

mRNA-Based Therapeutics – Advances and Perspectives

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Abstract—In this review we discuss features of mRNA synthesis and modifications used to minimize immune response and prolong efficiency of the translation process *in vivo*. Considerable attention is given to the use of liposomes and nanoparticles containing lipids and polymers for the mRNA delivery. Finally we briefly discuss mRNAs which are currently in the clinical trials for cancer immunotherapy, vaccination against infectious diseases, and replacement therapy.

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Besides common small molecule drugs that are widely used in clinical practice, monoclonal antibodies and peptides – so-called biopharmaceuticals – were recently introduced. Also great expectations are anticipated to gene therapy. Although for 30 years gene therapy has been expected to become a breakthrough approach in the treatment of many diseases, only recent considerable changes open the application of nucleic acids (NA) therapy for human in the near future.

There are two completely different approaches for NA based therapy from historical and methodological viewpoint: 1) inhibition or enhancement of gene expression; 2) excise or replacement of genes within the genome. In the late 1970s, Zamecnik et al. [1, 2] proposed a so-called antisense technology based on blocking of the mRNA translation by complementary synthetic oligonucleotides. After substantial development, this

approach yielded in the chemically modified oligonucleotides, which have been approved by FDA as drugs [3, 4]. Upon discovery of previously unknown natural mechanisms, novel applications of the nucleic acids in therapy have been proposed. For example, after discovery of RNA interference in 1998 many pharmaceutical companies started development of small interfering RNAs (siRNAs) as the therapeutics. Despite the experience obtained within the 1990s during development of antisense oligonucleotides and ribozymes, the majority of the projects failed due to poor stability of RNAs *in vivo* and inefficient delivery. In the late 2000s most of these issues were partially resolved that lead to a renaissance in the field of therapeutic NAs [5]. Currently many siRNAs are at the different phases of the clinical trials [6]. Data of the last years give a hope that 3-5 NA drugs will be approved by FDA in near future.

Technologies allowing gene editing within the genome should be discussed separately due to significant differences in overall [7, 8]. First attempts in this field were made with meganucleases and restriction endonucleases containing zinc finger motifs (ZFN). Later fusion proteins consisting of bacterial effectors and FokI restriction endonuclease were created (TALEN – transcription activator-like effector nucleases). Despite significant success demonstrated in model systems, their wide *in vivo* use is limited by insufficient specificity and efficacy. In

Abbreviations: CRISPR-Cas9, system of clustered regularly interspaced short palindromic repeats and Cas9 nuclease; dsRNA, double stranded RNA; FDA, Food and Drug Administration (USA); HIV, human immunodeficiency virus; LNP, lipid nanoparticles; mRNA, messenger RNA; NA, nucleic acids; ORF, open reading frame; PEG, polyethylene glycol; siRNA, small interfering RNA; TALEN, transcription activator-like effector nuclease; TLRs, Toll-like receptors; UTR, untranslated region; ZFN, zinc finger nuclease.

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2013, a novel approach for genome editing with the CRISPR-Cas9 system (clustered regularly interspaced short palindromic repeats and Cas9 nuclease) was proposed [9]. Superior efficiency of CRISPR-Cas9 in comparison to earlier developed systems resulted in the explosive growth of its application in various areas ranging from biomedicine to agricultural biotechnology [10]. Nonetheless, it should be emphasized that these systems are used mainly for *in vitro* and *ex vivo* engineering due to moderate efficacy. The reasons are probabilistic pattern of homology-directed recombination or nonhomologous ends joining as well as the low availability of tentative target genes because of poor understanding of chromatin structure and dynamics [11]. However there are examples of CRISPR-Cas9 application *in vivo* with the correction rate 0.4–6.0% [12, 13] that gives promise for the development of the efficient genome editing system *in vivo* in near future.

In the early 1990s the possibility of using plasmid DNA-encoding genes to induce antibody synthesis as well as to trigger T- and B-cell response was demonstrated, that led to the start of the DNA vaccines development [14, 15]. Moreover, it was shown that plasmids might be used for therapy of inherited and multifaceted diseases [16, 17]. In the therapeutic terms, mRNA delivery is a novel alternative approach for treatment of such diseases [18]. Compared to the use of plasmid DNA, several advantages can be emphasized: in case of mRNA delivery there is no risk of developing mutations and integration into genome; mRNA can be delivered to non-dividing cells as it does not need to get into the nucleus as DNA plasmids. In addition, it was shown that the efficacy of the cell transfection with mRNA in the liver *in vivo* might reach up to 100% while plasmid DNA was less efficient [19]. This data is very important for the future development of mRNA based replacement therapy. In comparison to protein pharmaceuticals mRNA gives more prolonged therapeutic effect. It is worth mentioning that mRNA can be used for the proteins and peptides expression within the intercellular space or for the generating pluripotent stem cells [20]. Furthermore, mRNA can encode nucleases such as ZFN, TALEN, and CRISPR-Cas9, which can be used for genome editing [21, 22]. Today marked progress in using mRNA as therapeutics at least in clinical trials is definitely noted. In this review, we will discuss this application for mRNAs translated *in vitro* and pay the main attention to a structural optimization and use of modified nucleosides as well as targeted delivery. Delivery approaches based upon physical methods [23] such as electroporation, micropipetting, ultrasound, laser radiation, and the gene gun system are beyond the scope of our review and will not be discussed. In addition, viral vectors [24, 25] as highly efficient system for delivering nucleic acids will not be covered. Also we will omit discussing mRNA transfection *in vitro* presented in a recent excellent review by Andreev et al. [26].

