Biomedical applications of mRNA nanomedicine

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ABSTRACT

As an attractive alternative to plasmid DNA, messenger RNA (mRNA) has recently emerged as a promising class of nucleic acid therapeutics for biomedical applications. Advances in addressing the inherent shortcomings of mRNA and in the development of nanoparticle-based delivery systems have prompted the development and clinical translation of mRNA-based medicines. In this review, we discuss the chemical modification strategies of mRNA to improve its stability, minimize immune responses, and enhance translational efficacy. We also highlight recent progress in nanoparticle-based mRNA delivery. Considerable attention is given to the increasingly widespread applications of mRNA nanomedicine in the biomedical fields of vaccination, protein-replacement therapy, gene editing, and cellular reprogramming and engineering.

1 Introduction

Messenger RNA (mRNA) is a transient carrier of genetic information from genes to ribosomes for protein synthesis. During the first decade after its discovery in 1961 [1], most studies focused on understanding the structure, function, and metabolic activity of mRNA in eukaryotic cells. In the 1970s, the translation of protein from isolated mRNA was first achieved in living cells [2]. The concept of using nucleic acid as a drug was not proposed until 1990 when Wolff et al. demonstrated that direct injection of in vitro transcribed (IVT) mRNA resulted in the expression of the encoded protein in mouse muscle [3]. Since then, mRNA has been explored as a new class of nucleic acid therapeutics for the treatment of various diseases, such as cancer and genetic diseases.

As a promising alternative to conventional plasmid
DNA (pDNA) therapy, mRNA-based therapy possesses multiple unique features. First, since mRNA is translated in the cytoplasm, it does not need to enter the nucleus to exert its bioactivity. Second, unlike pDNA, mRNA has a negligible chance of integrating into the host genome, which may avoid the risk of insertion mutagenesis [4, 5]. Third, mRNA has more consistent and predictable protein expression kinetics than the random onset expression kinetics of DNA [6–8]. Fourth, mRNA is only transiently active, which may be beneficial for applications where transient protein expression is required [9]. Finally, in vitro synthesis of mRNA is relatively convenient and robust, which further enhances the prospect of mRNA being used in therapeutics.

Despite these unique features of mRNA, its clinical application has been limited due to its poor stability, propensity for immunostimulation, and the lack of an efficient delivery system. As a single-stranded polynucleotide, mRNA is easily degraded by the highly active nucleases during production in vitro and delivery in vivo. Moreover, the negatively charged, large mRNA molecules have difficulty in directly crossing the cellular membrane. Such challenges have greatly hindered the clinical translation of mRNA therapeutics.

Recently, however, along with the increasing knowledge of the relationship between mRNA structure and its translation efficacy, a variety of chemical modification methods have been developed to improve mRNA stability and reduce its immunostimulation [10–12], thus potentiating its therapeutic utility. In parallel with these developments, nanotechnology has emerged as a promising method allowing these nucleic acids to withstand multiple extracellular and intracellular barriers (e.g., protecting them from enzymatic degradation, improving cytosolic transportation, and reducing renal filtration) [13–15]. The rise of mRNA nanomedicines is rapidly advancing their applications in a wide range of biomedical fields, such as vaccination, protein-replacement therapy, gene editing, and cell reprogramming and engineering. In this review, we discuss the chemical modification methods of mRNA, summarize the nanoparticle platforms for mRNA delivery, and provide an overview of their diverse biomedical applications.

2 Chemical modification of mRNA

Typically, mRNA is synthesized by an in vitro transcription method in a cell-free system. This process involves a linearized DNA template containing a phage promoter sequence (T7, T3, or SP6) and target gene sequence [16] (Fig. 1). Since IVT mRNA is not as biologically viable due to its instability and immunogenicity, it was originally considered to be disadvantageous compared to other therapeutic agents. In the past thirty years, substantial efforts have been devoted to developing effective modification strategies to optimize IVT mRNA synthesis [17]. Here, we discuss these possible strategies for mRNA modification that contribute to increased resistance to nuclease degradation and reduced recognition by the immune system.

2.1 Improvement of mRNA translation and stability

Similar to the mature mRNA in eukaryotic cells, IVT mRNA contains the following five structural elements (Fig. 1): 5’ cap, 5’ untranslated region (5’ UTR), protein-encoding open reading frame (ORF), 3’ UTR, and 3’ poly(A) tail [18]. Modification of these elements during mRNA synthesis in vitro can improve mRNA stability and lead to an efficient and stable expression of target proteins.

2.1.1 5’ cap

Natural eukaryotic mRNA molecules have a cap structure of 7-methylguanosine (m7G) linked to the
5′-end of pre-mRNA via a 5′-5′ triphosphate bridge. A functional 5′ cap structure is a prerequisite for the efficient translation of mRNA, since its binding to eukaryotic translation initiation factor 4E (eIF4E) promotes the initiation of translation [19], whereas its binding to mRNA decapping enzymes regulates mRNA decay [20]. IVT mRNA can be capped either by performing post-transcriptional modification using recombinant vaccinia virus-derived capping enzymes [21], or by incorporating synthetic cap analogs into the in vitro transcription reaction [22]. However, the enzymatic capping method is only limited to the standard cap structure (m7GpppG) because of enzyme specificity. By contrast, co-transcription with the cap analogs allows for more cap structures and is a simple process. It is a commonly used method of synthetic mRNA preparation. However, this method also has limitations. First, due to the competition between cap analogs and native guanosine triphosphate (GTP), some of the mRNA remains uncapped, which severely affects its translation efficiency [23]. Second, since cap analogs can be attached to mRNA in two orientations, half of the capped mRNAs are reverse-oriented and are not recognized by the cap-binding protein [24].

To avoid the reverse cap orientation, anti-reverse cap analogs (ARCAs) were developed. In an ARCA, the 3′-hydroxyl (OH) of the native mRNA cap is replaced by a methoxy group (OCH3) [25, 26]. In addition to reducing reverse orientation, a new generation of ARCAs has been developed to reduce the binding between the mRNA cap region and the decapping enzymes, further improving the mRNA translation efficiency and stability. Specifically, phosphorothioate-modified cap [27], boranophospho-modified cap [28, 29], phoshoroselenoate-modified cap [30], methylenebisphosphonate-modified cap [31] and 1,2-dithiodiphosphate-modified cap [32], dihalogenmethylenebisphosphate-modified cap [33] are other examples of 5′-cap modifications that display resistance to decapping enzymes.

2.1.2 3′ poly(A) tail

Polyadenylation is an essential step in the production of functional mRNA in eukaryotic cells. The poly(A) tail plays an important role not only in facilitating nuclear export and translation initiation, but also in protecting the mRNA from nuclease degradation through the interaction with poly(A)-binding protein (PABP) [34]. Generally, IVT mRNA is tailed either by a polyadenylation reaction catalyzed by recombinant poly(A) polymerase [35–37], or by direct transcription from the poly(T) sequence encoded in the DNA template [38]. Although recombinant poly(A) polymerase derived from *Escherichia coli* is a facile method for poly(A) tail addition, the IVT mRNAs prepared by this method are a mixture of RNAs with different poly(A) lengths. By contrast, IVT mRNA generated from poly(T)-containing DNA template has a defined poly(A) length and enables reproducible batch control. Thus, this approach is more preferable in practical applications.

The length of poly(A) acts as an indicator of mRNA stability [39] and also contributes to mRNA translational regulation [40]. Several studies have demonstrated that relatively longer poly(A) length is advantageous for mRNA translation. For example, the protein expression in HeLa cells transfected with mRNA increased as the poly(A) tail length increased from 14 to 98 residues [41]. Optimized mRNA containing 120 adenosines showed maximum protein expression in dendritic cells (DCs) [38]. In another study, IVT mRNA with a poly(A) length of 100 nucleotides allowed for efficient protein expression [42]. A linear plasmid vector system, pEVL, was recently explored as the template for generating IVT mRNA with poly(A) tails of up to approximately 500 bases and with a defined length [43]. Besides the tail extension, incorporation of adenosine analogs, such as 3′-deoxyadenosine or 8-azaadenosine, provided better mRNA protection from 3′-exonuclease degradation, which could also be used to increase protein expression [44].

