Hurdles in the development of mRNA-based therapeutics; current position and future approach

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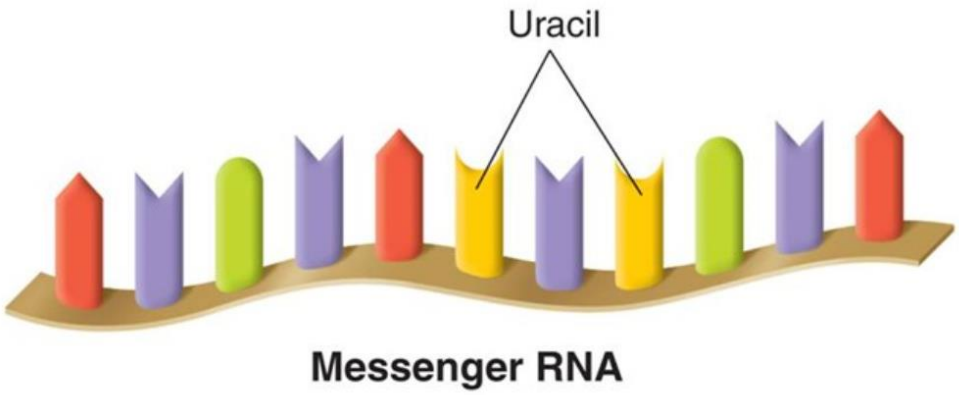

31-01-2017
MSc programme Biomedical Sciences
Rijksuniversiteit Groningen

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ABSTRACT

Gene therapy is the delivery of nucleic acids into a patient's cells to treat or prevent a disease. mRNA-based therapeutics have many advantages over both pDNA- and siRNA-based therapeutics. mRNA therapeutics can be applied in the treatment of genetic disorders, regenerative medicine and in the treatment of infective and neoplastic disorders.
Although mRNA was previously considered too instable to form a therapeutic, many techniques show effective mRNA transfection. This essay discusses the status quo in the field of mRNA modifications and a variety of mRNA delivery methods among which complex-forming delivery materials, techniques used for targeting of the mRNA, electroporation and gene gun delivery.
Although the general principles behind effective mRNA delivery have yet remained largely unclear, several techniques generated effective mRNA transfection. In combination with the broad range of applications, this pleads for more research in the field of mRNA-based therapeutics.

CONTENTS

1. INTRODUCTION

Gene therapy is the delivery of nucleic acids into a patient's cells to treat or prevent a disease. Several types of nucleic acids have been used for gene therapy in the past and they all have their up- and downsides. At the start of the development of gene therapy, plasmid DNA (pDNA) was the nucleic acid used most frequently. One of the advantages of pDNA is that by introducing a new gene, a mutated gene can be knocked out or replaced by a healthy gene. A disadvantage of pDNA delivery is that it requires crossing the nuclear membrane. A few years ago, delivery of small interfering RNA (siRNA) gained interest. siRNA forms a complex with certain proteins and this complex targets and cleaves messenger RNA (mRNA). Through this mechanism, siRNA mediates gene suppression. An advantage of siRNA is that it does not require delivery into the nucleus. However, a disadvantage is that siRNA can only decrease protein expression, whereas increased expression of a certain protein is often desired. For example, in case of a mutated gene resulting in a dysfunctional protein, this protein should be replaced. This gap can be filled by mRNA therapeutics.

Previously, mRNA was considered too instable to ever form the basis of an efficient transcript replacement method. However, recent studies showing efficient delivery of mRNA *in vivo,* prove that mRNA instability was overestimated[1-4]. With this insight, doors are opened for the development of mRNA-based therapeutics. By introducing synthetic mRNA, often referred to as transcript replacement therapy, expression of a desired protein is induced. In addition, mRNA does not require delivery into the nucleus as mRNA is translated in the cytoplasm. This spares crossing a very strict boundary, a troublesome task in pDNA delivery. Due to this advantage, mRNA enables the effective transfection of quiescent cells and cells that are generally hard to transfect[5]. Another advantage of mRNA over pDNA is that it does not integrate in the host genome thus preventing permanent mutagenesis[6]. Mutagenesis associated hazards forming problems in DNA transfection, e.g. leukemiogenesis, are therefore excluded in mRNA-based treatments. Finally, as mRNA is broken down within several days, the effects of mRNA transfection are transient[7,8]. This facilitates fine-tuning and a high degree of control over protein expression in time.

mRNA delivery is applicable for the treatment of genetic disorders (e.g. enzyme deficiencies like cystic fibrosis and congenital surfactant protein B (SP-B) deficiency[6]), regenerative medicine (mRNA can induce pluripotency or even differentiation into a desired cell type[8,9]) and for treating infective and neoplastic disorders (via mRNA-based immunotherapy a.k.a. vaccination)[6,10]. In the process of vaccination, antigen presenting cells are loaded with mRNA molecules coding for immunogenic epitopes, thus eliciting strong CD8+ and CD4+ T-cell responses[11]. These cytotoxic- and helper T-cells then act against cells expressing the immunogenic epitope. In contrast to classical vaccination, this method is not restricted to the human leukocyte antigen (HLA)-type, making personal tailoring superfluous[10]. As a transient boost of the immune system is more effective compared to longer lasting triggers, mRNA is more suitable for vaccination purposes compared to pDNA[10]. Among all applications for mRNA, clinical experience is currently solely available for vaccination[12].

Thus, mRNA-based therapeutics provide many opportunities. However, arranging efficient mRNA delivery is challenging. This essay describes the status quo in the field of mRNA delivery and points out topics that have yet remained unexplored. It discusses the current position of research on mRNA modifications, mRNA delivery methods and targeting of mRNA-based therapeutics. Analogies on siRNA and pDNA delivery are drawn where possible. In addition, an answer to the next question will be given: What is, with the present knowledge in mind, the most promising step towards the development of clinically applicable drugs for *in vivo* mRNA-mediated gene therapy?

