Aus der

Universitätsklinik für Thorax-, Herz- und Gefäßchirurgie Tübingen

The impact of the chemical modifications N1-methylpseudouridine and N4-acetylcytidine on messenger RNA translation, kinetics and cell viability

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Fleischhacker, Franziska Laura

2024

Dekan:	Prof. Dr. B. Pichler
1. Berichterstatter:	Prof. Dr. M. Avci-Adali
2. Berichterstatter:	Prof. Dr. J. Feucht
Tag der Disputation:	28.02.2025

Table of Contents

List of Figures

List of Tables

Abbreviations

2-5A	2’-5’-linked oligoadenylates
A549	Adenocarcinomic human alveolar basal epithelial cells
Ac4C	N4-acetylcytidine
ARCA	*Anti-Reverse Cap Analog*
ATP	Adenosine triphosphate
AUC	Area under the curve
bp	Base pair
°C	degree(s) Celsius
CARD	Caspase recruitment domains
cDNA	Complementary deoxyribonucleic acid
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cmRNA	Chemically modified ribonucleic acid
CTP	Cytidine triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
(d)NTP	(Deoxy)Nucleoside triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
dsRNA	Double-stranded ribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
eIF	Eukaryotic translation initiation factor
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FOXP3	Forkhead box protein 3
FSC	Forward scatter
FSC-A	Forward scatter area
FSC-H	Forward scatter height
g	Gramm(s)
GTP	Guanosine triphosphate

h	Hour(s)
IFN	Interferon
IκB	Inhibitor of NFκB
IL	Interleukin
IRF	Interferon regulatory factor
IVT	*In vitro*-transcription/*in vitro*-transcribed
kb	Kilobase
L	Liter(s)
LB	*Luria Broth*, lysogeny broth
Lipo2000	*Lipofectamine 2000*
m5C	5-methylcytosine
m6A	6-methyladenosine
m7G	7-methylguanosine
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation-associated protein 5
MFI	Mean fluorescence intensity
µg/ µL	Microgramm(s)/ Microliter(s)
µM	µmol/L, micromol/L
min	Minute(s)
mL	Milliliter(s)
mM	mmol/L, millimol/L
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response 88
N1Ψ	N1-methylpseudouridine
ng/ nL/ nm	Nanogramm(s)/ Nanoliter(s)/ Nanometer(s)
NFκB	Nuclear factor κB
OAS	2′-5′-oligoadenylate synthetase
ORF	Open reading frame
p53	Protein 53
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
pDNA	Plasmid deoxyribonucleic acid
PKR	Ribonucleic acid-dependent protein kinase
poly(A)	Polyadenylic acid
poly(U)	Polyuridylic acid
PRR	Pathogen recognition receptor
Ψ	Pseudouridine
Ψ-UTP	Pseudouridine triphosphate
qPCR	Quantitative polymerase chain reaction
RIG-I	Retinoic acid-inducible gene I
RLR	Retinoic acid inducible gene I-like receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
s2U	2-thiouracil
SD	Standard deviation
SDS	Sodium dodecyl sulfate
s	Second(s)
SPB	Surfactant protein B
SSC	Side scatter
ssRNA	Single-stranded ribonucleic acid
TAE	Tris-acetate-EDTA, tris(hydroxymethyl)aminomethane-acetate-ethylenediaminetetraacetic acid
TIR	Toll-IL-receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	Toll-IL-receptor domain-containing adaptor inducing IFN-β
tRNA	Transfer ribonucleic acid
UTP	Uridine triphosphate
UTR	Untranslated region
VEGFA	Vascular endothelial growth factor A

1. Introduction

1.1 The underlying concept of mRNA-based protein supplementation

mRNA-based approaches for protein supplementation use messenger ribonucleic acid (mRNA) as a vehicle to transfer genetic information into a patient's cells. In this way, previously missing, defective, or non-functional proteins can be replaced, or proteins with a specific therapeutic effect can be provided to the organism [1, 2].

mRNA for therapeutic purposes is commonly synthesized in a cell-free environment *in vitro* engineered in a way to mimic the structure of fully processed eukaryotic mRNA molecules [1] as close as possible. Mature eukaryotic mRNA is single-stranded, capped at the 5’ end, and contains a poly(A) tail at its 3’ end. A start and a stop codon circumscribe the open reading frame which encodes the protein of interest, and untranslated regions (UTRs) adjoin the 5' or 3' end of the coding sequence. The deoxyribonucleic acid (DNA) template for *in vitro* transcription (IVT) may either be a linearized plasmid or a polymerase chain reaction (PCR) product which encodes every component of mature eukaryotic mRNA except a 5’ cap structure [1]. In the presence of a mixture of ribonucleotides, IVT is generally carried out using SP6, T3, or T7 RNA polymerase [3, 4] followed by enzymatic 5’ capping. After IVT, the DNA template is digested by deoxyribonucleases (DNases) and the IVT mRNA is purified from the reaction mixture as the final step before transfection into the target cells.

Once the transfected IVT mRNA reaches the cytoplasm of the target cell, it is introduced into a physiological process in which it is translated into protein using the translation machinery of the transfected cell. The synthesized protein is subject to post-translational modification which is regulated through the same cellular mechanisms that take effect when translating and processing endogenous proteins, ensuring a properly folded and thus fully functional protein. Signal peptides encoded within the sequence of amino acids of the protein, either

naturally present or recombinantly inserted, target the protein to its desired functional compartment – either within the intracellular space in the cytosol or organelles, as a membrane protein or to the extracellular space [1]. Finally, IVT mRNA is degraded like endogenous mRNA via physiological cellular processes.

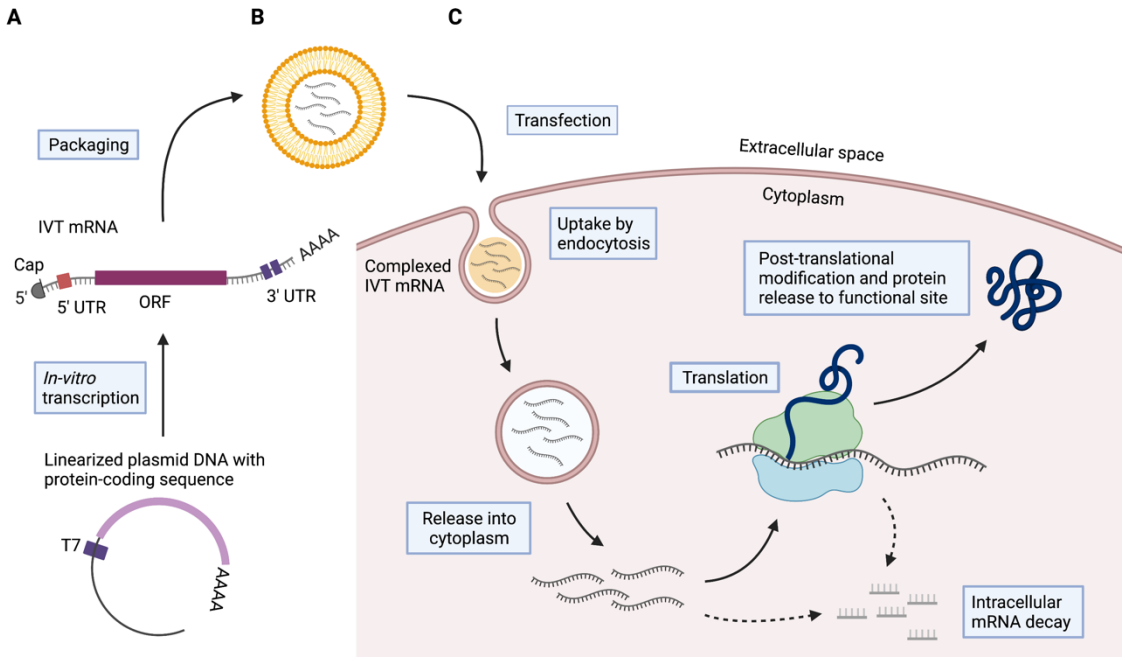

Figure 1: The basic concept of mRNA-based protein supplementation.
Adapted from Sahin U, Karikó K and Türeci Ö © 2014 Macmillan Publishers Limited. Reproduced with permission from Springer Nature. Created with BioRender.com.
(A) mRNA for therapeutic purposes is synthesized *in vitro* using the coding sequence of a linearized plasmid as a template for transcription. Processed IVT mRNA contains a protein-coding open reading frame (ORF) surrounded by 5' and 3' untranslated regions (UTRs), a cap structure seals the 5' end, and the 3' end is formed by a poly(A) tail. Each of those structural elements is a possible target for modifications and determines the mRNA's stability and translational activity when transfected into the target cells. **(B)** To prevent extracellular degradation by ubiquitously present RNases, IVT mRNA is complexed with nanocarriers. **(C)** Nanocarriers facilitate cellular uptake by spontaneous endocytosis. Protein-coding IVT mRNA is then released from endosomes into the cytoplasm and adopted by the host cell's machinery. Thus, cellular mechanisms within the host cell regulate the translation of the encoded protein which is accomplished by endogenous ribosomes. The newly synthesized protein is then post-transcriptionally modified and finally released to its intended functional site. Abbreviations: ORF = open reading frame, T7 = T7 promoter, UTR = untranslated region.

1.2 Advantages of mRNA-based pharmaceuticals over other approaches for protein supplementation

IVT mRNA as a therapeutic expressing biologically active protein holds numerous principal advantages over other strategies for protein supplementation [5].
Moreover, mRNA only needs to cross the plasma membrane to carry out its function. Once entering the cytoplasm, mRNAs are rapidly translated into proteins skipping complex and time-consuming steps such as nuclear import, transcription, and splicing. As no transport across the nuclear membrane is necessary, unlike DNA, mRNA does not require cell proliferation as it does not depend on nuclear envelope breakdown during cell division to reach its functional site [1]. Therefore, mRNA can express the encoded protein also in nondividing cells which makes mRNA an efficient tool for transfection of primary cells [6, 7].
Contrary to DNA-based approaches, the use of mRNA additionally minimizes the risk of genomic integration and insertional mutagenesis. Thus, it provides a safety advantage over DNA, avoiding adverse side effects that restrict the application of most DNA-based pharmaceuticals [6, 8].
Once considered a serious obstacle, the transient nature of mRNA along with its biodegradability via physiological metabolic pathways is now estimated as a major advantage. Shorter half-lives of mRNA molecules and their inability to replicate allow close control of the extent and duration of the encoded protein's expression and are an important feature to limit off-target effects [2, 6, 9]. In this way, protein expression from IVT mRNA is more stable and predictable than in DNA-based approaches [10-13].
In addition, manufacturing of mRNA-based pharmaceuticals bears advantages over protein-based therapeutics. Protein production is more complex than mRNA production from IVT and requires not only elaborate purification processes but also proper folding and in some cases the correct assembly of several different protein subunits. In contrast, mRNA synthesis is relatively simple and cost-effective as one DNA template can be transcribed many times, a process that is adaptable to be run continuously on large scale in bioreactors [1, 14]. Even to obtain different mRNAs for various proteins supplementation therapies, mRNA

synthesis can always take place in the same production facility using the same production process and only the sequence of the DNA template needs to be changed [5].

1.3 Potential therapeutic applications of IVT mRNA

Extensive research and constant progress in mRNA technology have aroused enthusiasm about mRNA as a therapeutic and have motivated preclinical as well as already clinical research on a broad variety of potential mRNA-based approaches [1, 2]. mRNA is not only a promising therapeutic candidate for protein supplementation but also in other fields of application. Most prominently, infectious disease vaccination [15, 16] has come under the spotlight in recent years with the development of mRNA-based Corona Virus Disease 2019 vaccines by biotech companies BioNTech/Pfizer [17] and Moderna [18], which have established the foundation for the rapid development of vaccines against emerging infectious diseases in the future. Furthermore, mRNA-based approaches hold huge potential in the development of modern cancer therapeutics. These include mRNA that encodes tumor antigens for the purpose of cancer vaccination, cytokines for cancer immunotherapy, engineered chimeric antigen receptors for T cell therapy, genome editing proteins for gene therapy, or mRNA-based replacement of mutated tumor suppressors to inhibit tumor growth.

Currently, there is no approved mRNA-based therapeutic yet available for protein supplementation. Nevertheless, the strategy of mRNA-based protein supplementation was preclinically applied to various disease models, ranging from common diseases to rare monogenetic disorders.

In 1992, Jirikowski et al. demonstrated the therapeutic effect of mRNA by reversing diabetes insipidus after intrahypothalamic application of vasopressin mRNA to the rat central nervous system [1, 19]. Approximately 20 years later, in 2011, Kormann et al. achieved expression of surfactant protein B (SPB) upon mRNA delivery in an SPB-deficient mouse model of congenital lung disease [1,

20]. Furthermore, the application of erythropoietin-encoding mRNA was shown to increase erythropoiesis, providing a new therapeutic approach for anemia [1, 20, 21]. Apart from SPB deficiency, mRNA-based protein supplementation is also being researched as a therapeutic option for other lung diseases. mRNA encoding the regulatory T cell transcription factor forkhead box protein 3 (FOXP3) rebalanced pulmonary T helper cell responses, thus preventing tissue inflammation and airway hyperresponsiveness in a mouse model of asthma [1, 22]. Additionally, several studies demonstrated the great potential of mRNA-based therapeutics in Cystic Fibrosis (CF) patients, regardless of the specific mutation underlying the disease [23]. For instance, Haque et al. achieved significantly improved Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) expression and recuperated lung function in CFTR-knockout mice [23, 24].

Moreover, mRNA-based approaches hold promise as a therapeutic tool to enable vascular regeneration in various cardiovascular diseases, which are a significant global health concern. Vascular endothelial growth factor A (VEGFA)-encoding mRNA has been proven to successfully enhance angiogenesis in a mouse model of myocardial infarction [1, 25]. As the first among mRNA-based protein supplementation therapies, it has entered clinical trials regarding therapeutic angiogenesis in diabetes mellitus (ClinicalTrials.gov Identifier: NCT02935712) [26] as well as in patients undergoing coronary artery bypass grafting surgery (ClinicalTrials.gov Identifier: NCT03370887) [26, 27].

Recent studies have highlighted the potential of mRNA-based protein supplementation in cancer treatment, specifically to replace mutated, and thus non-functional, tumor suppressor proteins such as protein 53 (p53). The p53 tumor suppressor gene is frequently altered in various cancers and its loss of function is associated with the initiation and advancement of tumor growth, as well as its resistance to treatment. Delivery of p53-encoding mRNA has been shown to restore p53 function and inhibit tumor growth in models of p53-deficient hepatocellular carcinomas and non-small-cell lung cancers [28]. In concordance, Raab et al. found that administering p53-mRNA to high-grade serous ovarian

carcinoma cell lines and orthotopic mouse models reduced tumor growth and inhibited tumor cell dissemination in the peritoneal cavity [29].

1.4 Obstacles and challenges in mRNA-based protein supplementation

As early as 1990, IVT mRNA was first introduced as a drug leading to protein expression upon direct injection of mRNA into target organs [30]. Already two years later, mRNA was first described as a therapeutic by Jirikowski et al. [19]. However, research primarily focused on plasmid DNA (pDNA) and viral vectors to deliver genetic information, while mRNA-based approaches for protein supplementation were not consistently pursued [6]. The significance and potential of mRNA as a new class of therapeutics were underestimated and the advantages of its use in protein supplementation were not recognized for a long time essentially for three major reasons. First, this was the instability of RNA in comparison to DNA and the consequent inefficiency of protein production from mRNA [6]. Additionally, the immunostimulatory effect of IVT mRNA, which is advantageous in the application for vaccination purposes [1, 31, 32], massively restricted the development of mRNA for protein supplementation therapies [6, 21].

