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Abstract

In single-gene disorders, such as α1-antitrypsin deficiency (AATD), hemophilia B (clotting factor IX deficiency), and lecithin-cholesterol acyltransferase deficiency (LCATD), a gene mutation causes missing or dysfunctional protein synthesis, which in turn can lead to serious complications for the patient affected. Furthermore, single-gene disorders are associated with severe, early-onset conditions and necessitate expensive lifelong care. Today, therapeutic treatment options remain limited, cost-intensive, or ineffective. Therefore, the novel mRNA-based therapeutic strategy for the treatment of single-gene disorders, which is based on the induction of de novo synthesis of the functional proteins, has extraordinary potential. After the delivery of the specific mRNA to the target cells, the desired protein is expressed by the cells’ own translational machinery, and hence, a fully functional protein replaces the defective or missing protein. mRNA therapy provides an innovative, highly promising, and inexpensive therapeutic approach and will thus lead to new advances in the treatment of single-gene disorders.

Keywords: α1-antitrypsin deficiency, hemophilia B, lecithin-cholesterol acyltransferase deficiency, mRNA therapy, single-gene disorders, messenger RNA, next-generation therapeutics, gene mutation

1. Introduction

The human body is made up of millions of cells, which have special, well-defined functions, such as the transportation of oxygen in the blood. Proteins carry out all these functions that are necessary for life. In most of the cells, the genetic information is encoded on the 23 pairs of
chromosomes found in the nucleus. On each chromosome, the information for the production of a great range of different proteins is contained in genes made up of DNA. There are approximately 25,000 protein-encoding genes in the human genome [1].

Protein synthesis takes place in two major processes. First, the DNA is transcribed into the mRNA in the nucleus followed by cytoplasmic mRNA translation into the protein.

Alteration in a single gene (i.e., a single-gene disorder) caused by a mutation in the gene’s DNA sequences leads to dysfunction of the gene. As a result, the protein the gene codes for is either altered or missing, which can result in serious complications in the human body.

Over the last few years, it has become apparent that single-gene disorders are far more numerous than previously assumed, and more than 1800 single-gene disorders have been identified [2]. Moreover, single-gene disorders are associated with severe, early-onset conditions necessitating lifelong care [3].

2. Examples of single-gene disorders

2.1. α1-Antitrypsin (AAT) deficiency (AATD)

AAT is a serine protease inhibitor belonging to the serpin superfamily, and it is predominantly synthesized in the liver and released into the bloodstream. By inhibiting neutrophil elastase, which is released from activated neutrophil granulocytes and macrophages, AAT plays a pivotal role in the prevention of proteolytic damage to the host tissue in the presence of inflammatory processes. Physiological AAT plasma concentrations are within the range of 20 to 53 μmol/L (150–300 mg/dL).

Mutations in the SERPIN1A gene are associated with AATD, one of the most common metabolic diseases in Europe. AATD, first described in 1963 by Laurell and Erikson, affects approximately 1 in 2000 to 1 in 5000 individuals [4, 5]. People suffering from AATD have AAT levels below 11 μmol/L (80 mg/dL) and are predisposed to severe lung diseases, such as chronic obstructive pulmonary disease (COPD), or liver diseases.

2.2. Hemophilia B

Hemophilia B, also called Christmas disease, is the second most common form of hemophilia, affecting approximately 20% of those diagnosed with hemophilia. It is a bleeding disorder that is typically inherited and characterized by a lack of clotting activity of factor IX (FIX; Christmas factor), a blood clotting factor that is synthesized in hepatocytes and plays a crucial role in blood coagulation. Depending on the bleeding phenotype of hemophilia B, which is classified as mild, moderate, or severe, and the sufferer’s overall health, symptoms vary from prolonged, partially excessive bleeding to serious bruising and joint pain [6].
2.3. Lecithin-cholesterol acyltransferase (LCAT) deficiency (LCATD)

The gene for LCAT is localized on chromosome 16 and primarily expressed in the liver. After secretion to the plasma, it is primarily found attached to circulating high-density lipoprotein (HDL) particles [7]. LCAT converts free cholesterol into cholesterol esters on the surface of HDL, thus removing cholesterol from the blood and tissues [8].