2. HURDLES IN mRNA DELIVERY

To be clinically applicable a mRNA therapeutic has to meet the following requirements:

I: A meaningful amount of mRNA molecules has to reach the cytoplasm without losing their functional structure.

II: The mRNA must produce a meaningful amount of protein.

III: The therapeutic should not induce excessive side-effects.

When designing mRNA therapeutics that meet all criteria, scientists come across several challenges. These scientific hurdles will be discussed in order of which they arise on the physical journey of the mRNA therapeutic, until the therapeutic effect has been achieved.

When mRNA is injected intravenously, the first environment it encounters is the blood. As mRNAs are unstable molecules, they will be degraded in the blood. As this jeopardizes meeting requirement I, **mRNA instability** forms the first hurdle. mRNA instability is mainly caused by its inability to form stable double beta-helix structures[13]. A hydroxyl group on the second carbon of the five-carbon sugar moiety causes sterical hindrance thus preventing the mRNA molecule to curl up into this structure that would protect it from degradation. This instability can be obviated by encapsulation of the mRNA to shield it from degrading enzymes. Modifications to the mRNA molecule itself can enhance stability as well.
Another factor complicating mRNA delivery, is the **immunogenicity of mRNA molecules**. As a result of the generation of a pathogenic immune response, meeting requirement III will fail as a result of the immunogenicity of mRNA. Although mRNA immunogenicity can be exploited for the previously mentioned vaccination purposes, activation of the immune system is not desirable in other applications and should be controlled in vaccination as well. This hurdle plays a role as mRNA is in the blood, but also during the travelling of the mRNA through other extracellular spaces. The mRNA-induced immune response is based on a pathway originally designed to combat viruses in which pattern recognition receptors (PRRs) of the innate immune system detect foreign RNA[6]. Among other PRRs, Toll-like receptors (TLRs) 3, 7 and 8 and retinoic acid-inducible gene-I (RIG-I) like receptors (RLRs) are activated. This leads to the indirect production of pro-inflammatory cytokines like interferon-I and nuclear factor κB (NF-κB)[14]. Interferon-I initiates transcription of over 300 genes indirectly inhibiting mRNA translation[14]. NF-κB on its turn induces cellular apoptosis. Also involved in mRNA recognition are nucleotide binding oligomerization domain (NOD)-like receptors (NLRs). NLRs also induce apoptosis, in this case by stimulating caspase-1 production. It is because of this immunological response that repeated treatment with mRNA therapeutics often requires simultaneous suppression of the innate immune system. This can be accomplished by administrating immunosuppressive drugs (e.g. B18R protein)[7,8] or by co-transfecting siRNAs knocking down mediators of the innate immune response. However, another approach will be to evade the immune response. Several possibilities lie in modifying the mRNA molecule itself in order to reduce its immunogenecity[6]. Encapsulation of the mRNA molecules can also serve as a strategy to avoid an immune response.
If the mRNA reaches the organ of interest without losing its functional structure and without inducing side-effects, the next step is to make mRNA evade the vascular system and reach the tissue of interest (still without losing its shape or inducing side-effects). Unless the vascular endothelium forms the tissue of interest, the vessel walls forms a boundary between the mRNA and the target cells. As a result of the natural presence of sinusoids and fenestrated endothelium in some organs, permeability for mRNA is enhanced at some places. However, evading the vessels on the remainder of sites remains problematic. As the mRNA will circulate through the body uniformly and evades the vessels at permeable places randomly, only a fraction of the administered dose will end up in the area requiring treatment. In addition, the therapeutic often depends on the cellular uptake of the cells in the target tissue for reaching the cytoplasm, and the cells displaying the highest cellular uptake might not be the cells of interest. As a result, mRNA molecules reaching the target tissue might not reach the target cells. With regard to requirement I, the fact that **without intervention, the mRNA ends up in many**

off-target cells forms another hurdle. In addition, the presence of therapeutics often induces side-effects, which should be minimized for meeting requirement III. This hurdle is partially addressed by drug targeting.
If all previous hurdles have been tackled and the mRNA therapeutic has reached the tissue of interest, the next challenge lies in **crossing the cell membrane** of the cells of interest. As mRNA molecules are large, positively charged and hydrophilic, passive diffusion through the membrane does not induce significant mRNA levels in the cytoplasm[15]. Examples of methods aiming on crossing the cell membrane are literally bombarding the cells with mRNA bound to heavy metal nanoparticles (NPs), disrupting the cell membrane by electrical pulses or encapsulating the mRNA in material inducing endocytosis. However, even after crossing the cell membrane by endocytosis, the cytoplasm is not always within reach, as a new barrier in the form of the endosomal membrane arises. This can be overcome by complexing the mRNA with specific compounds that release the mRNA in an acidic environment (the endosome).

After enabling mRNA to cross these membranes, it reaches yet another challenging environment; the cytoplasm with its high concentration of hydrolytic degrading enzymes[13]. These enzymes jeopardize meeting requirement II as the mRNA has to be able to spend some time in the cytoplasm before being degraded. Thus, mRNA instability forms a complication until the very end of the physical journey. Another factor complicating meeting requirement II, is the usually **inefficient translation of synthetic mRNA.** As mRNA cannot code for proteins whilst being encapsulated, encapsulation is not a suitable strategy to tackle these last two hurdles. Modification of the mRNA itself however could offer a solution.