2.1.3 5′ and 3′ UTRs

UTRs are the non-coding regions located at 5′ upstream and 3′ downstream of ORFs, which do not directly contribute to the protein sequences. However, they play a critical role in the regulation of mRNA stability and protein translation via the interaction with RNA-binding proteins (RBPs) [45, 46]. 5′ UTR is an important element for ribosome recruitment and start codon choice [47], and it may have a crucial impact on translation [48]. 3′ UTR determines protein expression...
levels through the regulation of mRNA stability and translation mediated by AU-rich elements, and also facilitates local translation through the control of mRNA localization [49]. The sequence, secondary structure, and length of UTRs influence the translation process. Therefore, incorporating 5' and 3' UTRs with regulatory sequences is another strategy to further improve the stability and translation of mRNA.

For instance, the presence of the internal ribosomal entry site in the 5' UTR enables effective mRNA translation even when the cell level of eIF4E is low [50]. mRNAs containing N6-methyladenosine (m6A) in their 5' UTR can be translated in a cap-independent manner [51]. The induction of the optimized Kozak sequence is an effective strategy to prevent fault initiation [52]. While the 5' UTR mainly influences the initiation of the translation process, the 3' UTR plays a critical role in stabilizing mRNA [53]. The 3' UTR of α and β-globin mRNAs are the best-characterized 3' UTR sequences that can enhance the stability and translation efficiency of mRNA [54]. The length of 3' UTR influences the localization of membrane proteins [55]. It was reported that the long 3' UTR of CD47 enables efficient protein expression on the cell surface, whereas the short 3' UTR primarily localizes the CD47 protein to the endoplasmic reticulum.

2.1.4 ORFs

An ORF is the coding region that provides genetic information for protein expression. The base composition may have an impact on translational activity and stability of mRNA [56, 57]. For example, reduction of the frequency of UU and UA dinucleotides results in increased mRNA stability and protein expression [58]. Besides this, replacing rare codons with frequently used synonymous codons is a common strategy to improve protein expression [59, 60]. Codon optimization in mRNAs has been successfully applied in the past decade [61, 62], although its accuracy in human therapeutics is still questioned [63]. Further studies are required to verify the outcome of codon adaptation for mRNA therapeutics.

2.2 Modulation of mRNA immunostimulation

IVT mRNA has an inherent immunostimulatory effect, as it can be recognized as exogenous RNA by pattern recognition receptors (PRRs) of the innate immune system, including toll-like receptors (TLRs) [64, 65] and cytoplasmic retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) [66]. Activation of these PRRs induces a downstream cascade of the immune response, resulting in the expression of proinflammatory cytokines and type I interferons (IFNs). IVT mRNA-induced immune activation is considered beneficial for vaccination, because it may provide an adjuvant activity to facilitate dendritic cell maturation as well as T cell activation [67, 68]. For non-immunotherapy applications, however, this immunostimulatory activity of IVT mRNA is a major disadvantage since it can seriously reduce the translation efficiency of mRNA [69]. Therefore, modulation of mRNA immunostimulation could provide promising opportunities to further improve the therapeutic effect of IVT mRNA.

The immunostimulatory activity of IVT mRNA can be reduced by incorporation of chemically modified nucleosides. Pseudouridine (ψ), a naturally occurring modified nucleoside, is one of the most common modifications used in IVT mRNA preparation. ψ-modified mRNAs reportedly diminish the activation of PRRs, leading to a low immune response and high protein expression [70, 71]. In addition, they reduced the activation of 2'-5'-oligoadenylate synthetase and show increased resistance to RNase L-mediated cleavage [72]. Meanwhile, incorporation of chemically modified nucleotides into mRNAs may potentially increase the translation efficacy of the mRNAs. In a recent study, ψ was employed as a suitable chemical modification for mRNA encoding AsCpf1 protein, a type-V CRISPR–Cas effector endonuclease from Acidaminococcus sp. [73]. The combination of modified crRNA (cr3'5F, containing five 2'-fluoro ribose at the 3' terminus) and fully ψ-modified AsCpf1 mRNA increased the gene-cutting efficiency by over 3-fold compared to the control group with unmodified nucleosides.

Besides ψ, other modified nucleosides commonly utilized in mRNA modifications (Fig. 2). They include N6-methyladenosine (m6A), N1-methyladenosine (m1A), 2-thiouridine (s2U), 5-methyluridine (m5U), 5-methoxyuridine (mo5U), N1-methylpseudouridine (m1ψ), 5-methylcytidine (m5C), 5-hydroxymethylcytidine (hm5C), 5-methoxycytidine (mo5C), and
Figure 2 The chemical structures of native nucleosides and modified nucleosides commonly utilized in mRNA modifications.

2-methylguanosine (m2G) [9, 74]. A recent study identified m1ψ, mo5U, and ψ as the top three favorable nucleosides for mRNA modification, which significantly augmented protein expression [75]. In addition, combining ψ and m5C in modified mRNA can produce higher protein expression compared to a single modification [76, 77]. In another study, the combination of 2-thiouridine (s2U) and m5C in modified mRNA considerably reduced activation of PRRs and extended the protein expression to four weeks [78]. m1ψ modification alone or in combination with m5C was superior to ψ or m5C/ψ modification in its ability to reduce innate immune response and increase protein expression [79].

Although many studies on chemically modified nucleosides have contributed to the development of mRNA modifications, the best modification remains unclear. Moreover, it should be noted that while most chemically modified nucleosides can reduce mRNA immunostimulation, translation efficiency of a modified mRNA can sometimes be decreased with respect to that of an unmodified mRNA [80]. Considering future in vivo studies and clinical applications, we need to screen the optimal modifications depending on the practical applications.

3 Nanotechnology platforms for mRNA delivery

Due to its physicochemical properties that include large size and highly negative charge, chemically modified mRNAs still face multiple extracellular and intracellular barriers. To overcome these barriers, a suitable delivery vehicle may be needed to facilitate the cell entry and endosomal escape of mRNA, protect it against enzymatic degradation, and prolong its circulation life when used for systemic delivery. Inspired by the experience from gene therapy and RNA interference (RNAi) therapy, viral and non-viral vectors have been explored as an mRNA delivery tool for in vitro and in vivo applications [81, 82]. However, viral vectors are associated with several inherent limitations, such as the risk due to their immunogenicity [83], limited loading efficiency, and difficulty of large-scale production [84, 85]. Alternatively, non-viral vectors, particularly those based on biocompatible nanotechnologies, are preferable for nucleic acid delivery due to their diverse properties. Moreover, non-viral vectors are easier to synthesize and modify. Although a number of non-viral vectors have been investigated for pDNA and RNAi delivery, these vectors have not always been effective for mRNA delivery due to their different structures. Here, we highlight the non-viral nanoplatforms for mRNA delivery, including lipid nanoparticles, polymer nanoparticles, polypeptide nanoparticles, hybrid nanoparticles, and gold nanoparticle-DNA conjugates (Fig. 3).

3.1 Lipid nanoparticles

Lipid nanoparticles (LNPs) represent a class of non-viral vectors formulated with synthetic or naturally derived lipids containing hydrophilic heads and hydrophobic tails. Cationic lipids are often used to complex the anionic nucleic acid via electrostatic interaction. The utilization of cationic lipids for mRNA
transfection dates back almost 30 years. Then, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) was demonstrated to condense and deliver luciferase-encoding mRNA into various cell lines [86]. Subsequently the DOTMA derivative 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) was extensively studied for mRNA delivery and better transfection efficiency was demonstrated [87, 88]. In a recent study, the zwitterionic lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was used in combination with DOTAP to facilitate endosomal escape, which enhanced gene expression [89]. A number of lipid-based transfection reagents, such as Lipofectamine\textsuperscript{TM}, are commercially available and have been widely used for mRNA transfection \textit{in vitro} [90]. However, these reagents are relatively toxic and have poor pharmacokinetics, limiting their \textit{in vivo} applications.