Mutations in the LCAT gene result in deficient or absent catalytic LCAT activity, leading to a reduction in the enzyme’s ability to attach cholesterol to lipoproteins. Hence, deficiency leads to the accumulation of unesterified cholesterol in different tissues (e.g., in the cornea, erythrocytes, or kidneys) and may lead to corneal opacities, renal failure, or hemolytic anemia [9]. Due to the accumulation of cholesterol in the lining of the arteries, LCATD sufferers have an increased risk for premature atherosclerosis.

2.4. Familial hypercholesterolemia (FH)

FH is a genetic disease characterized by high low-density lipoprotein (LDL) cholesterol levels in the blood. This is caused by a defect in the gene for the LDL receptor (LDLR) that prevents it from absorbing the LDL from the blood into the cell for metabolization. The symptoms in patients with FH range from harmless fatty skin deposits called xanthoma to life-threatening atherosclerotic vascular disease, which can culminate in myocardial infarction or stroke [10]. In patients suffering from the homozygous form of FH, such life-threatening complications occur in infancy. Statins are currently used for standard therapy, but their efficacy is controversial [11].

2.5. Available treatment options

Different treatment options exist depending on the genetic disorder. Treatment of AATD is currently achieved by aerosol or intravenous augmentation therapy of purified and pooled human plasma AAT protein [12]. Furthermore, the risks of proinflammatory stimuli to the lung need to be minimized by ensuring that the patient abstains from smoking, using bronchodilators, etc. Augmentation therapy is, however, very expensive, because the AAT protein derived from a healthy donor needs to undergo a rigorous screening process before it is ready to be infused [13].

Patients suffering from hemophilia B are routinely treated with recombinant FIX concentrates, which have greatly reduced the mortality associated with hemophilia B. However, there are still significant drawbacks of this existing therapy, including the necessity of multiple weekly infusions for patients with severe hemophilia B as well as repeated bleeding despite prophylactic therapy, which can cause long-term damage in joints and other tissues.

The therapeutic up-regulation of LCAT function has gained interest in recent years, not only as an enzyme replacement therapy for LCATD syndromes but also as a potential therapeutic strategy for reducing atherosclerosis [14]. In 2013, the first case report was published highlighting the success of LCAT replacement therapy using a recombinant enzyme form in a 53-year-old patient.
On the contrary, many gene therapy-based systems for the treatment of various genetic disorders have been developed and investigated during the last few years. Gene therapy promises the permanent expression of the functional protein after the incorporation of the corresponding gene into the host genome. However, as yet, gene therapy has not found wide clinical application, because, depending on the vectors used, it can be associated with risks for the patient, such as insertional mutagenesis, carcinogenic effects, immune responses, low gene-transfer efficiency, or protein misfolding [15].

3. mRNA as a novel therapeutic option

3.1. mRNA as a therapeutic agent

Next to gene therapy, wherein genetic defects are corrected by the introduction of specific DNA sequences into the genome, mRNA-based therapy promises new advances in the treatment of single-gene disorders. Via the delivery of a specific in vitro-generated mRNA to the target cells, the expression of a desired protein can be induced. The idea of using specific mRNAs to produce a protein of interest instead of protein replacement via DNA gene therapy was described 25 years ago by Wolff and colleagues [16]. At that time, however, the stability of the mRNA was poor and the immunogenicity was too high for the therapy to be practical. In recent years, the administration of the mRNA as a therapeutic agent has gained enormous potential in the fields of disease treatment, regenerative medicine, and vaccination [17–21].

Especially for monogenic diseases, mRNA therapy can be a highly beneficial alternative to classical gene therapy. Because monogenic diseases result in defective or missing protein synthesis, protein replacement therapy is primarily used for these kinds of diseases. The mRNA can be easily produced in large amounts for the protein of interest in comparison to pDNA, and if the mRNA is used, there is no need to integrate promoter and terminator regions in the sequence [22].

Moreover, the mRNA-based therapeutic strategy has significant advantages:

1. The introduced mRNA is translated by the cell’s own translational machinery under physiological conditions.
2. By introducing a specific mRNA, the physiological state of the cell is not altered, because the effect is transient and not mutagenic.
3. The protein synthesis can be controlled directly without intervening in the human genome.
4. The mRNA does not need to enter the nucleus for translation [15, 20, 22].

