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Strategies for modulating innate immune activation and protein production of in vitro transcribed mRNAs

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Abstract

Synthetic mRNA has recently shown great potential as a tool for genetic introduction of proteins. Its utility as a gene carrier has been demonstrated in several studies for both the introduction of therapeutic proteins and subunit vaccines. At one point, *synthetic* mRNA was believed to be too immunogenic and labile for pharmaceutical purposes. However, the development of several strategies have enabled mRNA technology to overcome these challenges, including incorporation of modified nucleotides, codon optimization of the coding region, incorporation of untranslated regions into the mRNA, and the use of delivery vehicles. While these approaches have shown to enhance performance of some mRNA constructs, gene-to-gene variation and low efficiency of mRNA protein production are still significant hurdles. Further mechanistic understanding of how these strategies affect protein production and innate immune activation is needed for the widespread adoption for both therapeutic and vaccine applications. This review highlights key studies involved in the development of strategies employed to increase protein expression and control the immunogenicity of synthetic mRNA. Areas in the literature where improved understanding is needed will also be discussed.

1. Introduction: mRNA as an advanced and unique tool for directed protein production

Synthetic messenger RNA *(*mRNA) produced via *in vitro* transcription (IVT mRNA) has emerged as an appealing tool for transient introduction of exogenous genes, both for therapeutic and vaccination applications. Protein expression driven by exogenously delivered nucleic acids, either mRNA or the more commonly used plasmid DNA, boasts unique characteristics compared to the use of recombinant proteins. One advantage of using nucleic acids to deliver protein is host-driven protein production. Appropriate post translational modifications of proteins in recombinant systems can be difficult to achieve (1), and using nucleic acids to drive protein production by the host often circumvents this problem. Additionally, protein introduction through delivery of nucleic acids offers different spatiotemporal characteristics compared to bolus protein injection. Construct design can potentially control the half-life and translation rate of IVT mRNA; qualities that are both desirable and difficult to achieve with bolus protein injection. Furthermore, nucleic acids can be made in nearly cell-free systems in large quantity, allowing for rapid and relatively easy production compared to recombinant proteins.

While protein expression driven by IVT mRNA and plasmid DNA share many characteristics, there are distinct differences between the two. IVT mRNA is clearly transient, which may be beneficial in certain applications, as improved control over protein expression kinetics and

dosing is possible. For some cell types, IVT mRNA may also lead to more effective protein production, as it only needs to enter the cytoplasm; where pDNA needs to enter the nucleus of cells to direct protein production (2) . This has particularly been demonstrated in non-dividing cells (3,4).

While the concept of using IVT mRNA as a medium for gene delivery was established in the late 1980s (5), protein expression was previously hampered by cellular antiviral responses characterized by high interferon expression and death. Since then, advances in IVT mRNA manufacturing and design have enabled more effective mRNA constructs. These advances include the substitution of modified nucleotides for their unmodified counterparts (6,7), codon optimization and the use of untranslated regions (UTRs) in the mRNA construct to enhance IVT mRNA functionality (8,9), the purification of IVT mRNA to remove immunogenic reaction contaminants from its synthesis (10), and enzymatic removal of 5' phosphates on uncapped IVT mRNAs (11). As a result, recent studies have demonstrated the translational potential of IVT mRNA.

A main thrust of development has been in IVT mRNA's utility as a carrier to delivery therapeutic proteins. This body of work has predominately benefited from incorporation of modified nucleotides in the mRNA, which show reduced interferon responses and enhanced protein production. A surge in landmark studies showing IVT mRNA's use for expressing therapeutic proteins began in 2011, where Kormann et al showed that mRNA substituting the modified nucleotides 2-thiouridine and 5-methylcytodine for uridine and cytosine, respectively, resulted in reduced immunogenicity and higher stability in mice. Their modified IVT mRNA was tested for functionality in two different disease models: (1) intramuscular injection of IVT mRNA encoding erythropoietin was shown to increase the hematocrit in mice, (2) aerosol delivery of IVT mRNA encoding surfactant protein B prolonged survival of mice in a lethal congenital lung disease model (7). Shortly after, a 2012 study by Kariko et al showed that HPLC purified, codon optimized IVT mRNA substituting the modified nucleotide pseudouridine for uridine reduced immunogenicity of IVT mRNA. The authors also made erythropoietin IVT mRNA and demonstrated that sub-microgram quantities of IVT mRNA could be used to attain functional outcomes (12). In 2013, another group showed that IVT mRNA incorporating the modified nucleotides 5-methylcytosine and pseudouridine and encoding for vascular endothelial growth factor A could assist in vascular regeneration of the heart (13) and have therapeutic benefits following myocardial infarction (14). Figure 1 outlines the elements of IVT mRNA as well as areas that can be engineered to affect mRNA regulation and function.

IVT mRNA's utility as a subunit vaccine has been demonstrated for cancer immunotherapy, allergy prevention, and infectious disease applications. For these applications, researchers have often opted to use unmodified nucleotides, as the innate immune activation by IVT mRNA is believed to be advantageous for directing immune responses against the encoded protein. IVT mRNA has shown efficacy as an immunotherapy in several preclinical cancer models, where it is now in clinical trials (15-19). Additionally, IVT mRNA has been explored for the use as a prophylactic vaccine against allergies. One study tested IVT mRNA vaccines for the ability to prevent allergy development in animal models for 29 different allergens, and showed that allergy could be reduced or prevented in a majority of the cases (20). Petsch et al showed that IVT mRNA encoding influenza antigens could elicit protective immunity when given intradermally. Here, IVT mRNA contained an open reading frame that was codon optimized for GC enrichment, and included beneficial UTRs; the IVT mRNA was further complexed to protamine to enhance innate immune activation. The vaccine showed a robust potency that rivaled the currently inactivated influenza vaccine in three different animal models (9). A myriad of investigations have also been conducted studying the ex vivo introduction of mRNA into cells.

These studies won't be discussed in this review, but more information is available in the literature (21,22).

While IVT mRNA has proven successful in multiple applications, there remain challenges that impede its widespread use. IVT mRNA is limited by the amount of protein it can generate and the fundamental reasons why are unclear. Further, innate immune activation is critical to vaccine applications: understanding how mRNA acts as an immunological activator will be important to designing mRNA based vaccines. The potential bottlenecks of IVT mRNA's functionality and areas for improvement will be discussed further in the review.

