Modified mRNA as an alternative to plasmid DNA (pDNA) for transcript replacement and vaccination therapy

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Introduction: Current gene therapy involves replacement of defective gene by delivery of healthy genetic material to precede normal function. Virus-mediated gene delivery is the most successful and efficient method for gene therapy, but it has been challenged due to serious safety concerns. Conversely, gene delivery using plasmid DNA (pDNA) is considered safer, but its transfection efficiency is much lower than virus-mediated gene transfer. Recently, mRNA has been suggested as an alternative option to avoid undesired insertion of delivered DNA sequences with higher transfection efficiency and stability.

Area covered: In this review, we summarize the currently available strategies of mRNA modification to increase the therapeutic efficacy; we also highlight the recent improvements of mRNA delivery for in vivo applications of gene therapy.

Expert opinion: The use of mRNA-based gene transfer could indeed be a promising new strategy for gene therapy. Notable advantages include no risk of integration into the genomic DNA, adjustable gene expression and easier modulation of the immune system. By reducing or utilizing the immunogenic properties, mRNA offers a promising tool for gene/or transcript replacement.

Keywords: chemical modification, delivery, gene therapy, mRNA

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1. Introduction

Strategies of current gene therapy include replacement of defective gene by delivery of healthy genetic material to precede normal function. For gene therapy, virus-mediated gene delivery has been considered to be the most successful gene replacement method with high efficiency. However, this method has been challenged due to serious safety concerns, including insertion mutagenesis and triggering of the innate immune response [1]. Conversely, non-viral vector-mediated gene delivery using pDNA is safer but has lower transfection efficiency compared to viral vectors because of insufficient nuclear transport. Modification of pDNA for nuclear localization and enhanced transcription by using a strong constitutive promoter increases the expression of therapeutic genes, but integration of delivered sequences into the nuclear DNA also induces unexpected genetic changes [1,2].

To overcome undesired integration of transferred pDNA, mRNA has been suggested as an alternative [3,4]. As sufficient amounts of in vitro transcribed mRNA...
can be easily obtained by commercially available kits, therapeutic gene delivery using mRNA is now an effective replacement for pDNA in gene therapy. Gene delivery using mRNA has several advantages compared to pDNA. First, unexpected insertion mutation and promoter dependency can be excluded because mRNA is works as a gene/or transcript replacement in the cytoplasm. Therefore, nuclear translocation and transcription is not required. Second, mRNA-mediated gene transfer occurs in non-dividing cells, while pDNA-mediated gene transfer is mostly effective in dividing cells. Third, immunogenicity can be easily modulated by chemical modification.

Although there are many advantages of mRNA-mediated gene delivery, mRNA was previously considered too unstable to be used as a therapeutic molecule. However, transfection efficiency of mRNA has been greatly improved and the half-life of mRNA has been dramatically increased, ranging from a few minutes to several hours by chemical modifications, which facilitates the use of mRNA for therapeutic gene transfer. By combining various mRNA modification and delivery methods, the efficacy of mRNA gene therapy could be greatly improved.

### 2. Modified mRNA

The main reason for mRNA instability is the presence of a hydroxyl group on the second carbon atom of the sugar moiety, which facilitates hydrolytic degradation. Either cis-acting or trans-acting factors can influence mRNA degradation. Mature eukaryotic mRNA consists of five significant portions, including the cap structure (m7GpppN or m7Gp3N [N: any nucleotide]), the 5' untranslated region (5'UTR), an open reading frame (ORF), the 3' untranslated region (3'UTR) and a tail of 100 – 250 adenosine residues (Poly(A) tails) (Figure 1A).

The cap structure is post-transcriptionally modified with methylated m7GpppN in the nucleus at the 5' ends of mRNA and plays an important role in normal mRNA function, for example, mRNA splicing, stabilization, transport, recruiting ribosomes and translational repression via microRNA. This structure contains an uncommon nucleoside, 7-methylguanosine (m7G) and is connected with the 5'-5' triphosphate bridge to the first transcribed nucleotide (Figure 1C).