Overall, this therapeutic strategy could be safely used in patients, and it is more cost-effective and easier to manipulate than gene therapy.

The standard in vitro procedure for mRNA generation begins with the plasmid, which contains the coding sequence of the protein. First, this sequence is amplified using polymerase chain
reaction (PCR). The generated DNA template contains all the important elements of the mRNA. Second, the amplified PCR product is used to generate the mRNA. Therefore, *in vitro* transcription (IVT) is performed using the T7 or SP6 RNA polymerase, which synthesizes the mRNA. After the purification and quality control steps, the mRNA is ready to use (Figure 1).

**Figure 1.** Generation process of the mRNA.

The mRNA is a single-stranded molecule containing a poly(A)-tail at the end and a 5′-cap at the beginning. The coding sequence for the protein is marked by a start codon and a stop codon. The untranslated regions (UTRs) are in between the cap/tail and the coding sequence (Figure 2) [16].

**Figure 2.** Schematic overview of the general structure of an IVT mRNA.

However, IVT mRNA is very sensitive to degradation by nucleases, which limits its suitability for transfections and therapeutic applications [21]. If the mRNA will be applied as a therapeutic agent, there is a need for special modifications. The modifications should be nonmutagenic and should not interfere with the translation machinery of the cell [23]. To improve the translation of the mRNA, the cap and the poly(A)-tail are important. The mRNA cap is responsible for the recognition of the ribosomes and binding to the ribosomes and for the initiation of the translation machinery [24]. It was reported that part of the unmodified cap structure could be bound in the wrong direction to the mRNA during IVT. Thus, the mRNA cannot be located by the cap-binding complex (CBC) of the ribosomes and the translation cannot follow [25]. By using an anti-reverse cap analog (ARCA) during IVT, this can be prevented. An ARCA contains a 5′-5′ triphosphate bridge, and the 3′OH is replaced by OCH$_3$, making it impossible for the cap to bind in the wrong direction [26]. The 5′-5′ bridge and the chemical modifications at the 3′-position lead to translatable mRNA of high quality and great translation efficiency [27, 28]. Furthermore, ARCA-capped mRNA is more resistant
to hydrolases [29]. The poly(A)-tail, which binds to the polyadenosyl-binding-protein (PABP), is also very important for the stability and translation of the mRNA [30, 31]. The PABP interacts with the CBC and makes a circular structure of the mRNA molecule [15, 32]. This circular structure minimizes the contact surface for nucleases [33]. If the poly(A)-tail is shorter than 12 adenine nucleosides or removed completely, the cap structure is cleaved and the mRNA is degraded. Thus, the poly(A)-tail is very important to obtaining the cap structure and delaying the degradation of the mRNA [15, 34]. A poly(A)-tail of more than 60 adenine monophosphates increases the translation efficiency of the mRNA [35]. Therefore, the poly(A)-tail and the cap structure contribute to the stability of the mRNA [27, 36]. The 5′- and 3′-UTRs include specific regulatory sequence regions that are necessary to modulate the stability and translation of the mRNA [16]. The 3′-UTR region contains α- and β-globin sequences that enhance the stability and translation of the mRNA [16, 37, 38]. Furthermore, the 3′- and 5′-UTR regions inhibit the decapping and degradation of the mRNA [16, 39, 40]. On the contrary, if limited protein production is desired, faster mRNA degradation is possible by integrating AU-rich areas in the 3′-UTR region [41]. The protein-coding region of the mRNA can be optimized in two ways. First, the use of optimized codons leads to improved translation of the sequence to the desired protein. Second, via the optimization of the bases, the endonucleolytic degradation can be reduced [16].

### 3.2. Potential immune response against IVT mRNA

In comparison to recombinant protein in the substitution therapy approach, mRNA-translated proteins are autologously produced by the cell’s own machinery. Usually, they undergo the correct modification and folding [16], but protein aggregation and immune reactions to the translated protein cannot be excluded. Therefore, it is necessary to investigate the activation of the immune system and distribution of the antibodies against IVT mRNA-translated proteins in clinical studies. Furthermore, the combination of the immune reaction to the translated mRNA protein and the foreign mRNA within the cells can lead to immunopathology in the cells or organs [16, 42].

