides with chemical modified species, such as m5C, t2C, ΨU and m1ΨU, is the most common approach, which inhibits the recognition of mRNA by innate immune receptors (see *Section 2.1.4* for detail).^{54, 110} High performance liquid chromatography (HPLC) purification to remove dsRNA contaminants was also reported to be effective in reducing mRNA immunogenicity.²²⁵ Along with these approaches focusing on mRNA, fine-tuned designing of mRNA carriers allows for the inhibition of mRNA recognition by immune receptors. After encapsulation of mRNA into PEGylated polymeric micelles (PMs), its recognition by TLR7 in endosome was suppressed, presumably through steric repulsive effect of PEG, leading to efficient *in vivo* delivery of mRNA with minimal inflammatory responses (see *Section 3.5* for detail).^{40, 174} Notably, even without mRNA chemical modification and HPLC purification, mRNA PMs exhibited successful outcome in tissue regenerative treatment to several diseases in model animals, such as osteoarthritis, intravertebral disc disease and spinal cord injury.²²⁶⁻²²⁸ Such immune regulation approach without relying on mRNA chemical modification is practically important, because mRNA chemical modification often leads to

1 impaired translational activity, compromising therapeutic potential of mRNA.^{61-63, 229}

2 These technology advances for alleviating mRNA immunogenicity opened the door for mRNA
3 application to protein replacement therapy. In the past decade, numerous reports demonstrated the
4 potential of mRNA for this therapeutic approach, with following 5 fields vigorously pursued: (1) Single
5 gene disorder treatment using mRNA encoding deficient genes, e.g. treatment of lung surfactant protein
6 deficiency,¹¹⁰ hemophilia,²³⁰ cystic fibrosis,^{231, 232} ornithine transcarbamylase deficiency,²³³ acute
7 intermittent porphyria,²³⁴ Fabry disease,²³⁵ and acute intermittent porphyria,²³⁶ (2) Production of
8 antibody, including single chain antibody (scFv),²³⁷ whole antibody,²³⁸⁻²⁴⁰ and bi-specific antibody,²⁴¹
9 targeting amyloid deposition, toxin, infectious diseases, and cancers; (3) Tissue repair and regeneration
10 using growth factors,^{227, 242-244} and transcriptional factors;^{226, 227} (4) Cellular reprogramming (see *Section*
11 *4.3*); (5) Cancer therapy (see *Section 4.4*).

12 While details of these applications were extensively reviewed elsewhere,^{1, 245-247} here, we
13 discussed therapeutic application of mRNA, by focusing on following 3 advantages of mRNA over DNA
14 in protein replacement therapy. (1) mRNA provides earlier onset of protein expression compared to DNA,
15 because mRNA does not require the processes of nuclear transport and transcription for protein
16 production (**Figure 10A**).^{40, 248} This time-dependent protein expression profile of mRNA is attractive for
17 targeting acute diseases. (2) Duration of protein expression from mRNA is at most one week, allowing
18 the use of mRNA to express therapeutic factors that would induce detrimental effects after prolonged
19 expression. (3) mRNA tends to induce protein expression in higher percentage of target cells compared
20 to DNA (**Figure 10B**), presumably because nuclear entry of DNA becomes a barrier for protein
21 expression in a large percentage of cells.^{176, 248} In the use of gene expressing secreted factors, their
22 expression in a small percentage of the target cells is sufficient for obtaining therapeutic outcome,
23 because untransfected cells also receives therapeutic factors secreted from transfected cells. On the
24 contrary, in the delivery of genes encoding intracellular factors, untransfected cells would obtain less

1 beneficial effect compared to transfected cells. Thus, mRNA is a more suitable option in this case,
2 because of its capability to induce protein expression in a large percentage of cells (**Figure 10B**).

