mRNA vaccines: the most recent clinical applications of synthetic mRNA

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Abstract Synthetic mRNA has been considered as an emerging biotherapeutic agent for the past decades. Recently, the SARS-CoV-2 pandemic has led to the first clinical use of synthetic mRNA. mRNA vaccines showed far surpassing influences on the public as compared to other vaccine platforms such as viral vector vaccines and recombinant protein vaccines. It allowed rapid development and production of vaccines that have never been achieved in history. Synthetic mRNA, called in vitro transcribed (IVT) mRNA, is the key component of mRNA vaccines. It has several advantages over conventional gene-expressing systems such as plasmid DNA and viral vectors. It can translate proteins in the cytoplasm by structurally resembling natural mRNA and exhibit various protein expression patterns depending on how it is engineered. Another advantage is that synthetic mRNA enables fast, scalable, and cost-effective production. Therefore, starting with the mRNA vaccine, synthetic mRNA is now in the spotlight as a promising new drug development agent. In this review, we will summarize the latest IVT mRNA technology such as new mRNA structures or large-scale production. In addition, the nature of the innate immunogenicity of IVT mRNA will be discussed along with its roles in the development of vaccines. Finally, the principles of the mRNA vaccine and the future direction of synthetic mRNA will be provided.

Keywords In vitro transcribed (IVT) mRNA · mRNA vaccines

Introduction

During the SARS-CoV-2 pandemic, mRNA vaccines, including mRNA-1273 and BNT 162b2, were developed by Moderna and Pfizer-BioNTech, respectively. They have been developed at an unprecedented speed in the history of vaccine development. The mRNA vaccines were approved by FDA under an Emergency Use Authorization in less than 1 year from identifying the sequences of the spike protein of SARS-CoV-2. The rapid development of the mRNA vaccines showed a significant impact on pandemic situation management. The mRNA was first discovered in 1961 while studying the protein synthesis mechanism of DNA (Brenner et al. 1961). In 1963, it was found that interferons are induced by mRNA, and in 1975, the cap structure of mRNA was identified (Isaacs et al. 1963; Muthukrishnan et al. 1975; Furuichi and Miura 1975). In 1978 and 1989, it was revealed that mRNA could be delivered into the cell by liposome or cationic lipid carriers to express proteins (Dimitriadis 1978, Malone et al. 1989). In 1990, in vitro transcribed mRNA (IVT mRNA) was directly injected into a mouse skeleton muscle, and it expressed target proteins (Wolff et al. 1990). Since the mRNA can induce both cellular and humoral immunity while presenting an mRNA-encoded antigen, it has been regarded as a powerful vaccine system. Therefore, mRNA has been widely applied in the development of therapeutic vaccines for cancers and prophylactic vaccines for influenza A virus and RSV (Petsch
et al. 2012; Geall et al. 2012). In 2020, COVID-19 mRNA vaccines became the first FDA-approved mRNA therapies for people (Polack et al. 2020; Baden et al. 2021).

Historically, vaccine development has enabled successful protection against various infectious diseases. Especially, smallpox, which had plagued people for 1000s of years, was declared completely eradicated by the WHO in 1980. Several types of vaccines have been developed to cope with infectious diseases. Vaccines using whole pathogens include live-attenuated and inactivated vaccines. The live-attenuated vaccine is a formulation of living bacteria or viruses with weakened pathogenicity. These can be obtained by culturing pathogens for a long time under abnormal culture conditions. Another way to weaken pathogens is to treat them with heat or chemicals. In this case, which is called inactivated vaccines, pathogens become completely impossible to grow within the host. However, there is still a risk of recovering pathogenicity. To overcome this, subunit vaccines are developed by the utilization of fragmented antigenic sites instead of whole pathogens. These approaches use toxoid, capsular polysaccharides, or recombinant protein antigens that are components of whole pathogens. Other types of vaccines include viral vectors and DNA vaccines that utilize genetic materials. They can induce immune responses by introducing gene sequences into the cell nucleus and subsequently expressing antigen proteins. Viral vector vaccines use low pathogenic viruses as their targets, and DNA vaccines use plasmid DNAs as non-viral vectors.

mRNA vaccines also use genetic materials expressing antigen proteins, but they have several advantages over the other types of vaccines that use genetic materials. While DNA vaccines require cellular entry into the nucleus to express encoded proteins, mRNA vaccines can express antigen proteins once they are delivered directly into the cytoplasm of cells (Sahin et al. 2014). Based on this difference, mRNA vaccines can express target proteins much faster with high translation efficiency compared to DNA vaccines. In addition, there is no risk of causing insertion mutagenesis because RNA cannot be inserted into the host genome (Sahin et al. 2014). The mRNA vaccines can be rapidly synthesized in a large scale using a cell-free system in a cost-effective manner. Thus, it has great potential in the development of vaccines against highly mutated viruses. Despite these advantages and current success in the development of the COVID-19 vaccine, mRNA vaccines still have some issues that need to be overcome to broaden their clinical applications. In this review, we provide a comprehensive overview of the structure and function of in vitro transcribed (IVT) mRNA, which is utilized in the development of mRNA vaccines. The methods of mRNA synthesis and large-scale production will be discussed. Then, a description of the innate immunogenicity of IVT mRNA will be provided. Finally, along with the principle of mRNA vaccines, we will introduce various applications of the IVT mRNA other than COVID-19 vaccines, including immunotherapies, protein replacement therapies, and genome engineering and reprogramming.

**Types of in vitro transcribed (IVT) mRNA**

In the mRNA vaccines, IVT mRNA is designed to encode antigen proteins to induce humoral and cellular immune responses. IVT mRNA has similar structural features resembling the natural eukaryotic endogenous mRNA (Sahin et al. 2014). Although linear type mRNA is the representative structure of IVT mRNA, new types of mRNA structures were designed to modulate protein expression patterns depending on the specific purposes. These include self-amplifying mRNA (SAM) and circular mRNA (Geall et al. 2012; Wesselhoeft et al. 2018).