Regarding protein replacement therapies, the activation of the immune system caused by mRNA administration might be disadvantageous. It is well known that foreign mRNAs as well as pDNAs activate the immune system via recognition through toll-like receptors (TLRs). TLRs recognize different pathogen patterns resulting in the expression of different cytokines. TLR3 and TLR7/8 are responsible for the recognition of different RNA types [43]. The mRNA and other RNA types, such as small interfering RNA (siRNA) or double-stranded RNA (dsRNA), are recognized by TLR3 [44–46], whereas TLR7/8 is activated by single-stranded RNA (ssRNA) [47]. In nonimmune cells, the recognition of the mRNA occurs through the retinoic acid-inducible gene I (RIG-I), which is activated by short RNA and dsRNA [48] and leads to interleukin-1β (IL-1β) production [49]. It is known that the IVT mRNA leads to a strong distribution of tumor necrosis factor-α (TNF-α) if the mRNA has no modifications [50]. Additionally, a strong type I interferon (type I IFN) response of the cells is induced upon contact with exogenous mRNAs. This is also induced by mRNAs that form secondary structures, such as loops or hairpins, or by mRNAs that bind to incompletely synthesized
mRNA fragments or incompletely degraded DNA fragments [43, 51]. IFNs then activate the antiviral genes in the genome and lead to a translation stop and the degradation of the mRNA [43]. However, the immune reaction can be avoided if the mRNA is purified and if modified nucleotides are inserted during IVT [50, 52–54].

The incorporation of different modified nucleotides, such as pseudouridine (pseudo-U), 2′-thiouridine (2′-tU), 5′-methyluridin (5′-mU), 5-methylcytidine (5′-mC), or N\textsubscript{6}-methyladenosine (N\textsubscript{6}-mA), can prevent the cellular immune response [50, 53]. These modified nucleotides incorporated in the mRNA help to avoid the activation of TLRs [50]. In particular, pseudo-U and 2′-tU make the recognition of IVT mRNA by RIG-I impossible [16, 54, 55]. To minimize the immune activation and optimize the translation efficiency of the mRNA to the protein, high-performance liquid chromatography (HPLC) purification should be applied to eliminate the dsRNA contamination that can still be present after the IVT process [56].

However, mRNA modification may also represent another risk. The naturally existing mRNA is degradable by the RNases in the extracellular space, but the modification of the IVT mRNA makes degradation more difficult [57]. Some of the modified nucleotides are associated with mitochondrial toxicities and hepatic failure and play a role in viral and tumor cell replication [58, 59]. Here too, further investigations and clinical trials are necessary to prove the risks and benefits of IVT mRNA modifications.

3.3. mRNA delivery systems and specific targeting

For clinical mRNA applications, a specific delivery system is needed, because otherwise the delivery of the mRNA to the target cells is unguaranteed and inefficient [27]. Therefore, the development and engineering of safe and effective delivery vectors for mRNA therapy is inevitable [60]. Viral and nonviral vectors can be used to bring the mRNA into the cells. For the direct translation of the mRNA to the protein, only positive-stranded viruses can be applied [61]. Negative-stranded viruses are not infectious and need an RNA-dependent RNA polymerase for mRNA translation [62]. However, viral vectors have some limitations; for example, they can be carcinogenic [63], they can activate immune responses [64], they can be difficult to produce [65], and they have a limited packaging capacity [66].