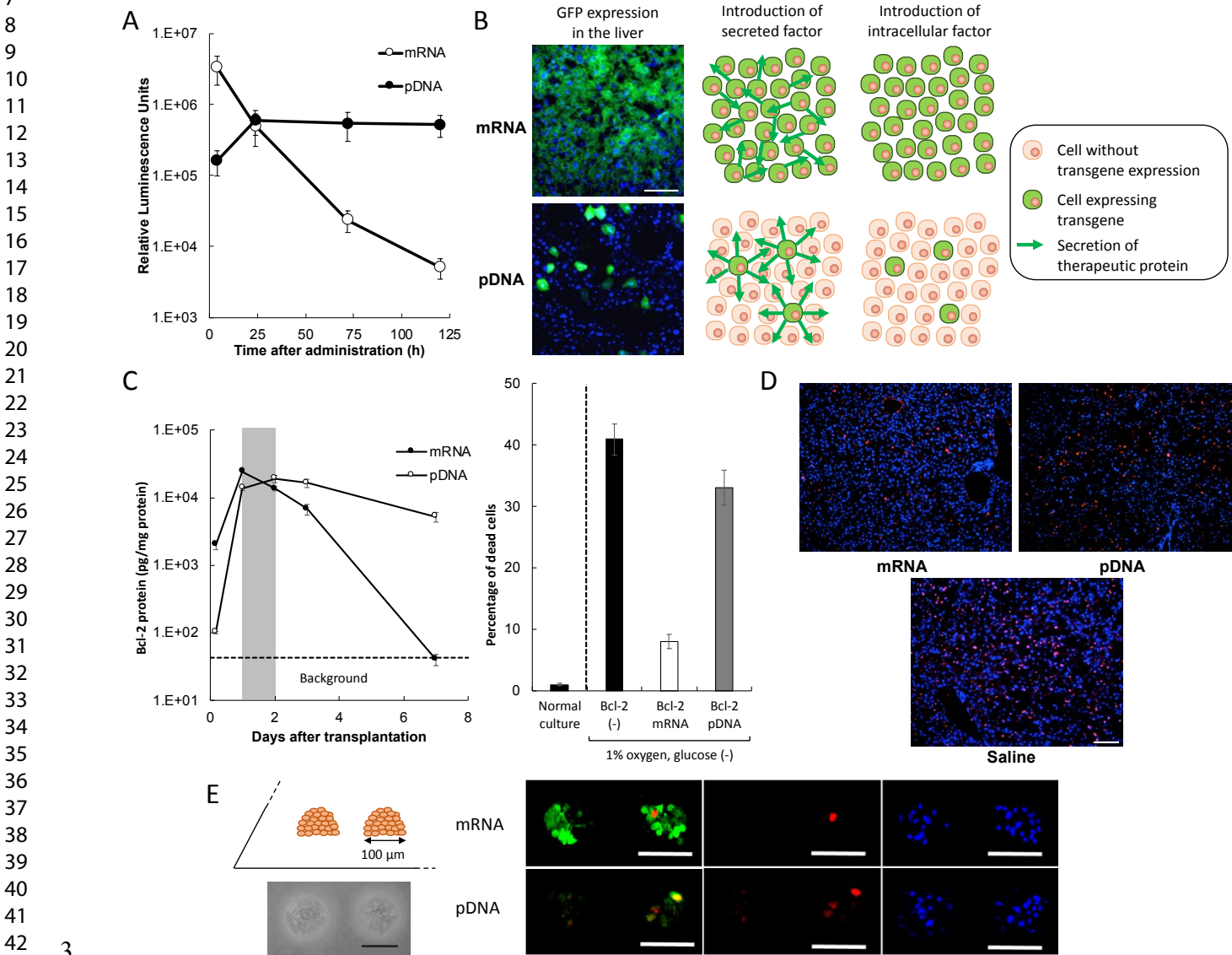


Figure 10. Comparison of mRNA and pDNA delivery. **A.** Time dependent protein expression profile. PEG-PAsp(DET)-based polyplex micelles (PMs, see *Section 3.5.*) loading luciferase mRNA or pDNA were injected to cerebrospinal fluid (CSF) of rats, followed by measurement of luciferase levels in CSF. The figure is adapted from [In Vivo Messenger RNA Introduction into the Central Nervous System Using Polyplex Nanomicelle](#) by S. Uchida, *et al.*⁴⁰ to show pDNA and mRNA. The figure is licensed under <https://creativecommons.org/licenses/by/2.0/>. **B.** Tissue distribution of protein expression from mRNA