In vitro mRNA SYNTHESIS – ESSENTIAL STRUCTURAL ELEMENTS AND THEIR OPTIMIZATION

Chemical automated synthesis of mRNA is impossible due to its length, which significantly exceeds common 100–150 nucleotides available by oligosynthesis, and the only way still relies on *in vitro* enzymatic synthesis or cell-free systems.

In eukaryotes, the structure of mature mRNAs can be divided into five major regions (Fig. 1): (i) cap – m⁷GpppN or m⁷Gp3N (N – any nucleotide); (ii) 5'-untranslated region (5'-UTR); (iii) protein-encoding open reading frame (ORF); (iv) 3'-untranslated region (3'-UTR); (v) 100–250 adenosine-containing region (3'-poly(A)-tail).

During posttranscriptional mRNA processing in the nucleus, the 5'-cap is incorporated into the mRNA molecule and plays an important role by stabilizing the structure of mRNAs as well as being involved in splicing, transport, and translation [27]. The 5'-cap is composed of an *N*⁷-methylguanosine (m⁷G) residue linked to the first transcribed nucleotide of mRNA via a triphosphate bridge (Fig. 1b). RNA polymerase can attach the m⁷GpppG to RNA in two orientations, making Gpppm⁷GpN as well as m⁷GpppGpN after linking the first nucleotide. This result presents in approximately half of *in vitro*-generated transcripts that would not be recognized by the ribosome inside cells. For solving this problem, several cap-analogs were synthesized that were unable to reverse bind, or it did not affect this process. For example, a symmetric cap-analog having both guanosine residues methylated at their *N*⁷-atoms, which are linked via tetraphosphate m⁷Gppppm⁷G (Fig. 1c, I), gave mRNA with high efficiency of the translation *in vitro* [28]. Alternatively, methylation or removal of the 3'-hydroxyl group of classic cap-region was proposed as well (Fig. 1c, II): such modification blocks elongation of the *N*⁷-methylated guanosine residue [29]. In addition to modifications blocking potential reverse orientation of the cap-region, the stability and efficacy of mRNA translation have been studied. For instance, introduction of a phosphorothioate cap group (Fig. 1c, III) results in downregulated binding between the cap-region and Dcp2 protein, which is involved in mRNA degradation, thereby increasing its stability without affecting its translational efficiency [30].

Mammalian cells possess components of innate immunity including a defense mechanism against viruses (Fig. 2). For instance, in the cytosol atypical RNA containing no cap- or 3'-poly(A)-region is recognized by RIG-I, MDA5, and RIG-I-like receptors. As a result, by acting via MAVS adaptor protein, they trigger a signaling cascade followed by release of type I interferons (IFN- α/β) and activation of several transcription factors such as NF- κ B, IRF3, and IRF7 [31–33]. This signaling pathway is transmitted through activation of I κ B kinase com-