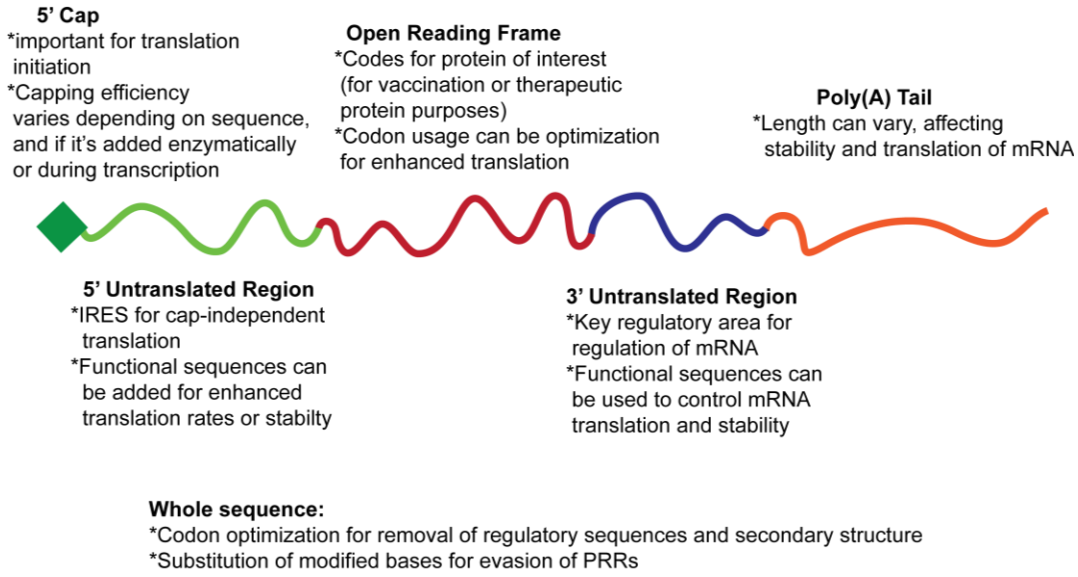

Figure 1. Diagram of IVT mRNA and areas for engineering its function. IVT mRNA consists of a 5' cap, 5' untranslated region, open reading frame, 3' untranslated region, and a poly(A) tail. Each of these areas can be engineered to affect IVT mRNA regulation and function.

2. Transcription and Translation of Endogenous mRNAs vs. Exogenously Delivered IVT mRNA

Translation of mRNA requires its interaction with host factors to form functional ribonucleoproteins (RNPs). These host factors regulate translation, localization, and half-life of mRNA in a complex and dynamic manner. Despite the importance of RNP formation for mRNA performance, it's unclear at what rate IVT mRNAs form RNPs, or how functional these RNPs are compared to endogenous RNPs. Here, we discuss the regulation of endogenous mRNAs to highlight some of the complexities involved in delivery of IVT mRNA.

2.1 Transcription and Translation of Endogenous mRNAs

Localization, half-life, and protein expression of endogenous mRNAs are tightly regulated. Regulation of endogenous mRNAs is dynamic and complex: at least several hundred different proteins are involved (23,24). Endogenous mRNAs are transcribed in the nucleus, where they concurrently bind to a variety of trans-acting factors, immediately becoming RNPs. In the nucleus, there is evidence that mRNAs undergo a process termed *mRNA imprinting*, which is believed to later assist the cell in differentiating host mRNAs from foreign mRNAs. The process of mRNA imprinting includes the addition of a 5' cap and binding of the cap binding complex;

addition of a poly(A) tail and binding of a nuclear form of the poly(A) binding protein (PABP); splicing; and the binding of a variety of other trans-acting factors. During splicing, as introns are removed from the mRNA, exon junction complexes (EJCs) are deposited upstream of adjoined exon-exon junctions and serine and arginine-rich proteins bind to the mRNA. These factors, along with other cis and trans acting factors can direct a variety of regulatory processes, including mRNA transport, translation, and surveillance mechanisms; even future localization of the encoded protein (25-27). Interestingly, mRNAs that undergo splicing are associated with higher translation rates compared to mRNAs that don't, an effect that is at least partially mediated by EJCs (26).

Once in the cytoplasm, mRNAs undergo a pioneering round of translation. Here, the nuclear cap binding complex facilitates ribosome binding to the mRNA. Following, the cap binding complex is replaced by eIF4e, EJCs may be displaced, and mRNA enters steady state translation. It's supposed that at this time the PABP and eIF4e mediate mRNA circularization to facilitate ribosome recycling- so that ribosomes that recently terminated translation are near the start codon to begin with the translation of a new protein (28). UTRs in mRNA may also affect its localization, translation, and half-life (29-31). A variety of decay mechanisms regulate mRNA half-life. Surveillance mechanisms check for abnormal or unwanted mRNAs and can lead to mRNA degradation.

2.2 Transcription and Translation of Exogenously Delivered mRNAs

One stark contrast between endogenously made and exogenously delivered mRNAs is that endogenous mRNAs are always known to be in RNPs in the cytoplasm, where IVT mRNA is typically delivered without any bound proteins. IVT mRNA has been shown to often enter the cell via the endolysosomal system (32,33). A study by Bire et al nicely followed cellular entry pathways of Polyethyleneimine-IVT mRNA nanoparticles, and showed that mRNA entered cells via both clathrin and caveolin dependent pathways. Their study suggested that endosomal escape poses a significant barrier to IVT mRNA effectiveness (33). Endosomal localization allows for IVT mRNA to interact with TLR7 and TLR3, PRRs that endogenous mRNAs do not normally come into contact with. Once escaped from the endosomal system, IVT mRNA may need to dissociate from a delivery vehicle, which could pose another barrier to achieving effectiveness. IVT mRNA may end up in stress granules, where it is either degraded or stored for future use (33,34).