(upper panels) and pDNA (lower panels). Left panels: GFP expression in mouse liver observed 24 h after hydrodynamic injection of PMs loading GFP mRNA or pDNA. Green: GFP, Blue: cell nuclei (DAPI). Scale bar: 100 μm . The figure is adapted from [Messenger RNA-based therapeutics for the treatment of apoptosis-associated diseases](#) by A. Matsui *et al.*¹⁷⁶ to show the difference between mRNA and pDNA. The figure is licensed under <https://creativecommons.org/licenses/by/4.0/>. Middle panels: Therapy using gene encoding secreted proteins. Protein expression in a small percentage of the target cells is sufficient for obtaining therapeutic outcome, because untransfected cells also receives therapeutic factors secreted from transfected cells. Left panels: Therapy using gene encoding intracellular proteins. Protein expression in a large percentage of the cells is required for therapy, as untransfected cells typically failed to receive therapeutic effects. **C.** Primary hepatocytes were transfected with Bcl-2 mRNA or pDNA for measuring of Bcl-2 protein expression levels (left). Bcl-2 mRNA and pDNA showed comparable levels of Bcl-2 protein expression from day 1 to day 2 after the transfection (highlighted with gray). In this period, cell death was induced by deprivation of oxygen and glucose. Bcl-2 mRNA showed enhanced pro-survival effect compared to Bcl-2 pDNA, although total Bcl-2 expression levels were comparable between these two groups. This result highlights the importance of obtaining Bcl-2 expression in a large percentage of cells. Reprinted with permission from reference²⁴⁹. Copyright 2018 Elsevier. **D.** Treatment of mouse model of fulminant hepatitis using Bcl-2 mRNA and pDNA. Liver sections 4 h post-treatment. Red: apoptotic cells (TUNEL). Blue: cell nuclei (DAPI). Scale bar: 100 μm . The figure is adapted from [Messenger RNA-based therapeutics for the treatment of apoptosis-associated diseases](#) by A. Matsui *et al.*¹⁷⁶ to show the difference between mRNA and pDNA. The figure is licensed under <https://creativecommons.org/licenses/by/4.0/>. **E.** Introduction of mRNA and pDNA to 3D cultured cells. Left: An array of mesenchymal stem cell spheroids, prepared using micropatterned plate.²⁵⁰ Right: Confocal microscopic images of the spheroids 24 h after introduction of GFP mRNA and pDNA. Dividing cells were labeled with 5-ethynyl-2-deoxyuridine (EdU, red), a nucleic acid analogue, which is

1 incorporated into the genome during S phase. Green: GFP, Blue: cell nuclei (DAPI). Scale bars: 100 μ m.

2 The figure is reproduced from [mRNA as a Tool for Gene Transfection in 3D Cell Culture for Future](#)
3 [Regenerative Therapy](#) by S. Uchida *et al.*²⁵¹ under <https://creativecommons.org/licenses/by/4.0/>.

4
5 These three advantages of mRNA provide strong rationale for the use of mRNA in some
6 therapeutic settings. For example, the treatment of acute myocardial infarction using *vascular endothelial*
7 *growth factor (VEGF)-A* mRNA utilized the advantages (1) and (2).²⁴² While both of *VEGF-A* mRNA
8 and plasmid DNA (pDNA) were effective in reducing infarct area through their angiogenic effects, only
9 mRNA improved the survival of model animals. Surprisingly, *VEGF-A* pDNA decreased the survival
10 even when compared to control groups, due to edema caused by prolonged VEGF-A expression. Thus
11 transient protein expression profile of mRNA was advantageous in this application.