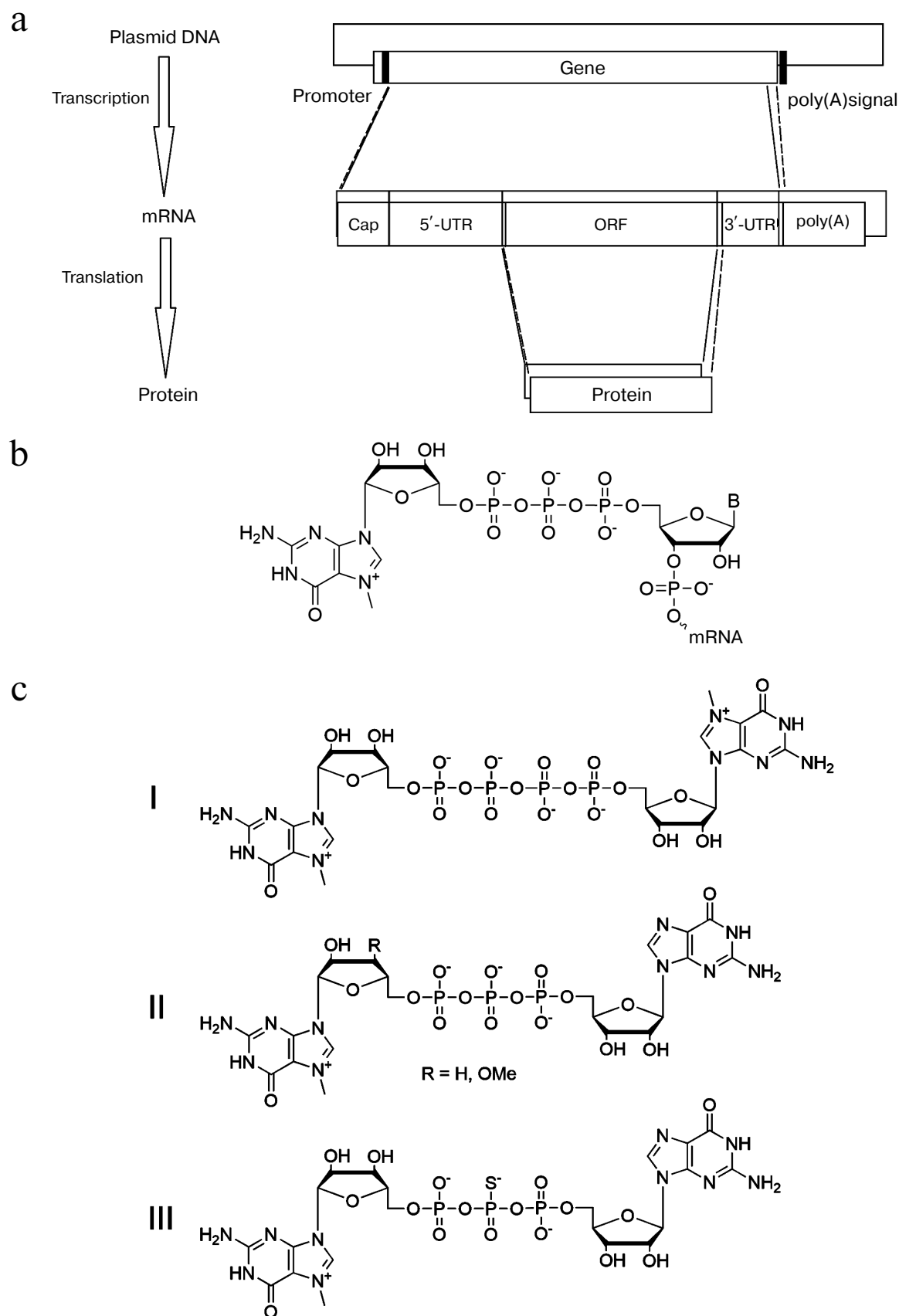


Fig. 1. a) General scheme depicting protein synthesis *in vitro* and the structure of mRNA; b) structure of 5'-cap in mRNA (B is a heterocyclic base); c) structure of modified cap-ends.

plex (IKK) and IKK-linked kinases (TBK1) and results in nuclear translocation of transcription factors NF- κ B, IRF3, and IRF7, which then induce expression of type I interferons. Additionally, NF- κ B activates expression of inflammatory cytokines such as TNF- α , IL-6, and IL-12 [34]. Type I interferons can be secreted into the intercellular space, where they bind to IFN receptors on surrounding cells with subsequently transduced signals informing the cell of detected foreign RNA and inducing immune response [35].

Recently it was demonstrated that NOD-like receptors are another representative of PRR family that are involved in the recognizing foreign mRNAs [36]. Mainly, they take part in regulating caspase 1 family proteins, whereas NLRP3 receptor is responsible for recognizing double-stranded (ds) RNAs. After that, the receptor forms a multi-protein complex with adaptor ASC and caspase 1 (the inflammasome), which is responsible for

proteolytic maturation of IL-1 β and IL-18 [37]. In addition, atypical dsRNAs can also activate autophosphorylation of protein kinases (PKR), which phosphorylates initiation factor eIF2a followed by inhibiting translation initiation and preventing replication of pathogens or synthesis of foreign proteins [38]. Thus, capping of mRNA at its 5'-end is necessary for minimizing activation of innate immunity. While performing mRNA capping *in vitro*, it is impossible to reach 100% efficacy of this reaction. Therefore, to reduce immunogenicity of mRNA, transcripts should be additionally treated with phosphatase to remove 5'-triphosphates [39].

The presence and length of the 3'-poly(A)-tail in mRNA also have crucial importance for efficient translation and increased stability of mRNAs. In mammalian cells, mRNAs containing 100-250 adenosine residues at the 3'-end are translated with the highest efficiency [40]. It was shown that by adding at least 150 nucleotides dur-