1.4.1 Innate immune response upon recognition of exogenous RNA

From an immunological point of view, the application of exogenous RNA is associated with viral infection of the host. Whenever the organism is confronted with foreign RNA, the innate immune system is called into action. This represents the first line of defense against invading pathogens and is triggered upon recognition of exogenous RNAs by their pathogen-associated molecular patterns (PAMPs). These may involve, for example, double-stranded RNA (dsRNA)

elements or the absence of a 5' cap structure. Such PAMPs naturally occur in viral RNA – or they may be present in unmodified IVT mRNA introduced by transfection. Thus, if unmodified IVT mRNA contains any of these PAMPs, it will elicit innate immune responses similar to the response to viral infection.

Specifically, Toll-like receptors (TLRs) as well as additional pathogen recognition receptors (PRRs), both physiologically involved in the defense against viral infections, are activated and subsequently initiate antiviral downstream signaling resulting in inflammation, inhibition of translation, and RNA degradation [33-35]. Figure 2 provides an overview of the signal transduction upon recognition of PAMPs associated with viral as well as unmodified exogenous RNA.

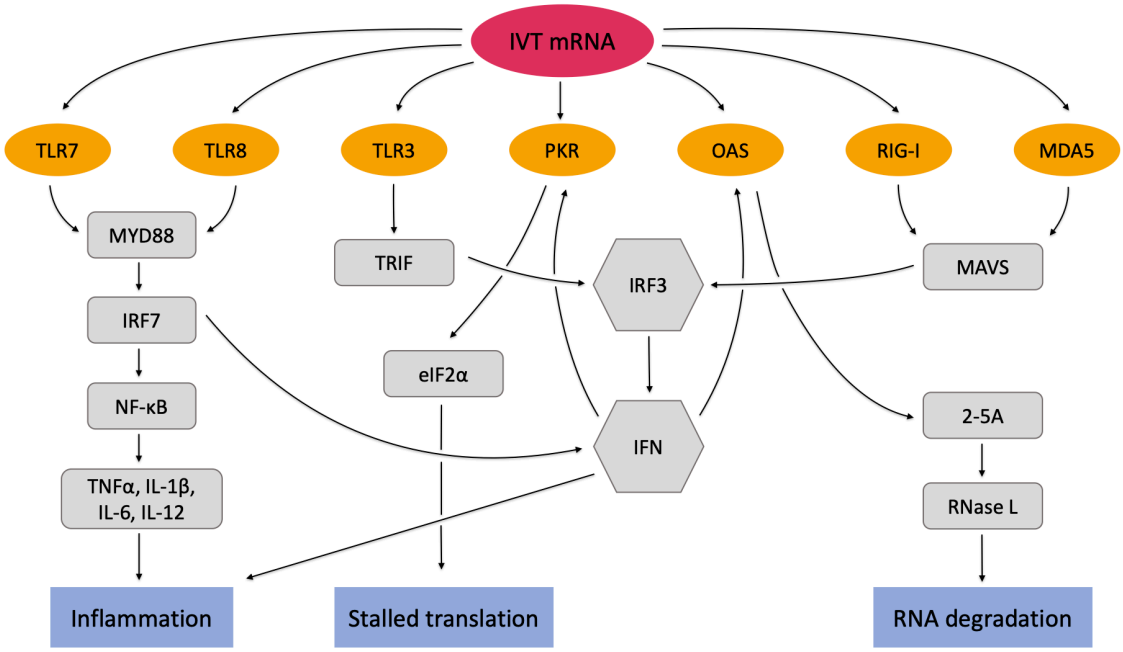

Figure 2: Innate immune response to exogenous RNA and downstream effects.
Adapted from Sahin U, Karikó K and Türeci Ö © 2014 Macmillan Publishers Limited. Reproduced with permission from Springer Nature.

Toll-like receptors. Human TLRs are expressed in distinct cells involved in innate immunity and partly also in other cell types. Four different types of the total of ten human TLRs are involved in the recognition of nucleic acids – TLR3, TLR7, TLR8, and TLR9 – all of which are found intracellular within the endosomal

compartment, thus sensing foreign synthetic nucleic acids present in the extracellular space or those taken up by cells via endocytosis, respectively.
While TLR3 is involved in recognition of double-stranded RNA (dsRNA) [33, 36], TLR7 and TLR8 are both activated by single-stranded RNA (ssRNA) [33, 36-38]. TLR9 signaling is activated by unmethylated CpG dinucleotides in DNA [33].
TLRs are single-pass transmembrane proteins whose extracellular domain is constructed for ligand binding. Their cytoplasmic tail carries a Toll-IL-receptor (TIR) domain. Ligand-induced dimerization or dimer conformational change enables TIR domains to interact with cytoplasmic adaptor proteins which determine further signaling. Among adaptor proteins, TIR domain-containing adaptor inducing IFN-β (TRIF) is the adaptor for TLR3 whereas signaling by TLR7 and TLR8 requires myeloid differentiation primary response 88 (MyD88) [1, 33, 39].
In TLR3 signaling, TRIF initiates binding of several kinases as components of a multiprotein complex. Thereupon, interferon regulatory factor 3 (IRF3) is phosphorylated, and thus activated, leading to induction of cytokines Interferon α (IFN-α) and Interferon β (IFN-β), referred to as type I interferons [1, 33, 39].
As mentioned, TLR signaling in TLR7 and TLR8 runs via MyD88. The signaling complex (Myddosome) recruits kinases that associate with interferon regulatory factor 7 (IRF7). IRF7 is activated via phosphorylation and induces type I interferon production. The most powerful interferon inducer is polyuridylic acid (poly(U)) which acts via TLR7 [1, 37, 40]. Additionally, the Myddosome involves further proteins resulting in nuclear factor κB (NFκB) activation and thus induction of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), Interleukin 1β (IL-1β), Interleukin 6 (IL-6) and Interleukin 12 (IL-12) [1, 33, 39].

RIG-I-like receptors (RLRs). RLRs are the main family of cytosolic RNA sensors [34]. Being expressed in the cytoplasm in almost all mammalian cell types, RLRs play a major role in recognition of exogenous RNA and immune response through interferon release [41]. The main members of the RLR family are retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5

(MDA5) which both have similar protein domain structures and thus very similar activation mechanisms [34].

Originating from its natural role in innate immunity, RIG-I recognizes ideally short pathogenic dsRNA by the absence of a 5' capping structure [42, 43] (as viral RNA usually lacks capping and carries an unmodified 5'-triphosphate instead). On the other hand, RNA recognition by MDA5 is considered to be dependent on RNA structures of high molecular weight containing both dsRNA as well as ssRNA patterns [44].

Sharing a common structure, RIG-I-like receptors contain a helicase-like domain for interaction with foreign RNA and additionally hold two caspase recruitment domains (CARDs) which allow interaction with adaptor proteins and activate downstream signaling upon ligand binding [45]. In the absence of pathogenic RNA, RIG-I and MDA5 remain in an autoinhibited configuration which is stabilized by interaction between the CARDs and the helicase domains [34, 45].

Once RNA binds the helicase domain, this interaction is disturbed, and the CARD associates with the downstream adaptor mitochondrial antiviral signaling protein (MAVS) [34, 46]. MAVS initiates recruitment of a multiprotein complex which, similar to TLR signaling, leads to type I interferon production and proinflammatory cytokine expression through activation of IRF3 and NFκB, respectively [34, 46].

RNA-dependent protein kinase (PKR). PKR is ubiquitously present in the cytoplasm of all mammalian cells and naturally holds a crucial position in regulation of translation within virus-infected cells. Activation of PKR is triggered by binding dsRNA elements which are widely present among a variety of RNA structures [47]. Upon dsRNA recognition, PKR is activated by auto-phosphorylation [48] and subsequently phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α) [49, 50] resulting in inhibition of translation. Additionally, PKR activates NFκB by initiating phosphorylation of inhibitor of NFκB (IκB) [51, 52]. Furthermore, PKR is connected to other signaling pathways as its phosphorylation activity is inducible by interferon [50, 52, 53] which is produced upon activation of TLRs and RLRs.

2′-5′-oligoadenylate synthetase (OAS) and Ribonuclease (RNase) L. OAS is a cytoplasmic sensor of dsRNA. Upon binding dsRNA, OAS produces unique 5'-end-phosphorylated 2'-5'-linked oligoadenylates (2-5A) from adenosine triphosphate (ATP) according to the formula $p_x5'A(2'p5'A)_n$; $x = 1–3$; $n > 2$ [54, 55]. RNase L is a latent endoribonuclease that is activated by 2-5A and, upon activation, cleaves all RNA (both self and non-self) within the cell.

The OAS/RNase L system naturally plays a major role in innate antiviral immunity and is closely linked to other antiviral signaling pathways. On the one hand, OAS expression is interferon-inducible [55, 56] and thus enhanced upon activation of TLRs and RLRs. On the other hand, RNase L amplifies interferon release by producing RNA fragments which are potent activators of RLRs. Thus, the OAS/RNase L pathway creates an extended state of defense against foreign RNA within the organism [55].

1.5 Approaches towards promising IVT mRNA pharmaceutics for protein supplementation

To bypass both endosomal TLRs and cytosolic PRRs and thus avoid innate immune reactions, it is crucial to exclude PAMPs in IVT mRNA for therapeutic applications. If such mRNA is successfully designed and produced, it reaches its target with minimal or even without activation of innate immunity. Thus, the goal for mRNA-based protein supplementation is to design non-immunogenic IVT mRNA (which at the same time remains translatable) to achieve highly efficient protein translation.

1.5.1 Modifications of structural elements of IVT mRNA

By chemical modification of mRNAs' structural elements, specifically focusing on 5' capping, the polyadenylic acid (poly(A)) tail, UTRs and codons, and by

modifying incorporated nucleotides, properties of IVT mRNA can be systematically improved, resulting in enhanced stability, reduced immunogenicity and eventually higher translational capacity efficiency [1, 23]. By this means, a substantial prolongation in the expression of the IVT mRNA-encoded protein is achieved over several days, which is important for protein supplementation therapies.

5' capping. Naturally, eukaryotic mRNA is capped by 7-methylguanosine (m7G) which is connected to the first cap-proximal nucleotide by a 5'-to-5' triphosphate bridge. 5' capping is crucial for the regulation of the translational process by binding eukaryotic translation initiation factor 4E (eIF4E) as well as to assure regulated mRNA decay by binding to decapping enzymes [1, 23, 57, 58].
Most commonly, IVT and 5' capping are conducted within one single reaction setup. In the context of an IVT reaction setup, m7G capping, as occurs in nature, is not suitable for capping because m7G competes with guanosine triphosphate (GTP) for incorporation, resulting in a considerable amount of uncapped and therefore immunogenic mRNAs [1, 23].
Therefore, early mRNA research was conducted with m7GpppG-capped IVT mRNA. However, these mRNAs exhibited reduced efficiency of translation as relevant proportions of m7GpppG caps were incorporated in reverse orientation [1, 30, 59]. This issue was remedied with the introduction of the Anti-Reverse Cap Analog (ARCA) which circumvents reverse incorporation and increases the efficiency of translation by escaping decapping enzymes and extending mRNA half-life [20, 23, 60, 61].

Poly(A) tail. Like 5' capping, the poly(A) tail at the 3' end is crucial for the regulation of mRNA stability, half-life, and thus efficiency of translation [23]. When producing mRNA via IVT, the poly(A) tail may either be encoded in the DNA template for transcription or it is enzymatically synthesized by a poly(A) polymerase after IVT. When IVT mRNA is produced for therapeutic purposes, the poly(A) tail is preferentially transcribed from the template to ensure even lengths

of poly(A) tails among mRNA molecules. The ideal length of the poly(A) ranges between 120 and 150 nucleotides [1, 5, 23, 60, 62].

5' and 3' UTRs. Regulatory sequence elements in UTRs identified in endogenous mRNA may be engineered for therapeutic IVT mRNA to customize stability and translation to specific therapeutic requirements. While some modifications in UTRs enhance mRNA stability and thus efficiency of translation, incorporated adenosine-uridine rich elements, for example, exert a destabilizing effect on IVT RNA. That way, mRNA is rapidly degraded and only leads to a short duration of protein expression – an effect which is advantageous if protein production is to be tightly limited in therapeutic application [1, 23, 63].

Codon optimization. Most amino acids are encoded by more than one synonymous codon, a notion referred to as the degeneracy of the genetic code. Systematic codon usage referred to as codon optimization can influence translational efficiency and comprises several methods, some of which are discussed in the following.

First, reutilization of the same transfer RNA (tRNA) and higher tRNA availability accelerate elongation and thus enhance translation efficiency. For that reason, the exchange of rare codons for more frequent ones encoding the same amino acid may increase protein yield. [1, 64, 65]. However, slower translation can be crucial to achieve proper protein folding [66]. Therefore, targeted use of rarer codons that slow down translation in crucial regions for protein folding can benefit the formation of accurate secondary and tertiary structures [1, 64, 67, 68]. Moreover, guanosine-cytidine enrichment of the coding sequence, for example using that codon with the highest guanosine and cytosine content, was shown to enhance protein yield when applied to unmodified or pseudouridine-modified mRNA [21, 23, 69]. Another method of codon optimization is uridine depletion. Cas9-encoding IVT mRNA was shown to result in higher Cas9 activity and reduced immunogenicity if the sequence was uridine depleted and remaining uridines were replaced by 5-methoxyuridine [35].

1.5.2 Chemical modifications of nucleotides in IVT mRNA

In 2005, a study by Karikó et al. revealed an immune evasion effect of chemically modified nucleotides when incorporated in naturally occurring mRNA [70]. While mammalian RNA commonly contains modified nucleotides beyond the basic adenosine, guanosine, cytosine, and uridine [71], they do not occur within bacterial RNA. Thus, chemical modification of nucleotides represents an important distinguishing feature between mammalian and bacterial RNA and thus, in a sense, “the foundation of the most ancient ‘immune’ mechanism” [70]. However, chemical modifications of nucleotides are frequent in viruses, most probably playing a role in avoiding immune activation of the host [5, 70, 72, 73]. The notion that chemically modified nucleotides in mRNA could reduce immunogenicity was a decisive scientific progress leading to increasing popularity of mRNA in the field of protein supplementation therapies. By eliminating this major obstacle of mRNA therapeutics, research in nucleotide modifications in IVT mRNA was further encouraged and nucleotide modifications were examined for their influence on IVT mRNA immunogenicity, stability, and efficiency of translation.

When mammalian mRNA is synthesized under physiological conditions, transcription is carried out using ATP, uridine triphosphate (UTP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP). Only after transcription, nucleotides are chemically modified at selected positions within the RNA transcript. To obtain IVT mRNA with modified nucleotides, the technically simplest and therefore most commonly used method is to replace a certain percentage of the canonical nucleoside triphosphates (NTPs) with corresponding modified NTPs [6, 70].

Far more than 100 nucleotide modifications occur in RNA. Pseudouridine (Ψ) was one of the first to be repeatedly shown to circumvent innate immune signaling upon application *in vitro* and *in vivo* when incorporated in mRNA. Specifically, Ψ incorporated in IVT mRNA blocks stimulation of TLR7 and TLR8 [70], it prevents innate immune signaling upon RIG-I activation [6, 42], Ψ-modified IVT mRNA escapes PKR-mediated inhibition of translation [47] and Ψ limits activation of

2′-5′-oligoadenylate synthetase (OAS) and impedes mRNA cleavage by RNase L [54]. Furthermore, the incorporation of Ψ increases translational capacity and leads to higher biological stability of mRNA [6]. Contributing factors are likely to be a promotion of base stacking by Ψ, and thus stabilized secondary structures [6, 74], and enhanced translation which might protect the mRNA through high ribosome occupancy [6].