**Linear mRNA**

Figure 1 shows the schematic illustration of conventional linear IVT mRNA. Linear IVT mRNA resembles the endogenous mRNA, consisting of a 5′ cap, 5′ untranslated region (5′ UTR), open reading frame (ORF), 3′ UTR, and poly A tail structure in the 5′ to 3′ direction. At the 5′ end of mRNA, there is a 5′ cap structure, and natural endogenous mRNA has a 7-methylguanosine cap structure linked to 5′-triphosphate bridge. The 5′ cap structure is critical for translation initiation because eukaryotic translation initiation factor 4E (eIF4E) binds to the 5′ cap of the mRNA. mRNA decaying enzymes such as DCP1, DCP2, and DCPS are also bound to the 5′ cap to regulate mRNA decay. The poly A tail regulates the stability and translation efficiency of mRNA (Gallie 1991). The most optimal length of the poly A tail is known to be 100–150 bp (Holtkamp et al. 2006; Mockey et al. 2006). However, according to some recent studies, mRNA with a poly A tail longer than 300 bp also shows high translation efficiency (Grier et al. 2016). In addition, many of the highly expressed genes in eukaryotes were found to have a short poly A tail, which seems to be related to the tail pruning of mRNA to make closed loops (Lima et al. 2017). Interestingly, a 5′ cap and a poly A tail work synergistically to make the closed-loop RNA structure. In the closed-loop RNA model, both ends of mRNA make strong interactions with each other through transcription initiation factors and poly A binding protein (PABP). Therefore, mRNA can be protected from enzymatic degradation and the re-entry of the ribosome is facilitated.

**UTRs regulate mRNA stability and translation efficiency through the RNA–protein interactions.** Naturally occurring UTR sequences of highly expressed genes such as α and β globin genes are widely used for synthetic mRNA
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(ROSS AND SULLIVAN 1985). However, UTR performance is often varied by species, cell type, and cell state. Artificially engineered UTRs, which are optimized for target cells and clinical applications, are being developed (Orlandini von Niessen et al. 2019). The presence of miRNA binding sites and AU-rich regions in the UTR promotes mRNA degradation (Chen and Shyu 1995). These elements can be incorporated into UTRs to translate proteins for short durations or to rapidly degrade mRNA in non-target tissues (Jain et al. 2018). Highly stable secondary structures of 5′ UTR, such as hairpin, can interfere with ribosome recruiting and scanning (Pelletier and Sonenberg 1985; Kozak 1986). Recently, bioinformatics or deep learning technology has been introduced to design novel UTR sequences and predict mRNA translation efficiency (Gaspar et al. 2013; Sample et al. 2019). The ORF is the coding genetic information of the target protein, translated from the IVT mRNA. The translation efficiency of the mRNA can be regulated by codon optimization (Mauro Chappell 2014; Hanson and Coller 2018). Replacing rare codons with frequently used synonymous codons often increases protein expression from the mRNA (Gustafsson et al. 2004). This is because cognate tRNA, which is abundant in the cytosol, is reused in the vicinity of ribosomes (Cannarozzi et al. 2010). Although codon optimization may increase the translation efficiency of the mRNA, some proteins require a slow translation rate for their proper folding (Kimchi-Sarfaty et al. 2007; Spencer et al. 2012). Therefore, different codon optimization strategies will be required for different target proteins.

**Self-amplifying mRNA (SAM)**

Self-amplifying mRNA (SAM) includes the RNA replication machinery of alphaviruses, aiming at high antigen protein expression with a lower amount of mRNA vaccine dose (Bloom et al. 2020). The genome of alphaviruses, which belong to positive-strand RNA viruses, has dual functions (Lundstrom 2018). Positive-strand mRNA can be translated into proteins immediately. Next, RNA-dependent RNA polymerase (RDRP) can be synthesized from non-structural genes. This RDRP can synthesize antigenome, a negative-strand mRNA, using a positive-strand mRNA genome as a template. In the later phase of infection, viruses require a large amounts of structural proteins for replication. In alphaviruses, RDRP can bind to the downstream promoter to synthesize the subgenomic RNA encoding structural proteins. Later, more structural proteins can be synthesized from the subgenomic RNA (Rayner et al. 2002).

Figure 2A shows the schematic illustration of the SAM structure. The major difference between SAM and conventional IVT mRNA is the incorporation of non-structural gene for the generation of RNA-dependent RNA polymerase. The SAM is designed in such a form that the non-structural protein of the alphaviruses’ genome is substituted with the gene of interest (Bloom et al. 2020). In other words, when the self-amplifying mRNA is introduced, it amplifies itself in the cell and induces a high level of target protein expression without the production of viral structural proteins. Therefore, the self-amplifying mRNA can theoretically generate comparable immune responses even at a lower dose than conventional linear mRNA (Vogel et al. 2018). In addition, the self-amplifying mRNA is especially suitable for applications in the mRNA vaccine development because of its inherent adjuvant effect. In the process of self-amplification in the cell, a transient double-stranded mRNA (dsRNA) structure is formed that triggers the pattern recognition receptors (PRR). This can result in strong antigen-specific immune responses in the host (Sahin et al. 2014; Pardi et al. 2018). In the past, viral vectors have been used to utilize synthetic self-amplifying mRNA as a vaccine (Zhou et al.
Later, non-viral delivery of self-amplifying mRNA has been reported using lipid nanoparticles (LNP) (Perri et al. 2003; Geall et al. 2012). Recently, self-amplifying mRNA for the SARS-CoV-2 vaccine was developed and showed high antibody production in mice (McKay et al. 2020). Self-amplifying mRNA can be easily scalable for mRNA vaccine production like that of linear IVT mRNA, and many clinical and preclinical self-amplifying mRNA vaccine studies are currently ongoing (Hekele et al. 2013; Bloom et al. 2020). However, there have been concerns about the safety issues of self-amplifying mRNA. Due to its inherent adjuvant effect, it is difficult to control the immune-modulating property of self-amplifying mRNA. Further studies are necessary to investigate the triggering of innate immune responses through the PRR. A bipartite vector system using trans-amplifying RNA (taRNA) may provide a solution to resolve these issues (Beissert et al. 2020).