12 mRNA delivery is also a preferable method to introduce pro-survival genes, as DNA-based
13 introduction might cause prolonged expression of pro-survival factors after genomic integration of the
14 gene, which is cancer promoting. In our previous report, mRNA encoding Bcl-2, an intracellular pro-
15 survival factor, was used for the treatment of acute hepatitis in mouse model, which utilizes 3 advantages
16 of mRNA described above.¹⁷⁶ When *Bcl-2* mRNA and pDNA was introduced *in vitro* to primary
17 hepatocytes treated with cell death stimuli, mRNA provided enhanced pro-survival effects to hepatocytes
18 compared to pDNA (**Figure 10C**).²⁴⁹ The enhanced potential of *Bcl-2* mRNA is attributed to its
19 capability to provide Bcl-2 protein expression and pro-survival effects to a larger percentage of cells
20 compared to *Bcl-2* pDNA. As a result, in the treatment of acute hepatitis, mRNA showed enhanced anti-
21 apoptotic effect in the liver compared to pDNA (**Figure 10D**). Along with such *in vivo* administration,
22 *Bcl-2* mRNA is applicable for *ex vivo* administration to improve the engraftment efficiency after cell
23 transplantation therapy²⁴⁹. In hepatocyte transplantation to mouse liver, *ex vivo* *Bcl-2* mRNA introduction
24 provided up to 5-fold increase in the hepatocyte engraftment efficiency with improved outcome in liver

functional support to mouse model of chronic hepatitis. Together, *Bcl-2* mRNA provides a safe and effective option to prevent cell death in a variety of therapeutic settings.

4.3 Cell reprogramming

mRNA therapeutics also provide an excellent tool for converting cell fates, *i.e.* generation of induced pluripotent cells (iPSCs), differentiation of stem cells, direct reprogramming, and genome editing, with several advantages over the other commonly used methods, such as the use of viral gene transduction.^{252, 253} iPSCs were firstly generated using retrovirus gene vectors,²⁵⁴ which cause random integration of the encapsulated genes into host genome. Genomic integration may result in the activation of oncogenes near the integration sites, as reported in a clinical trial.²⁵⁵ Moreover, prolonged expression of Yamanaka factors after genomic integration may hamper differentiation of iPSCs and also cause tumor formation.²⁵⁶⁻²⁵⁸ Although adenovirus vectors and non-viral pDNA transfection also allowed iPSC generation in a manner less prone to cause genomic integration,^{259, 260} thorough examination of genome sequence of iPSCs would still be required before clinical usage, to check the genomic integration, which occurs at certain rates after introduction of adenovirus vector and pDNA.^{261, 262} This issue of genomic integration also poses problems in gene transfection for stem cell differentiation, direct reprogramming, and genome editing. On the contrary, mRNA delivery would provide a safer option to cell reprogramming. In addition, transient protein expression profile of mRNA allows precise temporal control of protein expression, which is often needed for (trans-)differentiation of cells.

For iPSC generation, repeated mRNA transfection is needed due to short duration of protein expression from mRNA and relatively short intracellular half-life of transcriptional factor proteins. In repeated transfection, toxicity of each transfection procedure should be minimized to preserve cell viability and obtain efficient and prolonged protein expression. Thus, in the first successful report of mRNA-based iPSC generation, chemically modified mRNA with 100% substitution of C and U with