In addition, especially the frequently occurring modifications 6-methyladenosine (m6A), 2-thiouracil (s2U) and 5-methylcytosine (m5C) have aroused researchers' interest and have been investigated for their applicability in protein supplementation. Among other modifications, m6A, s2U, and m5C have been shown to block TLR and RIG-I stimulation [20, 23, 24, 70, 75]. Moreover, m6A holds a key position in regulation of mRNA metabolism [76, 77]. Similar to Ψ, m5C and/or s2U reduce PKR phosphorylation with m5C being the most favorable for incorporation in therapeutic IVT mRNA as it enables high translational capacity [23, 47]. Especially transcripts where U and C were completely replaced by Ψ and m5C showed the least immunogenicity and the most efficient translation [23, 78].

Recently, a study conducted by Andries et al. [79] revealed an even stronger effect on immunoescape, as well as further enhanced cell viability and protein translation through the use of N1-methylpseudouridine (N1Ψ) and m5C in mRNA. As a result, mRNA modified with N1Ψ and m5C replaced the former state-of-the-art Ψ- and m5C-modified mRNA and is now the most prevalent modification in recent studies [23, 79]. N1Ψ reduces immunogenicity and improves translational capacity by avoiding RIG-I activation [23, 75] and preventing inhibition of translation through eIF2α phosphorylation [80]. Also, N1Ψ was shown to increase ribosome density which ultimately contributes to high protein yield [80].

1.5.2.1 A newcomer among mRNA modifications: N4-acetylcytidine

N4-acetylcytidine (ac4C) as the first acetylated nucleotide has only recently been shown to be present in human mRNA. There, the amount of ac4C was shown to

decrease along the 5’ to the 3’ end of the mRNA with ac4C being primarily enriched within the coding sequence, especially in the wobble position of the codons [81]. Based on these findings and the notion that ac4C promotes Watson-Crick base pairing with guanosine in comparison to cytidine [81, 82], a role of ac4C in the interaction between codon and corresponding tRNA has been suggested [81]. Accordingly, it has been assumed that ac4C in mRNA enhances the decoding efficiency, i. e. the reading rate, during translation thus increasing translational efficiency [81, 83]. In addition, N4-acetylation of cytidines proved to increase intramolecular mRNA stability resulting in increased half-life of ac4C-enriched mRNA [81, 83].

Since ac4C modification in mRNA was shown to increase mRNA stability and an enhancing effect on translation efficiency was suggested, it is an interesting tool for mRNA-based protein supplementation therapy [84, 85].

1.6 Aim of the thesis

IVT mRNA as a new kind of therapeutic has become an increasingly attractive and promising tool for protein supplementation. Continuous improvements in its properties have made IVT mRNA auspicious for therapeutic application and since nucleotide modifications were found to further improve the properties of IVT mRNA, many modifications were investigated for their therapeutic applicability in protein supplementation.

This work focuses on the use of N1Ψ and the recently described ac4C for IVT mRNA modification. The aim of this thesis is to determine their impact on the kinetics of translation and cell viability. For this purpose, four different mRNAs are to be produced *in vitro*, all of which exemplarily encode the fluorescent protein *mKate2*: In addition to the N1Ψ/ac4C-modified mRNA, an unmodified mRNA and mRNAs with the standard modifications Ψ/m5C and N1Ψ/m5C are transcribed *in vitro* and included for comparison in each experiment. Using *in vitro* transfection experiments, the effect of the N1Ψ/ac4C modification on the kinetic profile of protein translation from mRNA and furthermore the translational capacity of N1Ψ/ac4C-modified IVT mRNA is to be determined. In addition, the effects of the N1Ψ/ac4C-modified IVT mRNA on mRNA half-life and cell viability will be investigated.

By introducing and investigating a novel modification in mRNA, this work builds the foundation for further research and contributes to the ultimate goal of safe and effective mRNA-based pharmaceuticals within the wide field of protein supplementation therapy.

2. Materials and Methods

2.1 Materials

In Table 1 the devices used in the experiments are listed.

Table 1: Devices

Device	Manufacturer
Accu-jet pro	BRAND
Agilent 2100 Bioanalyzer	Agilent Biotechnologies
Autoklav Systec VX-150	Systec
BD FACS X-20 Fortessa	BD Biosciences
Centrifuge 5430R	Eppendorf
Centrifuge Perfect Spin	Peqlab
HERAcell Vios 160i CO_2 incubator	Thermo Fisher Scientific
Darkhood DH-50	Biostep
EnSight Multimode Plate Reader	Perkin Elmer
Freezer MediLine (–20°C)	Liebherr
Freezer Model 905 (–80°C)	Thermo Fisher Scientific
Freezer ProfiLine (–20°C)	Liebherr
Fridge MediLine (4°C)	Liebherr
Fridge ProfiLine (4°C)	Liebherr
Heraeus Megafuge 8R	Thermo Fisher Scientific
Heraeus Pico 17	Thermo Fisher Scientific
Gel chamber model 40-0911	Peqlab
HERASAFE KS 12	Thermo Fisher Scientific
Incubator INFORS HT Ecotron	Infors
Kern EW 2200-2NM scale	KERN und SOHN
Laminar-flow hood BDK 6. 12S	BDK Luft- und Reinraumtechnik GmbH
Leica DMi1	Leica
MP-300V power supply	Major science
NanoDrop 2000c	Implen
Pipettes (0.1 – 2.5 µL, 0.5 – 10 µL, 2 – 20 µL, 10 – 100 µL, 20 – 200 µL, 100 – 1000 µL)	Eppendorf

Thermocycler peqSTAR 96 universal gradient	Peqlab
Thermocycler peqSTAR 2X universal gradient	Peqlab
Thermoshaker	Universal Labortechnik
Transferpette S (0.5 – 10 µL, 20 – 200 µL, 100 – 1000 µL)	BRAND
ViiA7	Applied biosystems
Vortex Genie 2	Scientific Industries
Waterbath	Neolab

In Table 2 the reagents and solutions used in the experiments are listed.

Table 2: Reagents and solutions

Reagent/Solution	**Manufacturer**
Seakam LE Agarose	Lonza Bioscience
Anti-reverse cap analog (ARCA) (100 mM)	Jena Bioscience
BD FACSFlow sheath fluid	BD Biosciences
DNA AWAY (4 L)	Molecular BioProducts
DNA ladder 1kb, Gene Ruler (0.5 µg/mL)	Thermo Fisher Scientific
DNA ladder 100bp, Gene Ruler (0.5 µg/mL)	Thermo Fisher Scientific
dNTP mix (10 mM each)	QIAGEN
Dulbecco's Modified Eagles Medium (DMEM) (1X)	Gibco
Dulbecco's phosphate-buffered saline (PBS) (1X)	Gibco
Ethanol absolute	PanReac AppliChem
FACS Clean Solution	BD Biosciences
FACS Rinse Solution	BD Biosciences
Fetal calf serum (FCS)	Biochrom GmbH
Gel Loading Dye Purple (6X)	New England Biolabs
GelRed Nucleic Acid Stain (10,000X in water)	Biotium
Glycerol	Carl Roth
Hydrochloric acid (2 N)	Carl Roth
Isopropanol	AnalaR NORMAPUR
Kanamycin sulfate	Thermo Fisher Scientific
Lipofectamine2000 (1 mg/mL)	Thermo Fisher Scientific
Luria Broth (LB) (25g/L)	Thermo Fisher Scientific
N^4-Acetylcytidine-5'-triphosphate, Sodium salt (100 mM)	Jena Bioscience

N^1-Methylpseudouridine-5'-triphosphate, Sodium salt (100 mM)	Jena Bioscience
opti-MEM, serum reduced medium	Gibco
Penicillin-streptomycin (10,000 U/mL)	Genaxxon bioscience
Power SYBR Green 2X Master Mix	applied biosystems
Pseudouridine-5'-triphosphate, Sodium salt (100 mM)	Jena Bioscience
RNase AWAY (4 L)	Molecular BioProducts
Sodium dodecyl sulfate (SDS) (≥ 99 %)	Carl Roth
TAE buffer (tris-acetate-ethylenediaminetetraacetic acid (EDTA) (10X)	Thermo Fisher Scientific
Thiazolyl blue (≥ 98 %)	Carl Roth
Trypan blue 0.4 %	Gibco
Trypsin-EDTA 0.25 %	Gibco
Turbo DNase	Thermo Fisher Scientific
Diethyl pyrocarbonate (DEPC)-treated water	Thermo Fisher Scientific

In Table 3 the consumables used in the experiments are listed.

Table 3: Consumables

Consumables	**Manufacturer**
Adhesive seal sheets	4titude
C-Chip, Neubauer Improved	Nano EnTek
Cell culture flasks (T-75, 75 cm^2)	Greiner
Cell culture plates (12-well, 48-well, 96-well)	Corning
Dual filter pipette tips (0.1 – 10 µL, 10 – 200 µL, 100 – 1000 µL)	Eppendorf
FACS tubes (5 mL, PS)	Corning
Falcon tubes (15 mL, 50 mL)	Greiner
MicroAmp Optical 384-well Reaction Plate	applied biosystems
Pasteur Capillary Pipettes	WU Mainz
PCR strips (8 tubes, 0.2 mL)	Nerbe plus
Pipette tips (10 µL)	Biozym
Pipette tips (200 µL)	Sarstedt
Pipette tips (1000 µL)	Greiner
Reaction tubes safe lock (0.5 mL, 1.5 mL, 2 mL)	Eppendorf
Serological pipettes (5 mL, 10 mL, 25 mL, 50 mL)	Corning
Filter tips (0.1 – 2.5 µL)	Biosphere

Filter tips (0.5 – 10 µL)	Nerbe plus
Filter tips (2 – 200 µL)	Nerbe plus
Filter tips (100 – 1000 µL)	Nerbe plus

In Table 4 the kits used in the experiments are listed.

Table 4: Kits

Kit	**Manufacturer**
Agilent 2100 Bioanalyzer RNA 6000 Nano Kit	Agilent Technologies
HiScribe T7 High Yield RNA Synthesis Kit	New England Biolabs
iScript cDNA Synthesis Kit	Bio-Rad
Monarch PCR and DNA CleanUp Kit (5 µg)	New England Biolabs
Monarch RNA CleanUp Kit (500 µg)	New England Biolabs
peqGOLD Xchange Plasmid Maxi Kit	VWR peqlab
Q5 Hot Start High-Fidelity DNA Polymerase	New England Biolabs
RNeasy Mini Kit	QIAGEN
RNase-free DNase Set (50)	QIAGEN

In Table 5 the plasmids used in the experiments are listed.

Table 5: Plasmids

Plasmid	**Insert**	**Manufacturer**
pVax.*mKate2*.A120	*mKate2*	cloning

In Table 6 the primers used in the experiments are listed. All primers were custom orders fulfilled by Metabion.

Table 6: Primers

Primer	**Sequence 5' → 3'**	**T_m[°C]**
mKate2 forward for qPCR	GGT GAG CGA GCT GAT TAA GG	60
mKate2 reverse for qPCR	TGC CGT ACA TGA AGC TGG TA	58
18S rRNA forward	GTA ACC CGT TGA ACC CCA TT	58

18S rRNA reverse	CCA TCC AAT CGG TAG TAG CG	60
pVax forward for IVT	AGC TCT CTG GCT AAC TAG AGA	59
pVax reverse for IVT	CTG CAG AAT TCC ACC ACA CTG GAC TA	76

2.2 Methods

2.2.1 Template production for *in vitro* transcription (IVT)

Plasmid preparation

Escherichia coli TOP10 from a glycerol stock transformed with pVax.*mKate2*.A120 (Figure 3) were cultured in 100 mL *Luria Broth* (LB) medium supplemented with 50 µg/mL kanamycin overnight at 37°C shaking at 160 rounds per minute (min).

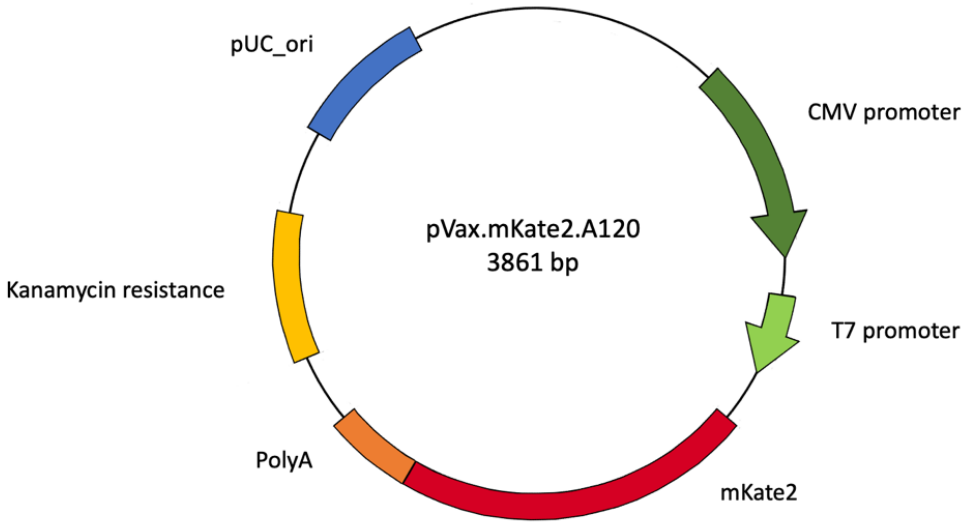

Figure 3: Plasmid map of pVax.mKate2.A120. Abbreviations: PolyA = poly(A) tail, pUC_ori = origin of replication.

Plasmid DNA was isolated from bacterial cultures using the peqGOLD Xchange Plasmid Maxi Kit. Isolation was conducted according to manufacturer's instructions for high copy-number plasmids adapted to the properties of the available centrifuge. The overnight culture was first centrifuged (5,000 x g, 15 min), then the pellet was completely resuspended in 12 mL Solution I/RNase A and 12 mL Solution II was added and gently mixed by inverting the tube. The lysate was neutralized in 12 mL pre-cooled (4°C) Solution III, mixed again by inverting, followed by 5 min incubation on ice. XChange Maxi column was equilibrated with 6 mL Buffer EQ and let empty by gravity flow. A filter was pre-wet with a few drops Buffer EQ before loading the bacterial lysate onto it. The clear lysate was collected and transferred to the equilibrated column to bind the pDNA. The column was then washed once with 32 mL DNA Wash Buffer before pDNA was eluted by applying 15 mL Elution Buffer directly to the binding matrix. Precipitation of the eluted pDNA was conducted with 11 mL isopropanol followed by centrifugation (7,197 x g, 60 min) at 4°C. Then, 5 mL 70 % ethanol was added to the pellet, briefly vortexed and centrifuged (7,197 x g, 10 min) at room temperature. Ethanol was carefully removed from the tube and the pellet was allowed to dry for 10 to 20 min before dissolution in 500 µL nuclease-free H_2O while shaking for 15 min. Concentration and purity of the pDNA lysate were measured by NanoDrop spectrophotometer.

Template amplification by PCR

Purified pDNA was subjected to PCR amplification to obtain a linear DNA molecule as template for IVT. The reaction components are outlined in Table 7 and PCR cycle conditions are listed in Table 8.

Amplified DNA was analyzed by agarose gel electrophoresis to verify the linearization. Agarose gel (1.5 %) was produced from 0.9 g agarose in 60 mL TAE (tris-acetate-EDTA) buffer and 3 µL GelRed (10,000X) were added as nucleic acid stain. The gel was kept at room temperature for 30 min until completely solidified. Meanwhile, 3 µL solution was diluted with 2 µL H_2O and stained with 1 µL Gel Loading Dye Purple. Samples and 4 µL of each a 1kb-ladder and a 100bp-ladder were loaded into separate wells of the gel.