Nonviral vectors have lower immunogenicity, they can deliver large genetic molecules, and they are easier to produce [60, 67, 68]. Nonviral delivery systems can be subdivided into direct and indirect delivery systems. Direct delivery is possible via electroporation or gene guns. Electroporation is an early and efficient method of transporting mRNA into cells [69], whereby electrical pulses make the cell membrane permeable for the entry of the mRNA into the cytosol. This method does not induce immune activation, which may occur when mRNA carriers are used [27, 69]. Transfection using a gene gun requires heavy metal particles to get the nucleic acid into the cell [70]. This method allows the delivery of the mRNA to mammalian organs with minimal damage and leads to transient protein expression in the target tissues [70]. Self-assembled complexation of negatively charged mRNAs and positively charged liposomes and polysomes to lipoplexes or polyplexes is the most widely used method of bringing mRNA into cells [60]. The lipoplexes can be taken up by the cells in two different ways. The first way is by the endocytosis of the lipoplexes, whereby 98% of the lipoplexes enter the cell. The second way
is through the fusion of the cell membrane and the lipoplexes, which results in the uptake of the remaining mRNA [71]. After the release of the mRNA into the cytosol, the protein translation can begin. The encapsulation of the mRNA into liposomes is a rapid, transient, and cell cycle-independent delivery method [27]. The translation of the mRNA to the protein can be measured 1 h after the transfection in nondividing cells [72].

For **in vivo** applications, the perfect mRNA delivery vector has to overcome various barriers:

1. Protection against nucleases,
2. Avoidance of nonspecific interactions with proteins or cells,
3. Prevention of renal clearance,
4. Permission of extravasation to target the tissue of interest, and
5. Increased entry into the cell [60].

For systemic delivery, the lipid and polymer complexes show protective properties against nucleases, whereby the mRNA is protected and the stability is increased [73]. However, liposome complexes sometimes interact with serum proteins. Together, they form aggregates or clots and are cleared rapidly [74]. The conjugation of the complexes with polyethylene glycol (PEG) helps to inhibit the nonspecific uptake and attachment to serum proteins [75].

Nanoscale delivery systems (10–200 nm) enhance the uptake efficiency and reduce the systemic toxicity [75]. The use of PEG coating of the liposomes increases the blood circulation time and avoids the detection of liposomes by immune cells [75, 76]. The liposomes have many advantages, such as low batch-to-batch variability, easy synthesis, biocompatibility, and scalability, over many other delivery systems [75]. The liposome surface can be functionalized by conjugation to chemically reactive lipid head groups [77]. This property makes it possible to functionalize the surface with ligands and thus enhance the target delivery [75, 78].

Different methods of application are tested for **in vivo** delivery. Polyplex nanomicelles applied with hydrodynamic intravenous injection have been shown to effectively deliver the mRNA to the liver in mouse models. This method shows a strong protein expression in nearly 100% of liver cells [79]. Intramuscular or intraperitoneal injection of erythropoietin (EPO)-coding mRNA complexed with cationic lipids leads to significantly high levels of EPO **in vivo** [20, 80]. Intratracheal and intranasal applications of Foxp3 mRNA show protective properties against asthma in mice [81]. Furthermore, it has been shown that intradermal [82] and intranodal [83] applications of the mRNA in animal models resulted in immunization against tumors. Many other methods of applying the mRNA have been described in publications in recent years, and this field is developing rapidly [19, 84]. Furthermore, patient-centered applications have improved, especially for mRNA therapy. Nebulization with the Pari-Boy® is the standard method used to apply the drugs to the lung. A study on the influence on mRNA transfection efficiency shows that the nebulization of complexed mRNA has no effects **in vitro** [85].
3.4. mRNA applications

Gene therapy allows the replacement of a defective gene through substitution and integration of the correct genetic code in the genome. The genome integration of this genetic code via viruses guarantees highly efficient gene replacement methods. However, undesirable effects, such as mutagenesis and innate immune response, may jeopardize the life or safety of the patient [27, 86]. Nonviral gene delivery is safer, but it is also associated with lower transfection efficiency due to insufficient nuclear transport. Furthermore, modifications of the pDNA, including adding a strong constructive promoter to improve transcription efficiency, may lead to unexpected alterations in the genome [27, 86, 87].

Protein-substitution therapy is associated with adverse reactions, such as headache, dizziness, and nausea [88], and high costs [89]. Recombinant proteins have been expressed in different microorganisms [90–92], plant cells [93], and human cells [94–96], but they are linked to disadvantages such as nonglycosylation or incorrect glycosylation of the product as well as product contaminations with endotoxins. Plasma-derived proteins, which are purified from human donor blood, are limited and prone to contamination.