**Circular mRNA**

Exogenous circular mRNA is designed to extend the expression duration of IVT mRNA (Wesselhoeft et al. 2018). Figure 2B illustrates the typical precursor structure of circular mRNA and their back-spliced products. Circular RNA is also found endogenously, which is produced by a back-splicing mechanism, and some of them play a role in expressing proteins in humans (Jeck and Sharpless 2014; Chen and Yang 2015; Wang and Wang 2014; Enuka et al. 2015; Legnini et al. 2017). Circular mRNA lacks free ends that can be recognized by exonuclease, so its lifespan is much longer than linear mRNA (Chen and Yang 2015; Enuka et al. 2015). Therefore, a method of circularizing IVT mRNA has been developed to increase the stability of mRNA and overcome the short half-life of linear IVT mRNA (Wesselhoeft et al. 2018). As a circularization strategy of exogenous IVT mRNA, a ribozymatic method is often utilized using self-splicing introns (Petkovic and Müller 2015). In the permuted group 1 catalytic intron-based system, the exon sequence is flanked by split introns, causing a double transesterification reaction in the presence of GTP and Mg$^{2+}$ cofactors (Petkovic and Müller 2015). Based on this system, circularization of longer IVT mRNA has been attempted. Inserting internal ribosomal entry site (IRES) and sequence of gene of interest have been conducted between exon fragment E1 and E2 present in permuted intron–exon (PIE) (Wesselhoeft et al. 2018).
Through the introduction of homology arms and spacers, it was possible to increase the circularization efficiency of mRNA with various lengths up to 5 kb (Wesselhoeft et al. 2018). Circular mRNA showed a stable protein expression profile over a longer duration than that of linear mRNA, confirming its enhanced stability (Wesselhoeft et al. 2018). However, there are still various concerns about the low production yield of circular mRNA. The back-splicing system clearly needs further optimization to overcome the difficulty of generating a full-length circular mRNA. Another problem is the relatively low protein expression efficiency as compared to that of the cap-dependent IVT mRNA. This is often related to the IRES-based mRNA systems that require translational initiation by the cap-independent process. There is still a need for in depth investigation into cap independent translational initiation and further identification of IRES sequences.

**Production of mRNA vaccines**

Figure 3 shows a general process to prepare IVT mRNA for mRNA vaccine development. This includes (1) antigen selection, (2) linear DNA template synthesis, (3) in vitro transcription, and (4) mRNA purification steps. When a target antigen is selected after the genome of a pathogen is sequenced, it is inserted into a plasmid DNA template. The production DNA template should contain sequences...
of antigen, 5′ and 3′ UTRs, and a T7 promoter upstream of the 5′ UTR. The in vitro transcription process requires a DNA template, modified or unmodified nucleosides, and T7 RNA polymerase. For the DNA template, linearized plasmid DNA or PCR product is often used. For in vitro transcription, T7 RNA polymerase is mostly used. However, some studies also utilized T3 or SP6 RNA polymerase. T7 RNA polymerase recognizes the T7 promoter of the DNA template and initiates the transcription of mRNA. The 5′ cap structure of IVT mRNA can be produced in two ways. One is to prepare it in one step by adding cap analogs during the in vitro transcription process, and the other is to synthesize it by enzymatic capping reaction after in vitro transcription of mRNA. Likewise, the poly A tail of IVT mRNA can be synthesized by two different methods. An enzymatic tailing method using poly A polymerase has been widely used to generate poly A tail. However, this method has the disadvantage that the length of the poly A tail is not homogenous among the produced IVT mRNA (Sahin et al. 2014).

As an alternative, direct incorporation of the poly A sequence can be attempted at the downstream of the 3′ UTR on the DNA template. In this method, T7 RNA polymerase synthesizes poly A tail during the in vitro transcription process. A DNA template with a poly A tail sequence can be prepared by performing the tail-PCR using a reverse primer with a 100–150 nt poly T sequence added to the 5′ end. This process ensures a homogenous poly A tail length on all produced IVT mRNA. However, there is a problem with the stability of the DNA template when encoding the long poly A tail sequence. To this end, Pfizer-BioNTech has improved the stability of BNT162b2 by inserting 10 nt UGC linkers between the poly A tail sequence (Chaudhary et al. 2021).

After in vitro transcription, the purification process must be performed to obtain the IVT mRNA at full length. The DNA template is removed by DNase, and if necessary, the immunogenic 5′ ppp of uncapped mRNA can be removed and converted to 5′-OH mRNA by phosphatase (Hornung et al. 2006). In addition, removal of dsRNA is necessary by high-performance liquid chromatography (HPLC). These dsRNAs are generated during in vitro transcription, and they are highly immune stimulatory. Removing dsRNA with HPLC consequently increases the overall expression of antigen protein and reduces inappropriate immune responses (Karikó et al. 2011). However, the problem with HPLC purification is the use of organic solvents such as acetonitrile and the high cost of the process. Purifying IVT mRNA using HPLC takes a lot of time, and the final production yield is only about 50%. So far, the biggest problem for the large-scale production of IVT mRNA is the purification step (Baiersdörfer et al. 2019).