Electrophoresis was performed with a current of 110 V for 50 min. Results were documented using the Darkhood DH-50 device and the Biostep argus X1 software and amplified DNA was reviewed for expected size.

After gel electrophoresis and verification of the PCR product, the amplified DNA was purified using the Monarch PCR and DNA CleanUp (5 µg) Kit. The purification was set up according to the manufacturer's instructions as follows: All centrifugation steps were carried out at 16,000 x g. The whole sample was diluted with 200 µL Binding Buffer, loaded onto the column, and centrifuged for 1 min. DNA was then washed twice with 200 µL DNA Wash Buffer and centrifuged for 1 min after each wash. DNA elution was conducted with 16 µL Elution Buffer pre-heated to 50°C and a 1-min centrifugation. Concentration and purity were determined by NanoDrop spectrophotometer.

Table 7: *mKate2* amplification PCR reaction components

Component	**Amount [µL]**
DNA pVax.*mKate2*.A120 (template)	50 ng
5X Reaction buffer	10
Primer forward (10 µM)	2
Primer reverse (10 µM)	2
GC Enhancer	2
dNTPs	1
Q5 Hot Start HF Polymerase	0.5
Nuclease-free water	ad 50
Total	**50**

Table 8: PCR cycle conditions for *mKate2* amplification

Step		**Temperature [°C]**	**Time [s]**
Initial denaturation		98	30
30 cycles	Denaturation	98	10
	Annealing	61	15
	Elongation	72	20
Final elongation		72	120

2.2.2 IVT

IVT was conducted in order to obtain mRNAs with unmodified nucleotides or selected nucleotide modifications, respectively, in a cell-free environment. Whenever modified nucleotides were used, they completely replaced their unmodified counterparts. IVT was performed using the HiScribe T7 RNA Polymerase Kit. The *mKate2* PCR product served as the DNA template. Reaction setup is outlined in Table 9. In Table 10 all synthetized mRNAs and modifications are listed.

Table 9: IVT reaction setup

Reagent	**Amount [µL]**	**Concentration [mM]**
ATP	3	100
CTP *or* 5-methyl-CTP *or* N4-acetyl-CTP	3	100
UTP *or* Ψ-UTP *or* N1-methyl-Ψ-UTP	3	100
GTP	1.5	100
ARCA	2.4	100
10X Reaction Buffer	4	
Template	2 µg	
Enzyme mix	4	
Nuclease-free water	ad 40	
Total	**40**	

Table 10: IVT mRNAs

Expressed protein	**Modifications [%]**	**Acronym**
mKate2	none	$mRNA^{mKate2}$
mKate2	N1-methylpseudouridine (100 %), 5-methylcytidine (100 %)	$cmRNA^{mKate2}$(N1Ψ/m5C)
mKate2	pseudouridine (100 %), 5-methylcytidine (100 %)	$cmRNA^{mKate2}$(Ψ/m5C)
mKate2	N1-methylpseudouridine (100 %), N4-acetylcytidine (100 %)	$cmRNA^{mKate2}$(N1Ψ/ac4C)

IVT reaction was incubated at 37°C for 2 hours. Afterwards, the DNA template was restricted by Turbo DNase treatment (1 µL added per IVT reaction setup) at 37°C for 15 min.

IVT mRNA was purified using the Monarch RNA CleanUp (500 µg) Kit according to the manufacturer's instructions. All centrifugation steps were carried out at room temperature at 16,000 x g. First, 10 µL of nuclease-free water was added to adjust the volume to the starting sample volume of 50 µL recommended by the manufacturer. After addition of 100 µL RNA CleanUp Binding Buffer and 150 µL ethanol (≥ 95 %) to the sample, it was loaded on the column and centrifuged for 1 min. mRNA was washed twice with 500 µL RNA CleanUp Washing Buffer and centrifuged for 1 min after each wash. For mRNA elution, the manufacturer's recommendation was adapted. mRNA elution was conducted with pre-heated Elution Buffer in two steps. First, 50 µL of Elution buffer was added to the column, followed by a 2-min incubation at 70°C, a 3-min incubation at room temperature, and centrifugation for 1 min. Secondly, 50 µL of Elution buffer was added to the column, followed by a 5-min incubation at room temperature and centrifugation for 1 min.

RNA integrity was determined by chip-based capillary electrophoresis using the Agilent 2100 Bioanalyzer RNA 6000 Chip Kit. The manufacturer's instructions were carried out and are outlined in the following. The analysis was conducted with 1 µL RNA solution from IVT diluted 1 to 15 with nuclease-free water. The RNA to be analyzed and the ladder were denatured at 70°C for 2 min and immediately stored on ice afterward. A gel-dye mix was prepared from 1 µL dye added to a 65 µL aliquot of filtered gel. It was vortexed and centrifuged (13,000 x g, 10 min). As described in the manufacturer's protocol, a chip was inserted into the priming station and prepared with gel-dye mix and RNA marker. RNA samples and the ladder were loaded onto the chip and the sample was analyzed in the Agilent 2100 Bioanalyzer. The mentioned dye, gel and RNA marker were included in the Agilent 2100 Bioanalyzer RNA 6000 Chip Kit.

2.2.3 Cell culture and transfection

Cell culture

All experiments were conducted with adherent adenocarcinomic human alveolar basal epithelial (A549) cells. A549 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 1 % penicillin-streptomycin in T-75 cell culture flasks and kept in a humidified atmosphere at 37°C and 5 % CO_2.

Whenever reaching 90 % confluency, cell culture medium was removed, cells were washed once with 1000 µL phosphate-buffered saline (PBS) and detached with 2 mL 0.25 % trypsin-EDTA solution during a 5-min incubation. Cells were then resuspended in 8 mL cell culture medium and 1 mL of cell suspension was used for passaging the cells in 10 mL fresh cell culture medium.

If a defined number of cells was needed for an experimental setup, cells were detached with 2 mL trypsin-EDTA solution during a 5-min incubation. Cells were then resuspended in 8 mL cell culture medium. Following, an aliquot of 10 µL of cell suspension was stained with 10 µL of trypan blue, and cells were counted in a Neubauer-improved cell counting chamber.

Seeding cells and transfection

In preparation for transfection, 24 hours in advance, cells were detached with trypsin-EDTA, stained, and counted as explained above. Cells were seeded at a density of 250,000 cells per well in 12-well culture plates or 62,500 cells per well in 48-well culture plates, respectively, and grown in antibiotic-free cell culture medium (DMEM + 10 % FCS) at 37°C and 5 % CO_2.

Lipofection of IVT mRNAs in A549 cells was performed with Lipofectamine 2000 (Lipo2000). The reactions per well were set up in two tubes per mRNA sample (tube A and tube B) as outlined in Table 11. Tube B was incubated at room temperature for 5 min, subsequently added to tube A, and the solution was further incubated at room temperature for 7 min. In the meantime, the pre-plated cells were washed once with 1000 µL PBS, and medium was changed to 500 µL opti-MEM in 12-well culture plates or 125 µL opti-MEM in 48-well culture plates,

respectively. Subsequently, the mRNA solution (tube B plus tube A) was added carefully drop by drop to the wells of the culture plate. After a 5-hour incubation at 37°C and 5 % CO_2, transfection complexes were removed and changed to cell culture medium. Cells were maintained in culture until evaluation.

Table 11: Components of IVT mRNA transfection

	Component	**12-well plate**	**48-well plate**
Tube A	IVT mRNA	1 µg	250 ng
	opti-MEM	ad 100 µL	ad 25 µL
Tube B	Lipofectamine 2000	3 µL	0.75 µL
	opti-MEM	97 µL	24.25 µL

Measurement of transfection efficiency and protein translation from IVT mRNA via flow cytometry

Transfection efficiency and protein expression from IVT mRNA were determined by flow cytometry by detecting the fluorescent protein *mKate2*. Cells were seeded in 12-well plates and transfection was carried out as explained above. Analysis was conducted 3, 6, 12, 24, 48, 72, and 120 hours post-transfection. Cells were washed once with 1000 µL PBS, detached by incubation with 200 µL trypsin-EDTA for 5 min, and afterward suspended in 800 µL FACS Buffer (PBS +10 % FCS). Cells were then collected by centrifugation at 500 x g for 5 min, the supernatant was removed, and the pellet was resuspended in 250-300 µL PBS. Measurements were conducted in the BD FACS X-20 Fortessa and 20,000 events were recorded per sample. Untreated A549 cells only exposed to opti-MEM were used as a control. For cell viability assays, an additional control sample was included, only treated with Lipo2000 under opti-MEM exposure. Raw data were analyzed using the FlowJo Software.

2.2.4 Quantification of intracellular RNA decay

Lysis and RNA purification from A549 cells

RNA purification was conducted using the RNeasy Mini Kit according to the manufacturer's instructions. All centrifugation steps were carried out at room temperature at 8,000 x g unless stated otherwise. Following flow cytometry, the remaining cell suspension was centrifuged (500 x g, 5 min), supernatant was removed and 350 µL of Buffer RLT was added. The lysate was mixed well with 350 µL of 70 % ethanol. The sample was loaded onto an RNeasy Mini spin column and centrifuged for 1 min. Subsequently, optional on-column DNase digestion was carried out with the RNase-free DNase Set. After adding 350 µL Buffer RW1, the column was centrifuged for 1 min. Then, 80 µL DNase I incubation mix (10 µL DNase I stock solution added to 70 µL Buffer RDD) was directly applied to the column membrane and incubated at room temperature for 15 min. Once more, 350 µL Buffer RW1 was added to the column followed by centrifugation. The column was washed twice with 500 µL of Buffer RPE in each wash. Washing step 1 was followed by a 1-min centrifugation. After washing step 2, the column was centrifuged for 2 min to dry the membrane. For elution, 32 µL RNase-free water pre-heated to 70°C was added directly to the column membrane. RNA was eluted by a 1-min centrifugation. Concentration and purity of the isolated RNA were determined by NanoDrop spectrophotometer.

Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was conducted in two steps. Reverse transcriptase (RT)-dependent conversion of RNA into complementary DNA (cDNA) was carried out with the iScript cDNA Synthesis Kit. The reaction was set up according to the manufacturer's instructions (outlined in Table 12). The complete reaction mix was incubated in a thermal cycler according to the manufacturer's protocol (see Table 13) including the optional step. Produced cDNA was stored at -20°C if not directly used for quantitative PCR (qPCR).

Table 12: Reverse transcription reaction setup

Component	Amount [µL]
5X iScript Reaction Mix	4
iScript Reverse Transcriptase	1
RNA template	200 ng
Nuclease-free water	ad 20
Total	**20**

Table 13: Reverse transcription reaction conditions

Step	Temperature [°C]	Time [min]
Priming	25	5
Reverse Transcription	46	20
RT inactivation	95	1
Optional step	4	hold

RT-qPCR allows to estimate the quantity of starting mRNA in a sample. The following reaction used *mKate2* cDNA as target for amplification running against 18S cDNA as endogenous reference. cDNA from untransfected cells functioned as control template. cDNA templates were diluted 1 to 20 with nuclease-free water. The reaction setup was prepared in a 384-well plate in triplicate for each sample with the components outlined in Table 14. The no-template control contained 5 µL nuclease-free water instead of template.

Table 14: Real-time quantitative PCR reaction setup

Component	Amount [µL]
2X Power SYBR Green	7.5
Primer forward (10 µM)	0.9
Primer reverse (10 µM)	0.9
Nuclease-free water	0.7
Template	5
Total	**15**

The plate was sealed with foil, shortly centrifuged and run in the ViiA7 device with the qPCR protocol outlined in Table 15 followed by the recording of a melting curve. The measured data was analysed using the ViiA7 RUO Software.

Table 15: qPCR conditions

Step	Temperature [°C]	Time [min]
Stage 1	50	2
	95	10
Stage 2 (40 cycles)	95	15 s
	60	1
Stage 3	95	15 s
	60	1

2.2.5 Cell viability assay

Cell viability after transfection was determined via MTT Assay. Analysis was conducted in triplicates 6, 24, 48, and 72 hours post-transfection of mRNA^{mKate2} and cmRNA^{mKate2} variants in A549 cells, including a control of untreated cells and cells treated with Lipo2000 only. A 12 mM MTT stock solution was prepared by dissolution of 1 g of MTT in 200 mL PBS and stored at -20°C until further use. Before application of MTT, culture medium was removed and replaced by 200 µL of fresh culture medium. Thereafter, 20 µL of MTT solution were added to each well including a negative control (blank) of 20 µL MTT solution added to 200 µL of cell culture medium alone. MTT-treated cells were incubated at 37°C and 5 % CO_2 for 4 hours. Afterwards, 200 µL SDS-HCl solution (0.1 g/mL 0.02 N HCl) was added to each well and mixed thoroughly followed by overnight incubation at 37°C and 5 % CO_2. Samples were then mixed again and 200 µL of each were transferred to the wells of a 96-well plate for measurements. Absorbance was read at 570 nm in the EnSight plate reader and measured data was analyzed with Kaleido 1.2 software.

2.2.6 Statistics

Data points are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 8.4.0. To identify statistically significant differences among different mRNA groups, Kruskal-Wallis test with Dunn's test for multiple comparisons was used. Differences were considered significant when $p \leq 0.05$.

3. Results

3.1 Integrity analysis of unmodified and chemically modified mRNAs

All mRNAs were produced by IVT of the *mKate2* template which was obtained from pVax.*mKate2*.A120 by PCR amplification. Additional to an mRNA containing only unmodified nucleotides, different chemically modified mRNAs were transcribed using chemically modified nucleotides for IVT. Whenever chemically modified nucleotides were used for IVT, the modified nucleotides completely replaced their unmodified counterparts. An overview of all IVT mRNAs synthesized for this thesis is presented in Table 10.

Prior to transfection, all mRNAs were examined for integrity using the Agilent Bioanalyzer 2100. The electropherogram of each IVT mRNA is shown in Figure 4.

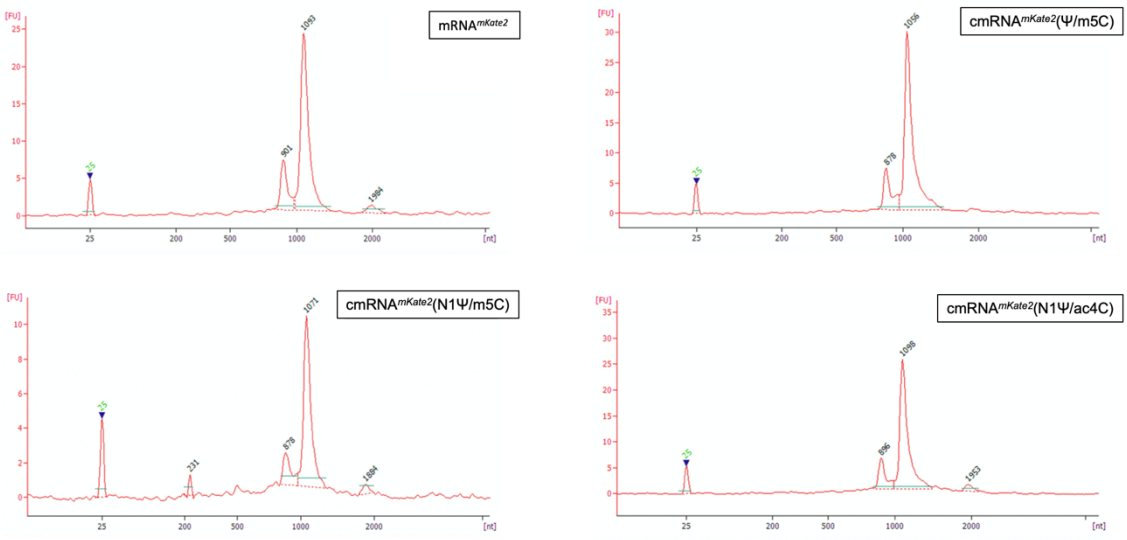

Figure 4: Electropherograms of *mKate2*-encoding mRNA or cmRNAs. Expected size of *mKate2*-encoding mRNA or cmRNAs is represented by the respective sharp peak just below 1100 bp in length. Method-derived marker peak at 25 bp in each sample. Note the different dimensions and labeling of the y-axis. FU = Fluorescence unit.