In addition to intravenous injection, administration routes frequently used for mRNA-delivery are intraperitoneal injection, inhalation, or injection directly into the target organ. Although these routes parry travelling through the blood and enable a more restricted bio distribution, most of the hurdles listed above play a role in these administration routes as well. Oral administration comes with even more hurdles as mRNA instability is tested even more in the digestive system since it is full of degrading enzymes and another boundary is added in the form of the intestinal epithelium.
Next to *in vivo* delivery, mRNA can also be delivered to cells which are taken out of the body temporarily. After *ex vivo* mRNA delivery, the cells are placed back into the body. With *ex vivo* delivery the mRNA is placed directly in or near the target cells. As the mRNA does not have to travel through the blood and other extracellular environments, *ex vivo* delivery is less challenging compared to *in vivo* delivery. *Ex vivo* delivery is particularly suitable for blood cells as these cells are easily placed back.

3. CURRENT TECHNIQUES IN mRNA THERAPY

There are different approaches when tackling the hurdles faced in mRNA delivery (Table 1).

Table 1. Current techniques in mRNA therapy and the hurdles they (partially) address

	mRNA instability	mRNA immunogenicity	mRNA ending up in off-target	Crossing the cell-	Inefficient translation
mRNA modifications	x	x			x
Complex-forming delivery materials	x	x		x	
Techniques for targeting			x		
Electroporation			x	x	
Gene gun delivery			x	x	

In the next chapters, the techniques addressing the first encountered hurdle on the mRNA journey are discussed first, and so on. As the techniques often address more than one hurdle, the hurdles are occasionally discussed earlier.

3.1 mRNA MODIFICATIONS

Synthetic mRNA molecules are instable, immunogenic and possess a lower translation efficiency compared to endogenous mRNA. Modifications to the synthetic mRNA molecule itself can serve as a solution to these problems.

Some modifications for synthetic mRNA are inspired by post-translational modifications of endogenous mRNA. These modifications focus mainly on the ends of the molecules. The 5' end of endogenous mRNA is post-translationally tailored by addition of a methylated cap structure. The 3'end is modified post-transcriptionally as well; a series of adenine nucleotides is added thus forming a poly(A) tail. Both modifications play major roles in transcription efficiency and mRNA stabilization and can be added to synthetic mRNA while it is being produced *in vitro* as well[16]. However, these modifications are still being optimized.

Other modifications focus on the nucleotide sequence of the mRNA. Some non-coding nucleotide sequences increase mRNA instability. By replacing them with stable sequences, mRNA half-life is improved[13]. Some coding sequences increase mRNA stability as well. However, these sequences cannot be entirely replaced, as this will change the sequence of amino acids building the protein. However, instable sequence can be modified by replacing single nucleosides with naturally occurring modified nucleosides in a way that a different codon coding for the same amino acid is formed[17,18]. This type of modifications also decrease immunogenicity. For example, replacement of the nucleoside cytidine with the naturally occurring nucleoside 5-methylcytidine decreases mRNA immunogenicity[6]. As all mRNA molecules coding for different proteins have a distinct nucleotide sequence determining stability and immunogenicity, they all need individual optimization.

mRNA modifications aiming on increasing cellular uptake or endosomal escape have not been reported until now.

In addition to modifying the synthetic mRNA, purification of the mRNA mixture is also important in reducing immunogenicity because aberrant (e.g. truncated) mRNA molecules are highly immunogenic[19].

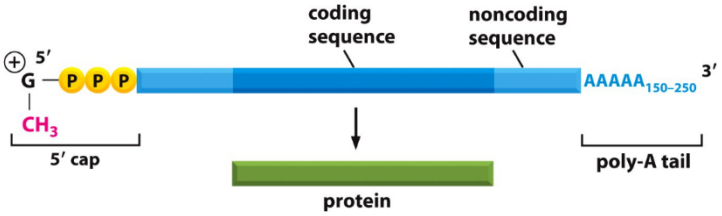

Figure 1: RNA capping and polyadenylation in endogenous mRNA
Reference: B. Alberts, Essential Cell biology, 3rd edition

3.2 mRNA DELIVERY METHODS

In contrast to mRNA modifications, mRNA delivery methods do not interfere with the structure of the mRNA itself. Delivery methods can be subdivided into viral and non-viral methods. This review will not discuss viral methods as they are associated with potential integration into the genome, the size of the transported mRNA is limited and these methods can elicit strong immunological responses[5]. Non-viral methods can be subdivided into chemical and physical methods.

Chemical delivery methods include mRNA encapsulation using protamine, polymers or lipid(like) materials. Although these methods are not optimized for mRNA delivery yet, they show high *in vivo* applicability in siRNA delivery.

Physical methods of mRNA delivery include gene gun delivery, electroporation, microinjection, laser-based transfection, magnetofection and sonoporation[20]. The microinjection method directly injects nucleic acids into the cytoplasm or the nucleus of the cell. However, since this method is highly cytotoxic and very labour-intensive it is not often used. Using laser-based transfection, transient pores can be made at every opted spot and this method is therefore very suitable for single cell transfection of small cells[20]. However, the method is expensive and rarely used *in vivo*. Sonoporation is an

ultrasound mediated transfection method which has very low transfection efficiency[21]. Therefore, this essay will not elaborate on these methods. Physical methods are directly applied to the tissue of interest. Therefore, the mRNA does not have to face the gut, the blood and other barriers. However, physical methods are therefore only applicable for superficial tissues. The methods of electroporation and gene gun delivery will be discussed.

3.2.1 COMPLEX-FORMING DELIVERY MATERIALS

Protamine, cationic polymers and lipids interact with negatively charged nucleic acids through electrostatic interactions thus forming condensed complexes with mRNA[22]. These complexes are popular in the field of nucleic acid delivery, as they reduce the immunological response and aid in stability. Most important, however, is the fact that these complexes have a slightly positive net charge which promotes interaction with the negatively charged cell membrane and subsequent endocytosis thus enabling mRNA to cross the cell membrane[23].