The mRNA is an alternative to overcome the disadvantages of pDNA and direct protein substitution [15]. The mRNA does not need to enter the nucleus for transcription, because the mRNA is directly translated in the cytosol of the cells; thus, the insertion of exogenous DNA into the genome poses no risk. Furthermore, the mRNA uses the cell’s own translation machinery and requires no strong promoter. Effective mRNA transfer can also take place in nondividing cells, and immunogenicity can be overcome by different modifications of the mRNA molecule [27]. Moreover, compared to protein-substitution therapy, the expression of receptors and intracellular molecules can be induced with specific mRNAs (Figure 3).

Figure 3. The way from encapsulated mRNA to protein expression in the target cell.
Several studies describe that the use of different mRNAs leads to an increase in the respective protein \textit{in vitro} and \textit{in vivo}. The first application of the mRNA was performed in 1992 [97]. However, the broad application of IVT mRNA only became possible as modified nucleotides were used; thus, a reduction of immune reactions and an increase of mRNA translation efficiency were achieved [50, 56]. For example, the successful expression of surfactant protein B (SP-B) of therapeutically relevant levels was shown in a mouse model after the application of SP-B-encoding mRNA via direct administration to the lung. The results showed that the inflammation of the lung (and the inevitable respiratory failure and death) was prevented [20, 98]. Likewise, the expression of the regulatory T-cell transcription factor (FOXP3) led to the prevention of asthma in a mouse model. After the administration of FOXP3-coding mRNA to the lung, the expressed protein protected the lung from allergen-induced inflammation. This mRNA approach can be used as a preventive and therapeutic drug [81]. In a different study, the expression of the AAT protein was shown after the transfection of cells with AAT-encoding mRNA. The level of AAT was measurable in the cellular supernatant 48 h after transfection, and the functionality of the protein was proven. All these studies show that mRNA therapy also promises a novel therapeutic strategy in the treatment of single-gene diseases such as AATD [99].

In the case of FH, it is also possible to induce the expression of functional LDLR after the transfection and thus regulate LDL metabolism. In this case, functional LDLR expression can help to prevent secondary diseases, such as stroke and atherosclerotic plaques. Although the induction of the expression of proteins, such as LCAT and FIX, can be performed similarly to the approach of AATD-mRNA therapy, different organs are targeted. Regarding hemophilia B and LCATD, hepatocytes should be transfected, which can be achieved by intravenous injections of the complexed protein-encoding mRNA.

Depending on the application method and the genetic defect, it is important that the cell type of interest is targeted and transfected with IVT mRNA. Additionally, the dose-effect relationship could be a challenge \textit{in vivo}, whereby the bioavailability and individual variations also play a pivotal role and potentially make individual dose adaptation necessary [16]. Overall, mRNA application promises an effective and low-cost therapeutic strategy with the potential to efficiently correct serious monogenic diseases.

3.5. Summary

Overall, the mRNA as a novel therapy for monogenic diseases has many advantages over the currently existing treatment options such as substitution therapy or gene therapy. Using the mRNA, the expression of nearly all proteins can be induced. In this way, not only extracellular proteins, but also proteins, which are important for the function of the cells, such as growth factors or receptors can be generated. Furthermore, the mRNA does not need to penetrate into the nucleus. The effect of the mRNA is transient, thereby enabling the precise control of protein expression. Depending on the introduced modifications, the half-life of the respective mRNA and thus a reduction or increase in mRNA stability can be determined. Developments in recent years, such as modification and purification methods, have made it possible to use the mRNA as a therapeutic agent, because it became possible to control the immune reaction, which is
typically triggered by exogenous mRNA. Further developments increased the extracellular stability and made mRNA transfection of the cells with nonviral vectors possible and efficient. The mRNA can be produced in large batches and under good manufacturing practice (GMP) conditions without batch-to-batch variations. Thereby, the production of specific mRNA is significantly cheaper compared to the production of the corresponding protein for substitution therapy.

The mRNA as a therapeutic agent could be a great help for patients suffering from monogenic diseases. The flexibility and variability of proteins that can be replaced by the cell’s own translational machinery through the use of the mRNA is nearly unlimited. This makes mRNA to a unique therapeutic molecule, which will revolutionize therapeutic options for affected patients in the coming years.

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References