To increase the efficiency of mRNA translation, an anti-reverse-cap analogue (ARCA), which contains a modified cap structure containing a 5'-5' triphosphate bridge, has been suggested (Figure 1D and E). In vitro transcription performed in the presence of a cap analog may be initiated by an RNA polymerase from either guanosine (G) or m7G to produce correctly-capped (m7GpppG) or reversely-capped (Gpppm7G) mRNA, respectively. The mRNAs bearing reversely-capped structures are poorly translated and more readily degraded. Only the 5'-5' triphosphate linkage yields a translatable mRNA molecule. Introducing a chemical modification at the 3'- (or 2'-) position of the cap analog prevents the reverse incorporation and improves both mRNA quality and translation efficiency. ARCA results in attachment in the correct direction only, which is recognized by eukaryotic initiation factor 4E (eIF4E), leading to ribosome recruitment and translation. In addition, it has been reported that a high number of cap modifications and elongated 5'-5' phosphate bridges in the ARCA improves translation efficiency and stability of mRNA.

The length of the poly(A) tail is also crucial for efficient translation and enhancing mRNA stability. In mammalian cells, most actively translated mRNAs contain 100 – 250 poly(A)s. For exogenous application, at least 20 poly(A)s are required for effective translation. Generally, translation efficiency is dependent on the number of poly(A)s. The poly(A) tail binds to numerous polyadenosyl-binding proteins (PABP), which recruit eukaryotic initiation factor 4G (eIF4G), leading to circular mRNA by increasing affinity to the mRNA cap. This synergistic effect of the cap structure and the poly(A) tail has been explained by a cap-eIF4E-eIF4G-PABP-poly(A) closed loop structure, which could facilitate the recycling of ribosomes and protect from nucleolytic degradation.

RNA degradation is initiated by shortening of the poly(A) tail and includes a de-capping process (Figure 1F). The cap structure and the poly(A) tail in the mRNA cooperatively function to maintain the stability of the mRNA. The mRNA cap binds to the cap-binding protein (CBP) complex, which regulates mRNA transport from the cytoplasm to the nucleus. Degradation of mRNAs takes place in the cytoplasm at sites called P-bodies, which contain 5'-3' exonucleases, de-capping and de-adenylating enzymes. Once the poly(A) tail has been shortened to less than 12 residues, mRNA degradation then occurs by de-capping, along with 5'→3' exo or 3'→5' cleavage.

Further optimization of the mRNA structure can be achieved by replacing unstable non-coding sequences with non-coding sequences of mRNAs that are known to be stable. Most eukaryotic mRNAs contain mRNA decay signals in...
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Figure 1. Typical gene deliveries for therapeutic application. A. pDNA or mRNA-mediated gene transfer is illustrated. pDNA contains the multiple cloning site (MCS), which is used for restriction endonuclease recognition to insert transgene. Mature eukaryotic mRNA consists of five significant portions, including the cap structure ([m7GpppN or m7Gp3N (N: any nucleotide)], the 5’ untranslated region (5’UTR), an open reading frame (ORF), the 3’ untranslated region (3’UTR) and a tail of 100 – 250 adenosine residues (Poly(A) tails). B. Regions of mRNA modifications for increasing their stability. C. Chemical structure of mRNA CAP. D. Standard dinucleotide cap analog. E. Anti-reverse cap analogs (ARCA). F. mRNA degradation pathways. Both major pathways of mRNA decay are initiated by deadenylation (continued).
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their 3’ untranslated regions (3’ UTRs), which affect mRNA stability. Various AU-rich sequences in the 3’ UTR of mRNA have been reported to be involved in the removal of the poly(A) tail [32]. The half-life of mRNA increases when AU-rich sequences are replaced with sequences of 3’ UTR from stable mRNA [10]. Other specific sequences in the 3’ UTR are the so-called iron responsive elements (IREs), which regulate mRNA stability in the 3’ UTR, and affect translation in the 5’ UTR [33]. 3’ UTRs of the human globin gene have also known as stable and insertion of two sequential 3’ UTR and poly (A) tail reported to show enhancement of mRNA stability in DC cells [34].

The coding region of mRNA sometimes facilitates mRNA degradation. Natural coding sequences often show less efficient codon usage for translation in specific organisms. Since different organisms usually show particular preferences for one of the several codons that encode the same amino acid [35], optimized codon usage improves translational efficiency [36]. As ribosomal traffic is modified by change of synonymous codon, mRNA turn-over and stability can be modified by altering RNases accessibility to target sites.