Similar to LNP formulations used in small interfering RNA (siRNA) delivery, other components can be incorporated to optimize LNPs for mRNA delivery, which may significantly improve their transfection efficiency. Typically, optimized LNPs are composed of four components (Fig. 3(a)): (a) a cationic or ionizable amino lipid to complex the nucleic acid and enhance endosomal escape; (b) a helper phospholipid to support the bilayer structure and facilitate cell uptake; (c) cholesterol to enhance the stability and promote membrane fusion; and (d) a polyethylene glycol (PEG) conjugated lipid to reduce aggregation, avoid reticuloendothelial clearance, and decrease non-specific uptake [91]. However, mRNA has a structure distinct from siRNA and may differ in its packing affinity with nanoparticles. Therefore, it is critical to optimize the parameters for formulating LNPs specifically for mRNA delivery. To this end, a generalized strategy was developed to optimize LNP formulations for mRNA delivery \textit{in vivo} using Design of Experiment (DOE) methodologies including Definitive Screening and Fractional Factorial Designs [92]. Erythropoietin mRNA-loaded C12-200 LNPs optimized for mRNA delivery were screened by varying their formulation parameters (composition ratios or phospholipid types). This optimization resulted in a 7-fold higher potency than the previously used formulations for siRNA delivery.

New ionizable lipid or lipid-like materials have also been developed to improve mRNA delivery. A class of N\textsubscript{1},N\textsubscript{3},N\textsubscript{5}-tris(2-aminoethyl) benzene-1,3,5-tricarboxamide (TT), which contains a phenyl ring, three amide linkers, and three amino lipid chains, was utilized to formulate LNPs for mRNA delivery \textit{in vivo}. Among them, TT3 was identified as the lead material for the most optimized formulation, improving the efficacy of luciferase-mRNA delivery \textit{in vitro} by over 350-fold with significantly reduced experimental workload [93]. More recently, this formulation was employed to encapsulate both mRNA and gadolinium-based contrast agents [94]. These dual-functional LNPs showed comparable or slightly higher delivery efficiency for mRNA than the original TT3 LNPs. In another study, a series of bioinspired alkenyl amino alcohol ionizable lipids were synthesized to formulate LNP together with cholesterol, DOPE, and C14-PEG-2000 for human erythropoietin coded mRNA delivery [95].

To successfully engineer the next generation of highly potent LNPs, the intracellular delivery mechanisms of these LNP-encapsulated mRNA should be understood. A recent study identified the late endosome/lysosome formation as an essential process for
functional mRNA delivery [96]. In addition to providing the structural backbone of the LNPs, lipids can serve as signaling molecules that regulate endosome biogenesis and degradation [97]. Thus, incorporating bioactive lipids enriched with endo/lysosomal compartment into LNPs can boost intracellular delivery of LNP-encapsulated mRNA and protein expression [96].

LNPs are currently the most popular non-viral delivery system in clinical trials for genetic drugs [98, 99]. In fact, Alnylam has just announced positive data from the Phase III APOLLO trial (NCT01960348) of Patisiran (an RNAi therapeutic formulated using LNP technology) for the treatment of transthyretin (TTR)-mediated amyloidosis. This represents the first RNAi therapy that has been success in a Phase III clinical trial. It is expected to be approved by the Food and Drug Administration this year. The success of this LNP formulation in siRNA delivery has bolstered confidence in the potential of the approach for similar mRNA delivery applications. Along with the recent development of novel lipids or lipid-like materials, LNPs remain promising candidates for the clinical translation of mRNA nanomedicines.

3.2 Polymer nanoparticles

Similar to cationic lipids, cationic polymers have also been commonly used to complex with mRNA and generate polyplex nanoparticles through electrostatic interactions (Fig. 3(b)). Polymers are easy to synthesize and possess a high degree of chemical flexibility, which makes them attractive for nucleic acid therapy. These cationic polymers often consist of amine groups that are protonated in the acidic environment of endosomes (approximate pH 5.5), allowing the polyplexes to achieve endosomal escape due to the “proton-sponge” effect [100, 101].

Polyethyleneimine (PEI) has a high density of amine groups. PEI is the most studied cationic polymer and is the gold standard control in gene transfection [102]. However, the potential toxicity associated with its high molecular weight and lack of degradability has historically prevented its broader application. To counteract this, researchers have attempted to develop a modified low molecular weight PEI for mRNA delivery. In one study, 2 kDa PEI conjugated to cyclodextrin was used as a safe carrier for mRNA encoding human immunodeficiency virus (HIV) gp120. Strong systemic and mucosal anti-HIV immune responses as well as production of cytokines were demonstrated [103]. In another study, 1.8 kDa PEI with its primary amines modified by different aromatic domains was evaluated. Only the salicylamide-modified PEI was a reliable carrier for mRNA delivery in HeLa and U87 cells [104]. As an alternative to PEI, poly (β-amino esters) (PBAEs) exhibit several advantages, such as easy synthesis, relatively low cytotoxicity, and good degradability, and have been demonstrated as an effective delivery system for nucleic acids [105, 106]. However, serum instability may be one of the drawbacks of PBAEs for systemic administration. To address this problem, PBAEs with alkyl side chains were developed for non-viral gene delivery. Nanoparticles formed from these PBAE terpolymers exhibited significantly increased stability in physiological conditions [107]. Based on these findings, polymer-lipid nanoparticles based on the interaction of PBAE terpolymer and PEG-modified lipid were developed and used for the intravenous delivery of functional mRNA to the lungs of mice [108].

Rapid developments in polymer chemistry have included controlled radical polymerization methodologies, which have been employed to construct a variety of cationic, multi-functional polymers with well-defined architectures and low material heterogeneity for gene delivery applications [109]. Among them, poly(dimethylaminoethyl methacrylate) (p(DMAEMA)) is one of the most intensively investigated gene vectors. However, whereas p(DMAEMA) bound strongly to pDNA, its observed binding to mRNA was weak [110]. Incorporation of PEG to the side chains of p(DMAEMA) increased its ability to complex mRNA and improved the transfection efficiency. Another study used reversible addition-fragmentation chain transfer (RAFT) polymerization to design a series of multifunctional triblock copolymers for intracellular mRNA delivery [111]. These materials are composed of a cationic DMAEMA segment to condense mRNA, a hydrophilic PEG methyl ether methacrylate (PEGMA) segment to improve stability and biocompatibility, and a copolymer of diethylaminoethyl methacrylate (DEAEMA) and butyl
methacrylate (BMA) to facilitate cytosolic entry. The optimal architecture was that with the PEGMA block in the center of the polymer chain, which produced the greatest stability and highest transfection efficiencies. Recently, the concept of reductive decationizable cationic block copolymers was introduced in mRNA delivery. By combining RAFT copolymerization with post-polymerization modification, a cationic block copolymer bearing disulfide-linked primary amines was synthesized. This polymer could effectively condense mRNA and subsequently release it in a reductive cytoplasmic environment [112].

The dendrimer is another type of non-viral gene carrier that is being investigated. Recently, a dendrimer-based nanoparticle system was developed to deliver antigen-encoding replicon mRNA in mice to generate protective immunity against various lethal pathogen challenges, including H1N1 influenza, Toxoplasma gondii, and Ebola virus [113]. Besides the synthetic polymers discussed above, some naturally occurring polysaccharides, such as chitosan, can also be utilized for nucleic acid delivery [114]. With chitosan/hyaluronic acid nanoparticles as the carrier material, mRNA could successfully be delivered into the CD44-expressing HCT-116 cells [115]. The mRNA binding strength and the internalization rate of the nanoparticles were associated with chitosan molecular weight and the degree of deacetylation.

Although polymer nanoparticles are not as clinically advanced as LNPs and some have only been studied in vitro by analyzing reporter protein expressions, their potential in mRNA delivery is undeniable. Further research is needed to explore their in vivo applications. Moreover, further development and optimization of polymers with improved biocompatibility and transfection efficacy will facilitate the use of polymer nanoparticles for mRNA delivery.