Unfortunately, these materials have a downside. Their positive charge also attracts negatively charged proteins in the serum, like e.g. opsonins. The materials (containing mRNA) and the proteins form aggregates which are cleared rapidly thus losing mRNA[24]. In addition, the positive charge promotes non-specific interaction with non-target cells.

This problem can partially be solved by shielding the positive charge with hydrophilic, uncharged polymers such as polyethylene glycol (PEG). This approach is termed PEGylation and can improve drug solubility, circulating half-life, stability and resistance to proteolytic degradation[25]. However, PEGylation typically reduces pDNA transfection efficiency. PEGylation decreases translation efficiency of mRNA complexed in lipid-like nanoparticles (LLNs) as well[26,27]. However, PEGylation improves transfection efficiency in mRNA-polymer-based nanoparticles (NPs)[28]. This shows that PEGylation might affect different nucleic acids in different ways and that the material of the NP influences this effect as well. By adding a certain targeting ligand to the distal end of the PEG polymers, the NP can actively target certain cells. In this way, non-specific interaction with non-target cells is reduced, thus minimizing side-effects. In addition, the dose-response curve is optimized as a higher percentage of the dose will reach the target cells. Wang et al. demonstrated *in vivo* anticancer activity using this PEG-ligand method for the delivery of mRNA to sigma-receptor overexpressing cancer cells[29].

Figure 2: The formation of a polyplex from cationic polymers and anionic nucleic acids. Reference: Kauffman KJ, Webber MJ, Anderson DG. Materials for non-viral intracellular delivery of messenger RNA therapeutics. J Control Release. 2016

3.2.1.1 Protamine-based delivery materials

Protamine, a cationic arginine-rich peptide weighing ca. 4 kDa, interacts with negatively charged nucleic acids thus forming complexes[22]. Although these nanoparticles protect the mRNA from nucleases[30], they do activate the Toll-like receptors of the immune system more than naked mRNA molecules do thereby initiating side-effects[31]. The upsides of protamine-mRNA complexes is that they are well-tolerated[32] and have already been used for vaccination against the influenza virus in animals[33] and the treatment of melanoma[34], lung- and prostate cancer in humans[32].

3.2.1.2 Polymer-based delivery materials

Cationic polymers form complexes with anionic nucleic acids termed polyplexes (Fig. 2). A lot of research has been performed on the use of cationic polymers for the delivery of siRNA and pDNA. The classes of polymers used most frequently in these studies are polyethyleneimine (PEI), poly(L-lysine) (PLL) and poly(2-dimethylaminoethyl methacrylate) (P(DMAEMA)). In the last few years, several studies investigated the potential role of these polymers in mRNA delivery. Additionally, poly(β-amino esters) (PBAEs), poly(glycoamidoamine) (PAAG), chitosan, synthetically modified polyaspartamides and poly(ε-caprolactone) (PCL) have been investigated for mRNA delivery.

Combining different monomers into one optimized copolymer is also possible[35]. For example, different segments responsible for either mRNA condensation, stability, biocompatibility and endosomal escape can be combined into one copolymer[35]. In addition, the combining different (co)polymers in one NP can increase transfection rates. Polymers can even be combined with protamines and lipids resulting in hybrid NPs, some of which have already proven their effectivity[36]. In this chapter, the different classes of polymers will be discussed.

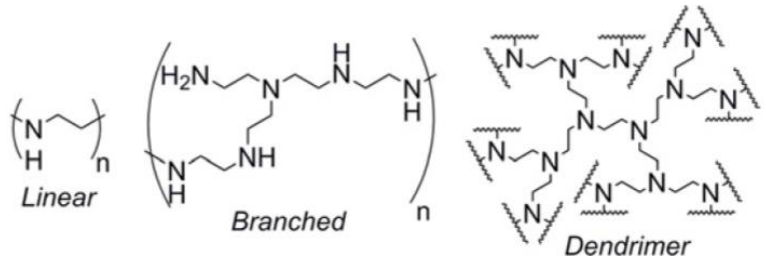

Figure 3: Types of polyethyleneimine (PEI) used for nucleic acid delivery. Reference: Kauffman KJ, Webber MJ, Anderson DG. Materials for non-viral intracellular delivery of messenger RNA therapeutics. J Control Release. 2016