General guidelines for large-scale production

In the case of epidemic infection, scalable production of IVT mRNA is the most important factor for rapid vaccination. Several methods have been developed. For example, one-pot synthesis using cap analogs and rapid purification through tangential flow filtration is favorable. Removing immunogenic dsRNA through the adsorption of cellulose can be an alternative to using HPLC. All these approaches enable the scalable production of mRNA vaccine (Baiersdörfer et al. 2019).

One-pot system with cap analogs

With the development of the synthetic cap analog, one-pot synthesis of capped IVT mRNA became possible without an additional enzymatic capping step. The biggest advantage of one-step synthesis is that there is only one nucleic acid precipitation or purification process during the IVT. Therefore, the time and cost of production can be dramatically reduced, and the production yield can also be increased. However, the synthetic cap analog approach still has some problems. First, cap analog competes with GTP during the in vitro transcription process. Therefore, some translationally inactive uncapped mRNA is produced. Second, some cap analogs are inserted into mRNA in a reverse orientation. This limitation was much improved with the introduction of anti-reverse cap analogs (ARCA) (Jemielity 2003). In particular, ARCA-capped IVT mRNA showed a very high protein expression profile in various cell types (Mockey et al. 2006; Rabinovich et al. 2006). On the other hand, a cap analog approach has some disadvantages over an enzyme capping method. The capping efficiency of ARCA is relatively low (70%), and it produces an immunogenic cap 0 structure. However, in the two-step capping method using vaccinia virus-derived capping enzyme, capping efficiency is almost 100% (Martin et al. 1975). Eukaryotic mRNA has several types of 5′ caps: cap 0, cap 1, cap 2, and m6Am cap. Here, the cap structure of natural endogenous mRNA is mostly cap 1 or cap 2, and these have high translational efficiency. Unlike vaccinia virus-derived enzymes, which produce cap 1, ARCA has the cap 0 structure. Cap 0 mRNA is also capable of translation initiation by recruiting a ribosome, but translation is inhibited by PRR recognition such as IFN-inducible protein with tetratricoid repeats (IFIT) (Kumar et al. 2014). Recently, CleanCaps (Cap 1) have been developed to overcome both disadvantages of ARCA (Vaidyanathan et al. 2018). A cotranscriptional capping method using CleanCap AG shows a far enhanced production yield of IVT mRNA (5 mg/ml) with a 94%-cap 1 structure (Henderson et al. 2021). These advanced cap analogs clearly offer many advantages and may play important roles in the future development of mRNA therapeutics.
Tangential flow filtration

Tangential flow filtration (TFF), also called crossflow filtration, is a method of quickly and efficiently filtering and concentrating solutions containing biomolecules. TFF is distinct from dead-end filtration methods. In TFF, the feed containing biomolecule solution flows tangentially across the surface of the filter, whereas in dead-end filtration, the feed is passed through a membrane and the filtrate is released at the other end. The principal advantage is that TFF can be a continuous process, unlike dead-end filtration. Because the filter cake is washed away during the filtration process of TFF, the operating time of the filter unit is increased. TFF is typically used for feeds containing small particle size solids because solid materials can quickly block the filter surface in the dead-end filtration. The applied pressure causes the feed flow to pass through the filter, while the retentate is returned back to the feed reservoir for recirculation. The TFF technique can be used in the scalable production of IVT mRNA (Rosa et al. 2021). mRNA precipitation techniques, which have been used on the laboratory scale in the past, are not suitable for large-scale good manufacturing processes (GMP) because they are time-consuming and require organic solvents such as alcohols. Currently, instead of mRNA precipitation methods, the TFF method is used in many cases, including SARS-CoV-2 mRNA vaccine manufacturing (Corbett et al. 2020; Zhang et al. 2020; Ouranidis et al. 2021). The TFF technique is expected to be suitable for various IVT mRNA formulations, without affecting mRNA stability and compliance with GMP.

Removal of dsRNA by cellulose

As an alternative approach for the removal of dsRNA from IVT mRNA, a purification method using cellulose rather than HPLC is developed (Baiersdörfer et al. 2019). In fact, cellulose has been widely used to separate viral genomes for a long time (Franklin 1966; Su and Comeau 1999; Urayama et al. 2015). It is known that cellulose has a different degree of binding to nucleic acids depending on ethanol concentration. Although the principle is not completely known, it is assumed that 2-hydroxyl residue, which is frequently present in dsRNA, forms a hydrophilic interaction with cellulose (Urayama et al. 2015). At a higher ethanol concentration, more dsRNA can be removed, but the recovery of single-stranded IVT mRNA tends to decrease. As a result, 16% ethanol is considered to be the best choice for adding to a cellulose-containing column for IVT mRNA purification (Baiersdörfer et al. 2019). After removing dsRNA using cellulose chromatography with 16% ethanol, the translation efficiency of IVT mRNA improves and becomes comparable to that of HPLC (Baiersdörfer et al. 2019). On the other hand, the interferon-α level decreased due to the removal of dsRNA (Baiersdörfer et al. 2019). Cellulose purification can improve the protein expression of both linear IVT mRNA and self-amplifying mRNA (Linares-Fernández et al. 2021; Zhong et al. 2021). In particular, the vaccination efficacy of self-amplifying mRNA against Zika viruses is enhanced (Zhong et al. 2021). Therefore, cellulose purification may offer an alternative approach for the large-scale production of mRNA vaccines.