3.3 Polypeptide nanoparticles

Protamines are a family of small peptides. They readily condense nucleic acids via electrostatic interactions. Protamines were one of the first cationic materials explored for RNA delivery, in 1961 [116]. An earlier study reported that mRNA could be protected from RNase degradation after condensation by protamine, and described that these protamine/mRNA complexes can act as danger signals that activate several human blood cells in a toll-like dependent manner [117]. Another example of protamine/mRNA-based therapy is the RNAActive® technology, an mRNA vaccine from CureVac. The vaccine is composed of a mixture of naked mRNA for antigen expression and protamine/mRNA complexes for immune stimulation [118]. Several clinical trials have evaluated RNAActive® vaccines, one of which contains self-adjuvanted mRNA encoding four antigens associated with prostate cancer (PSA, PSCA, PSMA, and STEAP1) [119]. When intradermally administered, the vaccine was well tolerated and immunogenic towards patients with advanced castration-resistant prostate cancer. Poly(lysine), which bears amine groups on its side chains, has become widely used in pDNA and siRNA delivery since it was first investigated as a carrier material for nucleic acid. Its application was recently extended to tumor-targeted mRNA delivery [120]. In this study, mRNA was condensed by a mixture of cRGD-PEG-polylysine (PLys) (thiol) and poly(N-isopropylacrylamide) (PNIPAM)-PLys (thiol), forming a stable nanoformulation with a core consisting of PLys and mRNA cross-linked by redox-responsive disulfide linkage. The nanoformulation protected mRNA from degrading in harsh biological environments and improved tumor accumulation and gene expression in vivo.

Apart from the aforementioned traditional cationic peptides, much research has also been devoted to developing polypeptides with structures that can improve the efficiency of mRNA delivery. One noteworthy example is a synthetic mRNA delivery system based on a dendronized polypeptide (denpol) architecture that reportedly can efficiently deliver mRNA to various cells. The denpol system described in one study contained a backbone of L-lysine-dicysteine polymer with multiple lysine dendrons grown on the surface. The resulting system combined the advantages of the conformational flexibility of a linear polymer, the beneficial multivalent interactions of a dendrimer, and the reduced responsivity of the disulfide linkages in the polymer backbone [121]. In a separate study, a range of polypeptides was synthesized by N-carboxyanhydride polymerization of L-benzyl
aspartate, followed by an exhaustive amination of the ester groups to manufacture various cationic side chains for mRNA co-encapsulation as well as a recombinant form of PBAP. This co-delivery strategy produced a 20-fold increase in mRNA expression in vitro [94].

Except for the protamine/mRNA nanoparticles now undergoing clinical trials, most studies using polypeptide nanoparticles for mRNA delivery are still in their infancy. More studies are required to facilitate the use of this kind of material in mRNA delivery.

3.4 Hybrid nanoparticles

In addition to the mRNA delivery nanoplatforms based on a particular material, it is also important to highlight the hybrid nanoparticles combining various components, such as lipid, polymer, peptide, and even inorganic nanomaterials. As such, hybrid nanoparticles could integrate multiple beneficial features from their individual components, and may provide more functionality and flexibility to achieve efficient mRNA transfection.

Among these hybrid nanoparticles, lipid-polymer hybrid nanoparticles (LPNs) are an archetypal example of an emerging generation of therapeutic delivery vehicles [122–124]. LPNs have a classic core-shell structure comprised of polymer cores and lipid/lipid-PEG shells. They exhibit complementary advantages of both polymeric and lipid nanoparticles. Our group has developed various kinds of LPNs for systemic RNAi and cancer therapy [125, 126]. Similar platforms are being developed for the delivery of tumor suppressor encoding mRNA for cancer therapy [127]. Generally, two strategies have been used to design LPNs for mRNA delivery. One involves the condensation of mRNA with a cationic polymer or peptide into polyplexes, followed by envelopment with a “lipid” shell (Fig. 3(c)). In this approach, a protamine/mRNA complex core and DOTAP/cholesterol lipid bilayers were inserted into DSPE-PEG to generate liposome/protamine/RNA (LPR) nanoparticles. The LPR nanoparticles successfully delivered modified mRNA encoding herpes simplex virus 1-thymidine kinase to xenograft-bearing nude mice to produce superior tumor growth inhibition compared to the equivalent pDNA treatment by the same formulation [128]. In a recent study, biodegradable hybrid nanoparticles composed of PBAE/mRNA core and PEG-lipid were developed for the systemic delivery of luciferase-encoding mRNA to the lungs [108]. Here, PEG-lipid contributed to improved in vivo serum stability of the hybrid nanoparticles. Another strategy for loading mRNA in hybrid nanoparticles is to absorb them onto the lipid surface layer through electrostatic interactions (Fig. 3(d)). One study used hybrid nanoparticles containing a pH-responsive PBAE core to promote endosomal escape and a phospholipid shell to minimize the toxicity of the polycationic core [129]. Negatively charged mRNA absorbed onto the surface of the cationic hybrid nanoparticles displayed efficient transfection in vitro and in vivo.

Organic-inorganic hybrid nanoparticles are another type of hybrid nanoparticles that have been widely investigated as a means of drug delivery [130]. Carbonate apatite developed by Akaike and colleagues is a pH-sensitive inorganic crystal that has a strong affinity for charged molecules. In their early studies using carbonate appetite as luciferase mRNA carriers, the researchers did not observe luciferase expression. However, applying inorganic carbonate apatite onto cationic DOTAP/mRNA complexes (Fig. 3(e)) substantially increased the luciferase expression in both mitotic and nonmitotic cells as compared to DOTAP/mRNA complexes [131]. The high transfection potency of the carbonate apatite/DOTPA/mRNA hybrid nanoparticles was mainly attributed to the enhanced cellular contact and internalization facilitated by the apparently higher gravitational force and the positive charge of the absorbed inorganic particles [87]. The transfection potency could be further improved by complexing fibronectin, an extracellular matrix (ECM) protein, into the hybrid nanosystem [132].

Different organic materials including polymers, lipids, dendrimers, peptides attached to diverse inorganic nanoparticles like gold, mesoporous silica, magnetic iron oxide, carbon nanotubes, and quantum dots have been widely used for efficient drug delivery and imaging [133]. With continuing research in this area, the repertoire of organic-inorganic hybrid nanoparticles for mRNA delivery will be expanded.
3.5 Gold nanoparticle-DNA conjugates

Gold nanoparticles (AuNPs) functionalized with thiol-terminated DNA (AuNP-DNA conjugates) (Fig. 3(f)) are another nanoparticle system that has been demonstrated to be an efficient and universal nanocarrier for drug and gene delivery due to their high cellular uptake [134–136]. AuNPs present another potential platform for cellular mRNA delivery with their conjugated DNA, which can be functional by having a sequence complementary to the mRNA of interest. The use of AuNP-DNA conjugates to deliver Bcl-2-associated X (BAX) mRNA to xenograft tumors in mice allowed for the effective expression of BAX protein, which inhibited tumor growth by inducing apoptosis [137]. With the rational design of a DNA oligomer, AuNP-DNA conjugates could also modulate the access and recycling time of ribosomes during mRNA translation, enhancing the in vitro translation efficiency of mRNA [138, 139].

4 Biomedical applications

The progress of mRNA technology along with the development of nanotechnology has enabled the utilization of mRNA for a wide range of therapeutic applications. In the following section, we highlight the application of mRNA nanomedicine in preventing or treating various diseases. The four major biomedical applications of mRNA nanomedicine include: 1) nanovaccines derived from antigen-encoded mRNA for the activation of the immune system; 2) protein-replacement therapy for the treatment of genetic disorder diseases and cancer due to the mutation or loss of protein expression; 3) gene-editing achieved by the co-delivery of Cas9-encoded mRNA and gRNA; and 4) cell programming and engineering through the introduction of mRNA encoding for transcript factors or other functional molecules. Some selected examples of mRNA nanomedicine for biomedical applications are summarized in Table 1.