5mC and ΨU was used with supplementation of B18R protein, a decoy receptor for type I interferon, to reduce immunogenicity of mRNA.²⁶³ Further, 5' triphosphate of uncapped mRNA contaminants, a potential substrate for an innate immune receptor, RIG-I, was enzymatically removed, and low oxygen culture was performed to improve iPSC generation efficiency. In subsequent researches, this protocol was modified to include additional reprogramming mRNA or micro RNA (miRNA).^{264, 265} Despite these advances, daily dosing of mRNA is still needed. To circumvent this troublesome procedure, self-amplifying RNA (saRNA) encoding all four Yamanaka factors were employed (see *Section 2.2* for detail about saRNA), which induced protein expression for more than a week.²⁶⁶ With supplementation of B18R protein to reduce immune responses, single administration of saRNA successfully generated iPSCs.

mRNA also demonstrated its potential for differentiation of stem cells. In the first report of mRNA-based iPSC generation described above, *MyoD* mRNA transfection to iPSCs allowed their differentiation to myotube.²⁶³ Several reports succeeded in osteogenic differentiation of mesenchymal stem cells (MSCs) *in vitro* using mRNA encoding *bone morphogenetic protein-2 (BMP-2)*, and also *in vivo* bone regeneration after *BMP-2* mRNA delivery to the region of bone defect in animal models.^{243, 267} Alongside with such *in vivo* delivery of differentiation factor mRNA, transplantation of cells after *ex vivo* differentiation using mRNA transfection is a promising approach of regenerative therapy. As three-dimensional (3D) culturing is a wide-spread method to potential cell transplantation therapy,²⁶⁸⁻²⁷⁰ we undertook *ex vivo* differentiation of 3D-cultured cells using mRNA. Interestingly, in osteogenic differentiation of MSC spheroids using runt-related transcription factor 2 (*Runx2*), transfection of *Runx2* mRNA exhibited improved outcomes compared to that of *Runx2* pDNA.²⁵¹ In mechanistic analysis using a reporter gene, reporter protein expression efficiency from mRNA in MSC spheroids was higher than that from pDNA (**Figure 10E**), while both of mRNA and pDNA induced comparable level of the protein expression in MSCs in monolayer culture. This result is explained by the observation that MSCs in spheroids showed lower rate of cell division compared to those in monolayer culture, and mRNA induced

more efficient protein expression in non-dividing cells compared to pDNA. This study demonstrated additional advantage of mRNA over pDNA in transfection to 3D culture cells.

Various reports demonstrated the utility of mRNA for direct reprogramming, *e.g.* from fibroblasts to hepatocyte-like cells,²⁷¹ endothelial progenitor cells,²⁷² neurons,²⁷³ and neural precursor cells,²⁷⁴ from pancreatic exocrine cells to insulin-producing cells,²⁷⁵ and from MSCs to neural stem cells.²⁷⁶ Notably, *in vivo* direct reprogramming to neurons was achieved by injection of 4 types of mRNA to brain parenchyma, leading to alleviation of Parkinson's disease symptoms in mouse models.²⁷³ Other exciting targets of cell programming include hematopoietic and immune cells. In CAR-T cell therapy, *Foxo1* mRNA was used to increase the fraction of central memory T cells before transplantation, which resulted in improved anticancer activity of CAR T cells.²⁷⁷ *Musashi-2* mRNA facilitated *ex vivo* proliferation of hematopoietic stem cells (HSCs) without differentiation, which would solve the problem of HSC shortage for clinical use.²⁷⁷ Because prolonged overexpression of *Musashi-2* is cancer promoting,²⁷⁸ its transient expression using mRNA is desirable, as is the case in *Bcl-2* (see Section 4.2).^{176, 249} Regulation of macrophage polarization attracts much attention in various therapeutic fields, with M1 macrophages desirable for cancer treatment, and M2 macrophages for tissue regeneration. Several reports succeeded in M1-to-M2 and M2-to-M1 transition *in vivo*, providing a new tool for macrophage polarization.^{279, 280} Other interesting strategies of macrophage potentiation include mRNA-based introduction of anti-microbial peptide in lysosome for treating bacterial infection.²⁸¹ In this strategy, mRNA encodes anti-microbial peptide connected with a cathepsin B, which translocates the peptide to lysosome after its translation in cytoplasm, and transplantation of the mRNA-treated macrophages was effective in treatment of multidrug-resistant bacteria-induced sepsis in mice.