Innate immunogenicity of IVT mRNA

As an innate immune response, the PRR plays an important role in identifying pathogen-associated molecular patterns (PAMP). Exogenous mRNA, such as viral mRNA, is recognized by the PRR and triggers complex cascades of intracellular signaling pathways. There are two types of PRRs, which are present in the endosomes or the cytosol. Therefore, PAMPs can be recognized at two different levels (Linares-Fernández et al. 2020). Toll-like receptors (TLRs) can recognize the viral mRNA at the endosomal level. TLR3 recognizes the dsRNA with 40–45 bp length (Alexopoulos et al. 2001; Botos et al. 2009). Moreover, TLR 7 and TLR8 recognize GU-rich regions of single-stranded mRNA (Diebold et al. 2004; Heil et al. 2004). The PRRs recognizing the exogenous mRNA at the cytosol level include melanoma differentiation-associated-5 (MDA-5), retinoic acid inducible gene I (RIG-I), and RNA-dependent protein kinase (PKR). MDA-5 and RIG-I recognize dsRNAs with lengths of more than 2 kb and 5′-triphasphate short dsRNA, respectively (Kato et al. 2008; Schlee et al. 2009; Binder et al. 2011). These PRRs commonly activate the interferon type I pathway. Secretion of interferons α and β, which belong to the type I interferon family, upregulates protein kinase R (PKR) and oligoadenylate synthetase (OAS) (Linares-Fernández et al. 2020). PKR is a serine-threonine kinase that also acts as a dsRNA sensor and blocks mRNA translation by phosphorylating eukaryotic initiation factor 2 (eIF2) (Clemens and Elia 1997). OAS is also a dsRNA sensor, and it activates RNase L to degrade mRNA (Pulit-Penaloza et al. 2012). Therefore, if the IVT mRNA is recognized as an exogenous mRNA, the translation of the target protein as well as its therapeutic efficacy will be greatly decreased.

Incorporating modified nucleosides can improve the therapeutic potency of IVT mRNA by avoiding the innate immune response (Karikó et al. 2008; Pardi et al. 2017; Richner et al. 2017; Vaidyanathan et al. 2018; Freyn et al. 2020; Laczkó et al. 2020). Modified nucleosides can inhibit the recognition of mRNA by PRRs and decrease the generation of dsRNA during the in vitro transcription process (Karikó et al. 2005; Durbin et al. 2016; Mu et al. 2018). Natural endogenous mRNAs contain modified nucleosides, and the innate immune system has evolved to recognize
unmodified mRNA as a viral mRNA (Chaudhary et al. 2021). Therefore, modified nucleosides decrease the risk of being recognized as an exogenous mRNA. Since TLR7 and TLR8 bind to the GU-rich regions of mRNA, modified uridine such as pseudouridine and N1-methylpseudouridine can inhibit the recognition of IVT mRNA by the PRRs (Karkikó et al. 2008; Vaidyanathan et al. 2018). Both mRNA-1273 and BNT 162b2, developed by Moderna and Pfizer-BioNTech, respectively, contain modified uridine nucleosides to ensure proper antigen expression in cells as well as to reduce undesirable innate immune-triggering effects (Chaudhary et al. 2021).

Since many viral RNA sensors recognize the dsRNA, it is important to remove the dsRNA from IVT mRNA. The main cause of dsRNA formation is the T7 RNA polymerase (Wu et al. 2020). In the “on-pathway” process, when T7 RNA polymerase operates accurately, the enzyme binds to the T7 promoter to synthesize IVT mRNA. In this case, the T7 RNA polymerase generates a run-off product that is a single-stranded mRNA (Gholamalipour et al. 2018). However, if the T7 RNA polymerase operates promoter abnormally, the dsRNA by-products are generated by the process called “off-pathway” or “pseudo-on-pathway” (Wu et al. 2020). When the run-off transcripts accumulate through the “on-pathway,” T7 RNA polymerase competitively binds to the DNA templates and the run-off transcripts. In the “off-pathway”, 3′-extension occurs by rebinding of T7 RNA polymerase to the run-off transcripts. Then, 3′-extended products become the dsRNA by intramolecular interaction (Triana-Alonso et al. 1995; Gholamalipour et al. 2018; Wu et al. 2020). The “pseudo-on-pathway” also occurs in a promoter-independent manner such as “off-pathway,” but it is different because of how the DNA template is recognized, not the 3′-end of the run-off transcript. In the “pseudo-on-pathway,” the T7 RNA polymerase binds to the end of the DNA template without the T7 promoter and synthesize anti-sense mRNA that is complementary to normal sense mRNA (Mu et al. 2018; Wu et al. 2020). Through this process, dsRNA is formed by the hybridization of sense-mRNA and anti-sense mRNA.

The dsRNA is recognized by multiple PRRs (TLR3, MDA-5, and RIG-I) and induces the secretion of type I interferons. Therefore, it is important to remove the dsRNA from IVT mRNA products. The dsRNA can be removed by the HPLC purification or the cellulose purification after the IVT process (Karkikó et al. 2011; Baiersdörfer et al. 2019). Besides, it is also possible to reduce the generation of dsRNA during the IVT process. These efforts include the use of modified nucleosides, applying high-temperature IVT, incorporating the template-encoded poly A tailing, and the use of low Mg2+ concentration (Mu et al. 2018; Wu et al. 2020). Thermostable T7 RNA polymerase can synthesize the IVT mRNA at 48 °C, which is higher than the average temperature of the cell. Among the two dsRNA generation mechanisms, high-temperature IVT reduces “off-pathway” by altering either the rebinding of the T7 RNA polymerase or the folding back of the RNA that results in the self-primed extension (Wu et al. 2020). The template-encoded poly A tailing can also inhibit the dsRNA generation by regulating the “pseudo-on-pathway” (Wu et al. 2020). It has also been proven that lowering Mg2+ concentration during the IVT process can reduce the dsRNA generation (Mu et al. 2018). However, Mg2+ concentration also affects the overall yield of the IVT process (Wu et al. 2020). Currently, various purification technologies are used to remove the dsRNA after the IVT process (Karkikó et al. 2011; Baiersdörfer et al. 2019). Cellulose purification has been developed for the scalable production of mRNA, but it is unclear whether this method can distinguish between the dsRNA by-products and the intrinsic secondary structure of mRNA (Wu et al. 2020). The combination of high-temperature IVT and template-encoded poly A tailing can synthesize high-purity IVT mRNA without dsRNA purification after the IVT process (Wu et al. 2020). Therefore, the optimizing IVT condition and process for the low generation of dsRNA can be a new alternative to post-synthesis purification. Moreover, reducing dsRNA generation by optimizing IVT conditions could be a new alternative to post-synthesis purification.