Throughout its extracellular, endosomal, and cytosolic presence, IVT mRNA may interact with pattern recognition receptors (PRRs), inducing antiviral immune responses, which is further discussed in Section 3. IVT mRNA then needs to interact with translation initiation factors and ribosomes for protein expression. Unfortunately, there is little understanding regarding mRNA cytosolic entry into cells, factors regulating its degradation, and its ability to form functional RNPs for protein translation.

3. Pathogen Recognition Receptors Sensing of mRNA: Benefits and Drawbacks

Subunit vaccines require effective immune stimulation to elicit protective immune responses (35), and many studies have touted the self-adjuvant effect of mRNA as a compelling motivation to use it as a vaccination platform. However, some of the cellular innate immune responses to IVT mRNA inhibit its protein production and can lead to cell death. Table 1 outlines PRRs that are known or suspected to be involved in responding to exogenously delivered mRNA, and their

cellular location and functionalities are described in Figure 2. This section of the review will discuss the relative costs and benefits of different PRR activation.

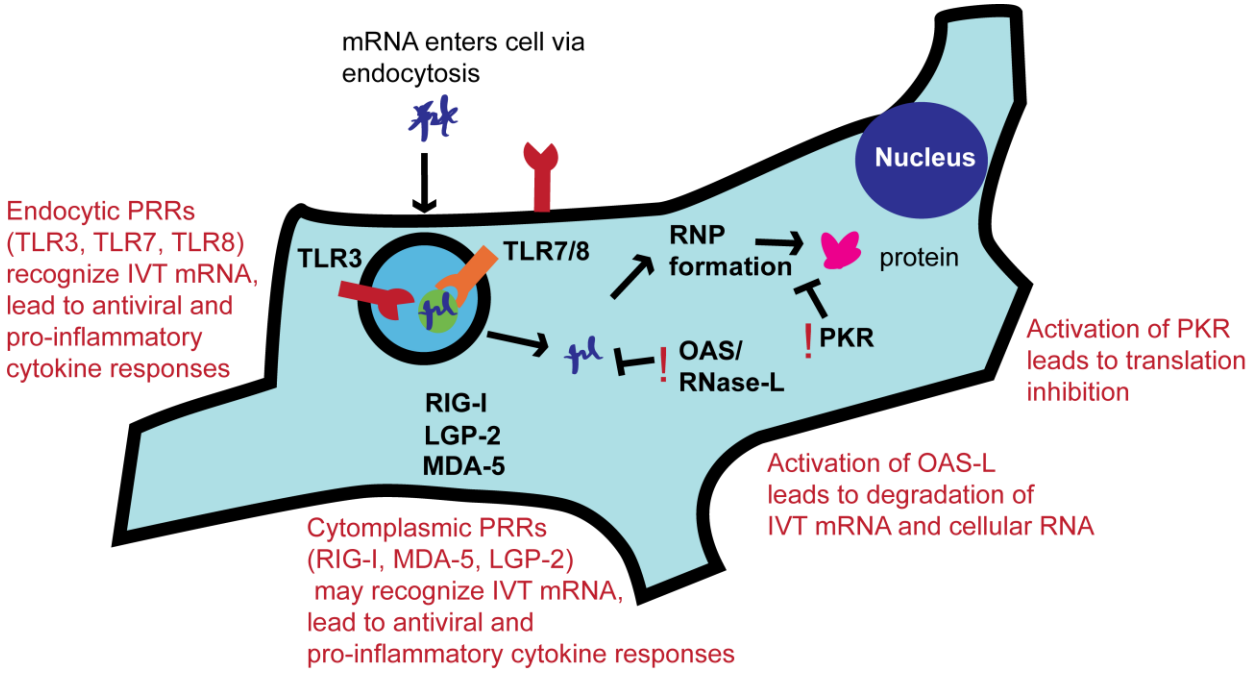

Figure 2. IVT mRNA enters into the cell via the endolysosomal system. IVT mRNA may interact with PRRs in the extracellular space (TLR3) or in the endosomal system (TLR3, TLR7, or TLR8). Once escaped, cytosolic IVT mRNA is available for interaction with additional PRRs, including RIG-I, MDA-5, LGP-2, PKR, and OAS-L. Activation of the OAS pathway and PKR lead to degradation of RNA and inhibition of protein production, possibly causing cell death. Activation of other PRRs leads to generation of type I interferon as well as inflammatory cytokines. This triggers a positive feedback loop where PRR expression is upregulated.

IVT mRNA activates antiviral defenses through type I interferon and NF-κB pathways. This initiates a positive feedback loop through autocrine and paracrine signaling to heighten cellular antiviral defenses (36). As a result, the dsRNA-dependent protein kinase (PKR) and 2'-5'-Oligoadenylate Synthetase-Like (OAS-L), along with other PRR genes, are upregulated (11). Both PKR and OAS-L recognize unmodified IVT mRNA (37,38). PKR and OAS-L pathways are likely the most detrimental antiviral pathways to IVT mRNA delivery, as they both inhibit protein expression (both of endogenous mRNA and IVT mRNA). When PKR is activated, it phosphorylates the initiation factor eIF2α, arresting initiation of protein translation, both from delivered and host mRNA. OAS-L responds to IVT mRNA detection by activating the latent RNase-L, which degrades rRNA and mRNA—reducing ectopic protein expression, cell proliferation, and potentially causing cell death (11).

The deleterious effects of an overactive innate immune response to mRNA delivery were demonstrated by Pollard et al. The group showed that an mRNA vaccine for HIV could generate potent T cell immunity against the HIV gag protein. However, they were curious by the high interferon responses initiated following IVT mRNA injection. To explore this, they vaccinated either interferon alpha receptor deficient or wild type mice with their vaccine and showed that mice interferon alpha receptor had significantly increased gag-specific T cell responses (39). This finding clearly demonstrates that certain aspects of IVT mRNA's adjuvant effect may be too strong for certain vaccinations strategies. However, Pollard et al did not identify why interferon responses inhibited vaccination efficacy.