Genome editing is another promising strategy of cellular reprogramming. Transient protein expression profile of mRNA is beneficial for genome editing, because prolonged expression increases the rate of off-target genome cleavage.²⁸² In clinical trials, gene encoding *C-C chemokine receptor 5*

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3 1 (*CCR5*), a receptor for HIV entry, was disrupted *ex vivo* using zinc finger nucleases (ZFN) mRNA, for
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5 2 making the transplanted cells resistant to HIV infection (ClinicalTrials.gov Identifier: NCT02500849,
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7 3 NCT02225665). Recently, research focus was shifted to RNA-based delivery of clustered regularly
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9 4 interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) 9. In co-delivery of
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11 5 *Cas9* mRNA and single guide RNA (sgRNA), sgRNA was degraded in cytoplasm before translation of
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13 6 sufficient amount of Cas9 protein, leading to reduced genome editing efficiency.²⁸³ Intracellular
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15 7 degradation of sgRNA was effectively prevented by chemical modification of sgRNA, which provided
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17 8 a boost in genome editing efficiency.^{115, 284} By *in vivo* co-delivery of *Cas9* mRNA and sgRNA, several
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19 9 reports succeeded in the disruption of disease-related genes in the liver, such as *proprotein convertase*
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21 10 *subtilisin/kexin (PCSK9)*, a target gene of familial hypercholesterolaemia,^{115, 285, 286} *transthyretin (TTR)*,
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23 11 a target gene of TTR amyloidosis,²⁸⁷ and hepatitis B virus (HBV) DNA.²⁸⁸ Notably, up to 60% of indel
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25 12 in the liver was achieved even after single administration of *Cas9* mRNA and sgRNA,^{115, 286} while
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27 13 achieving such a high efficiency is difficult in pDNA delivery.²⁸⁹ This may be partially attributed to
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29 14 mRNA capability to induce protein expression in higher percentage of cells compared to pDNA
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31 15 transfection (see *Section 4.2*).^{176, 248, 249}

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40 17 **4.4. Cancer**

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42 18 mRNA-based vaccines were the first application to be considered for using mRNA against
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44 19 tumors.^{116, 290-293} After the vaccination, the immune system can recognize cancer cells to elicit cell-
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46 20 mediated antitumor responses. Thus, mRNA-based cancer vaccines can be directed to promote immunity
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48 21 against tumor-associated antigens,²⁹⁴ or against neoepitopes due to cancer mutations.²⁹⁵ As the
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50 22 neoepitope signature, *i.e.* mutanome, is exclusive for each patient and the mRNA molecules can encode
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52 23 several neoantigen epitopes in their sequence, Sahin et al. proposed to use mRNA-based vaccines as
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54 24 personalized therapeutics.^{295, 296} This concept was demonstrated in first-in-human application by

1 immunizing metastatic melanoma patients against 10 epitopes.²⁹⁷ This initial success has triggered
2 several clinical trials against melanoma and other tumors, as reviewed elsewhere.³

