Strategies to Minimize Innate Immune Stimulation to Maximize Messenger RNA Bioavailability

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Abstract

Messenger RNAs (mRNAs) are an exciting new class of nucleic acid therapeutics entering the clinic. mRNAs are useful in difficult to transfect, non-dividing cells. In contrast to plasmid or viral vectors, there is no risk of insertional mutagenesis or oncogenesis. Transient mRNA expression is also desirable for cellular reprogramming, genome editing (ZFNs, TALENs, CRISPR/Cas9) and vaccines.

For maximal expression in cells or target organs, transfected mRNAs must avoid detection by pattern recognition receptors (PRRs) that evolved to sense pathogenic non-self RNAs. These include PRRs that recognize improperly capped RNAs and double stranded RNA. PRR activation leads to cytokine production, translational arrest and cell toxicity or death. Mammalian mRNAs are modified |post-transcriptionally to contain nucleotides with 2′-O-methyl residues, pseudouridine (Ψ) and N6-methyladenosine (^{m6}A). These modifications can reduce activation of PRRs and allow maximal translation of the transfected mRNA.

During mRNA capping, Cap 0 (^{m7}GpppN) is formed as an intermediate. Methylation of the 2′ position of the first and sometimes second nucleotide forms Cap 1 and Cap 2, respectively. Recombinant enzymes used to generate Cap 1 mRNA are expensive, do not always go to completion and the RNA must be purified prior to capping. We developed a novel co-transcriptional capping method that yields Cap 1 with high efficiency and lower costs in a "one pot" reaction. CleanCap® also facilitates modification of the 5′ end of mRNAs with diverse functional groups, including ^{m6}A. We developed a capping assay that allows direct assessment of mRNA capping. Capping efficiencies as high as 95% were obtained.

We synthesized several Ψ derivatives to explore the impact on innate immune stimulation: N1-ethyl-Ψ (Et1Ψ), N1-fluoroethyl-Ψ (FE1Ψ), N1-propyl-Ψ (Pr1Ψ), N1- isopropyl-Ψ (iPr1Ψ) and N1-methoxylmethyl-Ψ (MOM1Ψ) 5′-triphosphates. As a control, we tested 5-methoxy-uridine alongside the pseudouridine derivatives because it previously gave good activity. Luciferase mRNAs were fully substituted with these modifications and translational potential was monitored in wheat germ extracts. Activity was also measured in the THP-1 monocyte cell line, which is a sensitive model for innate immune activation. A recent report showed that minimizing uridine content in mRNAs reduced immune stimulation by unmodified mRNAs. Here we show that incorporation of our modified uridine residues into uridine depleted luciferase resulted in equivalent activity relative to Ψ in THP-1 cells. Interestingly, translation in extracts did not mirror activity in THP-1 cells.

Background: Why mRNA Therapeutics?

- **mRNA is a popular new tool for gene expression**
  - » Does not have a risk of insertional mutagenesis
  - » Can transfect difficult cells such as non-dividing cells
  - » Is transient
- **Applications**
  - » Genome editing (Transposons, Cre, ZFNs, TALENs and CRISPR/Cas9)
  - » Gene replacement
  - » Vaccines
- **Limitations**
  - » Innate immune response to unmodified mRNA
- **Solutions**
  - » Proper capping
  - » Chemical modification of mRNA can prevent innate immune stimulation
  - » Removal of dsRNA

Innate immune sensors recognize mRNA

Transfection of cells with unmodified RNAs can lead to cell death due to activation of innate immune pathways

Toll-like receptors 3, 7 & 8 recognize different RNA forms
» Found in endosomes where some viruses enter cells

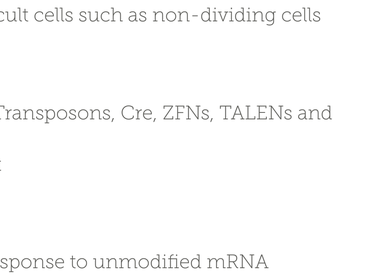
Cytosolic sensors
» Protein Kinase R (PKR): dsRNA
» MDA5: long dsRNA
» IFITs: unmethylated cap structures
» RIG-I: 5′-triphosphate

Figure 1: Cap 0, Cap 1 and Cap 2 Structures of 5′-Ends of mRNAs

Eukaryotic mRNAs have a Cap 1 or Cap 2 structure.

Sensing of proper cap structure is thought to be involved in self/non-self RNA recognition.