A sharp peak just below 1100 bp represents the expected size of *mKate2*-encoding IVT mRNA. Additionally, all *mKate2* mRNAs yielded a second peak minor in size of around 900 bp most likely resulting from secondary structures within the *mKate2* sequence. A varying quantity of fragments and by-products occurring in all IVT mRNAs was detected at approximately 200 and 2,000 bp indicated by lower peaks. The peak at 25 bp is a method-derived marker peak for alignment. Overall, these data indicate mostly full-length IVT mRNA.

3.2 Transfection efficiency and kinetics of protein translation from IVT mRNA via flow cytometry

Transfection of A549 was conducted by lipofection of unmodified and different chemically modified mRNAs, each encoding *mKate2* (listed in Table 10). To assess the kinetics of protein expression from IVT cmRNA^{mKate2}(N1Ψ/ac4C) and test for its ability to enhance translational capacity, IVT cmRNA^{mKate2}(Ψ/m5C) and cmRNA^{mKate2}(N1Ψ/m5C) were used for comparison additionally to unmodified mRNA. Thereby, cmRNA^{mKate2}(N1Ψ/m5C) and cmRNA^{mKate2}(Ψ/m5C) represent the current (N1Ψ/m5C-modified) and former (Ψ/m5C-modified) state-of-the-art chemical modification in mRNA for protein supplementation [23, 79].
The encoded protein *mKate2* as a fluorescent protein allows the researcher to validate transfection, assess transfection efficiency and determine the amount of translated protein via flow cytometry. Transfection efficiency is thereby defined as the proportion of *mKate2* positive cells among the singlet population. The flow cytometry gating strategy is exemplarily shown in Figure 5.

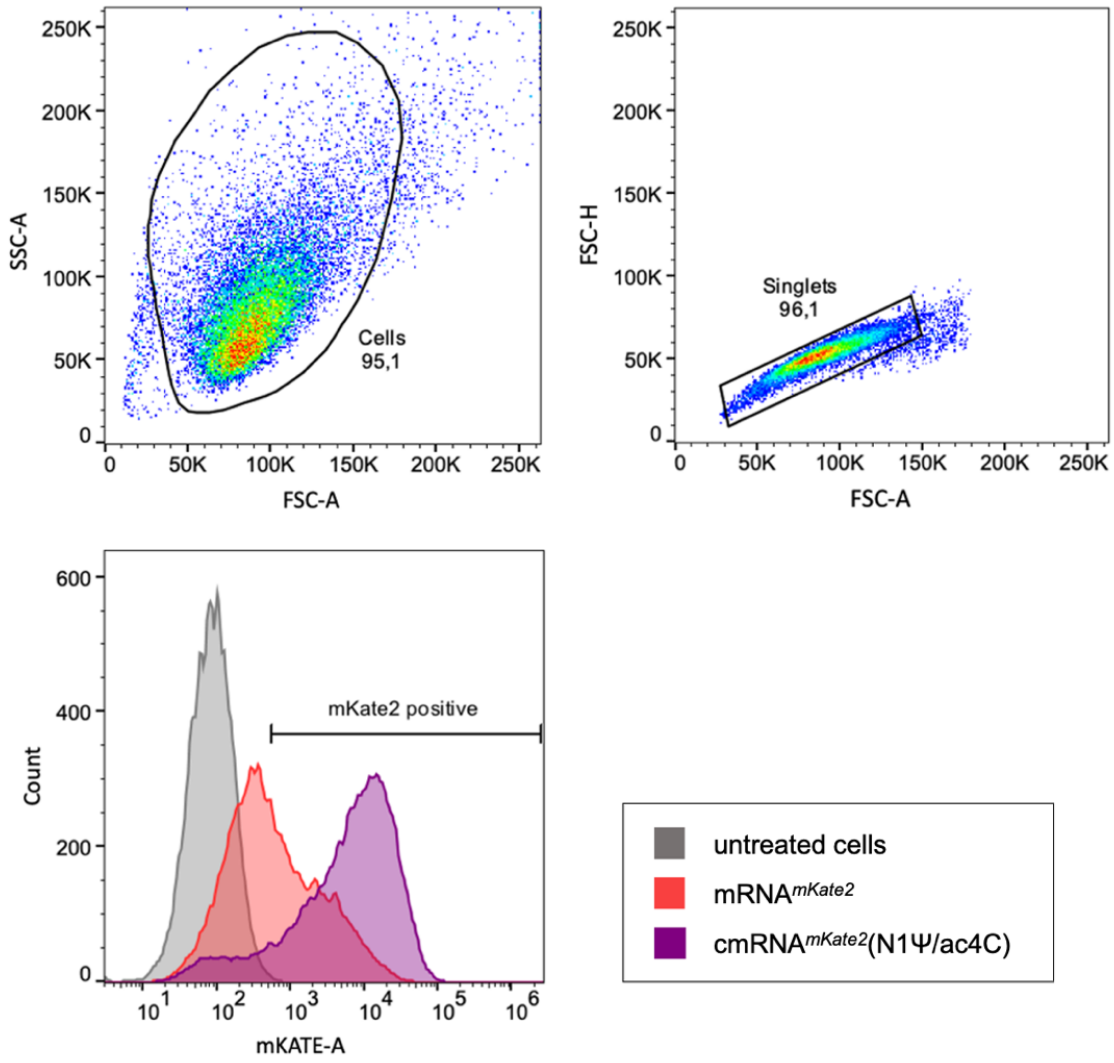

Figure 5: Flow cytometry gating strategy shown on the example of cmRNA^{mKate2}(N1Ψ/ac4C) and mRNA^{mKate2} 6 hours after transfection in A549. Abbreviations: FSC = forward scatter, SSC = side scatter, A = area, H = height.

3.2.1 Transfection efficiency

Transfection efficiency is represented by the percentage of *mKate2*-positive cells. High transfection efficiency, in a comparable order of magnitude across samples, was considered advantageous for the subsequent studies, as it does not only verify the pure transfection process (the Lipo2000-facilitated crossing of the cell membrane), but also the ability of the transfected cells in each sample to

effectively translate IVT mRNA into detectable protein *mKate2*. The proportion of *mKate2*-positive cells increased in all samples across modifications during the first 12 hours after transfection (Figure 6), which especially during the transfection process qualitatively displays the cellular uptake of transfected IVT mRNA that is immediately followed by translation. After 24 h, unmodified mRNA led to 87 % *mKate2*-positive cells, whereas the modifications reached 93 % (N1Ψ/m5C), 92 % (Ψ/m5C) and 96 % (N1Ψ/ac4C), respectively. Overall, transfection was highly efficient, and *mKate2*-positivity reached a comparable order of magnitude across unmodified *mKate2*-mRNA and its nucleotide modified counterparts.

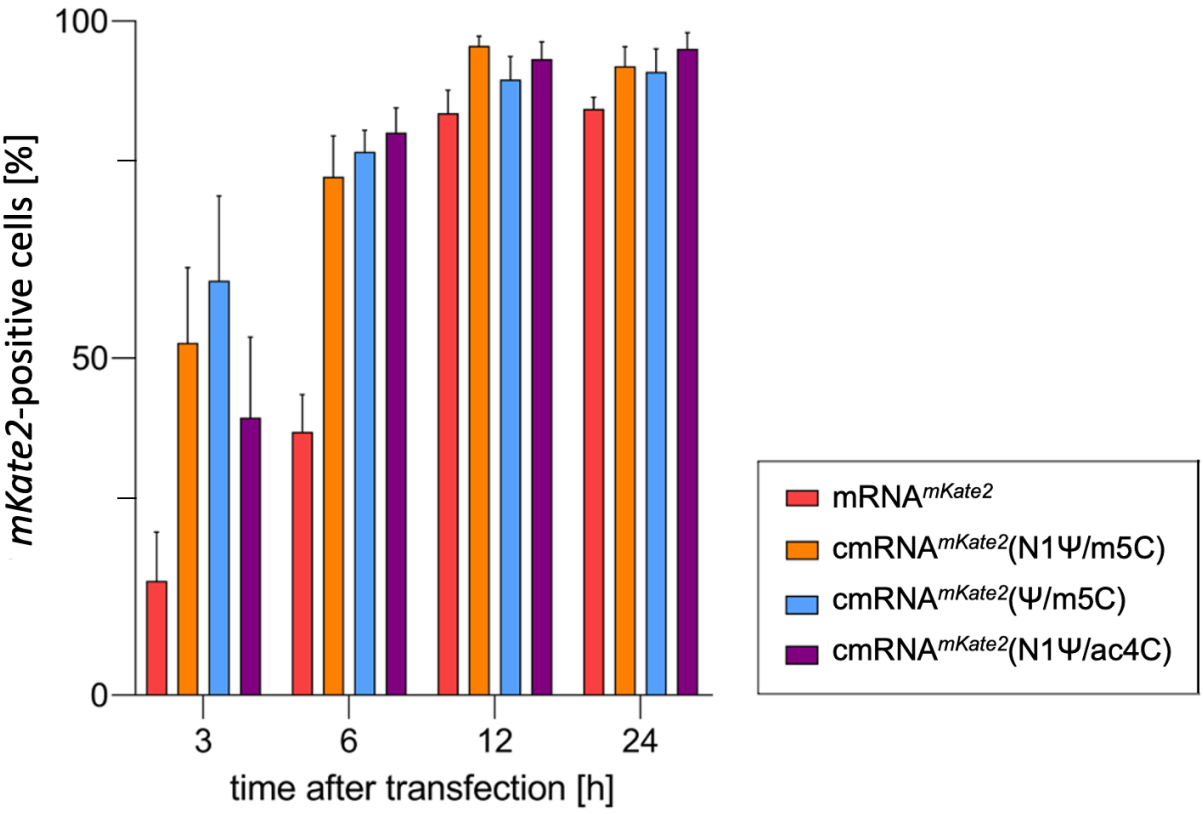

Figure 6: Proportion of *mKate2*-positive cells after lipofection of *mKate2*-encoding IVT mRNA in A549. Data is presented as mean ± SD for n = 4 samples.

3.2.2 Kinetics of protein translation from IVT mRNA

In addition to transfection efficiency, the quantity of translated protein from different mRNAs can be determined by flow cytometry detecting the fluorescent protein *mKate2*. Here, the mean fluorescence intensity (MFI) is a quantitative measure of the amount of translated protein. To understand the kinetics of protein translation from IVT mRNA and the differences between modifications herein, the MFI was determined at selected time points after transfection (Figure 7).

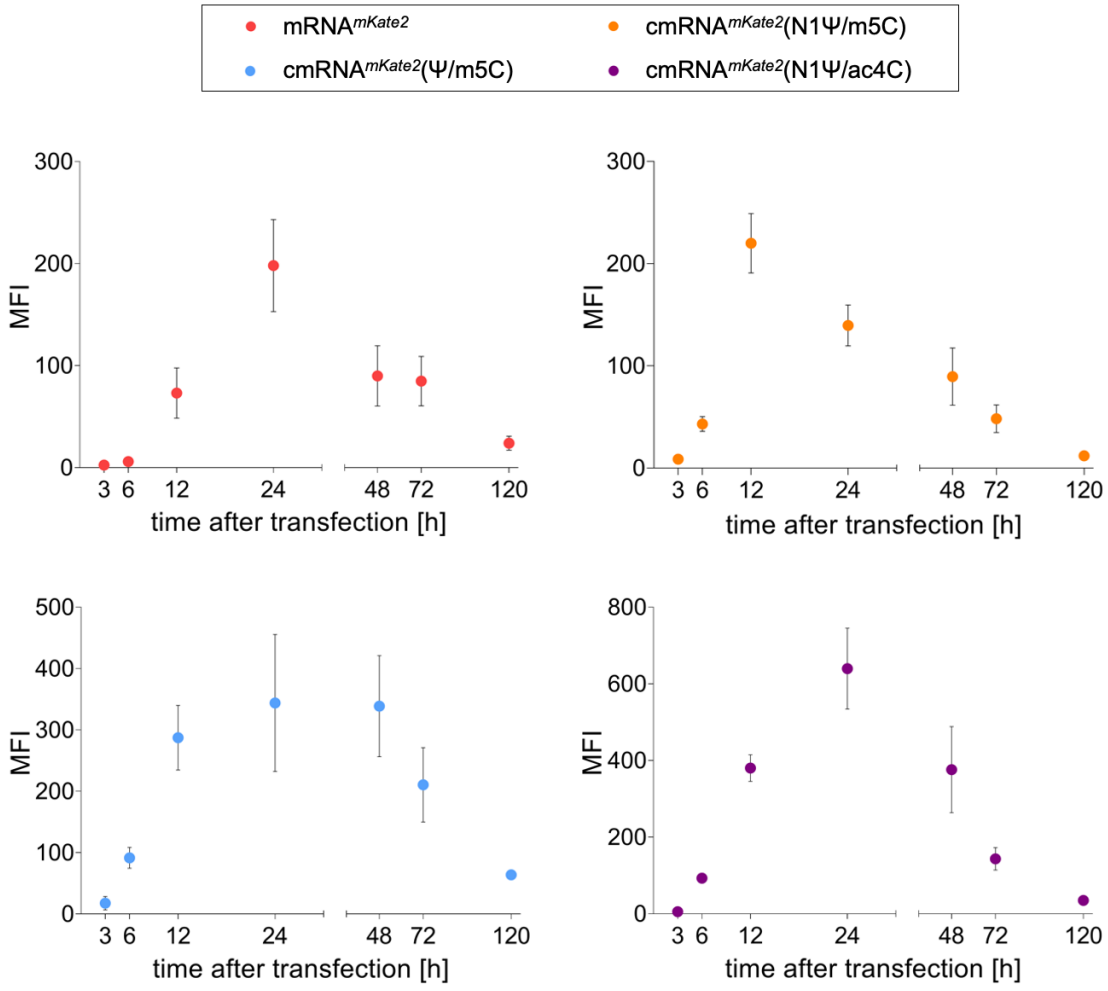

Figure 7: Kinetics of protein expression after lipofection of *mKate2*-encoding IVT mRNA in A549. Quantity of translated protein is defined through mean fluorescence intensity (MFI). Data are presented as mean ± SD for n = 4 samples. Abbreviations: MFI = mean fluorescence intensity.

In general, measured MFI increased across mRNA^{mKate2} and cmRNA^{mKate2} variants until 24 hours after transfection, except for cmRNA^{mKate2}(N1Ψ/m5C) which already shows its maximum MFI after 12 hours. After reaching maximum protein amounts, a decrease in MFI was recorded for all samples, and within 5 days after transfection the MFI has almost, however not yet completely, fallen to the initial level.

Individually assessed, the distinct mRNA modifications reveal different patterns of protein translation. In comparison to mRNA^{mKate2}, cmRNA^{mKate2}(N1Ψ/m5C) presented a relatively fast translation pattern, maximum MFI was shown already after 12 hours. By contrast, protein translation from cmRNA^{mKate2}(Ψ/m5C) was slower, but also long-lasting at its maximum level. Thus, maximum MFI only occurred after 24 hours with cmRNA^{mKate2}(Ψ/m5C), and it showed no significant decrease until the 48-hour measurement. cmRNA^{mKate2}(N1Ψ/ac4C) kinetics featured properties of both the expression patterns described above. On the one hand, transfection with cmRNA^{mKate2}(N1Ψ/ac4C) resulted in an early and strong increase in MFI during the first hours after transfection. Its maximum MFI was recorded with the 24-hour measurement point. On the other hand, although MFI decreased after 24 hours, cmRNA^{mKate2}(N1Ψ/ac4C) showed long-lasting high protein translation, which was most comparable to the kinetics of cmRNA^{mKate2}(Ψ/m5C).