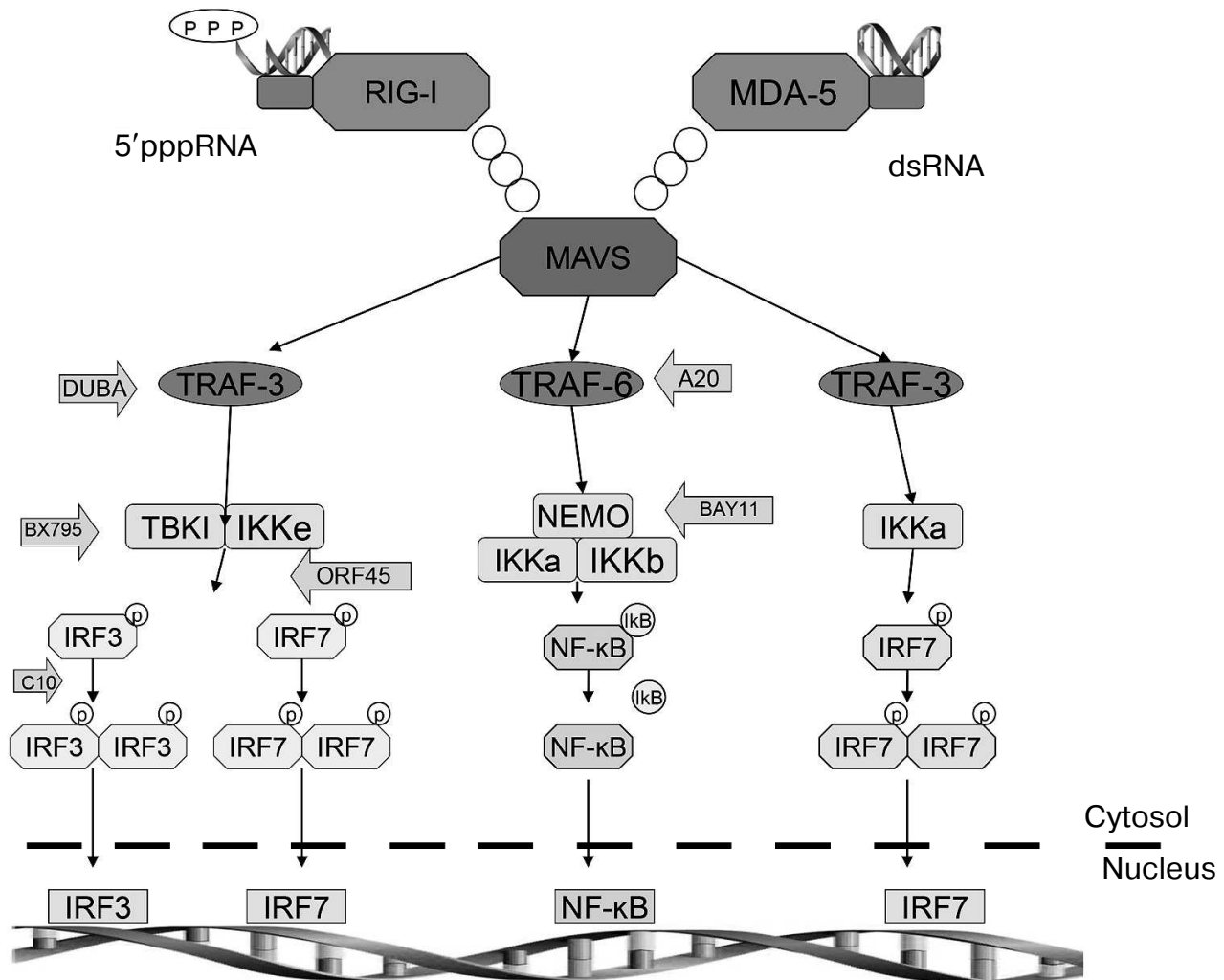


Fig. 2. Scheme depicting innate immune response to delivered artificial mRNA. dsRNA, double-stranded RNA; DUBA, deubiquitinase; IFN, interferon; IRF, interferon regulatory factor; NEMO, NF- κ B essential modulator.

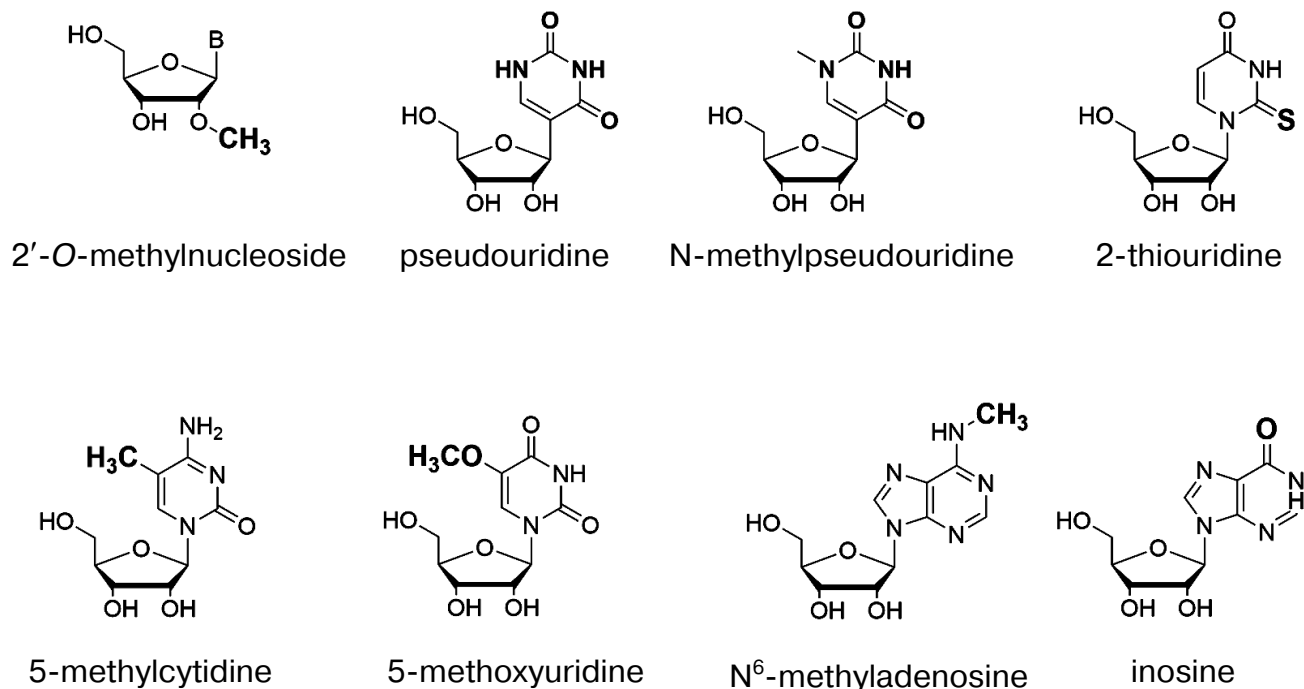


Fig. 3. Modified nucleosides decreasing immunogenicity of mRNA [21]. B is a heterocyclic base.

ing polyadenylation reduces mRNA immunogenicity [41]. It is known that poly(A)-binding proteins (PABPs) and translation initiation factor eIF4G bind to poly(A)-tail. By acting synergistically, the cap and poly(A)-tail of mRNA circularize the mRNA by forming a complex cap/PABP/eIF4E-eIF4G-PABP-poly(A), which improves binding to the ribosome and protects mRNA from degradation.