4.1 Vaccination

Over the past decades, nucleic acid-based vaccines have emerged as attractive alternatives to conventional vaccine strategies. With the major technological innovation and research investment in exploring mRNA as an immunotherapeutic tool, the field of mRNA-based vaccines is developing rapidly, and some are currently in clinical trials [4, 5]. Since mRNA is a non-infectious, non-integrating therapeutic agent with excellent translation efficiency, mRNA-based vaccines are safer and more efficacious than live, attenuated virus and DNA-based vaccines. Vaccination in vivo can be achieved using nanoparticle-based systems as the mRNA carrier. Vaccination with an mRNA-based nanovaccine is a multifaceted and

| Table 1 Selected biomedical applications of mRNA nanomedicinea |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Application                      | Nanoplatform    | mRNA encoding   | Delivery route  | Species         | Target           | Refs.           |
| Nanovaccines for infectious diseases | Dendrimer nanoparticle | H1N1 virus HA; EBOV gp; six Toxoplasma gondii specific antigens | i.n.              | Mouse           | H1N1 influenza Virus; EBOV; Toxoplasma gondii | [113] |
|                                 | LNP             | RSV-F           | i.m.            | Mouse, cotton rat | RSV           | [142] |
| RNAntive platform               | Rabies virus gp | i.d.            | Mouse, pig      | Rabies virus    | [149] |
| PSA nanomicelle                 | HIV-1 gag       | s.c.            | Mouse           | HIV             | [152] |
|                                 | LNP             | H10N8 or H7N9 virus HA | i.d., i.m.     | Mouse ferrets, monkey human | H10N8 and H7N9 influenza viruses | [153] |
|                                 | Dendrimer nanoparticle | prM-E             | i.m.            | Mouse           | ZIKV           | [147] |
|                                 | LNP             | prM-E           | i.m.            | Mouse           | ZIKV           | [154, 155] |
| Application                          | Nanoplatform          | mRNA encoding                     | Delivery route | Species | Target                        | Refs. |
|-------------------------------------|-----------------------|-----------------------------------|---------------|---------|-------------------------------|-------|
| Nanovaccines for cancer            | RNAActive platform    | Six melanoma-associated antigens  | i.d.          | Human  | Metastatic melanoma           | [157] |
|                                     | LNP                   | gp100, TRP2                        | s.c.          | Mouse  | Melanoma                      | [159] |
|                                     | Lipoplex              | Four melanoma antigens            | i.v.          | Mouse; Human | Melanoma                      | [162] |
|                                     | LNP                   | OVA                               | i.n.          | Mouse  | Lymphoma                      | [160] |
|                                     | LCP nanoparticle      | MUC1                              | s.c.          | Mouse  | Triple negative breast cancer | [164] |
| Protein-replacement therapy        | LPR                   | HSV1-tk                           | i.v.          | Mouse  | Lung cancer                   | [128] |
|                                     | PEG-PAsp(TEP)-Chol nanomicelle | sFlt-1                       | i.v.          | Mouse  | Pancreatic cancer             | [171] |
|                                     | LNP                   | hMUT                              | i.v.          | Mouse  | Methylmalonic academia        | [172] |
|                                     | LNP                   | hEPO or hFIX                       | i.v.          | Mouse  | Hemophilia B                  | [175] |
|                                     | PEG-PAsp(DET)-nanomicelle | Bcl-2                        | i.v.          | Mouse  | Fulminant hepatitis           | [178] |
| Tailor-made lipoid nanoparticle     | ACE2                  |                                   | i.v.          | Mouse  | Liver and lung fibrosis       | [181] |
| Gene editing                        | LNP                   | Cas9                              | i.v.          | Mouse  | Hereditary tyrosinemia        | [199] |
|                                     | LNP                   | Cas9 or sgRNA                     | i.v.          | Mouse  | Hypercholesteremia            | [200] |
|                                     | TT3 LLN               | Ca9 or sgRNA                      | i.v.          | Mouse  | HBV and hypercholesteremia   | [201] |
|                                     | ZAL nanoparticle      | Ca9 and sgRNA                     | i.v.          | Mouse  | N/A                           | [202] |
|                                     | LNP                   | Cas9 and sgRNA                    | i.v.          | Mouse  | N/A                           | [203] |
| Cellular reprogramming              | Lipofectamine         | Oct4, Lin28, Sox2, Nanog          | —             | In vitro | Generation of iPSCs from fibroblasts | [207] |
|                                     | GO-PEI                | KLF4, c-MYC, OCT4, SOX2           | —             | In vitro | Generation of iPSCs from fibroblasts | [210] |
|                                     | C-Lipo                | Gata4, Mef2c, Tbx5                | —             | In vitro | Generation of cardiomyocyte-like cells from cardiac fibroblasts | [211] |
| jetPEI cationic vehicle             | PDX1, NGN3, MAFA      |                                   | —             | In vitro | Generation of β-cells from AR42J cells | [213] |
| Cellular engineering               | Lipofectamine         | PSGL-1, FUT7, IL-10               | —             | In vitro | MSC engineering               | [215] |
|                                     | Lipofectamine         | ITGA4                             | —             | In vitro | MSC engineering               | [216] |

*ACE2: angiotensin-converting enzyme 2; Chol: cholesterol; C-Lipo: polyarginine-fused heart-targeting peptide and lipofectamine complex; EBOV: Ebola virus; gp: glycoprotein; FUT7: α-(1,3)-fucosyltransferase; gp100: tumor-associated antigens glycoprotein 100; GO: graphene oxide; HA: hemagglutinin protein; hEPO: human erythropoietin; hFIX: human factor IX protein; hMUT: human methylmalonyl-CoA mutase; HSV1-tk: herpes simplex virus 1-thymidine kinase; i.d.: intradermal; IL-10: interleukin-10; i.m.: intramuscular; i.n.: intranasal; iPSCs: induced pluripotent stem cells; i.v.: intravenous; LCP: lipid-coated calcium phosphate; LLN: Lipid-like nanoparticle; LNP: lipid nanoparticle; LPR: liposome-protein-RNA; MMA: methylmalonic acidemia; MSc: mesenchymal stem cell; MUC1: glycosylated type 1 transmembrane mucin; OVA: ovalbumin; PAsp(DET): poly(N′(N′-2-aminoethyl)-2-aminoethyl)aspartamide; PAsp(TEP): poly[(N′(N′-2-aminoethyl)-2-aminoethyl)2-aminoethyl]-2-aminoethyl)]aspartamide); PEG: poly(ethylene glycol); PEI: polyethylenimine; PSGL-1: P-selectin glycoprotein ligand-1; prM-E: premembrane and envelope proteins; PSA: polyethyleneimine-stearic acid copolymer; RSV: respiratory syncytial virus; RSV-F: respiratory syncytial virus fusion glycoprotein; sgRNA: single guide RNA; s.c.: subcutaneous; sFlt-1: soluble fms-like tyrosine kinase 1; TRP2: tyrosinase-related protein 2; TT: N,N,N′,N′-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide; ZAL: zwitterionic amino lipid; ZIKV: Zika virus
multistep process. First, the nanoparticle system should be engineered to efficiently deliver antigen-encoding mRNA into antigen presenting cells (APCs). Second, mRNA is translated into antigenic protein in the cytosol of APCs, and consequently processed into peptide epitopes with the aid of proteasomes to bind with the major histocompatibility complex (MHC) class. Finally, the MHC-peptides are transferred to the cell surface, where they present the peptide epitopes to either CD4+ T cell or CD8+ T cells, resulting in corresponding immune responses (Fig. 4). Remarkable progress has been made to date in developing mRNA-based nanovaccines for both infectious diseases and cancer.

4.1.1 mRNA-based nanovaccines for infectious disease

The earliest attempt to use mRNA-based nanovaccine was reported in 1993. Anionic liposomes were utilized as a carrier for mRNA that encoded influenza virus nucleoprotein. Virus-specific cytotoxic T cell responses were induced when the nanovaccine was administrated intravenously or subcutaneously [140]. Since then, more mRNA nanovaccines have been developed to induce protection from several viral pathogens via antigen-specific antibody and cellular immune responses.

One of the commonly used mRNA vaccines is the self-amplifying mRNA (SAM) platform based on an alphavirus genome [141], which contains an RNA replication machinery gene with the structural protein genes replaced by those for protein antigens. After immunization, the antigen-encoding RNA is capable of replication and amplification in the cytoplasm of the transfected cells, thereby generating a large amount of antigen even with a low vaccine dose. An early study demonstrated that a dose as low as 0.1 μg of SAM vaccine encoding respiratory syncytial virus fusion glycoprotein (RSV-F) in an LNP delivery system produced effective cellular and humoral immune responses in mice, and 1 μg elicited potent immune responses and conferred protection from further RSV infections in cotton rats [142]. Immunization with SAM vaccines encoding influenza virus hemagglutinin (HA) in a cationic nanoemulsion protected mice from influenza virus challenges [143, 144]. In other studies, SAM vaccines encoding influenza antigens were successfully delivered to DCs by chitosan-nanoparticles and PEI-based polyplexes, which were also reported to successfully induce humoral and cellular immune responses in mice [145, 146]. Besides influenza antigens, protection against several other viruses by SAM-based nanovaccines has been demonstrated in various species. For example, a modified dendrimer nanoparticle system was developed to deliver multiple mRNA replicons to generate protective immunity against a broad spectrum of lethal pathogens including Ebola, H1N1 influenza, and *Toxoplasma gondii* [113]. In another study, mRNA encoding Zika virus premembrane (prM) and envelope (E) proteins were encapsulated into the same nanoparticle system as a vaccine candidate against Zika virus. The approach successfully elicited antigen-specific antibody and CD8+ T cell response in mice [147].