PEI can form either linear, branched or dendrimer (repetitive branched) polymers (Fig. 3). PEI has clear downsides as it is relatively toxic and is not biodegradable. When using linear PEI polymer lengths that are efficient in pDNA delivery (25 kDa for PEI and 54 kDa for PLL), the mRNA release is compromised[10]. This is caused by the increased electrostatical interaction between the mRNA molecule and the polymer[37]. This reduced mRNA release is demonstrated in **PLL** (Fig. 4) comprised NPs as well. Unfortunately however, when shortening the polymers (2 kDa for PEI and 2,4 kDa for PLL) the NPs lose their endosomolytic activity resulting in a decreased transfection effectivity. When inducing endosomolysis by adding chloroquine (an agent that prevents endosomal acidification and fusion of endosomes and lysosomes[38]), the short polymers show great effectivity. This effectivity is illustrated by the fact that short PEI of PLL induce expression levels which are 5 times higher compared to levels induced by mRNA-lipoplex DOTAP (discussed in next chapter)[37]. Thus, short chain polymer complexes are taken up in the cell very efficiently. However, as chloroquine is toxic and is therefore not suitable for *in vivo* application, another method to disrupt the endosomal membrane has to be developed.
Chains slightly longer than 2 kDa might even perform better as NPs composed of these polymers might regain part of their endosomal escape properties.
The polymer **P(DMAEMA)** (Fig. 4) has been investigated for *in vitro* mRNA delivery as well[28]. When combined with oligo(ethyleneglycol)methylether-methacrylate (OEGMA), transfection efficiency was as good as transfection using the Lipofectamine™ 2000, which is a standard method for mRNA transfection *in vitro*[28]. *In vivo* effectivity has yet to be shown.
The class of polymers composed of **PBAEs** (Fig. 4) has already shown *in vivo* mRNA delivery effectivity[39]. Intranasal administration of PBAE-based NPs induced expression of the reporter protein within six hours. Efficiency in systemic delivery has not been shown yet. Therefore, PBAEs are currently most promising for vaccination purposes. However, in another study it was demonstrated that NPs comprised of lipids are more immunogenic and thus more suitable for vaccination compared to PBAE-based polyplexes[40].
Anderson et al. demonstrate potential for **PAAG** (Fig. 4) NPs as delivery vehicles by showing efficient mRNA delivery in mice[41]. The NPs induce a serum level of the reporter protein which is five times higher compared to NPs comprised of C12-200, a frequently used highly effective lipid. In addition, poly(glycoamidoamine)-based NPs are well tolerated[41].
Another polymer that has shown effectivity *in vivo* is **chitosan**, a bipolymer composed of repeating D-glucosamine and N-acetyl-D-glucosamine units (Fig. 4)[42]. Chitosan coated poly-D,L-lactide-co-glycolide NPs carrying mRNA coding for nuclease correct SP-B deficiency in mice when injected intratracheally[42].
Poly(aspartamide) is often synthetically modified when deployed for nucleic acid delivery[33]. For example, Baba et al. built a copolymer consisting of poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)) (Fig. 4) and PEG. mRNA carrying NPs comprised from this copolymer induce protein expression in the area surrounding the ventricle when administered by intracerebroventricular infusion in mice[43]. The induced protein was neprilysin, a protein important for

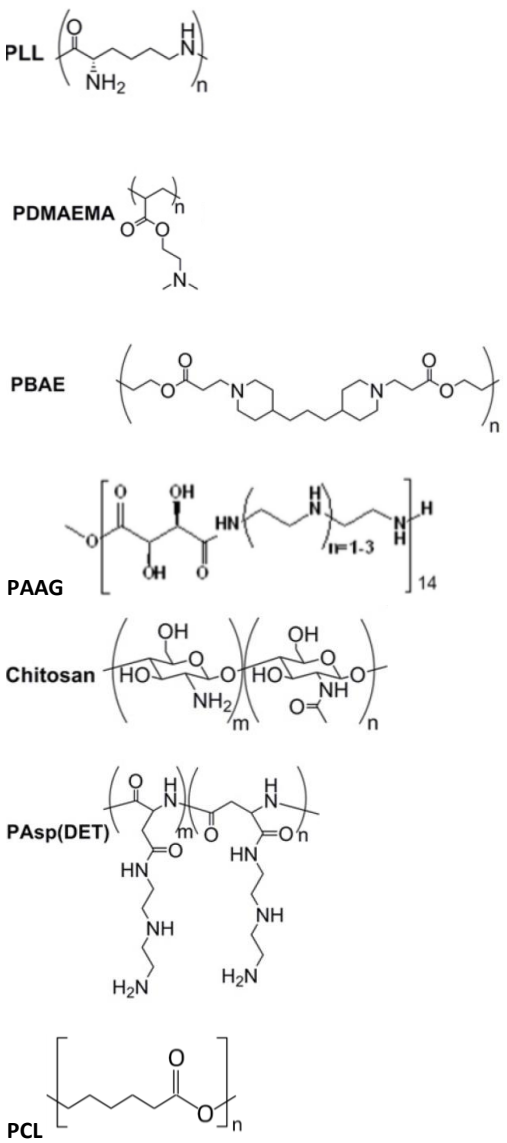

Figure 4: Structure formulas of poly(L-lysine) (PLL), poly(2-dimethylaminoethyl methacrylate) (P(DMAEMA)), poly(β-amino esters) (PBAEs), poly(glycoamidoamines) (PAAG), chitosan, the poly(aspartamide) poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)) and poly(caprolactone) (PCL)

Adapted from: Kauffman KJ, Webber MJ, Anderson DG. Materials for non-viral intracellular delivery of messenger RNA therapeutics. J Control Release. 2016
and Dong Y, Dorkin JR, Wang W, et al. Poly(glycoamidoamine) brushes formulated nanomaterials for systemic siRNA and mRNA delivery in vivo. Nano Lett.

amyloid-beta clearance in the brain. Induction of neprilysin reduces the amount of amyloid-beta that was supplemented in the mouse brains[43]. This PAsp(DET)-PEG copolymer was used in another neurological study as well. Intranasal administration of PAsp(DET)-PEG copolymer NPs carrying mRNA encoding brain derived neurotrophic growth factor improved recovery of sensory function after experimentally induced olfactory dysfunction in mice[1]. Systemic administration of mRNA-polyaspartamide NPs has not yet been reported.

In addition to cationic polymers, neutrally-charged polymers can contribute to nucleic acid delivery as well. For example, the biodegradable neutrally-charged polyester **PCL** (Fig. 4) has been used to encapsulate mRNA/protamine complexes[44]. The PCL shell protects the NP from proteolytic degradation by enzymes in the stomach and can thus be of great value in attaining oral administrable mRNA therapeutics.