Another approach to optimizing an mRNA sequence is based on replacing unstable regions in untranslated areas of the mRNA by sequences with the stable structure. For instance, the presence of adenosine/uridine-rich sequences within the 3'-UTR contributes to removal of the poly(A)-region upon degradation of mRNAs. Replacing the AU-rich regions by sequences from the 3'-UTR of stable mRNA significantly increased the half-life of the mRNA [42]. Additionally, iron-responsive elements (IRE) are found within the 3'-UTR, which can also regulate mRNA stability. Therefore, to increase stability and translational efficiency, the 3'-UTR derived from α - and β -globins are inserted when synthesizing mRNAs *in vitro*. The stabilizing effect can be enhanced by using two 3'-UTRs from β -globin in head-to-tail orientation [43, 44].

In addition, the order of codons also influences the efficiency of translation. Replacement of rare codons for synonymous codons is one of the ways to optimize the sequence of the coding region in mRNA. However, this approach should be used with care, as rare codons are necessary for correct folding in some cases.

Use of various modified nucleotides for *in vitro* mRNA translation dramatically alters its stability and immunogenicity. Modified nucleotides presented in mRNA influence on its recognition by Toll-like receptors (TLRs) involved in cellular immune response against foreign RNA. In humans and mice, 13 TLRs have been identified. In particular, TLR3, TLR7, and TLR8 localized in endosomes are responsible for recognizing foreign mRNA. For example, uridine-rich single-stranded RNA activates TLR7 [45], whereas double-stranded RNA acts on TLR3 [46]. It is known that mRNAs are able to form various secondary structures, hairpins, double-stranded regions, thereby making them proper ligands for both TLR7 and TLR3. Activated TLRs trigger signaling cascades via adaptor proteins (for TLR7 and TLR8 – via MyD88, for TLR3 – via TRIF) eventually resulting in activation of transcription factors NF- κ B, IRF3, and IRF7 [14].

It was demonstrated that activation of TLR3, TLR7, and TLR8 can be minimized by inserting into RNA 2'-O-methylnucleosides, 5-methylcytidine (m5C), N⁶-methyladenosine (m6A), 5-methyluridine (m5U), pseudouridine, and 2-thiouridine [47]. The same modified nucleotides hinder recognition of mRNA by RIG-I receptors. Moreover, insertion of phosphorothioate groups into RNA results in two-fold decrease in RIG-I activation by single-stranded RNA [48] (Fig. 3).

Interestingly, mRNA containing pseudouridine modifications exhibit higher translation activity compared to unmodified. It was found that pseudouridine

residues increase biological stability of mRNA, perhaps by stabilizing secondary RNA structure. In addition, it should be noted that pseudouridine occurs naturally, and it does not increase mRNA toxicity [49]. Apart from pseudouridine, translational activity can also be upregulated by inserting *N*-methylpseudouridine and 5-methoxyuridine [50]. In particular, it was found that combination of the modified nucleotides, e.g. by substituting 25% of uridine and cytosine residues by 2-thiouridine and 5-methylcytosine, can reduce the mRNA recognition by pattern recognition receptors (PRRs) such as TLR3, TLR7, TLR8, RIG-I as well as by dsRNA-activated protein kinases (PKR) [51]. In the process of the modified mRNA design it is necessary to balance between decreased immunogenicity and potentially lowered mRNA stability. For instance, inserting *N*⁶-methyladenine decreases mRNA lifetime, as this modification results in binding of YTH family proteins, which stimulate RNA degradation [52]. Also HPLC purification of mRNA decreases its immunogenicity parameters due to removing dsRNA fragments. Along with inducing immune response, dsRNA can also be used for RNA interference for targeted downregulation of RNA translation [53].

***In vivo* delivery of mRNA.** First attempts to use mRNA without any delivery vehicle revealed that this approach might be applied for vaccination with strongly induced immune response lacking side effects [54]. Although the vast majority of mRNA is degraded in the bloodstream and cell lysosomes, some amount of mRNA was still delivered to the cytosol followed by translation. It should be taken into consideration that positive results were obtained mainly after subcutaneous or intramuscular administration of non-optimized mRNA, whereas i.v. route results in rapid mRNA degradation by ribonucleases and in activation of the immune system. This approach is now widely used for the development of mRNA-containing vaccines currently going through various phases of clinical trials [55, 56].

As common direct route of delivery suffers from poor efficiency and does not provide a significant expression of target protein, it can be applied only for vaccination. In case of replacement therapy delivery vehicles must be used. Overall, application of synthetic NAs in clinical practice is determined by: (i) selection of chemical modifications of NAs that provide resistance to nucleases without affecting functionality of the NAs, and (ii) efficiency of targeted delivery to a tissue of interest and inside cells. Hence, the delivery process is a sophisticated task, because NA should first be delivered to a certain tissue, then passed through the cell membrane, and finally released into the cytosol. The ability to pass across the cell membrane was always considered as the main issue in targeted NA delivery. As NAs are large anionic hydrophilic molecules, they cannot penetrate directly through the hydrophobic cell membrane. Moreover, many defense

mechanisms against pathogens intended to recognize foreign NAs have evolved in cells. Therefore, the optimal NA delivery method must provide its transfer across the membrane, protection from nucleases, and release from endosomes into cytosol for subsequent translation without activating immune system. For long time the use of mRNAs was shadowed by plasmids and siRNAs, so a large number of *in vivo* delivery methods were first studied for these NAs. However, any vehicles cannot be directly transferred for mRNA delivery – thorough optimization is always needed [57-59].