For in vivo applications that use mRNA as an alternative to pDNA, the immune response should be considered. Both DNA and RNA stimulate the mammalian innate immune system through activation of Toll-like receptor (TLRs). Thirteen TLRs have been identified and four of them (TLR3 for dsRNA, TLR7 and 8 for U-rich ssRNA, TLR9 for CpG DNA motif) are involved in nucleic acid recognition. It has been reported that mRNA can be recognized by TLR3 [37] and in vitro transcribed RNAs induce a strong TNF-α response in dendritic cells (DC) [38]. There are other receptors for TLR-independent immune response, RIG-I like receptor (RLR) such as retinoic acid inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5) trigger type I IFN by IFN-regulatory factor 3 (IRF3)-dependent pathway, and they can be stimulated by ds/mRNA.

The use of modified nucleosides (Figure 2), such as 2’-O methyl nucleoside for in vitro transcription, shows dramatic suppression of TLR-mediated DC activation [38], but the effects of modified nucleosides on the TLR-independent immune response are still unknown. As many modified nucleosides are present in mammalian RNAs, such as pseudouridine, 2-thiouridine, 5-methylcytidine, 6-methyladenosine, inosine and many 2’-O-methylated nucleosides at the 5’-terminal cap, these nucleosides can be used for modified mRNA to reduce the immune reaction [3].

On the other hand, the strong immune-stimulatory effect of mRNA can in fact be exploited for use in vaccination. Targeting antigen-presenting cells (APCs) with mRNA has been reported to induce tumor or antitumor immunity by activating T and B cells [39,40]. Although mRNAs from
organelles like mitochondria are less modified than mRNAs from nuclear and bring stronger immune response, several reports have shown that mRNA-based gene transfer has a higher antigen (e.g., melan A or influenza matrix protein M1) loading efficiency to stimulate cytotoxic T cells [6,41].

3. Delivery systems for modified mRNA

Because the spontaneous uptake of naked nucleic acids by cells is very inefficient, nucleic acid delivery has been developed, adopting both viral and non-viral systems [42]. Replication-deficient recombinant viruses are commonly used vehicles for gene transfer. Use of viral vectors for pDNA has been intensively studied, but only a few cases of mRNA delivery using RNA viruses have been investigated. Generally, viruses which carry therapeutic DNA or RNA can copy to DNA in the cytosol after transfection, and enter into the nucleus. Some RNA viruses have shown localized replication and expression in the cytosol. For mRNA delivery, positive-strand viruses can be directly used and translated into therapeutic proteins [43] but negative-sense RNA should be coupled with its RNA-dependent RNA polymerase so that the positive-sense virus is translated because the negative-sense virus is not infectious [44].

In addition, various transfection reagents have been developed for non-viral delivery, which can be classified into two different groups. One is mRNA delivery across the cellular membrane by physical disturbances, and the other is generation of mRNA endocytosis by cationic carriers. Various physical manipulations have also been used to improve efficiency for direct transfection [45]. Methods utilizing electroporation, gene guns, ultrasound or high-pressure injection can be applied for direct delivery of nucleotides to the cells. These methods can also be used for in vivo as well as in vitro applications. In particular, the use of electroporation is one of the most efficient methods for mRNA delivery [6,46,47]. As the mRNA does not have to enter the nucleus, soft electrical pulses may be applied to reduce cellular toxicity. Another advantage of electroporation is the direct delivery of mRNA into the cytosol, which could surpass the unwanted immune response. For therapeutic vaccination, mRNA-pulsed DCs can be used as APCs and modulate tumor immunity. Compared to transfection of pDNA, higher tumor antigen loading of DCs was observed in the gene transfer by mRNA in human [6,41,46]. The gene gun method uses high-velocity heavy metal particles such as gold, and this particle-mediated mRNA delivery is applicable to most mammalian tissues [48]. Gene gun-mediated GFP mRNA delivery was reported to show detectable expression for a week, and mRNA encoding for the human epidermal growth factor (hEGF) showed a wound healing effect.