The other strategy is to use non-replicating mRNA for vaccination. RNActive® vaccines, developed by the CureVac Company, are a powerful technology for non-replicating mRNA vaccination by combining naked mRNA for expression of an antigen and protamine/mRNA complexes that stimulate the immune system. The RNActive® technology induces balanced humoral and cellular immune responses in several animal models. The first report of the protective antibody response achieved by RNActive® vaccines was published in 2012, in which direct intradermal injection of a mixture of naked mRNA encoding various influenza infection virus and a protamine/
A considerable number of mRNA complexes adjuvant resulted in protective immunity against influenza infection in multiple animal models including mouse, ferret, and pig [148]. Another study demonstrated that RNActive® vaccine encoding non-replicating rabies virus glycoprotein was capable of inducing potent neutralizing antibodies in mice and pigs and protecting mice from lethal intracerebral challenges [149]. A first-in-human phase I clinical trial (NCT02241135) is currently underway to evaluate the safety and immunogenicity of the RNActive® rabies vaccine [150]. Non-replicating mRNA-based nanovaccines can also be directly administrated to induce an immune response in vivo. One example is HIV-1 gag encoding mRNA complexed with DOTAP/DOPE, PEI or polyethyleneimine stearic acid copolymer in mice, which reportedly elicited antigen-specific CD4+ and CD8+ T cell responses [151,152]. Cyclodextrin-PEI 2k conjugate-complexed mRNA encoding HIV-1 gp120 also led to strong mucosal and systemic immune responses [103]. LNP-formulated, modified mRNA encoding HA protein of H10N8 or H7N9 virus generated rapid and robust immune responses in mice, ferrets, non-human primates, and humans [153]. Encouraged by these results, a phase I trial (NCT03076385) is evaluating the safety and immunogenicity of H10N8 vaccines in humans. In addition, a Zika virus vaccine has been engineered by encapsulating modified mRNA encoding Zika virus prM-E into LNP. Immunization by the direct injection of the nanovaccine into the spleen of mice elicited both anti-gp100 antibody and CTL responses against B16 melanoma [156].

4.1.2 mRNA-based nanovaccines for cancer

Cancer immunotherapy aims to exploit the benefit of the activated immune system that can recognize and kill cancer cells through humoral and cellular immune responses. The most widely used application of mRNA vaccines in cancer immunotherapy is to transfect mRNA encoding tumor-associated antigens (TAAs) into patient-derived DCs in vitro and then re-administer these activated DCs to the patients. DC-loaded mRNA vaccines against cancer are the most widely investigated nanovaccine variety in clinical trials [4]. Although this intervention shows good efficacy in eliciting a tumor-reducing immune response in cancer patients, it involves a complex manipulation process that may complicate its practical application regarding production costs, doses used, and intrinsic phenotypic variability.

As an alternative, the direct injection of mRNA-based cancer nanovaccines is a simpler way to improve the vaccination effects in vivo. The nanoparticle platform protects mRNA from degradation by nuclease in body fluids and facilitates its uptake by APCs. In 1999 an mRNA nanovaccine was formulated by encapsulating gp100 into hemagglutinating virus of Japan (HVJ)–liposome. Immunization by the direct injection of the nanovaccine into the spleen of mice elicited both anti-gp100 antibody and CTL responses against B16 melanoma [156].

Other delivery routes, such as intradermal, subcutaneous, and intranasal injection, have also been used to administer mRNA-based nanovaccines in cancer therapy. Due to the prevalence of APCs in the skin, intradermal injection is a favorable route for mRNA cancer vaccination. In a trial reported in 2007 (NCT00204607), one of seven patients with metastatic melanoma displayed a complete clinical response after being treated by the intradermal administration of protamine-stabilized mRNA coding for six melanoma-associated antigens [157]. Intradermal injection of the RNActive® vaccine CV9103, which encodes four prostate specific antigens, to patients with castrate-resistant prostate cancer produced a safe but unexpectedly high level of cellular immunogenicity [158].

Subcutaneous administration also has been explored, in which the mRNA nanovaccines are administered to the APCs that are prevalent in lymph nodes through the lymphatic system. The subcutaneous regions between the skin and skeletal muscles can be easily accessed, making it a convenient vaccination route. LNP-encapsulated mRNA coding for the tumor-associated antigens glycoprotein 100 (gp100) and tyrosinase-related protein 2 (TRP2) was used for melanoma vaccination. Subcutaneous immunization of the vaccine with a single dose delayed tumor growth and prolonged overall survival in a B16F10 melanoma.
mouse model [159].

By intranasal administration, mRNA-based nanovaccines can be directly delivered into immune cells within lymphoid tissues located in the nasal cavity, while avoiding the systematic barrier. Since the intranasal immunization route is needle-free and non-invasive, it enables repeated administrations and leads to favorable patient compliance. In one study, nasal vaccination was demonstrated as an effective administrated route for cancer vaccination with ovalbumin (OVA) encoding mRNA nanoparticles [160].

Furthermore, systemic delivery of mRNA can be achieved by administering mRNA-based nanovaccines intravenously. Nanotechnology engineering of mRNA enables its stability in the bloodstream and ensures its delivery to DCs of the target organs, with the aim of inducing a subsequent anticancer immune response. In one study, mice were intravenously vaccinated with MART-1 melanoma antigen-encoding mRNA in mannosylated and histidylated lipopolyplexes. The strategy resulted in the efficient delivery of the mRNA to splenic DCs and led to a significant inhibition of B16F10 melanoma growth [161]. In another study, RNA-lipoplexes (RNA-LPX) were developed by complexing liposomes with mRNA encoding four tumor antigens. The RNA-LPX were intravenously injected to lymphoid DCs in mice. Strong effector and memory T-cell responses were successfully induced, which mediated potent interferon-gamma-dependent rejection of progressive tumors [162]. An ongoing phase I dose-escalation trial (NCT02410733) is testing RNA-LPX that encode shared tumor antigens in patients with advanced malignant melanoma.

Combination of mRNA-based immunotherapy with other therapeutic approaches may exert a synergistic effect in cancer therapy. For instance, combining tumor-specific RNAActive® mRNA vaccine with local radiation therapy produced a strong synergistic antitumor effect with the efficient eradication of large established E.G7-OVA tumors and Lewis lung cancer tumors [163]. Combination immunotherapy of glycosylated type 1 transmembrane mucin mRNA nanovaccine and anti-cytotoxic T-lymphocyte-associated protein 4 monoclonal antibody significantly enhanced the anti-tumor immune response for the treatment of non-immunogenic triple-negative negative breast cancer [164]. More recently, the same nanoparticle system was employed to co-deliver mRNA encoding TRP2 and siRNA silencing programmed death-ligand 1. The combinational therapeutic approach downregulated the expression of programmed death-ligand 1 in the tumor antigen-presenting DCs and significantly promoted T cell activation and proliferation, resulting in an enhanced immune response against established melanoma [165].

The field of mRNA nanovaccines is experiencing a very exciting phase with several clinical studies in progress. However, while preclinical studies showed encouraging results for the potential of mRNA nanovaccines in animal models, a recent clinical trial reported that vaccination with the CV7201 RNAActive® rabies vaccine in humans resulted in only modest immune responses [150]. Further research is needed to understand the different immune responses and mechanisms between animal species and humans. Another challenge of mRNA nanovaccines is the limited nanoplatforms for mRNA delivery. Although the RNAActive® platform and LNPs have shown some potential for clinical use, the bottleneck has not yet been overcome for most formulations. Detailed knowledge of the mechanisms of mRNA delivery and antigen presentation of mRNA nanovaccines is needed to screen suitable formulations and doses for optimized immune responses. Addressing these issues will help to achieve the true potential of mRNA nanovaccines in clinical translation.