Common mRNA delivery may be improved just by adding Ca²⁺-containing solution prior to administering mRNA *in vivo* [60]. This significantly elevated the level and duration of mRNA translation. Historically, protamines (arginine-rich ~4 kDa proteins), which form complexes with NAs, were used as the first RNA delivery system [61]. In such complexes, mRNA is protected from nucleases [62], but its immunogenicity was increased [63]. The latter is an unfavorable factor for replacement therapy, but, undoubtedly, is a positive feature for developing mRNA vaccines. Currently, CureVac AG (Germany) has commercialized this approach.

For a long time, various lipids have been used to deliver different drugs *in vivo*. Liposomes, lipid nanoparticles, and lipoplexes consisting of cationic lipids are used for delivering NAs. Additionally, cationic nanoemulsions are also described [64]. Lipid nanoparticles (LNP) possess crucial differences from liposomes in terms of forming periodic multilamellar structure between lipids and NAs [65]. According to transmission electron microscopy data [66], such structures in case of mRNA are formed in LNP only partially and single liposomes with mRNA can be also found in LNP. At the beginning, simple liposomes containing DOTAP (1,2-dioleoyl-3-(trimethylammonium)propane) [67] or DOTAP-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) were used [68]. Later, more efficient systems such as lipid nanoparticles based on DLinDMA (1,2-dilinoleyloxy-3-dimethylamino-propane) and PEGylated lipids [69] or lipoplexes – polyplexes covered with lipids (discussed later) were developed. Cationic lipids *per se* are not quite efficient NA delivery vehicles. Therefore a number of helper lipids is needed – fusogenic phospholipids for improving transfection, PEGylated lipids for minimizing particle aggregation and decrease of nonspecific immune response, cholesterol for increasing of particle stability [70]. Besides reducing immune response, the proportion of PEGylated lipids contained in the mixture determines the size of particles, which should be ranged within 50-100 nm. On one hand this decreases nonspecific immune response, on the other hand it prevents nanoparticles from undergoing glomerular filtration in the kidneys and renal escape. Moreover, surface charge on nanoparticles is also an important parameter. While circulating in the blood, neutral charge is optimal to exclude non-specific interac-

tions, whereas positive charge of nanoparticles is critical for disruption of endosome membranes and release of mRNA into the cytosol. Thus, pH-sensitive lipids with pK_a 6.0–6.5 were developed [71], which are more effective for delivery due to neutral charge in the bloodstream followed by protonation upon lowered pH in endosomes that lead to membrane disruption. Later, biodegradable lipids containing ester or acetal groups were synthesized with significantly reduced toxicity [72].

Pardi et al. obtained important data on comparing expression kinetics *in vivo* of LNP packed mRNA applied *via* various administration routes [73]. In particular, subcutaneous or intramuscular vs. *i.v.* or intratracheal routes resulted in increasing halftime of protein synthesis, on average, by 3–4-fold (20–30 and 7–7.5 h, respectively). It is interesting to note that the intradermal route gave rise to two-fold increase in halftime compared to the subcutaneous route (29.6 and 14.7 h, respectively). In addition, it is important that the total amount of synthesized protein differed after administering mRNA via various routes at doses of 0.1 and 1 μ g. For instance, after administering 1 μ g mRNA via different routes, the final efficacy was comparable, but upon introducing it at the dose of 0.1 μ g via the *i.v.* or intratracheal route it was either lower up to 2–3 orders of magnitude or undetectable, respectively. In the latter case low protein level was most likely due to rapid degradation of mRNA by nucleases, in spite of protection by lipid nanoparticles. Again, this example shows the ultimate need to develop novel modified nucleotides that could be incorporated into mRNAs, compatible with ribosomal translation and able to enhance nuclease resistance.

Despite the fact that upon the *i.v.* route liposomes and lipid nanoparticles are mainly delivered to the liver [74], there are methods allowing targeting of lipid nanoparticles containing mRNA to dendrocytes [75, 76], retina [77], lung [78], and some other cells. Improvement of mRNA LNP delivery relies on both designing novel lipids [79, 80] as well as optimizing their composition [81, 82]. On the other hand lipid conjugation with ligands of cellular receptors involved in receptor-mediated endocytosis is another important way for LNP optimization. It is worth mentioning the use of lipids containing *N*-acetylgalactosamine residues [83, 84] – ligands of asialoglycoprotein receptor in hepatocytes – ensures accelerated delivery of lipid nanoparticles. In addition, mannosylated liposomes containing RNA were able to transfect dendritic cells more efficiently due to interaction with mannose receptor in comparison to common liposomes [85]. Still this approach is rarely used due to the difficulties in the synthesis of such lipids.