Self-assembled lipo- or poly-plexes are well-known transfection vehicles that are spontaneously generated by charge-to-charge interactions between the complexations of negatively charged mRNA and cationic lipids or polymers, such as lipoplexes, polyplexes, polycations and dendrimers [49]. Although various polycations, such as DEAE (diethylamino-ethyl)-dextran [50], DOTMA/DOPE (1,2-dioleoyl-3-trimethylammoniumchloride/1,2-dioleoyl-3-phosphoethanolamine) [5], poly L-lysine [51] and PEI (polyethylene imine) [49], showed an ability to transfect mRNA, the most efficient was demonstrated to be DOTAP (1,2-dioleoyl-3-trimethylammonium propane) [9]. Single-stranded mRNA strongly binds to cationic polymers compared to pDNA, and cytosolic mRNA is involved in mRNA release from the cationic polymer [52]. As efficient cationic polymers that are used for pDNA transfection may not suitable for efficient mRNA transfection [3], the design of novel cationic polymers for mRNA delivery in human cells has to be carefully considered.

4. Systemic delivery of modified mRNA for gene therapy

In order to use mRNA as an efficient therapeutic agent for in vivo application, delivery routes also have to be considered. Direct delivery with naked mRNA at the targeted site was usually administrated by local injection, but indirect delivery using carrier-mediated mRNA with targeting moiety was recommended for systemic injection (Figure 3) [53]. For systemic delivery, the increased stability and the prolonged circulation of mRNA is important to improve therapeutic efficacy. In addition to chemical modification to prevent mRNA degradation, cationic lipid or polymer complexes showed successful protection against nucleases and increased stability of mRNA for systemic delivery in a mouse model. Net positively charged complexes showed increased stability of mRNA in vitro, although they sometimes interact with negatively charged serum proteins to form aggregates resulting in rapid clearance or creating clots [54]. To acquire the characteristic of prolonged circulation and to inhibit non-specific uptake by the reticuloendothelial system (RES), conjugation with polyethylene glycol (PEG) has been frequently suggested to play a role [55]. PEGylated lipids or polymers were reported to have higher stability by inhibiting attachment to serum proteins. PEGylation is very important to preserve the integrity of mRNA and to target tumor sites after prolonged circulation.

On the other hand, immune modulation by mRNA should also be considered for in vivo application. Although single-stranded RNA is reported to stimulate the innate immune system [56], therapeutic mRNA can be disguised by chemical modification to reduce the innate immune response by resembling the highly modified RNAs from organelles in mammalian cells [57-59]. In addition, the secondary structure of exogenous mRNA is reported to activate IFN-inducible protein kinase R, a global repressor of protein translation, resulting in a low transgene expression [60].

In an effort to improve efficiency of systemic delivery of modified mRNA for gene therapy, specific nanoparticle formulation using liposome-protamine-RNA (LPR) was
suggested [61]. To suppress immune activation, Wang et al. modified cytidine triphosphate and uridine triphosphate with 5’-methylcytidine and pseudouridine triphosphate (Figure 4) during the in vitro transcription of mRNA. Modified anionic mRNA was then mixed with polycation (protamine, 4 kDa) to generate an mRNA/protamine complex. Cationic lipid DOTAP/cholesterol was used to generate a liposome, which was then mixed with the mRNA/protamine complex. To reduce the attachment of serum protein and minimize uptake of RES for systemic injection, extensive PEGylation was applied to generate LPR. As sigma receptor overexpressed tumor cells (H460) were used, anisamide was also added to the distal end of PEG for specific targeting (Figure 4A). LPR is small in size (< 100 nm) for easy internalization. Herpes simplex virus 1-thymidine kinase/ganciclovir (HSV-tk/GCV) therapy, one of the most widely used suicidal gene/prodrug coupling for gene therapy, was used in this research. Tumor cells were transfected with LPR, which contains modified mRNA encoding HSV1-tk. After HSV-tk/GCV therapy, tumor cells showed reduced survival in vitro (Figure 4B), and tumor reduction was observed in an in vivo mouse xenograft model (Figure 4C). Interestingly, mRNA functions not only as an antigen-encoding molecule, but also as an adjuvant by enhancing immunological responses and antigen presentation. In addition, naked RNA vaccine administered into the lymph node was reported to show rapid selective uptake by lymph node DCs driven by micropinocytosis [68].