In addition to PKR and OAS-L, exogenous delivery of IVT mRNA is also known to either activate or interact with TLR3 (40), TLR7 (41), TLR8 (7) , and RIG-I (6). Activation of some of these PRRs has shown to be centrally important in efficacy of different vaccines (20,42-44). For example, Mleczek et al showed that for an anti-cancer vaccine, the use of mRNA delivery formulations that enhanced TLR7 activation (complexing mRNA to protamine) led to enhanced tumor regression and differential serum cytokines (41). It's important to keep in mind that different vaccines may require stimulation by different PRR(s) to induce immunological protection, and dual PRR stimulation may be also be highly beneficial (45). The ability to reduce certain PRR activation (such as PKR and OAS-L) while potentially enhancing activation of other PRRs (such as TLRs and RLRs) could prove beneficial for next generation mRNA vaccines.

It may be surprising how IVT mRNA is recognized by antiviral receptors while endogenous mRNAs are not. Both extracellular and endosomal presence of the mRNA may allow for enhanced PRR activation. Additionally, IVT mRNAs likely develop secondary structures, a pattern that many PRRs recognize. The formation of secondary structure in endogenous RNA may be hampered by RNP formation.

Table 1. PRRs known or suspected to respond to exogenous mRNA delivery

Pattern Recognition Receptor	**Ligand**	**Commentary**		**Key References**
PKR*	Uncapped mRNA, RNA with complex secondary structure such as a pseudoknot	Phosphorylation of EIF-2α (inhibition of translation)		(37,46,47)
OAS-L*	dsRNA	Leads to activation of RNase-L, mRNA and rRNA degradation, possibly apoptosis, and activation of RIG-I		(38,48-50)
MDA-5	Long dsRNA	Interferon, inflammatory cytokine production		(53)
RIGI-I*	5' triphosphate with dsRNA; complex secondary structure in RNA; RNase-L digestion products	Consistently expressed across cell types (51,52)		(7,54-56)
LGP2	dsRNA	Assists and modulates MDA-5 interaction with RNA		(57)
TLR3*	dsRNA	Active in the plasma membrane and endosomes	Expressed differentially across cell types	(7,40,58)
mTLR7*/ hTLR8*	ssRNA (GU rich RNA)	Active in endosomes		(7,59-61)
hTLR7*	Possibly ssRNA (G rich ssRNA)			(7,51,61,62)

*exogenously delivered IVT mRNA has been verified to activate or interact with the receptor

4. Strategies to modulate properties of mRNA:

4.1 Modified Bases: One way to modulate mRNA interaction with innate immunity and is by substituting modified bases into the mRNA strand for their unmodified counterpart. Endogenous

mRNAs (mRNA, rRNA, tRNA, and even viral RNA) are naturally modified (such as by addition of a methyl or thiol group) in over a hundred different ways (63). These modifications are known to influence RNA secondary structure (64-67), regulate gene expression (68-71), and influence RNA detection by PRRs (72-74). IVT mRNAs containing combinations of 5-methylcytosine (7,11), 2-thiouridine (7,75), 5-methyluridine (37), 6-methyladenosine (6,37), and most notably, pseudouridine (6,12,37,38) have been explored, and have each shown to impact IVT mRNA performance differently. Pioneering work by Kariko identified that that substitution of uridine with pseudouridine may diminish PKR activation (37), reduce RNase-L activity (38), and ultimately produce enhanced levels of protein (6,37). Further work has shown that additional incorporation of 5-methylcytosine, an analog of cytosine, can further lead to increased protein production (11). However, mRNA transgene protein production doesn't always seem to increase upon substitution of these modified bases; benefits associated with incorporation of modified bases are likely gene- and sequence-specific, as shown in (8).

While pseudouridine has been shown to markedly increase mRNA transfection, it can influence mRNA in other ways that may prove disadvantageous. Pseudouridine presence in the stop codon has been shown to allow non-canonical base pairing and suppression of translation termination (76-78). This is clearly an issue for IVT mRNA, but as delivered mRNA are degraded within cells and the nucleotides available for endogenous transcription, this may be an issue for endogenous mRNA production as well. As IVT mRNA, to date, is synthesized by incubating a DNA template, RNA polymerase, and the ribonucleotides of interest in a single reaction, there is no control over pseudouridine location in the mRNA strand, and it is always included in the stop codon. If pseudouridine use is to be pursued, further work is needed to either direct locations of RNA modifications or improve our understanding of translation suppression and how it can be avoided for *in vitro* transcribed mRNAs. Inclusion of modified bases in IVT mRNA has also been shown to reduce functionality of UTR sequences (8). Modified bases have been shown to also affect translation initiation, localization, and siRNA binding in endogenous genes, and likely affect these processes in IVT mRNA as well (72).

4.2 Codon Optimization: The degeneracy of the genetic code allows for multiple codons to be specific for the same amino acid. Codon choice has been known to affect transgene expression for decades, and has been studied extensively. In some cases, codon optimization of DNA plasmids has improved protein yield over 1,000 fold (79). Codon choice may influence protein expression in a variety of ways, including mRNA export from the nucleus, mRNA stability in the nucleus, rate of translation, error rate in the protein sequence, mRNA half-life (80), and even protein folding and function (81-83). Most commonly, codon optimization is algorithms focus on matching the codon usage bias of a species, along with reducing mRNA secondary structure (84-86), enhancing GC content, and avoiding regulatory sequences in the mRNA (87,88). Several excellent reviews on the topic are available here (82,89,90).

There has been some exploration regarding the use of codon optimization specifically for IVT mRNA. Kariko et al used a codon optimized sequence for IVT mRNA encoding the hormone erythropoietin, and showed that it performed better than the wild type sequence. Interestingly, the benefit of codon optimization only held true when pseudouridine was substituted for uridine in the transcript; when unmodified bases were used, codon optimization did not enhance protein production (12). A paper written by researchers at CureVac showed that GC enrichment of an mRNA sequence encoding for either luciferase or erythropoietin could produce markedly increased levels of protein. Similar to Kariko's paper (12), CureVac also explored how incorporation of pseudouridine influenced transgene expression. However, the authors found a reverse trend from Kariko's paper: for both luciferase and erythropoietin encoding mRNA, pseudouridine substitution enhanced protein production in the non-optimized sequences, but

reduced protein production in the codon optimized sequences (8). The disparate influences pseudouridine incorporation and codon optimization has on IVT mRNA sequences, suggests there remains more to learn regarding how codon optimization and pseudouridine substitution influences expression of exogenously delivered mRNA.