In addition to comparing kinetics, it is important to also consider the varying levels of protein translation among different *mKate2*-mRNA modifications. For this purpose, in Figure 8 the two measuring points around the maximum protein levels (12 and 24 hours) were selected from Figure 7 for a direct comparison of protein amounts. It is remarkable that both at 12 and 24 hours after transfection, cmRNA^{mKate2}(N1Ψ/ac4C) resulted in the highest MFI among the *mKate2* mRNAs. The amount of protein translated from cmRNA^{mKate2}(N1Ψ/ac4C) exceeded not only mRNA^{mKate2}, where it achieved significantly higher MFI than its unmodified counterpart at both 12 hours and 24 hours after transfection. In addition, cmRNA^{mKate2}(N1Ψ/ac4C) also outperformed the standard modification cmRNA^{mKate2}(Ψ/m5C) and significantly outperformed the state-of-the-art cmRNA^{mKate2}(N1Ψ/m5C) after 24 hours.

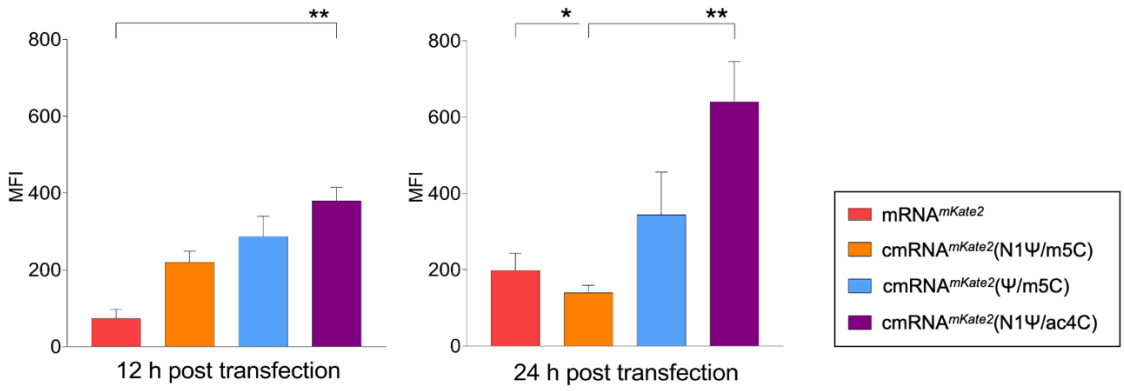

Figure 8: Levels of protein *mKate2* translated after lipofection of *mKate2*-encoding IVT mRNA in A549. Quantity of translated protein is defined through mean fluorescence intensity (MFI). Data are presented as mean ± SD for n = 4 samples. p-values are shown for significant differences in MFI compared to mRNA^{mKate2}. * $p \leq 0.05$, ** $p \leq 0.01$

3.3 Intracellular IVT mRNA decay

To quantify the amount of intracellular *mKate2* mRNA over time as an indicator for mRNA half-life, cells were lysed at selected time points after lipofection of mRNA^{mKate2} and cmRNA^{mKate2} variants in A549. Subsequently, RNA was purified followed by RT-qPCR. qPCR was performed targeting the *mKate2* sequence with 18S cDNA as an endogenous control.

For both the mRNA^{mKate2} and cmRNA^{mKate2} variants, relative expression showed an overall decrease over the observation period. In particular, the 120-hour measurement (end of the observation period) yielded only about 1-2 % of the initial relative expression measured directly after completion of the transfection process (represented by the 6-hour value). Unexpectedly, no substantial difference in relative mRNA levels was observed between *mKate2* mRNA modifications (Figure 9).

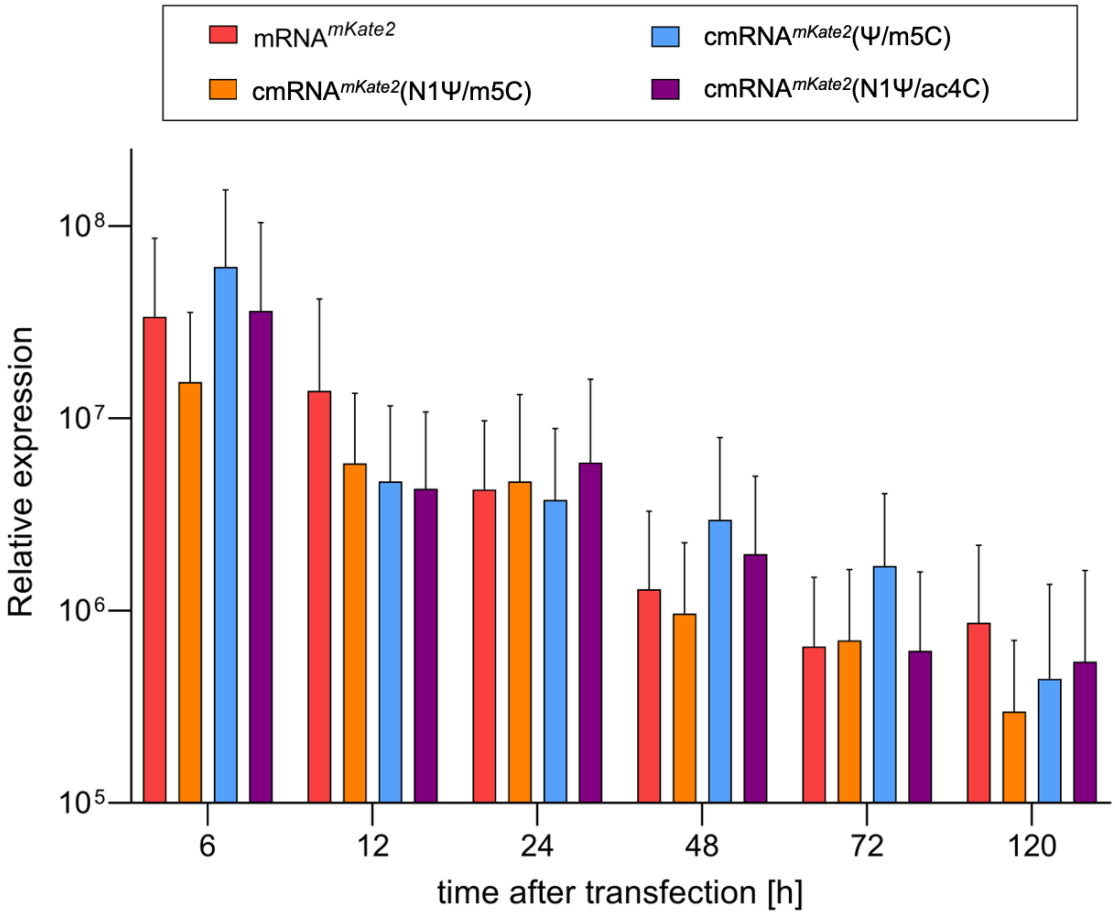

Figure 9: Kinetics of intracellular IVT mRNA decay after lipofection of *mKate2*-encoding IVT mRNA in A549. Quantity of present IVT mRNA is defined through relative expression $R = 2^{-\Delta\Delta Ct}$ [86]. Data are presented as mean ± SD for n = 5 samples.

3.4 Cell viability

Flow cytometry observations visually showed unequal amounts of debris (with especially low FSC-A in the FSC vs. SSC density plot), thus suggesting varying degrees of impairment on cell viability between mRNA modifications. An MTT assay was therefore performed at selected time points after transfection to validate cell viability in A549 cells over time and to better understand the side effects of IVT mRNA transfection. To quantify the isolated effect of Lipo2000 treatment on cell viability, cells treated with Lipo2000 alone were included as a

reference in addition to untreated cells as control. In this way, the impairment of cell viability between Lipo2000 and IVT mRNA could be better differentiated and an idea of the extent of the actual pure IVT mRNA effect could be gained (Figure 10).

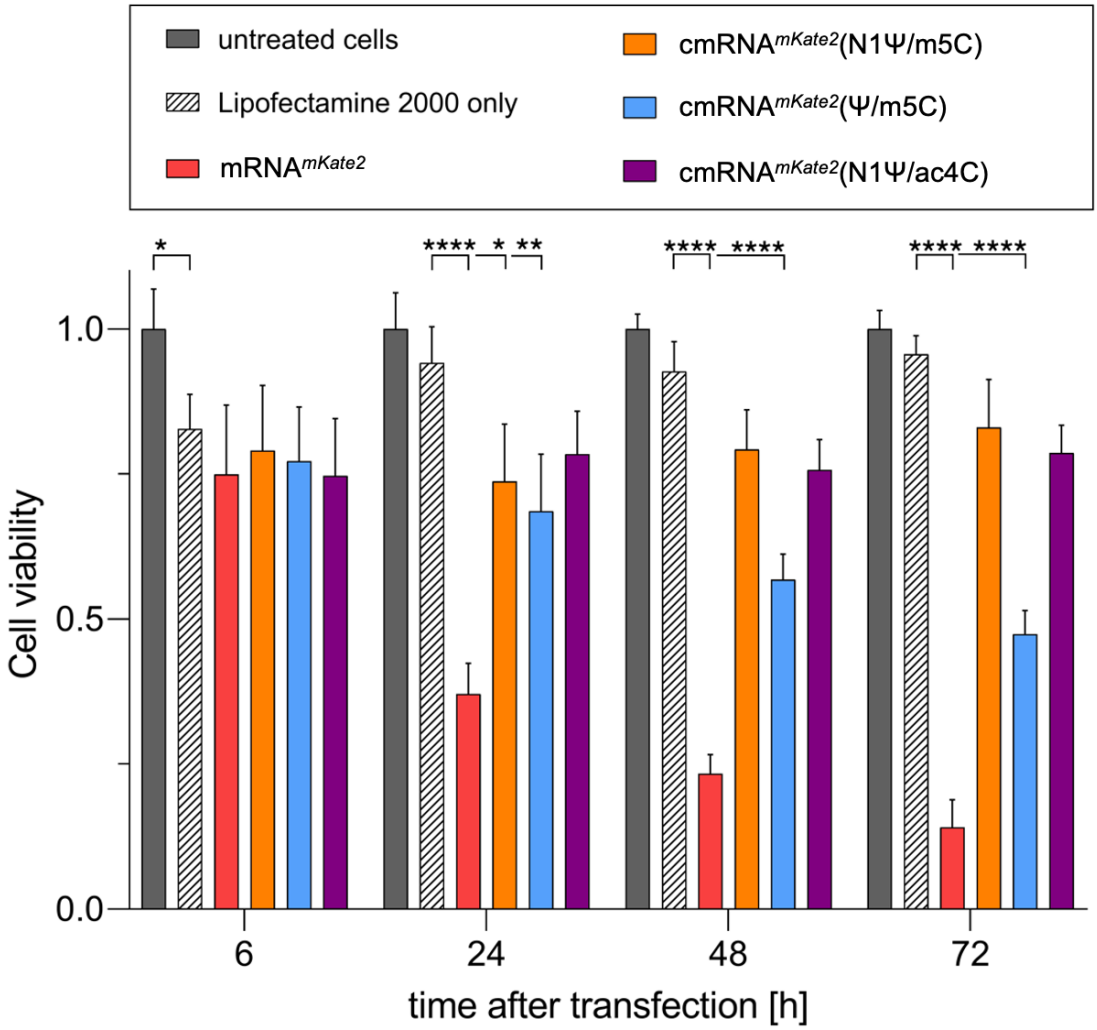

Figure 10: Cell viability after lipofection of *mKate2*-encoding IVT mRNA in A549. Cell viability is shown relative to control (untreated cells). Data are presented as mean ± SD for n = 4 samples. p-values are shown for significant differences in cell viabilities compared to Lipofectamine 2000 alone. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

After completion of the transfection process (6-hour measurement), Lipo2000-treated cells presented significantly lower cell viability than untreated cells.

However, samples transfected with *mKate2* mRNA and cmRNA variants did not show any significant additional decrease in cell viability compared to Lipo2000-treated samples after 6 hours.

When transfecting cmRNA^{mKate2}(N1Ψ/ac4C), no significant decrease in cell viability was observed at any measurement time point compared to Lipo2000 alone and cell viability remained relatively constant after the completion of the transfection process. The least also applies to cmRNA^{mKate2}(N1Ψ/m5C), although it showed some cell impairment with significantly lower cell viability than Lipo2000 alone after 24 hours.

In contrast, mRNA^{mKate2} showed a significant decrease in cell viability. Comparably, cmRNA^{mKate2}(Ψ/m5C) showed a notable decrease in cell viability, although not as pronounced as for mRNA^{mKate2}.

4. Discussion

4.1 General remarks

During the last three decades, IVT mRNA has been extensively studied as a therapeutic tool and has evolved from an unappreciated molecule to a desirable and promising method for protein supplementation which is now entering clinical trials. Through continuous modification of the molecules' structural elements thereby optimizing their properties, mRNA-based therapeutics have been brought step by step closer to therapeutic application. A particularly decisive contribution to scientific progress in this field was made by Karikó et al. who demonstrated a strategy for deimmunization of mRNA through the incorporation of modified nucleotides into IVT mRNA [70]. This has expanded the application possibilities of IVT mRNA beyond vaccination, thus paving the way for successful mRNA-based protein supplementation.

This thesis provides insight into the impact of incorporating the newly introduced nucleotide modification ac4C in combination with N1Ψ on the properties of IVT mRNA. In the experiments conducted, cmRNA^{mKate2}(N1Ψ/ac4C) showed high transfection efficiency and its kinetic profile portrays long-lasting translation shown in high protein levels over at least 5 days. In terms of translational capacity, cmRNA^{mKate2}(N1Ψ/ac4C) outperformed the comparative unmodified and modified mRNAs yielding superior amounts of protein upon transfection. Together with only marginal cytotoxicity upon transfection of cmRNA^{mKate2}(N1Ψ/ac4C), these results make cmRNA^{mKate2}(N1Ψ/ac4C) a highly desirable tool, particularly suitable for protein supplementation when defective or missing protein needs to be replaced. This conclusion shall be discussed in the context of the experiments on the kinetics of translation and cell viability that have been performed to characterize the impact of N1Ψ and ac4C on the properties of mRNA.

4.2 Kinetics of cmRNA translation and cmRNA half-life

Comparative measurements on mRNA kinetics rely on successful transfection across IVT mRNAs to ensure equal starting conditions. Transfection efficiency was therefore initially determined in each experiment for verification, showing only insignificantly small differences between mRNA variants.
The kinetic analysis of protein translation provides data on the relative amount of protein present at specific time points. If continuously plotted over time in an ideal setup, these measurements would allow an exact mapping of the translation kinetics and the determination of the total amount of translated protein obtained by calculating the area under the curve (AUC). Realistically, however, this is not methodically feasible, and the kinetic profile can only be studied approximately through the targeted selection of measurement points, as was the case in this work. Thus, it can be assumed but not given complete certainty that the kinetics are within this range. Furthermore, this type of representation does not allow for the calculation of an AUC. To provide an indication of how the IVT mRNA variants compare in terms of translational capacity, the 12- and 24-hour values were selected, although these are of course only the maximum values measured but possibly not the true maximum levels of protein.