4.2 Protein-replacement therapy

Abnormal protein expression is a frequent cause of many diseases. The ability to normalize protein expression in vivo has a great potential in treating these diseases. RNAi technology has been widely used to treat diseases characterized by over-expression of specific proteins [166]. Various siRNA-based nanomedicines are now being assessed in clinical trials [167]. Alternatively, mRNA-based nanomedicine is a new approach to diseases characterized by protein loss or malfunction, using IVT-mRNA as the source of therapeutic proteins. The use of modified nucleotides and improved purification methods in IVT mRNA preparation reduces the immunogenicity of IVT-mRNA, which is critical for its application in protein-
replacement therapy. As shown in Fig. 5, after mRNA-loaded nanoparticles are internalized into the cell, the mRNA needs to be released and then recognized by the translation machinery in the cellular cytosol to finally bind with the ribosomal complex. The ribosomal complex scans the mRNA sequence in the 5′ to 3′ direction and begins translation when it recognizes the start codon (initiation). The amino acids are added to the elongating peptide and the translation process continues until the ribosome recognizes the stop codon (termination). The newly synthesized peptide is released from the complex for post-translational modifications, yielding the new protein for replacement therapy.

Several diseases have been studied in which the in vivo protein production resulted following the systemic delivery of mRNA nanoformulation. Inducible Hsp70 is the most protective of the heat shock proteins against subsequent hypoxia or ischemia. In one study, cationic lipids were used to deliver mRNA encoding Hsp70 to the central nervous system by intrathecal injection. Lipid/mRNA complexes were able to protect the mRNA from degradation in human cerebrospinal fluid in vitro and the expression of reporter protein was successfully detected in coronal sections throughout the rat brain [168]. This was one of the earlier attempts at using mRNA-based nanoparticles for protein-replacement therapy.

Cancer is characterized by multiple genetic disorders in the cancer cell genome, which drive cancer pathogenesis and development. Correction of the cancer cell genetic disorders by, for instance, upregulation of tumor suppressor genes could be a promising approach for cancer therapy [127]. Bax is a pro-apoptotic molecule that functions as a tumor suppressor in various cancers. The use of cationic liposomes to transfer Bax mRNA has resulted in a stronger anti-tumor effect against malignant melanoma in vitro and in vivo compared to the pDNA-based therapy in the same formulation. Enhanced expression of Bax protein was observed and caspase-3 activity increased significantly in HMG cells following transfection with liposome-Bax mRNA complexes [169]. Anti-angiogenesis therapy is another important strategy for inhibiting tumor growth, which has been intensively investigated in cancer treatments [170]. In a recent study, soluble fms-like tyrosine kinase 1 (sFlt-1), an anti-angiogenic protein, was efficiently expressed in a pancreatic tumor-bearing mouse model after intravenous injection of mRNA encoding sFlt-1 encapsulated in a nanomicelle system stabilized by cholesterol. Using this mRNA-based nanomedicine for systemic anti-angiogenic therapy, a remarkable anti-cancer effect was observed for the first time in an intractable pancreatic cancer model [171].

Another promising application of mRNA-based nanoparticles is to restore or augment metabolic enzymes that are associated with metabolic diseases. A codon-modified mRNA encoding human methylmalonyl-CoA mutase (hMUT) was encapsulated in a biodegradable LNP system as a remedy for methylmalonic acidemia [172]. Intravenous administration of the therapeutic into two murine models of methylmalonic acidemia resulted in robust hepatic MUT expression and improved growth and survival of mice. In a recent study, a hybrid mRNA delivery system comprising a lipid nanoparticle for mRNA protection and a polymer micelle for hepatocytes targeting was used to deliver human ornithine transcarbamylase mRNA in a murine model of ornithine transcarbamylase deficiency, which led to pronounced
synthesis of the desired protein in the liver and prolonged survival [173].

Apart from deriving the intracellular protein from the delivered mRNA in the cytosol, the translated protein could instead be secreted into the extracellular space to systemically treat the protein deficiency. In two dependent studies, intravenous administration of nanoparticle-formulated mRNA encoding human erythropoietin in mice, non-human primates, and even pigs resulted in elevated human erythropoietin protein levels in the serum [174, 175]. Hemophilia B is a disease caused by a single defective protein Factor IX that normally produced by the liver. LNPs were used to deliver human FIX (hFIX) mRNA to the liver to treat hemophilia B in a FIX knockout mouse model. The hFIX mRNA was effectively translated into functional FIX protein by hepatocytes and secreted into the circulation where it alleviated the clotting defect of the mice [176].

Several other studies have also described the use of mRNA nanoparticles for protein replacement therapies in various therapeutic areas including neurological disorders [177], fulminant hepatitis [178], bone defects [179, 180], and liver and lung fibrosis [181]. Considering the diversity of proteins that may be potential candidates for replacement therapy, together with the proven feasibility of nanotechnology for systemic mRNA delivery, mRNA nanomedicines are a promising candidate for therapeutics targeting any kind of functional protein deficiency. However, it must be noted that these endeavors are still at preclinical stages of their development. One of the major hurdles may be the lack of understanding of the exact pharmacokinetic and pharmacodynamic properties of these nanoparticle systems in humans. Moreover, while there has been much success in vitro, the challenges of delivering a therapeutically active dose of mRNA in vivo to the hard-to-access cells or tissues have not been thoroughly addressed.

4.3 Gene editing

In the past several decades, genome editing has emerged as a powerful tool for therapy of genetic diseases since it can precisely delete, replace, and insert a DNA sequence at a specific site in the genome [182]. Traditional nuclease-based gene targeting technologies, such as zinc finger nucleases and transcription activator-like effector nucleases, have been applied to generate a variety of animal and cellular models [183]. However, these nucleases recognize the target gene sequence by protein-DNA interactions, and thus require a customized protein for each target, which is a complex and time-consuming process. More recently, clustered regularly interspaced short palindromic repeats-CRISPR-associated protein (CRISPR-Cas) technology, which was originally identified as a prokaryotic adaptive immune system that protects bacteria against foreign DNA invasions [184], has provided a more precise platform for the gene editing of mammalian cells [185, 186]. The most widely used CRISPR-mediated gene editing technology is the CRISPR-Cas9 system derived from Streptococcus pyogenes [187]. The Cas9 nuclease can be directed to cleave the target gene at a precise location using a single guide RNA (sgRNA) that is complimentary to the target DNA sequence [188]. The power of CRISPR-Cas9 lies in the fact that Cas9 nuclease can target any site in the genome simply by modifying the sequence of sgRNA. Moreover, it is also possible to simultaneously edit multiple independent genes using multiple sgRNAs [189].

Effective gene editing by the CRISPR-Cas9 system requires delivery of a functional Cas9-sgRNA ribonucleoprotein complex to the cell nucleus. Delivery of a plasmid DNA encoding both the Cas9 protein and sgRNA is a simple and convenient format for the CRISPR process [190]. However, this approach risks nonspecific editing and off-target effects due to the extended presence of Cas9 in the cell [191] and gene editing can be delayed by natural transcription and translation mechanisms. Direct delivery of the ribonucleoprotein complex seems to be the most straightforward alternative that may potentially minimize the off-target cleavage [192]. However, this approach also suffers the challenge of delivering the large Cas9 protein across the two cellular barriers of the cell membrane and nuclear membrane. Thus, co-delivery of sgRNA and Cas9 mRNA provides an alternative method of CRISPR gene editing with the following benefits [193]. mRNA delivery leads to transient protein expression, which may be favorable
in limiting the off-target editing. In addition, this approach avoids the challenge of crossing the nuclear membrane, as mRNA does not require nuclear entry to exert its effect. In one study, the gene editing efficiencies of these three delivery formats (pDNA, Cas9 mRNA/sgRNA, and direct RNP delivery) were compared. Cas9 mRNA/sgRNA delivery was superior to that of pDNA delivery in all cell lines tested, likely due to the quicker onset of gene editing by the relatively stable Cas9 protein directly translated from the mRNA [194].