**Principles of mRNA vaccines**

The general mechanism of mRNA vaccines is illustrated in Fig. 4. mRNA vaccines are administered intramuscularly (IM injection) and lead to uptake by nearby cells at the injection site such as muscle cells and dendritic cells (DC). IVT mRNA escapes from the endosome and then gets translated by ribosomes to generate target antigen proteins in the cytosol. IVT mRNA can stimulate the innate immune response by interacting with PRRs and is quickly degraded after being translated into the target antigens. Since the synthesized target antigen proteins are not used in cells, they are degraded by the proteasome after ubiquitination. Some of the peptides from the degradation by the proteasome are moved to the rough endoplasmic reticulum (rough ER) by the transporter associated with antigen processing (TAP) protein. Additionally, secreted antigen proteins can be uptake and degraded on the inside of endosomes. Antigen peptides can be presented by the major histocompatibility complex (MHC) class I or MHC class II to activate the immune system. While most cells only have MHC class I, APCs have both MHC class I and MHC class II. When antigen peptide fragments are presented to the cell membrane of an APC by MHC class II, helper T cells can be activated. Subsequently, helper T cells activate cytotoxic T cells and B cells. The B cells with antibodies can bind to the antigen-presenting cells (APC) and produce a large amount of antibodies, inducing an adaptive
immune response against pathogens. If antigen peptide fragments are presented by MHC class I, activated cytotoxic T cells can secrete cytotoxic molecules such as perforin and granzyme to kill infected cells. After the immune response, T cells and B cells each produce memory cells for the recognized antigens. The memory cells protect the body before the pathogen breaks the homeostasis by quickly producing immune cells when the same infection occurs again (Chaudhary et al. 2021).

One of the characteristics of mRNA vaccines is their inherent adjuvant properties, and these can be either beneficial or detrimental (Linares-Fernández et al. 2020). The mRNA vaccines induce strong interferon type I secretion at the injection site. As stated previously, interferon type I induced by exogenous mRNA-PRR interaction can inhibit translation and degrade the IVT mRNA (Clemens and Elia 1997; Pulit-Penaloza et al. 2012). However, interferon type I can enhance the immune response directly or indirectly (Broos et al. 2016). It can regulate DCs and cytotoxic T cells (Broos et al. 2016). Interferon type I induces DC maturation by upregulating co-stimulatory molecules of DCs (Ceppi et al. 2005; Hervas-Stubbs et al. 2011; Pantel et al. 2014). Furthermore, it can modulate cytotoxic T cell immunity by affecting survival and differentiation (Hervas-Stubbs et al. 2011; Broos et al. 2016). According to the timing of interferon type I signal, cytotoxic T cells induce a stronger immune response through proliferation and differentiation (De Beuckelaer et al. 2017). However, on the contrary, apoptosis of cytotoxic T cells can be triggered by a strong interferon type I signal, and the mechanism is still unclear (De Beuckelaer et al. 2017). Indirect effects of interferon type I involve their ability to trigger the induction of chemokines and cytokines to recruit various immune cells (Broos et al. 2016). Further research will be needed on how interferon type I affects vaccine-mediated immunity; however, so far, it seems that these responses are highly related to its amount, appropriate time, and location (Broos et al. 2016). To improve the immune response of

Fig. 4 Mechanism of the mRNA vaccines. Upon the introduction of IVT mRNA into cells, various TLRs and PRRs recognize the presence of exogenous mRNA and trigger innate immune responses. IVT mRNA delivered in the cytoplasm of cells directly utilizes the host translation system to express target proteins. Expressed proteins are further processed by proteasomes and moved to the cellular membrane by MHC class I
the mRNA vaccines, studies have been conducted to induce interferon type I by inserting dsRNA structures into IVT mRNA (Loomis et al. 2018; Uchida et al. 2018). Hybridizing a short poly-U sequence to poly A tail induces more secretion of interferon-β and interleukin-6 without affecting the translation of antigens in the DCs (Uchida et al. 2018). As the dsRNA structure within the poly A tail induces an innate immune response, co-stimulatory molecules of DCs such as CD80, CD86, and CCR7 are upregulated, which further activate DCs (Uchida et al. 2018). Recently, co-delivery of TLR-agonists with mRNA has been suggested as an adjuvant (Verbeke et al. 2017). However, if the inherent adjuvant properties of IVT mRNA are appropriately exploited, it will be possible to elicit a strong immune response without additional adjuvants.

**Conclusions and future directions**

mRNA vaccines for infectious diseases are the most recent applications of IVT mRNA therapeutics. Currently, starting with SARS-CoV-2, several mRNA vaccines against infectious diseases such as HIV-I, Zika, and rabies are undergoing clinical trials (Chaudhary et al. 2021). Tables 1 and 2 summarize the data from the current clinical trials of various mRNA vaccines (Chaudhary et al. 2021).

In addition to the treatment of infectious diseases, IVT mRNA-based therapeutics can be used for immunotherapies, protein replacement therapy, and genome engineering and reprogramming. Here, we would like to conclude our review by briefly outlining the current and future directions of these approaches.