Generally, mRNA half-life along with its ability to increase the efficiency of translation are the most important determinants of the kinetic profile of translation and the overall protein yield from IVT mRNA [1].
On the one hand, a high protein yield relative to the amount of IVT mRNA introduced in a setup is a strong indication of a highly effective translation process that generates large amounts of protein per mRNA molecule and time. This applies particularly to cmRNA^{mKate2}(N1Ψ/ac4C), which resulted in superior amounts of translated protein upon transfection compared to the other IVT mRNA variants. The finding, that cmRNA^{mKate2}(N1Ψ/ac4C) features enhances translational capacity, agrees with Arango et al. who suggested that ac4C has a promoting effect on translational efficiency [81]. Furthermore, it demonstrates the

enhancing effect on translational capacity also for ac4C incorporated in IVT mRNA together with N1Ψ.

On the other hand, the decrease in translated protein towards the end of the multi-day experiment can be attributed to the degradation of both the reporter protein and the protein-encoding mRNA, which is then no longer available to replace the decayed protein. As all mRNAs encoded the same protein in this study, only the process of mRNA decay can account for the varying rates of decline in protein levels whereas protein half-life can be disregarded as an influencing factor on the kinetic profile in this case.

Alongside spontaneous mRNA decay, mRNA immunogenicity is an important determinant of mRNA half-life. Specifically, the incorporation of modified nucleotides in mRNAs is considered a strategy to reduce immunogenicity, resulting in decreased susceptibility to intracellular immune processes and subsequent minor mRNA degradation compared to unmodified mRNA. Indeed, distinct translation patterns observed in this study pointed to significant differences in half-life between individual IVT mRNA variants, which however could not be confirmed through measurements of intracellular mRNA levels over time in this work. As long-lasting translation and protein detection cannot be exclusively explained by superior translational capacity, in this context, the lack of contrast between the half-life periods is most likely attributable to a methodological issue.

Despite strong indicators for a differing half-life between the IVT mRNA variants in their expression patterns, the experiments did not show a difference in mRNA half-lives as expected. Thus, the presumed enhanced stability and longer half-life of the nucleotide-modified mRNAs, especially cmRNA^{mKate2}(N1Ψ/ac4C), could not be demonstrated.

The likely cause of this failure is the calculation method of relative expression, $R = 2^{-\Delta\Delta Ct}$ [86] which amplifies even minor differences between the Ct values. Although the measured values were internally consistent within biological replicates, the calculated relative expression varied significantly across replicates (partially by several magnitudes), resulting in a conspicuously high standard deviation and no permanent decrease in relative expression. It remains to be

proven in future studies whether the combination of N1Ψ and ac4C has a stabilizing and immune-evading effect on IVT mRNA, thereby prolonging its half-life.

Further methodological limitations are associated with the data obtained for the comparative cmRNA^{mKate2}(N1Ψ/m5C) in this work restricting its informative value as a reference. The kinetic profile of protein translation for cmRNA^{mKate2}(N1Ψ/m5C), displaying an early peak in protein levels, was consistent with the laboratory's preliminary results. However, previous studies have demonstrated that the translational capacity of cmRNA(N1Ψ/m5C) outperforms cmRNA(Ψ/m5C). These results were in line with the findings of Andries et al.'s research which showed superior translation of cmRNA(N1Ψ/m5C) compared to cmRNA(Ψ/m5C) [79]. Surprisingly, cmRNA^{mKate2}(N1Ψ/m5C) exhibited lower translational capacity and a relatively rapid decay compared to cmRNA^{mKate2}(Ψ/m5C) in the experiments conducted for this thesis.

On the one hand, these findings were consistent across all biological replicates, indicating a failure likely already at the level of cmRNA(N1Ψ/m5C) IVT. Although mRNA integrity was assessed prior to transfection, it is important to note that Bioanalyzer analysis has limitations and cannot verify mRNA activity, functionality, or the encoded sequence. Therefore, the analysis is an indicator, however not a completely reliable predictor of mRNA performance upon transfection. Decreased concentration of cmRNA^{mKate2}(N1Ψ/m5C) at correct length, alongside a relatively high amount of by-products in the sample, provides evidence of substandard mRNA performance. As mostly mRNA with impaired sequence or other structural damage account for the level of by-products, and were not purified from intact mRNA before transfection, these may be the cause for overall higher instability and earlier decay. Furthermore, mRNA encoding an inaccurate sequence can result in defective *mKate2* which may display reduced or absent fluorescence.

On the other hand, it is possible that different proteins cannot always be translated equally well from mRNA carrying the same modifications. Therefore,

while N1Ψ/m5C has been shown to be an advantageous combination of modifications increasing total protein yield for some proteins in previous studies, it may not necessarily have the same effect on *mKate2*.
Both factors may contribute to the lower values observed in MFI and the reduced translational capacity for cmRNA^{mKate2}(N1Ψ/m5C) in this thesis.
Despite these limitations for the comparative cmRNA^{mKate2}(N1Ψ/m5C) in terms of translational capacity and half-life, its kinetic profile of protein translation resulted as expected. Thus, cmRNA^{mKate2}(N1Ψm/5C) remains a suitable reference to provide context for the kinetic profile of cmRNA^{mKate2}(N1Ψ/ac4C) in this work.

4.3 Cell viability after transfection

Cell viability is an indispensable factor in determining the suitability of mRNA for pharmaceutical use in protein supplementation. Balancing the need for efficient protein expression with the imperative of maintaining cellular viability is decisive for the successful translation of mRNA-based therapeutics from *in vitro* studies to clinical applications. Cell viability assays conducted for this thesis highlight different patterns of cell viability impairment by IVT mRNA transfection. In the case of mRNA^{mKate2} and cmRNA^{mKate2}(Ψ/m5C) treatments, the evidence strongly indicates that cell death primarily arises from the mRNA itself. In contrast, Lipo2000 treatment was the main driver of cell death when cells were transfected with cmRNA^{mKate2}(N1Ψ/m5C) and cmRNA^{mKate2}(N1Ψ/ac4C) – IVT mRNA treatment itself only exerted minor cytotoxicity in overall terms.
In accordance with prior research, it is most likely that lower cytotoxicity of IVT mRNAs containing N1Ψ is due to a modulation in innate immune signaling: N1Ψ-incorporating IVT mRNA has been shown to evade immune activation in previous studies [79], avoiding RIG-I activation [23, 75] and preventing eIF2α phosphorylation [80]. Therefore, it is highly probable that also in combination with ac4C, immune evasion by N1Ψ is at least partly responsible for the reduction of immunogenicity and thus lesser cytotoxicity of cmRNA^{mKate2}(N1Ψ/ac4C).

The present study underscores that cell viability is a critical determinant influencing cellular outcomes and its preliminary evaluation is essential for accurately determining the potential of a given mRNA modification for *in vivo* protein supplementation.

4.4 Outlook

4.4.1 Future research on ac4C-modified therapeutic IVT mRNA

Ac4c was only recently added to the list of nucleotide modifications naturally incorporated in human mRNA [81]. This study now provides a first insight into the properties of ac4C incorporated into IVT mRNA together with N1Ψ for potential use in therapeutic protein supplementation. Here, mRNA modified with N1Ψ/ac4C exhibited a favorable kinetic profile with high protein yield efficiency and only minor cytotoxicity, making it a promising tool for mRNA-based protein supplementation.

Further research beyond this first approach is certainly required to gain a more comprehensive understanding of the properties of ac4C-modified IVT mRNA. Future studies should focus on investigating the mechanisms by which ac4C enhances translation efficiency, mediates immune evasion, and prevents cytotoxicity. To achieve precise characterization of ac4C, it will be necessary to conduct transfections with IVT mRNAs containing ascending percentages of ac4C, both alone and in combination with other nucleotide modifications.

Moreover, it will be crucial to perform transfection experiments in cell lines other than A549 to obtain a more generalized understanding of the effects of ac4C, at least *in vitro*. Being cancer cells, A549 are only suitable as a model for primary cells to a limited extent as many immune mechanisms do not function without restriction. Therefore, it cannot be excluded that transfection of N1Ψ/ac4C-modified mRNA in primary cells differs from that in A549 with respect to kinetics, translational capacity, half-life, and cytotoxicity.

Finally, transitioning from *in vitro* to *in vivo*, including the evaluation of the kinetic profile and considering the complex process of administration *in vivo*, will be an essential step towards clinical translation.

4.4.2 Challenges and opportunities in mRNA-based protein supplementation

As with the development of any other drug, safety aspects must be adequately considered for mRNA-based protein supplementation. The issue of immunogenicity of mRNA molecules has largely been addressed by the incorporation of modified nucleotides into therapeutic IVT mRNA. However, the variations in cell viability observed between modifications in this study emphasize the importance of carefully selecting mRNA modifications, critically considering their potential impact on cell viability.

Due to limited experience from clinical studies, it is currently unclear whether an immunogenic effect of the protein translated from IVT mRNA must be expected, as can occur with recombinant protein [1, 87, 88]. Unlike recombinant protein, IVT mRNA-encoded protein is translated in the recipient's cells, where it is folded and post-translationally modified similarly to endogenous proteins. Therefore, adverse events due to immunogenicity are unlikely in mRNA-based protein supplementation, yet dose escalation protocols in clinical trials and close patient monitoring are critical to ensure adequate safety.

In the context of clinical trials and future clinical application, additional challenges may arise. *In vivo* IVT mRNA delivery commonly requires nanosized drug formulations with complexing agents to protect the mRNA from degradation by ubiquitously present RNases, and to facilitate cellular uptake [1, 23]. Moreover, tissue-selective delivery needs to be precisely coordinated to ensure best preconditions for highly efficient transfection [89]. Finally, the achievement of the intended therapeutic dose level must be ensured at the target organ or the target cells. This requires precise knowledge of how much protein can be generated

from a certain amount of IVT mRNA *in vivo*, and whether this value is consistent between individuals.

mRNA-based protein supplementation therapies hold great potential for personalized medicine. Simplified manufacturing processes in contrast to complex traditional therapeutics may reduce production expenses and increase the accessibility to innovative treatments. Additionally, mRNA technology allows a wide range of applications – from treating rare genetic disorders by specific protein supplementation to vaccination and various approaches to cancer therapy. Especially the latter offers the possibility of therapeutic mRNA tailored to the patient's individual genetic profile. For example, personalized mRNA-based cancer vaccines can be developed that code for tumor neoantigens and are designed according to the specific mutation signature.

Given the broad experience recently gained in mRNA-based infectious disease vaccination, research may profoundly benefit from knowledge transfer between these neighboring fields and is further encouraged to explore novel treatment modalities across various medical areas. The promising advancements in mRNA technology emphasize its transformative potential to become a widely applied therapeutic strategy to address critical health challenges. Thorough characterization and thoughtful selection of mRNA modifications ensure both efficacy and safety in the development of mRNA-based therapies, paving the way for a new era of personalized therapeutics.

5. Abstract

The concept of mRNA-based protein supplementation relies on introducing synthetic mRNA as a transient carrier of genetic information into an organism, where it is translated into the desired therapeutic protein using the cell's machinery. mRNA-based approaches have become increasingly popular as an alternative to DNA-based gene therapy for replacing absent or faulty protein. The landmark discovery that RNA immunogenicity can be reduced by incorporating modified nucleotides initiated the advancement of mRNA from an underappreciated molecule to a promising new class of therapeutics.

This thesis investigates the impact of the chemically modified nucleotides N4-acetylcytidine (ac4C) and N1-methylpseudouridine (N1Ψ) incorporated in IVT mRNA on the kinetic profile of protein translation. *mKate2*-encoding synthetic mRNA modified with N1Ψ and ac4C was transfected into A549 cells *in vitro*. For comparison, mRNA modified with Ψ and m5C as well as mRNA modified with N1Ψ and m5C, which represent the former and current state-of-the-art modifications to enhance translational capacity and cell viability, were included in the investigations in addition to unmodified mRNA. Subsequently, their translational kinetics, mRNA decay, and their influence on cell viability were examined over time.

mRNA modified with N1Ψ and ac4C demonstrated superior translational capacity that lasted for at least 5 days and resulted in a higher yield of reporter protein *mKate2*. Additionally, N1Ψ- and ac4C-modified mRNA caused only marginal cytotoxicity, resulting in minor losses in terms of cell viability. These results indicate the potential of modifying IVT mRNA with N1Ψ and ac4C as a promising tool for mRNA-based protein supplementation.

6. Zusammenfassung

Das Konzept der mRNA-basierten Proteinsupplementation basiert darauf, synthetische mRNA als transienten Träger genetischer Information in einen Organismus einzuschleusen, wo diese mithilfe der zelleigenen Maschinerie in das gewünschte therapeutische Protein translatiert wird. Als Alternative zur DNA-basierten Gentherapie, um fehlendes oder fehlerhaft gebautes Protein im Organismus zu ersetzen, ist der Ansatz der mRNA-basierten Proteinsupplementation zunehmend in den Vordergrund gerückt. Insbesondere die richtungsweisende Entdeckung, dass die Immunogenität einer mRNA durch den Einbau modifizierter Nukleotide herabgesetzt werden kann, ermöglichte die Entwicklung der mRNA zu einem vielversprechenden neuen Therapiekonzept.

Diese Arbeit untersucht, welchen Einfluss der Einbau der chemisch modifizierten Nukleotide N4-Acetylcytidin (ac4C) und N1-Methylpseudouridin (N1Ψ) auf das kinetische Profil der Translation ausübt. Hierzu wurde für das Protein *mKate2* kodierende, mit N1Ψ und ac4C modifizierte synthetische mRNA *in vitro* in A549-Zellen transfiziert. Zum Vergleich wurden Ψ- und m5C-modifizierte sowie N1Ψ- und m5C-modifizierte IVT-mRNA (früherer und aktueller Standard zur Verbesserung der Translationskapazität und Zellviabilität), zusätzlich zu unmodifizierter mRNA in die Untersuchungen miteinbezogen. Im Anschluss wurden die Translationskinetik, der mRNA-Abbau sowie der Einfluss auf die Zellviabilität im Zeitverlauf untersucht.

N1Ψ- und ac4C-modifizierte mRNA zeigte dabei eine gesteigerte Translationskapazität, die über mindestens 5 Tage anhielt und zu einer vergleichsweise hohen Ausbeute des Reporterproteins *mKate2* führte. Darüber hinaus wies N1Ψ/ac4C-modifizierte mRNA nur eine marginale Zytotoxizität auf und verursachte so nur geringe Einbußen in Bezug auf die Zellviabilität. Diese Ergebnisse demonstrieren das Potential der Modifikation von IVT mRNA mit N1Ψ und ac4C als vielversprechendes Werkzeug für die mRNA-basierte Proteinsupplementation.