3 mRNA can also be used to produce therapeutic proteins with antitumor activity, such as anti-
4 angiogenic proteins or antibodies,^{41, 241} thereby, using the organs and cells in the body as temporary
5 factories. This approach could be used to promote the bioavailability of agents that otherwise are rapidly
6 cleared from the body, thereby, reducing the number of injections and decreasing the burden for patients.
7 Moreover, producing the proteins *in situ* could avoid cumbersome procedures for synthesizing and
8 purifying protein drugs in clinical grade. For example, Stadler et al. developed mRNA-loaded
9 lipopolyplexes encoding bispecific tandem single-chain variable fragments (scFv), *i.e.* bi-(scFv)₂
10 proteins, directed to simultaneously target both CD3 expressed on T cells and tumor-associated antigens,
11 *i.e.* claudin 6, claudin 18.2, and the epithelial cell adhesion molecule (EpCAM)).²⁴¹ These bi-(scFv)₂
12 work by connecting T cells to cancer cells for improving the cell killing efficiency. The mRNA-loaded
13 nanomedicines accumulated in liver to achieve effective mRNA translation and sustained release of bi-
14 (scFv)₂ for around 7-days. The nanomedicines encoding bi-(scFv)₂ directed to CD3 and claudin 6
15 eradicated large ovarian tumors, and their activity was comparable to that of the clinical recombinant
16 bispecific antibody. However, the nanomedicines required 3-fold less frequency than the antibodies to
17 exert the same antitumor effect.

18 mRNA-loaded nanomedicines can be applied to introduce specific proteins into cancer cells.
19 Such approach can be exploited to reduce cancer malignancy or sensitize cancer cells to other treatments.
20 For example, by using polymer-lipid hybrid nanoparticles comprised of PAMAM (generation 0)
21 dendrimer for complexation with mRNA, poly(lactic-co-glycolic acid) (PLGA) for increasing
22 hydrophobicity and ceramide-PEG for coating, it was possible to deliver mRNA encoding the tumor-
23 suppressor gene phosphatase and tensin homologue (*PTEN*) to tumors. The resulting expression of
24 functional PTEN in PTEN-null prostate cancer cells suppressed the phosphatidylinositol 3-kinase

(PI3K)–AKT pathway and increased apoptosis.²⁹⁸ In another report, mRNA encoding *p53*, a tumor suppressor gene, was introduced to *p53*-deficient tumors using polymer-lipid hybrid nanoparticles, comprised of cationic lipid-like molecules for mRNA complexation, disulfate-containing polymers for mRNA release in reductive intracellular environment and PEGylated lipids.²⁹⁹ The treatment with *p53* mRNA suppressed the growth of *p53*-deficient tumors, potentiated the function of a mammalian target of rapamycin (mTOR) inhibitor and ultimately exhibited successful outcomes in mouse models of orthotopic hepatocellular carcinoma and disseminated non-small cell lung cancer. More recently, PEI-based polyplexes were used to introduce mRNA encoding vascular endothelial growth factor-C (VEGF-C) into brain tumor cells to promote lymphangiogenesis and induce the infiltration of CD8 T cells into the tumors.³⁰⁰ The combination of these mRNA-loaded polyplexes with immune checkpoint inhibitors allowed the effective treatment of mice models of brain tumors.

5. Future perspectives

mRNA therapeutics are now gradually moving toward clinical applications. Vaccination against cancer is one of the most advanced fields, with the first report of the clinical trials dating back to 2002.³⁰¹ Targeting neoantigens, which are expressed only in cancer cells, is a good approach to improve the efficacy of vaccines by circumventing immune tolerance mechanisms. A recent clinical trial provided a proof of concept to this approach, by preparing mRNA expressing neoantigens, which were determined by deep sequencing of cancer samples from each patient.²⁹⁷ With advances in mRNA vaccine technologies, including RNA design, administration routes and delivery system, further improvement of therapeutic outcomes are expected. Such personalized therapy has the potential to revolutionize the methodology of cancer treatment.