**Table 1** Clinical trials of mRNA vaccines against infectious diseases (Chaudhary et al. 2021)

| Name      | Target                        | Vaccine type                        | Clinical trial phase | Clinical trial identifier                  | Funding source   |
|-----------|-------------------------------|-------------------------------------|----------------------|-------------------------------------------|------------------|
| mRNA-1647 | CMV                           | Nucleoside-modified mRNA-LNP        | Phase II             | NCT04232280, NCT03382405                 | Moderna          |
| mRNA-1443 |                               | Nucleoside-modified mRNA-LNP        | Phase I              | NCT03382405                              | Moderna          |
| mRNA-1893 | Zika                          | Nucleoside-modified mRNA-LNP        | Phase I              | NCT04064905                              | Moderna          |
| mRNA-1325 |                               | Nucleoside-modified mRNA-LNP        | Phase I              | NCT03014089                              | Moderna          |
| mRNA-1653 | hMPV/PIV3                     | Nucleoside-modified mRNA-LNP        | Phase I              | NCT04144348, NCT03392389                 | Moderna          |
| mRNA-1345 | RSV                           | Nucleoside-modified mRNA-LNP        | Phase I              | NCT04528719                              | Moderna          |
| mRNA-1777(V171) |                       | Nucleoside-modified mRNA-LNP      | Phase I              | Unregistered, Moderna/Merck              | Moderna/Merck    |
| mRNA-1172(V172) |                       | Nucleoside-modified mRNA-LNP      | Phase I              | Unregistered, Moderna/Merck              | Moderna/Merck    |
| mRNA-1851 (VAL-339851) |                   | Nucleoside-modified mRNA-LNP        | Phase I              | NCT03345043                              | Moderna          |
| mRNA-1440 (VAL506440) |                   | Nucleoside-modified mRNA-LNP        | Phase I              | NCT03076385                              | Moderna          |
| mRNA-1010 | Influenza A (H1N1, H3N2), Influenza B (Yamagata lineage, Victoria lineage) | Unknown                     | Phase I/II            | NCT04956575                              | Moderna          |
| MRT5400   | Influenza A (H3N2)            | Unknown                             | Phase I              | Unregistered, Translate Bio, Sanofi       | Translate Bio, Sanofi |
| MRT5401   | Influenza A (H3N2)            | Unknown                             | Phase I              | Unregistered, Translate Bio, Sanofi       | Translate Bio, Sanofi |
| mRNA-1944 | Chikungunya                   | Nucleoside-modified mRNA-LNP        | Phase I              | NCT03829384                              | Moderna          |
| mRNA-1388 (VAL-181388) |                   | Nucleoside-modified mRNA-LNP        | Phase I              | NCT03325075                              | Moderna          |
| CV7201    | Rabies                        | Unmodified mRNA complexed in RNActive | Phase I              | NCT02241135                              | CureVac          |
| CV7202    |                               | Unmodified mRNA-LNP                 | Phase I              | NCT03713086                              | CureVac          |
| GSK3903133A |                               | Self-amplifying mRNA in cationic nanoemulsion | Phase I              | NCT04062669                              | GSK              |
Table 2  Clinical trials of mRNA vaccines against SARS-CoV-2 (Chaudhary et al. 2021)

| Name                        | Vaccine type | Clinical trial phase | Clinical trial identifier                          | Funding source                  |
|-----------------------------|--------------|----------------------|---------------------------------------------------|---------------------------------|
| BNT162b2 (Tozinameran)      | Nucleoside-modified | Phase I              | NCT04839315                                      | BioNTech, Pfizer                |
|                             |              |                      | NCT04816643                                      |                                 |
|                             |              | Phase I/II           | EUCTR2020-001038-36, NCT04380701                  |                                 |
|                             |              |                      | NCT04889209                                      |                                 |
|                             |              |                      | NCT04588480                                      |                                 |
|                             |              | Phase II             | ISRCTN73765130                                   |                                 |
|                             |              |                      | NCT04894435                                      |                                 |
|                             |              |                      | NCT04761822                                      |                                 |
|                             |              |                      | NCT04824638                                      |                                 |
|                             |              |                      | NCT04860739                                      |                                 |
|                             |              |                      | EUCTR2021-001978-37                              |                                 |
|                             |              |                      | NCT04649021                                      |                                 |
|                             |              |                      | ISRCTN69254139                                   |                                 |
|                             |              |                      | NCT04907331                                      |                                 |
|                             |              |                      | NCT04895982                                      |                                 |
|                             |              | Phase II/III         | NCT04368728                                      |                                 |
|                             |              |                      | NCT04754594                                      |                                 |
|                             |              | Phase III            | NCT04805125                                      |                                 |
|                             |              |                      | NCT04816669                                      |                                 |
|                             |              |                      | NCT04800133                                      |                                 |
|                             |              |                      | NCT04713553                                      |                                 |
| mRNA-1273                   | Nucleoside-modified | Phase I              | NCT04785144                                      | Moderna, NIAID, BARDA           |
|                             |              |                      | NCT04813796                                      |                                 |
|                             |              |                      | NCT04839315                                      |                                 |
|                             |              |                      | NCT04283461                                      |                                 |
|                             |              | Phase I/II           | NCT04889209                                      |                                 |
|                             |              | Phase II             | ISRCTN73765130                                   |                                 |
|                             |              |                      | NCT04847050                                      |                                 |
|                             |              |                      | NCT04894435                                      |                                 |
|                             |              |                      | NCT04748471                                      |                                 |
|                             |              |                      | NCT04761822                                      |                                 |
|                             |              |                      | NCT04405076                                      |                                 |
|                             |              | Phase II/III         | NCT04649151                                      |                                 |
|                             |              |                      | NCT04796896                                      |                                 |
|                             |              | Phase III            | NCT04811664                                      |                                 |
|                             |              |                      | NCT04470427                                      |                                 |
|                             |              |                      | NCT04860297                                      |                                 |
|                             |              |                      | NCT04806113                                      |                                 |
|                             |              |                      | NCT04805125                                      |                                 |
| TAK-919                     | Nucleoside-modified | Phase I/II           | NCT04677660                                      | Takeda, Moderna                 |
| CVnCoV (Zorecimeran)        | Unmodified    | Phase I              | NCT04449276                                      |                                 |
|                             |              | Phase II             | ISRCTN73765130                                   | CureVac                         |
|                             |              |                      | NCT04515147, PER-054-20                           |                                 |
|                             |              | Phase III            | NCT04652102, EUCTR2020-003998-22                  |                                 |
|                             |              |                      | EUCTR2020-004066-19                              |                                 |
|                             |              |                      | NCT04674189                                      |                                 |
|                             |              |                      | NCT04860258                                      |                                 |
|                             |              |                      | NCT04848467                                      |                                 |
| ARCoV                       | Unmodified    | Phase I              | ChiCTR2000034112                                  | Walvax Biotechnology, PLA       |
|                             |              | Phase Ib             | ChiCTR2000039212                                  |                                 |
|                             |              | Phase II             | ChiCTR2100041855                                  |                                 |
|                             |              | Phase III            | NCT04847102                                      |                                 |
Immunotherapies