3.2.1.3 Lipid-based delivery materials

Complexes formed by interaction between cationic lipids and nucleic acids are termed lipoplexes. Transfection efficiency increases drastically when additional excipients are incorporated into the lipoplexes thus forming stabilized lipid nanoparticles (LNPs). Most commonly used excipients are cholesterol (increases stability), phospholipids (improves the bilayer structure), PEG derivatives (previously discussed) and ionizable lipids[45,27].

The cationic lipid **1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)** and the phospholipid **1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)** are frequently used in NPs. These compounds are also effective in mRNA delivery[4,26,27]. DOTAP is relatively cheap to formulate and is therefore studied extensively. DOTAP-mediated mRNA transfection has been improved by the addition of inorganic additives specifically enhancing endocytosis[46,47]. To be precise, *in vitro* transfection rates increased up to 15 fold compared to when using DOTAP exclusively[47]. Incorporation of fibronectin, a compound targeting α5β1 integrins, into DOTAP NPs enhanced mRNA expression in HeLa cells compared to non-targeted particles[48]. Incorporation of neutral lipids into the complex enhances the endosomal escape in DOTAP-mediated mRNA delivery. DOPE is one of these neutral lipids which facilitates destabilization of the endosomal membranes. In addition, it reduces aggregation of the lipid systems[23,49] thus improving gene expression[31].
DOPE, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) are all phospholipids widely used in nanoparticle formulations. LLNs

containing DOPE show higher efficiency compared to DSPC and POPE formulated nanoparticles[27]. Optimization of the formulation ratio of lipid-like compounds, phospholipids and cholesterol can improve *in vitro* delivery efficiency up to two times[27].

Ionizable lipids are neutrally charged at physiological pH but positively charged in acidic endosomes. This characteristic is proposed to facilitate quick cytosolic release of siRNA by disruption of the endosomal membrane[50]. The specific cationic or ionizable lipid or lipid-like structure that one chooses to incorporate in the NP highly influences both the rate of endocytosis and the rate of gene silencing in siRNAs[33,51]. In addition, transfection using lipid NPs yielded the highest transfection rates in mRNA delivery thus far. Therefore, these lipid- or lipid-like compounds are assumed to be of great importance in successful mRNA delivery. Lipid and lipid-like compounds are the structures most studied in mRNA delivery.
Compounds commonly used for pDNA and siRNA transfection have been improved for mRNA delivery and new compounds were designed as well. The collection of lipids/lipid-like materials used in NPs comprises many different structures ranging from the synthetic lipid **DLinDMA** with an oxygen core, (Fig.) to **amino-alcohol based lipids** (newly formulated by Anderson et al.) and **lipids with polyamine cores and multiple hydrophobic tails** (originally designed for siRNA).

An ionizable lipid which has shown efficiency in *in vivo* siRNA and pDNA delivery, is 1,2-dilinoleyloxy-3-dimethylaminopropane **(DLinDMA)**[52,53]. This lipid is also very potent for mRNA vaccination, as DLinDMA/mRNA complexes elicit immune responses comparable to those in viral delivery techniques in mice[54]. Whereas siRNA molecules commonly consist of ca. 20 bases, the mRNA molecule in this study comprised 9000 nucleotides. Thus, DLinDMA is successful at carrying extremely long RNA molecules; a valuable characteristic in mRNA delivery. Using rational design, DLinDMA has been further optimized for siRNA delivery, resulting in the synthesis of DLin-KC2-DMA, DLin-MC3-DMA, and L319[33]. These compounds are suitable for mRNA delivery as well. This is illustrated by a study by Thess et al. in which DLin-MC3-DMA was used to encapsulate and deliver mRNA coding for EPO in mice, pigs, and non-human primates[33,55]. This resulted in therapeutically relevant concentrations of EPO.

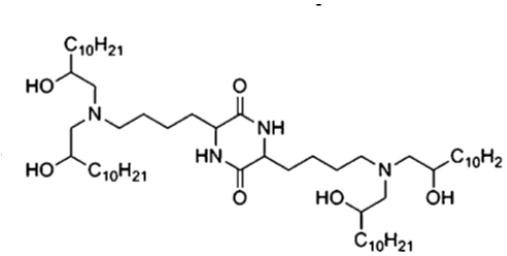

Figure 5: structure formula of cKK-E12 Adapted from: Dong Y, Love KT, Dorkin JR, et al. Lipopeptide nanoparticles for potent and selective siRNA delivery in rodents and nonhuman primates. Proc Natl Acad Sci U S A. 2014

The use of combinatorial synthetic methodology brought us another class of lipid-like materials for siRNA. This class is characterized by **polyamine cores and multiple hydrophobic tails** and comprises some of the most potent lipid-like materials for siRNA delivery[33]. Examples of lipids in this class are C12-200, cKK-E12 (Fig. 5), and 503O13. Although originally developed for siRNA, these materials can be optimized for mRNA delivery by varying the different parameters of LNP formulation. These parameters include lipid weight ratios, the type of phospholipid, and the composition of other excipients. Optimal adjustments increase *in vivo* potency of C12-200 LNPs over sevenfold[4]. The adjustments involved among others an increase in the lipid:mRNA ratio and the addition of DOPE to the NP. The fact that these adjustments did not improve siRNA potency underlines the importance of mRNA tailored delivery systems[4]. Another member of the polyamine-core-and-hydrophobic-tails class, C14-113, showed mRNA delivery efficiency in pigs[56]. Stabilized by distearoyl-phosphatidylcholine, cholesterol and a PEG-lipid conjugate, C14-133 LNPs induced reporter protein expression throughout the ventricular wall upon both direct myocardial injection and percutaneous intracoronary delivery[56].