4.3 The 3' and 5' Untranslated Regions: Engineering 5' and 3' UTR sequences into mRNA that regulate its functions is an obvious strategy for exerting control over exogenously delivered mRNA. UTRs in endogenous mRNAs can interact with trans acting factors to influence mRNA characteristics such as half-life and translation rates. Several studies have incorporated UTRs into mRNA molecules from mRNAs with known long half-lives. For example, Warren et al included the α-globin 3'UTR sequence (11); Thess et al included the 3'UTR from albumin (8), and Korman et al included the β-globin 3' UTR (7), Holtkamp et al included two sequential β-globin 3'UTR sequences (91). However, there has been little, if any, discussion regarding if these sequences influenced IVT mRNA in the way expected. As exogenously delivered mRNA likely does not come into contact with the trans acting factors in the same way that endogenously delivered mRNA does, it's possible that engineered UTRs will function differently in endogenous and exogenously delivered mRNAs.

A supplementary figure in a study by Thess et al showed that mRNA incorporating untranslated regions comprised of a 5' UTR from hydroxysteroid (17-beta) dehydrogenase 4 (HSD17b4), a 3'UTR from albumin, a poly(A) tail, and a histone stem loop led to increased protein levels compared to mRNA incorporating a different 3'UTR and poly(A) tail. Unfortunately, the study did not discuss a mechanism regarding how the UTR regions influenced transgene production. It's also unclear if these UTR sequences could be used universally used with any coding region. The authors further found that pseudouridine incorporation reduced the supposed effectiveness of the UTR sequences (8).

In the same study, the authors tested the functionality of incorporating an internal ribosome entry site (IRES) sequence. IRES sequences are best known to be a functional element in viruses to recruit ribosomes and facilitate cap independent translation. They facilitate protein translation while the cell is under stress, when cap dependent translation is impaired. Here, the authors constructed a bicistronic mRNA coding for luciferase downstream of an IRES sequence from the encephalomyocarditis virus and found that it allowed for cap independent translation. Again, pseudouridine was found to impair IRES functionality (8).

4.4 Delivery Strategies: Many degrees of freedom surround developing the appropriate delivery strategy for IVT mRNA. The route (intradermal, subcutaneous, etc), mechanism (electroporation, injection, gene gun, etc), and formulation (lipid nanoparticle, polymer nanoparticle, etc) can all be varied, and any combination may be warranted for a particular application. The ultimate goal of most IVT mRNA delivery applications is to produce adequate levels of the desired protein in the anatomical region of interest. To this end, delivery vehicles have been designed to mitigate nuclease degradation of IVT mRNA, enhance its cellular uptake, and facilitate it's escape from the endolysosomal system. While the field of IVT mRNA delivery is relatively young, many previously identified strategies for delivery of plasmid DNA or siRNA are applicable. Applicable reviews on DNA or siRNA delivery will be useful for designing mRNA delivery and some excellent ones are available (5,92-95).

Many concepts surrounding delivery of siRNA and plasmid DNA also apply to IVT mRNA; however, there are distinct differences that should be considered. Where IVT mRNA applications aim to introduce exogenous proteins into a host, siRNA applications aim to *reduce* endogenous proteins levels. This key difference may greatly affect delivery parameters for delivery of the nucleic acids. For siRNA to be most effective, applications may require its

introduction into as many cells as possible. Further, a controlled, steady release of siRNA into the cytoplasm of cells may be required. For IVT mRNA, achieving transfection in a wide variety of cells may not be as crucial, especially if the protein is secreted extracellularly and then dispersed throughout the body. Instead, the goal of IVT mRNA delivery may be to simply achieve high levels of ectopic protein expression in an appropriate area of the body for the given application. Also, while siRNA has been shown to stimulate the immune system through TLR7 recognition (96), concern of immunostimulation by IVT mRNA may be greater, as it is recognized by a more diverse array of PRRs(5).

Although the clinical applications of IVT mRNA overlap more with plasmid DNA compared to siRNA, there are still some key differences in the design requirements for optimal delivery each nucleic acid. Plasmid DNA needs to be delivered to the nucleus to be functional and thus targeted delivery to mitotic cells is important. IVT mRNA; however, does not have this limitation, as it is functional in the cytoplasm of cells. Also, plasmid DNA is immensely less susceptible to nuclease degradation and thus more stable than IVT mRNA. While nuclease protection and quick cytoplasmic intracellular uptake may be more important considerations for IVT mRNA, they may be less of a concern for delivery of plasmid DNA. As the lifetime of IVT mRNA is more transient than that of plasmid DNA, delivery vehicles may also aim to impart appropriate kinetics of IVT mRNA uptake and cytoplasmic release.

While, naked mRNA injected *in vivo* effectively leads to protein expression (97), the use of a delivery vehicle can improve IVT mRNA protein production both *in vitro* (98-101) and *in vivo* (2,8,98,101-103). Specific strategies employed for IVT mRNA delivery are depicted in Figure 3 and specific studies are outlined in Table 2. Previous work has focused on facilitating mRNA uptake (through receptor mediated endocytosis or the use of a cationic particle) and endosomal escape (through the incorporation of pH sensitive elements, (103), altering mRNA interaction with PRRs (through complexation of mRNA with protamine) (41,104), and conferring protection from nucleases (2,102).