The IVT mRNA can be used for cancer immunotherapy and allergy tolerization. Cancer immunotherapy has been studied for the longest time among IVT mRNA-based therapeutics (Boczkowski et al. 1996). In the field of cancer immunotherapy, IVT mRNA encodes tumor-associated antigens or antigen receptors such as chimeric antigen receptors (CAR) and T cell receptors (TCRs) (Beck et al. 2021). T cells play an important role in cancer immunotherapy. By delivering mRNA encoding the cancer-associated antigen to DCs, DCs stimulate T cells with antigen-specific T cell receptor (TCR) (Beck et al. 2021). Another approach is to deliver CAR mRNA to T cells extracted from the patients and then to re-administer it (Beatty et al. 2014; Tchou et al. 2017; Xiao et al. 2019; Lin et al. 2021). In both ex vivo methods, it is important to efficiently deliver IVT mRNA to the isolated cells and strongly express target proteins to generate engineered cells. (Perez and Palma 2019). More recently, direct in vivo delivery of CAR or TCR mRNA to the body has been attempted (Miao et al. 2019; Cafri et al. 2020). These approaches require further targeting strategies to ensure the delivery of CAR or TCR mRNA to the T cells. Therefore, targeting ligands such as antibodies and small-molecule sugar moieties are often utilized for this purpose. Next, IVT mRNA-encoding allergens can be used to alleviate type I allergic disease by two strategies. One is to modulate T cell response, and the other is to induce immunoglobulin G (IgG) antibodies that compete with immunoglobulin E (IgE) for binding to the allergens (Valenta et al. 2010; Weiss et al. 2012; Scheiblhofer et al. 2018). It has been proven that the DNA vaccine can alleviate various allergies by modulating the response of type 1 T helper cells (Raz et al. 1996; Chua et al. 2009). However, the DNA vaccines for allergic diseases have some safety issues because of their long-term gene expression, which can induce anaphylactic side effects (Slater et al. 1998; Weiss et al. 2012). IVT mRNA has emerged as a new candidate for allergy vaccine due to its short half-life and a strong immunostimulatory capacity to type 1 T helper cells such as the DNA vaccines (Roesler et al. 2009; Weiss et al. 2010).

Protein-replacement therapy

The basic principle of protein-replacement therapy is to administer IVT mRNA-encoding proteins that are not expressed or malfunction in the patients. The first preclinical application of IVT mRNA to protein-replacement therapy was performed in 1992, and further studies have been conducted since the introduction of modified nucleosides (Jirikowski et al. 1992; Karikó et al. 2012; Zangi et al. 2013; Sahin et al. 2014). Despite the introduction of...
modified nucleosides, there is a problem with the varied protein expression profile depending on cell types (Sahin et al. 2014). These include post-translational modifications such as glycol-conjugation and proteolytic processing and recognition of secretory signal peptides (Sahin et al. 2014). Currently, various UTR optimization has been under investigation for developing cell-specific mRNA for targeted protein expression. This will be an interesting next step for the design of the IVT mRNA.

**Genome engineering and reprogramming**

Genome editing can be achieved by expressing zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly spaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-CAS9) in cells (Miller et al. 2007; Hockemeyer et al. 2011; Mali et al. 2013). The major role of these approaches is the utilization of viral vectors such as adeno-associated virus (AAV). This severely limits the size of gene cas9 along with sgRNA sequences. A viral expression can also be associated with non-specific genome editing by the long duration of expression of genome-editing proteins in cells (Fu et al. 2013). Therefore, IVT mRNA can be an alternative choice to express genome-editing proteins in cells to overcome this issue since IVT mRNA transiently expresses the genome editing proteins in cells, which is clearly beneficial for reducing the risk of non-specific genome editing.

The IVT mRNA-based approach was successfully applied to site-specific genome modification (Shen et al. 2013; Wefers et al. 2013; Niu et al. 2014; Yang et al. 2014). In addition, IVT mRNA encoding transposase was also applied for transposon-mediated stable gene transfer, showing higher efficacy than plasmid DNA (Sumiyama et al. 2010). By expressing transcription factor proteins, IVT mRNA can also be utilized to reprogram cell phenotypes. In 2010, it was reported that IVT mRNA encoding Yamanaka stem cell factors can generate induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006; Warren et al. 2010). Later, iPSCs or other types of cells can be further reprogrammed into other types of cells (Warren et al. 2010; Guo et al. 2015; Preskey et al. 2016). Reprogramming cell fates using IVT mRNA has the advantage of safety because it does not produce residual transgenes (Warren et al. 2010). Therefore, IVT mRNA therapeutics will be useful for the reprogramming and direct differentiation of cells for regenerative medicines.

Finally, IVT mRNA is likely to be used in various therapeutic fields as a novel drug candidate. In particular, in pandemic situations, mRNA vaccines will play an extremely effective role because they enable fast, scalable, and cost-effective production. Although there are still issues about safety, cold storage requirements, and duration of antibody response, mRNA vaccines are expected to be the major types of vaccines in the future.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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