Among a wide variety of lipid-like compounds, **amino alcohol-based lipids** have shown superior activity in *in vivo* siRNA mediated gene silencing[57]. Anderson et al. synthesized novel bioinspired amino alcohol-based lipids for mRNA delivery[45]. This study revealed that alternative numbers and placement of double bonds in these lipids greatly influences *in vivo* transfection efficiency. It can even make the

difference between meaningful serum protein concentrations and negligible serum protein concentrations. One tested alkene with trans geometry did not promote *in vivo* efficacy, which could mean that only cis oriented alkenes in LNPs are suitable for mRNA delivery. However, to confirm this postulation, more cis oriented alkenes should be tested. Another finding was that the optimal number of double bonds is two per tail for mRNA delivery, which is true for siRNA NPs as well. In addition, the optimal placement of the double bonds is similar to optimized siRNA LNPs as well[45,58,52].
The amino-alcohol-based lipid that performed best in the study of Anderson et al. [49] is called OF-02 and induces a EPO secretion twice as high as cKK-E12 NPs[45], a frequently used ionizable lipid in the siRNA delivery field which is a member of the polyamine core lipids[59]. cKK-E12 differs from OF-02 by the absence of double bonds and the much shorter carbon chains in its tails. The structure formula for the tails of cKK-E12 is $C_{10}H_{21}$ (Fig. 5) where it is $C_{16}H_{29}$ for OF-02[45]. The increased protein expression induced by OF-02 is not caused by a variant biodistribution as the protein shows nearly identical distribution when with cKK-E12 NPs. These results plead for longer tails in amino-alcohol-based lipids. OF-02 also outperforms benchmark polyamine core lipids 503013 and C12-200 in terms of *in vivo* EPO production[45].
Next to the number and placement of double bonds, the placement of functional groups influences mRNA transfection rates in amino-alcohol based lipids as well[27]. Li et al. investigated this in a series of lipid-like derivatives containing the same six-membered ring 1,3,5-triazinane-2,4,6-trione (TNT). By exchanging the position of hydroxyl and amino groups, effects of placement of these functional groups on delivery efficiency were investigated. Optimal placement can improve *in vitro* mRNA delivery efficiency up to two times[27].

3.2.2 TECHNIQUES FOR TARGETING

A method that might overcome the problem of arrival in off-target tissues is formed by the targeted delivery of drug-loaded carriers. The two most advanced approaches of targeting are passive and active targeting. Passive targeting is based on the combination of longevity of the carrier-system in the blood, compromised vasculature and the consequential accumulation of the drug in pathological sites. An example of passive targeting is the increased accumulation of liposomes and NPs in tumors, referred to as the enhanced permeability and retention (EPR) effect. This accumulation is generally explained by the abnormal architecture of the endothelium of the tumorous neovasculature. Poor alignment of widely fenestrated endothelial cells and ineffective lymphatic drainage cause the drugs to accumulate inside the tumor[60]. *(Vladimir P. Torchilin, Volume 197 of the series Handbook of Experimental Pharmacology)* As a result of the natural presence of sinusoids and fenestrated endothelium, enhanced permeability for nanometer-sized particles is also seen in the liver and to a lesser extent in the spleen the kidney. However, passing the vascular wall on the right place does not necessarily lead to uptake by the target cells. Unfortunately, most nanoparticles end up in cells of the mononuclear phagocytic system. In the liver, the Kupffer cells tend to endocytose most nanoparticles. Therefore, a lower percentage of the drug will end up in hepatocytes. In rodents, intravenously administrated nanometer-sized particles (100–300 nanometer) labeled with ^{14}C or ^{111}In mainly accumulate in the liver and the spleen[61-63]. Although the lungs are the first organs the NPs pass after intravenous injection, only a small percentage of the NPs accumulates in the lungs[64]. Targeting of the lung with lipids forming micron-sized aggregates carrying siRNA has shown to be efficient[65]. In addition, albumin-based NPs carrying methotrexate and doxorubicin-loaded solid lipid NPs are shown to accumulate in the lungs[66]. Another often used administration route for passive lung targeting is inhalation[67,68].
Nanoparticles smaller than 10 nanometer (InAs(ZnS) quantum dots) accumulate in the liver, kidney and the intestines within an hour after administration[69]. Bio-distribution to the lungs is extremely low for this size of nanoparticles. However, by varying the chain length of the PEG polymers coating these NPs, the organ-specific biodistribution can be altered[69].

In active targeting, specific ligands attached to the surface of the drug carrier-system recognize and bind specific cells. *(Vladimir P. Torchilin, Volume 197 of the series Handbook of Experimental Pharmacology).* Targeting to inflamed endothelial cells in the microvasculature is already proven to be

possible for siRNA[70]. The carriers used in this study are so called SAINT-O-Somes covered with antibodies against vascular cell adhesion protein 1 (VCAM-1). This technique might have value in the targeted delivery of mRNA as well.

3.2.3 ELECTROPORATION

Electroporation is the most widely used physical method for nucleic acid delivery[20]. In electroporation, external, low-voltage electrical pulses transiently destabilize the lipid bilayer thereby forming pores in the cell membrane. The electric pulses subsequently drive the nucleic acids towards the cells by electrophoreses causing the mRNA to enter the cell *via* the pores[71].

Electroporation is associated with a high cellular mortality rate. However, this method is sometimes preferred over lipid-based transfection as its efficiency is higher in some cell types like for example hematopoietic cells[72].

Electroporation is most commonly used *in vitro.* However, *in vivo* application is possible for superficial tissues as well, as has been shown for pDNA delivery[73,74].

The fact that crossing the nuclear membrane is not required for efficient mRNA delivery brings advantages for electroporation as a delivery method as well. mRNA electroporation requires lower voltages compared to pDNA electroporation[75]. mRNA electroporation is therefore associated with lower cell toxicity compared to pDNA electroporation as was demonstrated in myelogenous leukemic cells (a mortality rate of 15% compared to 51% respectively)[75]. In addition, transfection rates are higher for mRNA when compared to pDNA (89% versus 40% respectively).