Vaccine against infectious disease is another important field. In the pandemic of COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), several companies have

1 launched or planned to start clinical trials of mRNA vaccine against the viral spike protein
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3 1 (ClinicalTrials.gov Identifier: NCT04283461, NCT04368728), based on their promising results in
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7 3 preclinical or clinical studies of mRNA vaccine against other viruses, including Zika, influenza and
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9 4 rabies viruses.^{197, 302-305} Importantly, mRNA vaccine against a new infectious disease can be easily
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11 5 prepared just by changing the mRNA sequence in vaccine formulations developed for other viruses,
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13 6 which allowed fast start of clinical trials within a few months after sequencing of SARS-CoV2 genome.
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15 7 The utility of mRNA vaccine against pandemic will be evaluated within a year in large clinical trials.
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17 8 Notably, competing technologies of nucleic acid vaccines, such as electroporation of pDNA and
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19 9 adenovirus vector administration, are also being pursued for vaccination against SARS-CoV2.^{306, 307} Pros
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21 10 and cons of each technology should be assessed from wide perspectives, including effectiveness, adverse
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23 11 effects, production costs, quality control and large-scale production.

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26 12 Clinical trials of mRNA therapeutics were conducted also for protein replacement therapy and
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28 13 genome editing, with *VEGF-A* mRNA injection for heart regeneration (ClinicalTrials.gov Identifier:
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30 14 NCT03370887), cystic fibrosis transmembrane conductance regulator (CFTR) supplementation for
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32 15 cystic fibrosis (ClinicalTrials.gov Identifier: NCT03375047), and *ex vivo* ZFN-mediated disruption of
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34 16 *CCR5* gene for HIV treatment, as extensively reviewed elsewhere.^{308, 309} However, clinical trials in these
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36 17 fields are still much fewer than that those for mRNA vaccines, which may be partially due to the issues
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38 18 of mRNA delivery. Protein replacement therapies and genome editing often targets larger organs
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40 19 compared to the case of mRNA vaccine, which makes mRNA delivery difficult. While various delivery
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42 20 systems exhibited successful outcomes in small animals, their functions should be evaluated using larger
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44 21 animals for clinical translation. Because tremendous advances have been made in the core technologies
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46 22 of mRNA delivery as reviewed here, combination of these technologies may provide effective systems
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48 23 for clinical usage.

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50 24 Target diseases and genes should be properly selected based on advantages of mRNA
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therapeutics over competing methodologies, including viral gene therapy and protein therapeutics. As described above, mRNA provided a more proper option compared to viral gene vectors, when prolonged gene expression induces detrimental effects (see *Sections 4.2, 4.3*). Acute diseases are a promising target of mRNA therapeutics, as the onset of protein expression from mRNA is as early as a few hours after the transfection.^{40, 248} A major problem of viral gene therapy is the production of neutralizing antibody, which hampers repeated systemic administration of viral vectors.³¹⁰ mRNA delivery is less prone to produce neutralizing antibody, allowing multiple systemic injection without reduction of delivery efficiency.^{233, 234} On the other hand, transient profile of protein expression from mRNA should be taken into account in selecting target diseases. Repeated injections would be required for a long term to treat chronic diseases. When compared to protein therapeutics, mRNA therapeutics are more cost-effective due to their ability to produce numerous copies of proteins from single mRNA.³¹¹ While global market of antibody therapeutics is growing,³¹² mRNA may provide an inexpensive and effective option for antibody-based treatment.²³⁷⁻²⁴¹ Moreover, mRNA delivery is an easier approach to introduce intracellular and membrane proteins compared to protein delivery, expanding the candidates of therapeutic proteins for disease treatment.

6. Conclusion

Vigorous efforts were devoted to solve the problems of mRNA therapeutics, such as immunogenicity, short duration of protein expression, rapid enzymatic degradation, and limited cellular uptake. The strategies are classified into two orthogonal approaches, mRNA designing and nanomedicines, which cooperatively function to facilitate mRNA delivery and minimize safety issues. These technological advances allowed various therapeutic applications of mRNA including vaccinations, protein replacement therapy, cancer therapy and cell reprogramming in preclinical and clinical studies. Through further development of delivery system and proper selection of target diseases, mRNA

nanomedicines will solve various medical problems, which are intractable using conventional approaches, as well as promote unprecedented therapeutic strategies.

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