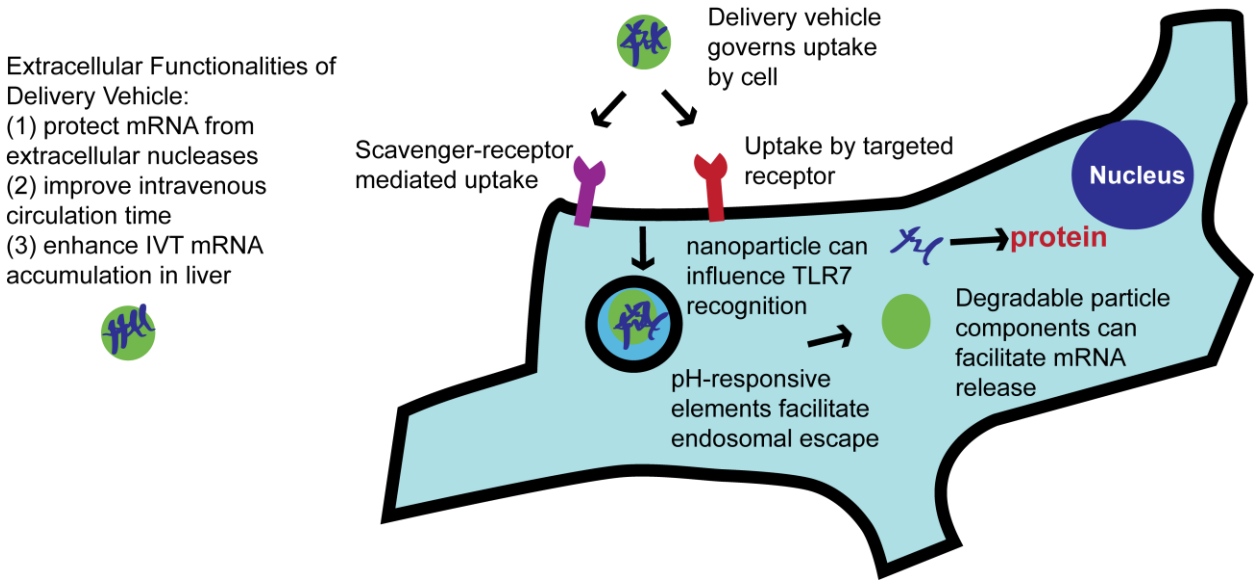

Figure 3. Strategies for effective delivery of IVT mRNA. Delivery vehicle may enhance in *vivo* circulation and distribution of mRNA, target certain cellular receptors for selective uptake, allow for endosomal disruption and IVT mRNA escape, and facilitate IVT mRNA release from the particle.

Especially important for vaccination applications, delivery vehicles may impact mRNA interaction with PRRs. Studies from CureVac showed that complexing mRNA to protamine

leads to heightened TLR7 dependent immune responses compared to naked mRNA delivery (105). To a similar end, Nguyen et al screened a library of lipid-like materials for delivery of siRNA with enhanced immune stimulatory properties. The authors show that lipid nanoparticle characteristics could have a dramatic effect on siRNA's stimulation of the immune system. The effects were shown to be both TLR7 independent and dependent (106). In both cases, it's unclear if delivery vehicles enhance immune responses due to improved cellular uptake of mRNA, enhanced endosomal retention of mRNA, or if mRNA complexation to protamine facilitates PRR recognition of the nucleic acid. Conversely, electroporation may transport IVT mRNA into the cell in a route that bypasses endosomal TLRs, reducing certain innate immune responses. This as well as other delivery strategies that function to bypass mRNA detection by PRRs may be warranted for IVT mRNA's use as tool to induce therapeutic protein expression.

Table 2. Delivery Vehicles Employed for IVT mRNA Delivery

Nanoparticle Composition	**Injection Route**	**Application/Comments**	**Reference**
Lipoplex formulation consisting of the cationic lipids MLRI, TransFast and DOTMA	intraventricular	Conferred protection of mRNA from RNases	(102)
Positively charged triblock polymer (DMAEMA, PEGMA, DEAEMA, and BMA)	Studied *in vitro*	pH responsive segment for endosomal release, cationic segment for mRNA complexation, and a hydrophilic segment for in vivo stability	(107)
Poly(β-amino ester) core enveloped by a phospholipid bilayer	intranasal	pH responsiveness of poly(β-amino ester) allowed for endosomal disruption	(103)
mRNA-protamine associated with cationic liposome	intravenous	Conferred protection of mRNA from RNases and, prolonged circulation time, enhanced uptake by cancerous cells	(2)
mRNA-protamine complexed with poly(ε-caprolactone)	studied *in vitro*	pH responsiveness facilitates endosomal release	(99)
Ionizable cationic lipid/phosphatidylcholine/cholesterol/ polyethylene glycol (PEG) lipid	Intravenous	pH responsiveness facilitates endosomal release	(8)
(PEG)-polyamino acid block copolymer, polyplex nanomicelle	intrathecal injection	pH responsiveness facilitates endosomal release; PEG facilitates enhanced stability	(108)
Mannosylated and histidylated lipopolyplexes	intravenous	Mannose on nanoparticles enhanced dendritic cell uptake of mRNA	(109)
Protamine	intradermal	Conferred protection of	(41,105,110)

		mRNA from RNases, enhanced detection of mRNA by TLR7	
Polyacridine-PEG-peptide	hydrodynamic tail vein injection	Peptide binds mRNA; poymers confer in vivo stability	(111)

Injection of IVT mRNA is currently under clinical evaluation for anti-cancer immunotherapy treatments. CureVac has ongoing clinical trials using their RNActive technology, where a portion of IVT mRNA is complexed to protamine (15,110). Here, protamine is expected to enhance activation of PRRs by IVT mRNA. Other clinical trials have tested IVT mRNA delivered in a free form when supplemented with granulocyte-macrophage colony stimulating factor as an adjuvant (112). Other applications of IVT mRNA are currently in preclinical testing. While clinical trials have shown that IVT mRNA is tolerated without serious side effects, all of the these trials included efforts to better stimulate innate immunity, suggesting that the self-adjuvant effect of IVT mRNA may not be substantial enough for eliciting appropriate immunity.

The delivery route of IVT mRNA is also an important factor to consider for both therapeutic and vaccination purposes. The use of IVT mRNA as a vaccination platform has predominated been studied by injection of IVT mRNA intradermally. While the exact rationale for why this route is so heavily explored is uncertain, but this may be because intradermal injection expose IVT mRNA to higher amount of $TLR7^+$ cells, allowing for enhanced stimulation of innate immune responses compared to other routes (113). Intranodal delivery for cancer applications has also been a focus of recent work. A study by Kreiter et al explored how delivery route impacted an anti-cancer IVT mRNA vaccine. The authors compared subcutaneous, intradermal, near nodal, and intranodal injection routes, and found that intranodal delivery led to both the greatest amount of detectable protein expression as well as heightened T cell responses (114).