Ex vivo mRNA transfection using electroporation has already proven its efficiency. For example, dendritic cells transfected with mRNA encoding prostate-specific antigen (PSA) stimulated cytotoxic T cell responses against prostate metastases in a clinical study[76]. Moreover, over 50 clinical trials involving electroporation-mediated DNA vaccination were conducted in 2015[77]. Next to dendritic cells, *ex vivo* transfection of other blood cell types is efficient as well. For example, transfection percentages for mRNA encoding GFP were 35% in hematopoietic progenitor cells, 90% in mesenchymal cells and 50% in PHA-stimulated T cells[78].

In vivo electroporation-mediated transfection of mRNA has not been performed yet. However, mRNA might outperform pDNA in *in vivo* electroporation.

3.2.4 GENE GUN DELIVERY

Gene gun or biolistic delivery is a method in which heavy metal particles are coated with nucleic acids. Using a hand-held device, superficial tissues are bombarded with these particles resulting in penetration of the cell membrane and subsequent transfection[13]. Already back in 1996, this technology enabled the *in vivo* transfection of three mRNA molecules encoding different reporter genes in rodent liver- and epidermal tissues[79]. In the same study, gene gun delivery of alpha-1 antitrypsin mRNA induced a strong cellular immune response in mice[79]. In 2001, gene gun delivery of mRNA encoding human epidermal growth factor facilitated wound healing in rats[80]. A more recent study implicated apossible role for gene gun mRNA delivery in treating the sensory epithelium of the inner ear[3]. This method is especially suitable for this tissue as it works well with terminally differentiated cells like cells of the sensory epithelium. However, this study also showed that gene gun delivery is associated with many disadvantages of which low transfection efficiency due to the many adjustable variables is the most important. In addition, gene gun delivery can cause mechanical damage to the ear if not carefully finetuned[3]. Gene gun-mediated delivery of RNA has shown relevance in the skin disease dystrophic epidermolysis bullosa as well[81]. Efficient transfection of skin cells with a circular 5' RNA molecule efficiently corrects the aberrant transcript resulting from the genetic mutation in mice[81]. Gene gun mRNA delivery is currently applied very little. However, more research has been performed on gene gun delivery of pDNA delivery. A clinical trial on vaccinating patients suffering from a certain type of cervical intraepithelial neoplasia using gene gun delivery is currently being performed (https://clinicaltrials.gov/ct2/show/NCT00988559).

As the heavy metal particles used in gene gun delivery are commonly (non-degradable) gold particles, several studies aimed on finding biodegradable and less expensive nanoparticles for pDNA gene gun delivery. For example, a study performed in 2016 demonstrates that bacterial spores could replace the

heavy metal particles in *in vivo* gene gun delivery of pDNA vaccines[82]. The spores would thereby form an easy and low cost alternative.
A major downside to gene gun delivery is its restriction to superficial tissues. This is not a problem for vaccination purposes however, as here the antigen presenting cells located in the epidermis can be targeted[82]. As mRNA has the advantage of inducing transient protein expression which is perfect for vaccination purposes and does not induce threatening permanent mutagenesis, gene gun delivery of mRNA might be of even greater therapeutic value compared to pDNA gene gun delivery.

4. CONCLUSION

The amount of mRNA-based therapeutics that is taken up in the cell differs between cell types. Cell types that generally do not take up much mRNA-therapeutics are treated best with physical delivery methods, provided that the cell type is physically attainable or can temporarily be taken out of the body for *ex vivo* treatment. As high transfection rates in hematopoietic cells are especially achieved using electroporation, this method is the most suitable method for mRNA transfection for vaccination. As more research has been performed on electroporation than on gene gun delivery, and electroporation is cheaper, electroporation is the most promising physical method for *ex vivo* mRNA delivery. More research has to be performed on the *in vivo* application of physical methods for mRNA delivery.
Cell types that do take up a meaningful amount of mRNA-based therapeutics through endocytosis and are not suitable for physical methods are best treated with chemical delivery methods. This is because chemical methods are less toxic and allow systemic application.
The type of delivery material (protamine, polymers or lipids) that is most potent differs between cell types. For example, lipid-based materials can be most suitable for transfection of cell type A while polymer-based materials are most suitable for cell type B. However, as there is a lot of variation between the potency of different compounds of one class of delivery materials, matching a cell type to a certain delivery material might be too simplistic. In fact, adding a new compound or changing the ratio of the compounds in a NP can drastically influence the potency of a NP as well. It is hard to find general principles in mRNA delivery as so many variables are involved. However, finding out why a certain material/ratio/modification works best is very important, as it can help designing delivery units matching certain cell types in a directed way.
The transfected mRNA molecules will finally have to reside in the cytoplasm, no matter which delivery method has been used. Modifications stabilizing the mRNA molecules and improving translation efficiency are therefore valuable in combination with every delivery method. Exploration of different categories of modifications has been started, but there is much room for optimization. Co-transfection of compounds increasing stability or translation efficiency might be an option.
As the research on mRNA delivery is still in its infancy, for now it is the best to keep experiment with all materials, excipients and mRNA modifications previously mentioned in this essay. By adjusting only one variable at a time we might unravel the rationale behind well performing NPs. Because active targeting influences cellular uptake as well, it is important to already integrate the targeting approaches in upcoming research.
In conclusion, although the general principles behind effective mRNA delivery remain largely unclear, several techniques generated effective mRNA transfection. As mRNA-based therapeutics have many applications this pleads for more research in this field.

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