A polyplex is a complex of mRNA with various polymers, which represents a valuable alternative to liposomes and lipid nanoparticles for efficient delivery of mRNA *in vivo*. Usually, cationic polymers are used to make stable complexes with NA for generating polyplexes. In particular, it was shown that mRNA inside a polyplex is signifi-

cantly protected from ribonucleases and interactions with TLRs *in vivo* [86], and its expression was higher by an order of magnitude compared to free mRNA. Polyplexes have been known for more than 50 years [87, 88]; however, for a long time, due to high toxicity, clinical trials never proceeded further than phase I–II. Linear and branched polyethylenimine [89, 90], poly(2-(dimethylamino)ethyl methacrylate), and poly(L-lysine) [91] are classic examples of cationic polymers. Despite being frequently used for delivery of NA *in vitro*, their relatively high toxicity and poor transfection capacity *in vivo* do not allow considering them as optimal vehicles for delivery of mRNA. Recently, a variety of novel polymers have been proposed that are more efficient and less toxic, primarily due to their biodegradability. Among them, poly(β -amino esters) [92, 93] and polyaspartamides [94] should be noted. Interestingly, Kataoka et al. described how the structure of a polymer might influence translation efficiency [95]. In particular, it was demonstrated that by decreasing the number of aminoethylene units contained in various polyamines used to modify poly(β -benzyl-L-aspartate), it profoundly reduced translation of delivered mRNA via downregulated binding of translation initiation factor eIF4E. Apart from regulating translation, this further confirms that polymer-bound rather than free mRNA is transferred from endosomes into the cytosol. Special attention should be given to the report of Phua et al. [96], where translation kinetics for delivered polyplex-bound vs. free mRNA was examined *in vitro* and *in vivo*. It should be noted that if a polyplex was superior in the *in vitro* setting, then upon subcutaneous administration *in vivo* the half-life of translation of free mRNA was notably higher. This study shows that any novel vehicle must be thoroughly investigated, as unexpected results may be obtained in case of different routes of administration.

Also there are polyplexes containing functional conjugated molecules, e.g. cholesterol [97] or ligands for cell receptors such as sugar residues [98, 99] or their mimetics [100, 101]. For instance, introduction of a cholesterol residue significantly improved circulation of a polyplex in mice that lead not only to undetectable level of the antiangiogenic protein sFlt-1, but also to reduction of the tumor size in the pancreatic cancer xenograft model. As already discussed for lipids, application of receptor-mediated endocytosis allows significantly decrease the amount of administered mRNA with preserving level of translation. It should be emphasized that polyplexes can be used for delivering mRNA not only to dendritic cells, but for example, to bronchial epithelial cells in aerosol [102] or to the central nervous system [103, 104].

Moreover more complex constructs containing a polyplex with mRNA with subsequent formation of a lipid shell have been increasingly used [64, 88]. This lead to high efficiency of mRNA release from endosomes as well as minimizing toxicity of the entire delivery vehicle due to polymer masking.

Therapeutic applications of mRNA. Therapeutic use of mRNA is promising for cancer immunotherapy, vaccination against infectious diseases, inducing tolerance to type I allergy [105], and replacement therapy as well as regenerative medicine.

Antigenic epitopes bound to major histocompatibility complex (MHC) class I and II molecules are exposed on the surface of human dendritic cells, which can simultaneously trigger humoral and cell-mediated immune response. mRNA induces release of proinflammatory cytokines such as TNF- α , IL-1 β , IL-12, IL-6, and IL-8. Moreover, after delivery of mRNA into peripheral blood cells, it also stimulates secretion of type I interferons (IFN- α and IFN- β), chemokines GRO, MCP-1, RANTES, and MDC [106]. Overall, such substances activate both innate and adaptive immune cells: the latter is achieved via stimulating maturation of antigen-presenting cells (dendritic cells, B-cells, activated macrophages). On one hand, this may complicate the use of mRNA in replacement therapy. On the other hand, such particular properties underlie the usage of mRNA for immunotherapy and vaccination upon its delivery to dendritic and T- and B-cells *in vivo* or *ex vivo*. At the present time, various anticancer mRNAs, which are able to stimulate immune response, have been examined in preclinical and clinical trials. The main advantage of this approach is an opportunity to develop personalized anticancer therapy by taking into consideration individual mutations.

One of the first attempts of usage mRNA for the therapy of metastatic prostate cancer has an approach, when patients were administered autologous dendritic cells *ex vivo* transfected with mRNA encoding prostate-specific antigen (PSA). This resulted in detection of PSA-specific T-cells in peripheral blood (9 out of 9 examined patients), thereby allowing the use of this approach for modulating T-cell immune response in humans [107]. Nonetheless, a recent clinical trial with mRNA vaccine against prostate cancer (stage I/IIa) [108] has already been based on applying several mRNAs encoding four different antigens, and the data obtained suggest a fifth candidate antigen. Thus, this approach becomes significantly more complicated and begins to resemble use of total tumor-derived RNA for stimulation of polyclonal tumor-specific T-cell immunity [109, 110]. However, the use of mRNA vaccines undoubtedly wins in terms of safety. For instance, this approach was applied to stimulate T-cell immune response in prostate cancer (upregulated T-cell response was observed in 12 out of 19 patients) and melanoma (10 out of 19 patients) [111, 112]. Alternatively, mRNA can be administered directly via the intradermal route. A two-component vaccine containing mRNA encoding antigen supplemented with mRNA–protamine complex for enhancing immune response was developed by CureVac AG (Germany) based on studies on stimulating immune response via direct mRNA vaccination con-

taining granulocyte–monocyte colony stimulating factor [113]. It was demonstrated during preclinical studies that this vaccine produced good results, and it is currently being used in clinical trials with prostate cancer patients [114]. In this review, we just outline a few examples from such a broad field, which, undoubtedly, have great potential for rapid introduction into clinical practice [115].