For therapeutic protein production applications, IVT mRNA should be delivered in a way to facilitate cellular uptake by cells that will produce protein and localize it appropriately for the end application. Efforts have predominately focused on nanoparticle mediated intravenous delivery, where IVT mRNA accumulates in the liver. Nanoparticle mediated delivery can increase circulation time by bypassing glomerular filtration and enhancing IVT mRNA uptake in the liver (115). IVT mRNA is translated and the ectopic protein is secreted into the bloodstream (5). Effective delivery of IVT mRNA has also been shown upon aerosol mediated delivery to the lungs (7), intramuscular delivery (7), subcutaneous(98), and intradermal (112). Phua et al showed that delivery of naked IVT mRNA subcutaneously led to more sustained protein expression compared to nanoparticle mediated intravenous delivery (98). However, while subcutaneous delivery may have produced more detectable protein, it may not allow for similar systemic delivery of protein compared to intravenous delivery.

A significant hurdle for the development of delivery vehicles is the variability between in vitro and in vivo results. These inconsistencies are illustrated in a study Phua et al. This study showed that while IVT mRNA nanoparticles transfected cells in vitro it did not lead to protein expression upon subcutaneous administration to mice. Conversely, transfection was unsuccessful when naked IVT mRNA was delivered to cells in vitro, but was successful when delivered to mice subcutaneously. However, upon intranasal and intravenous delivery, naked IVT mRNA produced less protein than nanoparticle mediated delivery (98). Others have also identified these inconsistencies and have shown that while cationic nanoparticles function well in vitro, they are less effective in vivo (116,117). This discrepancy may be explained by the

interaction of nanoparticles with the extracellular matrix in vivo. Cationic delivery vehicles may interact with negatively charged serum proteins or negatively charges within the extracellular matrix. Further, the extracellular matrix may also limit the diffusion of larger particles, inhibiting interaction with their cellular targets (118). Thus, delivery vehicles intended for in vivo use require either optimization and testing in vivo, or the use of in vitro systems that appropriately mimic the specific route delivery.

Additionally, a better understanding of the bottlenecks associated with IVT mRNA may better direct development. Delivery vehicles can be effectively designed to overcome different barriers associated with IVT mRNA delivery. However, the relative importance of each barrier is not well understood. Thus, the relative importance of different design requirements for IVT mRNA delivery is unknown. For example, engineering a delivery vehicle that facilitates endosomal disruption may not be effective if IVT mRNA is rapidly degraded by nucleases. Thus, the community could benefit from biodistribution studies of IVT mRNA as well as an enhanced understanding of the difficulties associated with IVT mRNA delivery. Together, this information may allow for more rational and effective approaches to IVT mRNA delivery.

Conclusions, Future Directions, and Adding to the Toolbox for IVT mRNA:

IVT mRNA technology shows great potential as a platform for both therapeutic protein delivery and subunit vaccines. Incorporation of modified nucleotides has greatly reduced interactions of IVT mRNA with PRRs, and as a result, effectively enhances transgene protein levels. Incorporation of certain untranslated regions also shows promise in enhancing mRNA expression and may allow researchers better control over regulating protein expression kinetics and characteristics. However, the modified base pseudouridine, while beneficial in many respects, impedes function of engineered UTR sequences, and may alter characteristics of the expressed protein as well. These factors need to be carefully considered.