7. References

1. Sahin, U., K. Kariko, and O. Tureci, *mRNA-based therapeutics--developing a new class of drugs.* Nat Rev Drug Discov, 2014. **13**(10): p. 759-80.
2. Kariko, K., *In vitro-Transcribed mRNA Therapeutics: Out of the Shadows and Into the Spotlight.* Mol Ther, 2019. **27**(4): p. 691-692.
3. Jorgensen, E.D., et al., *Specific contacts between the bacteriophage T3, T7, and SP6 RNA polymerases and their promoters.* J Biol Chem, 1991. **266**(1): p. 645-51.
4. Krieg, P.A. and D.A. Melton, *Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs.* Nucleic Acids Res, 1984. **12**(18): p. 7057-70.
5. Kallen, K.J. and A. Thess, *A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs.* Ther Adv Vaccines, 2014. **2**(1): p. 10-31.
6. Kariko, K., et al., *Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability.* Mol Ther, 2008. **16**(11): p. 1833-40.
7. Van Tendeloo, V.F., P. Ponsaerts, and Z.N. Berneman, *mRNA-based gene transfer as a tool for gene and cell therapy.* Curr Opin Mol Ther, 2007. **9**(5): p. 423-31.
8. Hacein-Bey-Abina, S., et al., *LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1.* Science, 2003. **302**(5644): p. 415-9.
9. Newbury, S.F., *Control of mRNA stability in eukaryotes.* Biochem Soc Trans, 2006. **34**(Pt 1): p. 30-4.
10. Qureischi, M., et al., *mRNA-based therapies: Preclinical and clinical applications.* Int Rev Cell Mol Biol, 2022. **372**: p. 1-54.
11. Leonhardt, C., et al., *Single-cell mRNA transfection studies: delivery, kinetics and statistics by numbers.* Nanomedicine, 2014. **10**(4): p. 679-88.
12. Ligon, T.S., C. Leonhardt, and J.O. Radler, *Multi-level kinetic model of mRNA delivery via transfection of lipoplexes.* PLoS One, 2014. **9**(9): p. e107148.
13. Yamamoto, A., et al., *Current prospects for mRNA gene delivery.* Eur J Pharm Biopharm, 2009. **71**(3): p. 484-9.
14. Breckenridge, N.C. and R.H. Davis, *Optimization of repeated-batch transcription for RNA production.* Biotechnol Bioeng, 2000. **69**(6): p. 679-87.
15. Qin, S., et al., *mRNA-based therapeutics: powerful and versatile tools to combat diseases.* Signal Transduct Target Ther, 2022. **7**(1): p. 166.
16. Gao, M., et al., *Synthetic modified messenger RNA for therapeutic applications.* Acta Biomater, 2021. **131**: p. 1-15.
17. Polack, F.P., et al., *Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine.* N Engl J Med, 2020. **383**(27): p. 2603-2615.
18. Baden, L.R., et al., *Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine.* N Engl J Med, 2021. **384**(5): p. 403-416.
19. Jirikowski, G.F., et al., *Reversal of diabetes insipidus in Brattleboro rats: intrahypothalamic injection of vasopressin mRNA.* Science, 1992. **255**(5047): p. 996-8.
20. Kormann, M.S., et al., *Expression of therapeutic proteins after delivery of chemically modified mRNA in mice.* Nat Biotechnol, 2011. **29**(2): p. 154-7.

21. Kariko, K., et al., *Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin.* Mol Ther, 2012. **20**(5): p. 948-53.
22. Mays, L.E., et al., *Modified Foxp3 mRNA protects against asthma through an IL-10-dependent mechanism.* J Clin Invest, 2013. **123**(3): p. 1216-28.
23. Sahu, I., et al., *Recent Developments in mRNA-Based Protein Supplementation Therapy to Target Lung Diseases.* Mol Ther, 2019. **27**(4): p. 803-823.
24. Haque, A., et al., *Chemically modified hCFTR mRNAs recuperate lung function in a mouse model of cystic fibrosis.* Sci Rep, 2018. **8**(1): p. 16776.
25. Zangi, L., et al., *Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction.* Nat Biotechnol, 2013. **31**(10): p. 898-907.
26. Gan, L.M., et al., *Intradermal delivery of modified mRNA encoding VEGF-A in patients with type 2 diabetes.* Nat Commun, 2019. **10**(1): p. 871.
27. Anttila, V., et al., *Synthetic mRNA Encoding VEGF-A in Patients Undergoing Coronary Artery Bypass Grafting: Design of a Phase 2a Clinical Trial.* Mol Ther Methods Clin Dev, 2020. **18**: p. 464-472.
28. Kong, N., et al., *Synthetic mRNA nanoparticle-mediated restoration of p53 tumor suppressor sensitizes p53-deficient cancers to mTOR inhibition.* Sci Transl Med, 2019. **11**(523).
29. Raab, M., et al., *Rescue of p53 functions by in vitro-transcribed mRNA impedes the growth of high-grade serous ovarian cancer.* Cancer Commun (Lond), 2024. **44**(1): p. 101-126.
30. Wolff, J.A., et al., *Direct gene transfer into mouse muscle in vivo.* Science, 1990. **247**(4949 Pt 1): p. 1465-8.
31. Bourquin, C., et al., *Immunostimulatory RNA oligonucleotides trigger an antigen-specific cytotoxic T-cell and IgG2a response.* Blood, 2007. **109**(7): p. 2953-60.
32. Sander, L.E., et al., *Detection of prokaryotic mRNA signifies microbial viability and promotes immunity.* Nature, 2011. **474**(7351): p. 385-9.
33. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.
34. Chen, N., et al., *RNA sensors of the innate immune system and their detection of pathogens.* IUBMB Life, 2017. **69**(5): p. 297-304.
35. Vaidyanathan, S., et al., *Uridine Depletion and Chemical Modification Increase Cas9 mRNA Activity and Reduce Immunogenicity without HPLC Purification.* Mol Ther Nucleic Acids, 2018. **12**: p. 530-542.
36. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.* Nature, 2001. **413**(6857): p. 732-8.
37. Heil, F., et al., *Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8.* Science, 2004. **303**(5663): p. 1526-9.
38. Diebold, S.S., et al., *Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA.* Science, 2004. **303**(5663): p. 1529-31.
39. Balka, K.R. and D. De Nardo, *Understanding early TLR signaling through the Myddosome.* J Leukoc Biol, 2019. **105**(2): p. 339-351.
40. Diebold, S.S., et al., *Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides.* Eur J Immunol, 2006. **36**(12): p. 3256-67.
41. Yoneyama, M., et al., *The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses.* Nat Immunol, 2004. **5**(7): p. 730-7.
42. Hornung, V., et al., *5'-Triphosphate RNA is the ligand for RIG-I.* Science, 2006. **314**(5801): p. 994-7.

43. Pichlmair, A., et al., *RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates.* Science, 2006. **314**(5801): p. 997-1001.
44. Pichlmair, A., et al., *Activation of MDA5 requires higher-order RNA structures generated during virus infection.* J Virol, 2009. **83**(20): p. 10761-9.
45. Kell, A.M. and M. Gale, Jr., *RIG-I in RNA virus recognition.* Virology, 2015. **479-480**: p. 110-21.
46. Wu, B. and S. Hur, *How RIG-I like receptors activate MAVS.* Curr Opin Virol, 2015. **12**: p. 91-8.
47. Anderson, B.R., et al., *Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation.* Nucleic Acids Res, 2010. **38**(17): p. 5884-92.
48. Galabru, J. and A. Hovanessian, *Autophosphorylation of the protein kinase dependent on double-stranded RNA.* J Biol Chem, 1987. **262**(32): p. 15538-44.
49. Ehrenfeld, E. and T. Hunt, *Double-stranded poliovirus RNA inhibits initiation of protein synthesis by reticulocyte lysates.* Proc Natl Acad Sci U S A, 1971. **68**(5): p. 1075-8.
50. Balachandran, S., et al., *Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection.* Immunity, 2000. **13**(1): p. 129-41.
51. Kumar, A., et al., *Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B.* Proc Natl Acad Sci U S A, 1994. **91**(14): p. 6288-92.
52. Garcia, M.A., et al., *Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action.* Microbiol Mol Biol Rev, 2006. **70**(4): p. 1032-60.
53. Samuel, C.E., *Mechanism of interferon action: phosphorylation of protein synthesis initiation factor eIF-2 in interferon-treated human cells by a ribosome-associated kinase processing site specificity similar to hemin-regulated rabbit reticulocyte kinase.* Proc Natl Acad Sci U S A, 1979. **76**(2): p. 600-4.
54. Anderson, B.R., et al., *Nucleoside modifications in RNA limit activation of 2'-5'-oligoadenylate synthetase and increase resistance to cleavage by RNase L.* Nucleic Acids Res, 2011. **39**(21): p. 9329-38.
55. Chakrabarti, A., B.K. Jha, and R.H. Silverman, *New insights into the role of RNase L in innate immunity.* J Interferon Cytokine Res, 2011. **31**(1): p. 49-57.
56. Kristiansen, H., et al., *The oligoadenylate synthetase family: an ancient protein family with multiple antiviral activities.* J Interferon Cytokine Res, 2011. **31**(1): p. 41-7.
57. Li, Y. and M. Kiledjian, *Regulation of mRNA decapping.* Wiley Interdiscip Rev RNA, 2010. **1**(2): p. 253-65.
58. Sonenberg, N., *eIF4E, the mRNA cap-binding protein: from basic discovery to translational research.* Biochem Cell Biol, 2008. **86**(2): p. 178-83.
59. Malone, R.W., P.L. Felgner, and I.M. Verma, *Cationic liposome-mediated RNA transfection.* Proc Natl Acad Sci U S A, 1989. **86**(16): p. 6077-81.
60. Mockey, M., et al., *mRNA transfection of dendritic cells: synergistic effect of ARCA mRNA capping with Poly(A) chains in cis and in trans for a high protein expression level.* Biochem Biophys Res Commun, 2006. **340**(4): p. 1062-8.
61. Stepinski, J., et al., *Synthesis and properties of mRNAs containing the novel "anti-reverse" cap analogs 7-methyl(3'-O-methyl)GpppG and 7-methyl (3'-deoxy)GpppG.* RNA, 2001. **7**(10): p. 1486-95.
62. Holtkamp, S., et al., *Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells.* Blood, 2006. **108**(13): p. 4009-17.
63. Chen, C.Y. and A.B. Shyu, *AU-rich elements: characterization and importance in mRNA degradation.* Trends Biochem Sci, 1995. **20**(11): p. 465-70.

64. Mauro, V.P., *Codon Optimization in the Production of Recombinant Biotherapeutics: Potential Risks and Considerations.* BioDrugs, 2018. **32**(1): p. 69-81.
65. Yu, C.H., et al., *Codon Usage Influences the Local Rate of Translation Elongation to Regulate Co-translational Protein Folding.* Mol Cell, 2015. **59**(5): p. 744-54.
66. Stein, K.C. and J. Frydman, *The stop-and-go traffic regulating protein biogenesis: How translation kinetics controls proteostasis.* J Biol Chem, 2019. **294**(6): p. 2076-2084.
67. Kimchi-Sarfaty, C., et al., *A "silent" polymorphism in the MDR1 gene changes substrate specificity.* Science, 2007. **315**(5811): p. 525-8.
68. Angov, E., et al., *Heterologous protein expression is enhanced by harmonizing the codon usage frequencies of the target gene with those of the expression host.* PLoS One, 2008. **3**(5): p. e2189.
69. Thess, A., et al., *Sequence-engineered mRNA Without Chemical Nucleoside Modifications Enables an Effective Protein Therapy in Large Animals.* Mol Ther, 2015. **23**(9): p. 1456-64.
70. Kariko, K., et al., *Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA.* Immunity, 2005. **23**(2): p. 165-75.
71. Boccaletto, P., et al., *MODOMICS: a database of RNA modification pathways. 2017 update.* Nucleic Acids Res, 2018. **46**(D1): p. D303-D307.
72. Koski, G.K., et al., *Cutting edge: innate immune system discriminates between RNA containing bacterial versus eukaryotic structural features that prime for high-level IL-12 secretion by dendritic cells.* J Immunol, 2004. **172**(7): p. 3989-93.
73. Kariko, K. and D. Weissman, *Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: implication for therapeutic RNA development.* Curr Opin Drug Discov Devel, 2007. **10**(5): p. 523-32.
74. Davis, D.R., *Stabilization of RNA stacking by pseudouridine.* Nucleic Acids Res, 1995. **23**(24): p. 5020-6.
75. Durbin, A.F., et al., *RNAs Containing Modified Nucleotides Fail To Trigger RIG-I Conformational Changes for Innate Immune Signaling.* mBio, 2016. **7**(5).
76. Huang, H., et al., *Recognition of RNA N(6)-methyladenosine by IGF2BP proteins enhances mRNA stability and translation.* Nat Cell Biol, 2018. **20**(3): p. 285-295.
77. Yang, X., et al., *5-methylcytosine promotes mRNA export - NSUN2 as the methyltransferase and ALYREF as an m(5)C reader.* Cell Res, 2017. **27**(5): p. 606-625.
78. Warren, L., et al., *Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA.* Cell Stem Cell, 2010. **7**(5): p. 618-30.
79. Andries, O., et al., *N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice.* J Control Release, 2015. **217**: p. 337-44.
80. Svitkin, Y.V., et al., *N1-methyl-pseudouridine in mRNA enhances translation through eIF2alpha-dependent and independent mechanisms by increasing ribosome density.* Nucleic Acids Res, 2017. **45**(10): p. 6023-6036.
81. Arango, D., et al., *Acetylation of Cytidine in mRNA Promotes Translation Efficiency.* Cell, 2018. **175**(7): p. 1872-1886 e24.
82. Kumbhar, B.V., A.D. Kamble, and K.D. Sonawane, *Conformational preferences of modified nucleoside N(4)-acetylcytidine, ac4C occur at "wobble" 34th position in the anticodon loop of tRNA.* Cell Biochem Biophys, 2013. **66**(3): p. 797-816.
83. Dominissini, D. and G. Rechavi, *N(4)-acetylation of Cytidine in mRNA by NAT10 Regulates Stability and Translation.* Cell, 2018. **175**(7): p. 1725-1727.

84. Karthiya, R., S.M. Wasil, and P. Khandelia, *Emerging role of N4-acetylcytidine modification of RNA in gene regulation and cellular functions.* Mol Biol Rep, 2020. **47**(11): p. 9189-9199.
85. Jin, G., et al., *The Processing, Gene Regulation, Biological Functions, and Clinical Relevance of N4-Acetylcytidine on RNA: A Systematic Review.* Mol Ther Nucleic Acids, 2020. **20**: p. 13-24.
86. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
87. Kromminga, A. and H. Schellekens, *Antibodies against erythropoietin and other protein-based therapeutics: an overview.* Ann N Y Acad Sci, 2005. **1050**: p. 257-65.
88. Casadevall, N., et al., *Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin.* N Engl J Med, 2002. **346**(7): p. 469-75.
89. Sergeeva, O.V., V.E. Koteliansky, and T.S. Zatsepin, *mRNA-Based Therapeutics - Advances and Perspectives.* Biochemistry (Mosc), 2016. **81**(7): p. 709-22.

8. Erklärung zum Eigenanteil

Die Arbeit wurde in der Universitätsklinik für Thorax-, Herz- und Gefäßchirurgie unter Betreuung von Frau Prof. Dr. rer. nat. Meltem Avci-Adali angefertigt. Die Generation und Auswertung der Daten erfolgte in der Universitätsklinik für Kinder- und Jugendmedizin unter Betreuung von Prof. Dr. rer. nat. Michael Kormann.

Die Konzeption der Studie erfolgte mit Unterstützung von Brian Weidensee M. Sc. im Kormann Lab.

Sämtliche Versuche wurden nach Einarbeitung durch Brian Weidensee von mir eigenständig durchgeführt, bei der Durchführung der Transfektionen erhielt ich Unterstützung durch Brian Weidensee.

Die statistische Auswertung erfolgte nach Anleitung durch Brian Weidensee durch mich.

Ich versichere, das Manuskript selbstständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Stuttgart, den 16. Mai 2024

Franziska Fleischhacker

Acknowledgements

I would like to take this opportunity to thank everyone who has provided me with their great support in writing this thesis.

I would especially like to thank Prof. Michael Kormann for sparking my interest and enthusiasm for mRNA-based therapies and accepting me into his research group in the Kormann Lab.
My special thanks also go to Prof. Avci-Adali for kindly taking over the supervision of this thesis and supporting me during the writing process and final completion.

Furthermore, I would like to thank my lab tutor Brian Weidensee for introducing me to all laboratory methods and for the great guidance throughout my time in the Kormann Lab. Also, I would like to thank my lab supervisor Dr. Tahereh Mohammadian Gol for her valuable help with all my questions.

Finally, I would like to say thank you to both my family and friends for your support, encouragement, and your patience proof-reading this thesis.