Recently, transfection efficiency and transgene expression using mRNA by various injection routes have also been intensively investigated [69,70]. Figure 5 shows bioluminescence imaging to evaluate in vivo transfection efficiency of naked or nanoparticle (ovalbumin)-based mRNA with chemical modification (ARCA) mRNA delivery. Transfection was increased by OVA nanoparticle-mediated mRNA transfer compared to naked mRNA both in intranasal and i.v. injections.

5. Conclusions

The use of mRNA-based gene transfer could indeed be a promising new strategy for gene therapy. Notable advantages include no risk of integration into the genomic DNA,
adjustable gene expression and easier modulation of the immune system. Various chemical modifications increase the stability of mRNA and provide more chances for the possible use of mRNA-mediated gene transfer for gene therapy. However, one major challenge for the potential use of mRNA in gene therapy is to try to reduce inflammatory reactions after repeated treatment. Although repeated mRNA application seems to be feasible in principle, knowledge about application frequencies for long-term treatment is currently unknown. By reducing or utilizing the immunogenic properties, mRNA offers a promising tool for both gene therapy and vaccination approaches, respectively.

6. Expert opinion

Previously, the stability and transfection efficiency of mRNA was considered too low for it to be used as a therapeutic molecule. However, the efficacy of mRNA transfer has been greatly improved by combining various chemical modification and delivery methods. Since mRNA degradation is
initiated by shortening of the poly(A) tail and includes a de-capping process, mRNA structure has been optimized by replacing unstable non-coding sequences with stable non-coding sequence. Various physical manipulations and nanoparticle-mediated mRNA delivery have also been used to improve transfection efficiency. In addition, modification of the cap structure and optimization of codon usages for specific organisms have been frequently used to increase the efficiency of mRNA translation.

Use of mRNA as an alternative to pDNA for therapy has major advantages of its cellular localization; i) risk of insertion mutagenesis can be avoided because mRNA exerts its function in the cytoplasm; ii) promoter-dependent modulation of gene expression is not required because amount of delivered mRNA into cytosol is directly related to its function; iii) effective delivery of mRNA into non-dividing cells is possible because transfection efficiency of mRNA is independent from cell cycle and nuclear transport; iv) vector-induced immunogenicity can be avoidable; v) repeated application is possible.

Although mRNA-based gene transfer was considered as a safe application, induction of unwanted immune response by repeated treatment remains still critical for their in vivo application. By utilizing the immunogenic properties, mRNA-based gene transfer offers a promising tool for both transcript replacement and vaccination approaches, respectively. However, control of immunogenic properties of mRNA to reduce inflammation for mRNA based-transcript replacement therapy has not been carefully investigated to date. Especially, most of researches have been focused on short-term application because mRNA can only render a transient expression, but long-term treatment is required for the treatment of inherited disease. Repeated mRNA treatment seems to be feasible but knowledge about frequency, interval and duration for long-term application is currently unknown. It would be interesting to investigate how to control a protein expression pattern after mRNA delivery under the different pathologic situation.

Recently, mathematical models that predict kinetics and efficiency of mRNA delivery in vitro and in vivo were suggested by several reports [71-73]. Kinetics related to endosomal uptake and cytosolic release of exogenous mRNA in the cellular level should be studied to establish a compartment
model with rate equation. These studies established a great correlation between theories and efficiency of delivered mRNA; thereby provide useful information to optimize mRNA-based therapeutic application. Particularly, a more detailed and quantitative understanding of artificial mRNA delivery is important for in vivo application. The degree of predictive power describing synthetic mRNA expression level and timing will depend on the degree of accuracy with the transfection efficiency and kinetics for the experimental planning. Although mRNA delivery is inherently stochastic and the expression level of every single cell is different, measurement at the single cell level and analysis of the corresponding distribution of functions, in terms of successfully delivered and translated mRNA, is still necessary to acquire the true population response in the mRNA delivery. We are convinced that predictive modeling of mRNA delivery will provide dramatic advances in the theoretical as well as practical aspects of mRNA-based therapy.

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Declaration of interest

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