Perhaps one of the most prominent events in medical history was discovery of vaccination against infectious diseases, which can prevent later infection or ameliorate its course. Currently used live attenuated virus vaccines protect against poliomyelitis, rubella, smallpox, and other dangerous infections. Apart from them, safer recombinant vaccines containing pathogen-derived antigens are used as well. In some cases, pathogen-derived proteins administered to the host must be matured via processing by endoproteases. As an alternative, vaccines based on mRNA might also trigger both humoral and cell-mediated immune response. Compared to antibodies recognizing one viral subtype, which is developed after a standard vaccination, T-cell-mediated immune response might be triggered by various viral subtypes, thereby making mRNA vaccines potentially more universal. Moreover, in the case of pandemics, mRNA can be quickly adjusted to new mutant viruses and manufactured in sufficient amount for vaccination of many people.

In 1993 it was demonstrated for the first time that mRNA encoding influenza virus protein induced virus-specific T-cell immune response in mice [116]. This study laid the foundation for applying artificially synthesized mRNA in vaccination. In the case of influenza, a two-component vaccine containing an auxiliary mRNA and hemagglutinin-encoding mRNA was designed [117]. An alternative one-component anti-influenza mRNA vaccine was found to be effective as well [118]. Here, it is interesting to note an unsuccessful attempt to create anti-HIV mRNA-based vaccine. In particular, patients were administered dendritic cells containing mRNA that coded for HIV proteins and triggered antigen-specific CD8⁺ and CD4⁺ T-cell immune response [119, 120]. However, no antiviral effect was found in the vaccinated patients [120].

The possibility of using mRNA in replacement therapy depends on development of methods for chemical modification of mRNA for improving its stability and reducing immunogenicity. It was demonstrated that mRNA encoding surfactant protein-B (SP-B) delivered in aerosol into the lungs resulted in its marked expression and increased mice survival [31]. Delivery of mRNA encoding transcription factor FOXP2 efficiently down-regulated immune response in asthma model [121]. Also mRNA-based therapy might be used in therapy of cardiovascular diseases, primarily in regenerative cardiology [122]. For instance, after myocardial infarction, mice administered mRNA encoding Vascular Endothelial Growth Factor (VEGF) via the intramyocardial route

resulted in significant vessel regeneration and increased long-term survival [123]. However, it should be kept in mind that this approach currently has serious limitations related to poor stability and delivery of mRNA as well as high immunogenicity.

In addition we will describe several interesting approaches based on mRNA, some of which so far have been performed only *in vitro*. As expression of transcription factors can reprogram fibroblast differentiation [124], delivery of appropriate mRNAs can induce pluripotent stem cells [125]. Interestingly, in this case immunogenicity of mRNA had a positive effect, as TLR3 activation is necessary for efficient induction of pluripotency genes in the cells [126]. Moreover, use of mRNAs encoding nucleases required for genome editing (ZFN, TALEN, CRISPR-Cas9) may be another promising approach. Nonspecific insertion of DNA fragments resulting from a prolonged translation of nucleases from DNA vectors now represents a major problem during genome editing. There are studies reporting successful use of mRNA encoding nucleases *in vivo* [127-129], and most likely this approach will be applied more often in the future. In addition, mRNA encoding transposase Sleeping Beauty and piggyBac in genome editing has been reported [130-132]. So far, such studies are far from preclinical trials, but they may become a powerful tool in molecular and cell biology.

For managing protein expression, a large number of methods relying on nucleic acids have been described. Synthetic nucleic acids (NA) have been extensively investigated for use in clinical practice. More than 70 NAs are currently passing through different phases of clinical trials, and two of them have already been approved by the FDA. Now major efforts are directed to applying antisense oligonucleotides, siRNAs, and aptamers. Despite such a variety of NA-based technologies, use of synthetic mRNAs will undoubtedly find its niche in therapy. Obviously, in the near future mRNA vaccines will come into medical practice, especially in cancer immunotherapy. In addition, use of mRNAs holds promise for reprogramming somatic cells towards pluripotent stem cells [133].

The possibility of rapid and short-term increase in concentration of a target protein is a crucial point for the use of mRNAs in replacement therapy. Usually, the highest expression level is observed within the first 24 h after administration, and existing alternative approaches do not allow achieving such kinetics for protein accumulation and downregulation. However, efficient application of mRNAs for expression of therapeutic proteins or replacement therapy does not seem easily attainable at this time. Despite the data discussed above, there is a need to search for novel modified nucleosides to stabilize mRNA *in vivo* as well as to develop more efficient means for targeted delivery to lower the amount of applied mRNA and associated side effects. Based on these data, polymer and lipid

vehicles, notably conjugated with ligands of cell receptors, seem to be the most promising delivery systems. In the latter, sugar residues including mannose and N-acetylgalactosamine as well as folic acid derivatives hold the greatest promise. Similar to other NAs, release of mRNA from endosomes still represents a key problem for a delivery system at the cell level. This may be solved only by creating pH-dependent lipids or polymers able to break the endosomal membrane more efficiently than current ones. Therefore, if the above-mentioned problems could be solved, there is no doubt that mRNA will find applications for local short-term expression of therapeutic proteins and replacement therapy. Also we want to emphasize that a significant part of the studies on mRNA therapeutics is carried out in pharmaceutical companies – Moderna Therapeutics (USA), Arcturus Therapeutics (USA), PhaseRx (USA), Acuitas Therapeutics (USA). However their results are not available in the press now.

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