Cas9 mRNA has been widely utilized to generate gene-modified animal models by disrupting or inserting DNA sequences \textit{ex vivo} in embryonic cells (zebrafish, mouse, rabbit, and monkey) with high efficiency [195–197], as well as to generate modified chimeric antigen receptor T cells for enhanced cancer immunotherapy [198]. Nevertheless, the systemic delivery of Cas9 mRNA by nanoparticles for \textit{in vivo} applications was only reported two years ago. Optimal \textit{in vivo} genome editing is determined mainly by the delivery routes of Cas9 mRNA and sgRNA. These two components can be either encapsulated into two separate systems or co-administrated in a single dose (Fig. 6). An earlier study used LNP-mediated delivery of Cas9 mRNA in combination with adeno-associated viruses carrying a sgRNA and a repair template to generate fumarylacetoacetate hydrolase (\textit{Fah})-positive hepatocytes by correcting the causative Fah-splicing mutation gene mutation in a mouse model of human hereditary tyrosinemia [199]. In another study, Cas9 mRNA and a chemically modified sgRNA targeting \textit{Pcsk9} were separately encapsulated in LNPs. Simultaneous administration of these two formulations in a single dose enabled the nearly complete editing of the target gene in hepatocytes \textit{in vivo} [200]. Despite its success, Cas9 mRNA must first be translated into the Cas9 protein. Thus, the timing of delivery is another concern. A recent study demonstrated that the expression of Cas9 protein was robust 6 hours after the injection of the nanoparticle-formulated Cas9 mRNA and rapidly decreased at 12 hours. Thus, delaying the delivery of the sgRNA to 6 hours following mRNA injection may enhance the editing efficiency [201].

Since mRNA and sgRNA are both single-stranded RNA molecules, they are also readily co-delivered in the same nanoparticles (Fig. 6). This strategy guarantees that these two components are delivered to the same individual cells, and has achieved greater editing efficiency [202]. An LNP-based delivery system that co-formulated Cas9 mRNA and gRNA into a single particle for simultaneous delivery resulted in a significant editing of the mouse transthyretin gene in the liver, with > 97% reduction in serum protein levels that persisted for at least 12 months [203].

The development of CRISPR-Cas9 has revolutionized the genome editing field. While similar to the conventional mRNA-based gene therapy, the delivery of Cas9 mRNA together with sgRNA still faces many challenges that need to addressed. The rapid development of the CRISPR technology may provide a solution. For instance, the Cpf1 nuclease has been identified as an alternative to Cas9 [204]. Discovery and implementation of safe and efficient delivery systems or materials are critical for the successful application of the CRISPR technology in clinical settings.
4.4 Cellular reprogramming and engineering

Cellular reprogramming is the process of differentiated cells back into pluripotent cells. This has great potential in studies of normal development, constructing patient-specific disease models, and generating autologous tissues for cell-based therapies that can repair damages from injuries or illnesses. In 2006, Yamanaka and colleagues first demonstrated that adult somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by transfection with pDNA encoding four transcription factors (Oct4, Sox2, Klf4, and c-Myc; also known as Yamanaka factors) using retroviral vectors [205, 206]. The retroviral delivery strategy carries the risk of genomic integration, which may endow the derived iPSCs with tumorigenic characteristics due to the consistent expression of these oncogenic transcription factors. Therefore, considerable efforts have focused on developing a safe and effective means of directing the fate of iPSCs.

Since IVT mRNA is transient and does not enter the nucleus, it eliminates the risk of insertional mutagenesis and tumorigenesis. In addition, mRNA can be directly translated into transcription factors within the cytoplasm, which enables higher in vitro transfection efficiency than pDNA. As shown in Fig. 7, transcription factors are expressed from mRNA in the target cell, enter the cell nucleus, and bind to the enhancer or promoter sequences of genomic DNA to regulate specific gene expressions. These advantages of mRNA make it a powerful tool to modulate cell phenotype and function, suggesting great promise for regenerative medicine [74]. The pioneering use of IVT mRNA to mediate reprogramming of somatic cells was reported in 2010. IVT mRNA encoding four transcription factors (Oct4, Sox2, Lin28, and Nanog) was transfected using Lipofectamine to reprogram human foreskin fibroblasts into iPSCs [207]. In the same year, cocktails of mRNA encoding Yamanaka factors were also used to generate iPSCs, followed by transfection with MyoD mRNA to induce further differentiation into myogenic cells [208]. Subsequently, other researchers attempted to improve the mRNA-based reprogramming technology by optimizing the transcript factor cocktails [209] or employing graphene oxide-polyethylenimine as the mRNA delivery system [210].

Besides reprogramming somatic cells into iPSCs, somatic cells can transdifferentiate and directly transform into the desired functional cell types through the transfection of mRNA encoding specific transcription factors. For example, mRNAs encoding three cardiac reprogramming factors (Gata4, Mef2c, and Tbx5) were formulated into a C-Lipo delivery system composed of a heart-targeting peptide and Lipofectamine. Cardiac fibroblasts were partially reprogrammed towards a cardiomyocyte-like state via daily transfection of this mRNA nanoformulation [211]. A similar strategy has been applied to reprogram other types of cells. Pancreatic exocrine cells and human pancreatic duct-derived cells could be transdifferentiated into insulin-secreting β cells by transfecting with mRNA encoding pancreatic transcription factors [212, 213]. Human fibroblasts could directly be reprogrammed into hepatocyte-like cells by synthetically modified mRNA encoding HNF1A plus any two of FOXA1, FOXA3, or HNF4A in the presence of an optimized hepatic growth medium [214].

Another application of IVT-mRNA is to engineer mesenchymal stem cells (MSCs), which are attractive candidates for cell-based therapy to treat multiple diseases. mRNA transfection has been successfully used to engineer MSCs with enhanced homing properties. The engineered MSCs simultaneously expressed a combination of homing molecules (P-selectin glycoprotein ligand-1 and Sialyl-Lewisx and immunosuppressive cytokine interleukin-10) [215]. In another study, the delivery of ARCA 5’-cap analog modified mRNA encoding integrin α4 subunit in MSCs resulted in improved homing properties.

Figure 7 Delivery mechanism of transcription factor-encoding mRNA nanoparticles for cellular reprogramming.
in a successful production of integrin α4 protein. The strategy may enhance cell adhesion and MSC migrations [216].

IVT mRNA has great potential in cellular reprogramming and engineering, which will promote the development of regenerative medicine. Considerable progress has been made; however, a variety of limitations remain to be overcome. Due to the transient protein expression of IVT mRNA, repeated daily transfections are required for the successful transformation of functional cells, which may be time-consuming. One potential strategy to address this issue is to develop biomaterials or nanoparticles for sustained mRNA release and protein expression. Moreover, most of the reported studies were limited to in vitro cellular reprogramming using commercial transfection agents, with only a few studies having investigated their further applications in vivo. More efficient mRNA nanoparsonas are needed to prolong mRNA-mediated protein expression and facilitate their translation into practical application.

5 Conclusions and perspectives

mRNA-based nanomedicine holds great potential in gene-based therapies. In the past decades, substantial advances have been made in the chemical modification of mRNA, addressing the key challenges associated with its instability and immunostimulation. Another major issue for the in vivo application of mRNA is the efficient delivery of mRNA to the desired cells while reducing the systemic exposure. Advances in nanotechnologies and biomaterials for mRNA delivery have improved the stability of mRNA in a physiological environment and have realized an efficient strategy for the desired delivery of mRNA.

Nevertheless, the application of mRNA still faces a variety of challenges. First, the systemic administration of mRNA formulations sometimes results in poor accumulation in the target tissue and/or cell, or insufficient penetration in the lesion tissue, which lead to the inefficient expression of desired protein. More efforts are required to further optimize the materials and formulations used for mRNA delivery that would enable the efficient protein expression in hard-to-access tissue and/or cell. Second, since the chemically modified mRNA is not identical to the natural mRNA in eukaryotic cells, the immunogenicity of the protein derived from IVT mRNA may be another concern that needs to be considered. Through rational and individualized design, the synthetic mRNA should be closer to the natural mRNA while not affecting the therapeutic efficacy. In addition, the high cost of synthesized mRNA also limits its clinical application [13]. Continuing efforts are needed to develop more efficient synthesis methods of mRNA to reduce the production cost.

This review has presented four main categories of the recent applications of mRNA nanomedicine: vaccination, protein-replacement therapy, gene-editing, and cellular reprogramming and engineering. It is worth noting that mRNA-based nanovaccines have progressed rapidly for various infectious diseases and cancer, and some have progressed to clinical trials. However, other applications are still in preclinical stages. Given that the advances continue and interest is sustained in this field, we can expect to see more mRNA nanomedicines to enter clinical studies in the foreseeable future.

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