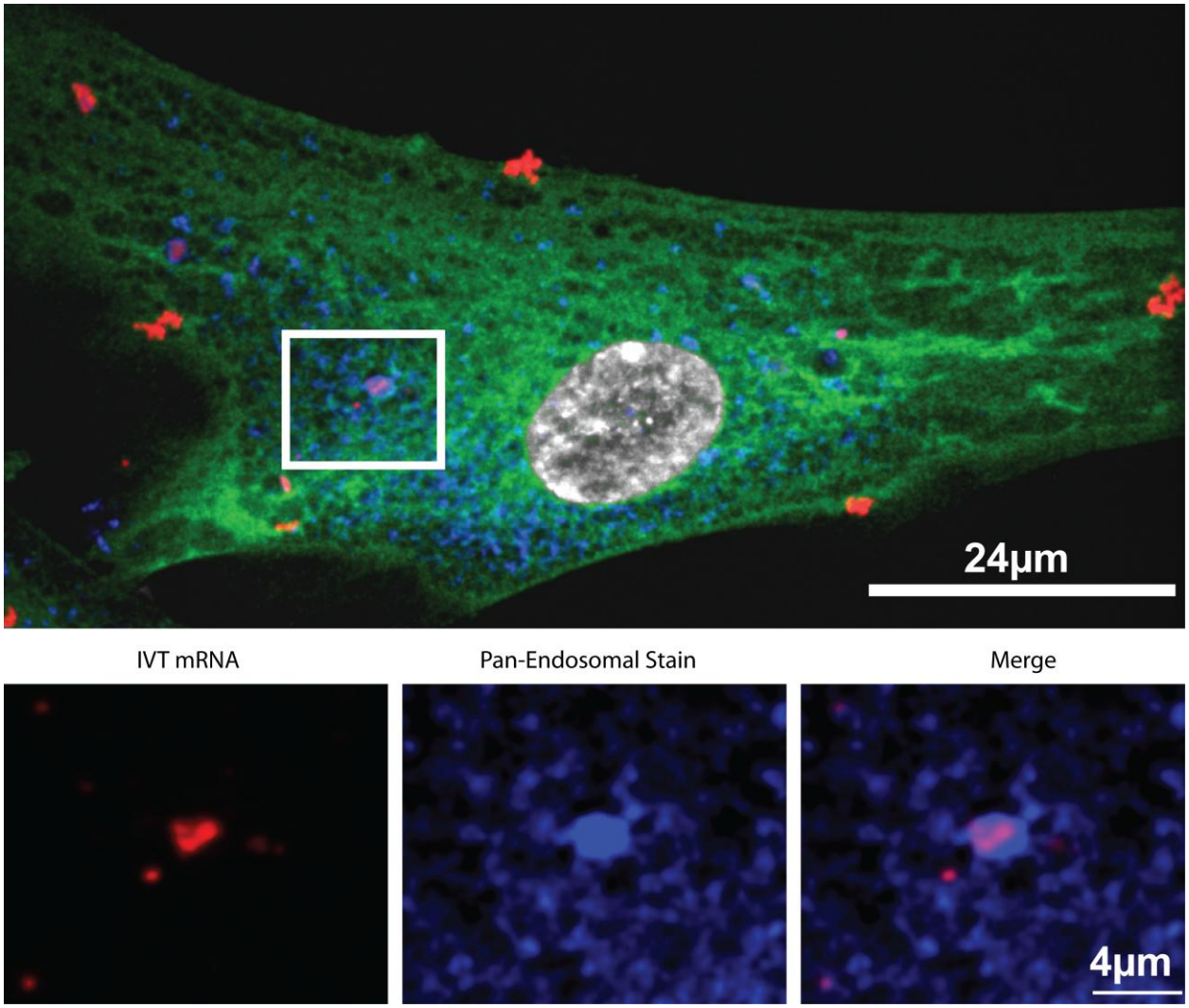

Figure 4. IVT mRNA can be detected and is present in endosomal compartments. Prior to transfection, IVT mRNA encoding for green fluorescent protein (GFP) was labeled with fluorescent probes developed in the Santangelo lab (119). IVT mRNA (red) is visible inside the cell, and its entrapment in endosomal compartments (blue), which was stained for with antibodies targeting CD63, EEA1, and LAMP1, can also be visualized. The nucleus is shown in white and GFP protein is green.

Furthermore, gene to gene variations exist in the efficacy of each strategy, as shown in the combination of GC enrichment and incorporation of pseudouridine. This suggests that each newly developed mRNA may need to be optimized individually, as no strategy appears to be universally effective. Currently, we lack structural and mechanistic understanding on how these strategies affect mRNA performance. Improved mechanistic understanding should allow for more rational sequence design and development of strategies that could be universally applied to mRNA constructs.

To date, mRNA performance has largely been gauged through transgene protein production and elicited cytokine responses. However, these measures don't describe bottlenecks to mRNA performance, including cell uptake, endosomal escape, RNP formation, translation rates, translation inhibition, and innate immune activation. In order to better understand these bottlenecks, we believe the adoption of more specific success criteria is important.

To better understand some of these concerns, our lab has recently focused on applying multiply-labeled tetravalent RNA imaging probes (MTRIP) technology (119,120). MTRIP technology consists of 2′-O-methyl RNA/DNA chimeric nucleic acid oligonucleotides that bind to complementary regions in IVT mRNA. Fluorophores or other reporter molecules are incorporated into the oligonucleotides to form MTRIPS and facilitate detection of IVT mRNA. MTRIPs, coupled with appropriate imaging devices, allows for visualization of IVT mRNA at the single molecule, cell, or whole organism level. We are able to detect IVT mRNA in cytosolic and endosomal compartments, to better understand how IVT mRNA delivery conditions affect cytosolic uptake. Further, quantification of intracellular or cytoplasmic IVT mRNA and ectopic protein expression can be correlated on a per cell basis. Figure 4 shows previously unpublished work from our lab using MTRIPs. Here, we can see that some, but not all transfected IVT mRNA is present in endosomal compartments. MTRIPs can also be modified to study mRNA-protein interactions, for example to visualize interaction with key RNA binding proteins or PRRs (120).

We believe that further investigation of RNP formation is also important for future IVT mRNA applications. Immunoprecipitation techniques that identify RNA-protein interactions could also assist in understanding aberrant binding of proteins to transfected IVT mRNAs (121,122). Additionally, there is limited understanding regarding how OAS-L pathway activation and PKR activation limit IVT mRNA performance. Studies that specifically probe how IVT mRNA delivery and sequence design affect these pathways could dramatically affect IVT mRNA performance. We expect that an improved understanding on the relative contributions of each "bottleneck," including (1) cellular uptake (2) subcellular location (3) interactions with PRRs, and (4) formation of functional RNPs will facilitate more rational approaches for improving IVT mRNA performance. Hopefully, this will ultimately contribute to the adoption of IVT mRNA for a variety of healthcare challenges.

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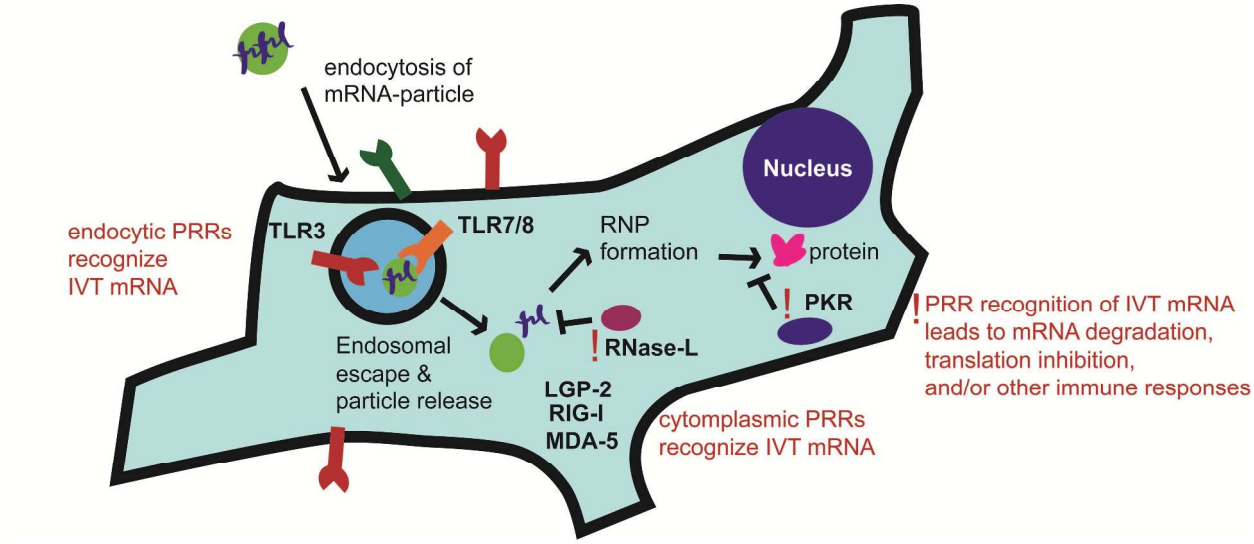

This review discusses the challenges associated with IVT mRNA therapeutics and vaccines as well as the current strategies employed to overcome these challenges.