Cap structure influences activation of PRRs
» RIG-I is activated by Cap 0 RNAs but not Cap 1 mRNAs (PMID: 18426922 and 20457754)
» IFIT1 binds Cap0 RNAs more tightly than Cap 1 mRNAs (PMID: 24371270)



Cap0: R^1 and R^2=H
Cap1: R^1=CH_3; R^2=H
Cap2: R^1=CH_3; R^2=CH_3
B^{1-3} = A, C, G, U or ^{m6}A (B^1)

Co-transcriptional capping with CleanCap® (Cap 1) helps evade an immune response

Figure 2: CleanCap®, Enzymatic and ARCA, Capping Comparison

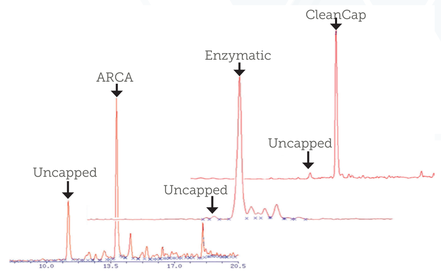
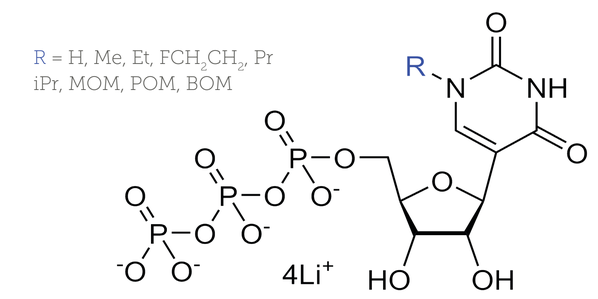


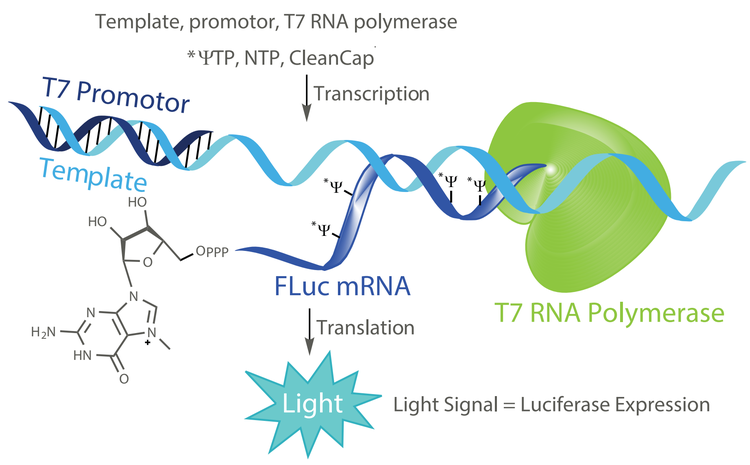
Figure 3: Pseudouridine 5'-Triphosphate Derivatives

» Incorporation of modified nucleosides in mRNA helps to evade an immune response
» Ψ or m5C/Ψ modifications of mRNA are current industry standard
» Several novel pseudouridine NTPs were synthesized and tested in firefly luciferase mRNA transcriptions



Pseudouridine 5′-triphosphate derivatives; H = pseudouridine, Me = N1-methyl, Et = N1-ethyl, FE = fluoroethyl, Pr = propyl, iPr = isopropyl, MOM = methoxy methyl, POM = pivaloxy methyl, BOM = benzyloxy methyl pseudouridine

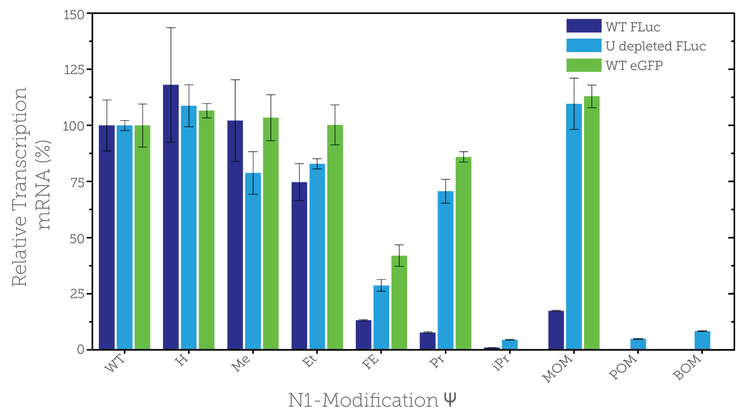
Figure 4: mRNA Synthesis by *In Vitro* Transcription



Schematic representation of transcription and translation with N1-modified pseudouridine 5′-triphosphate derivatives in the presence of CleanCap®: *N1-modified-Ψ

Figure 5: U Depletion of Primary Luciferase mRNA Sequence Improves Incorporation of N1-Substituted Ψ Derivatives by T7 Polymerase

» Some N1-substituted Ψ derivatives did not incorporate well in WT mRNA
» We decreased the number of uridine residues in the sequence by substituting synonymous codons
» U depletion resulted in good incorporation



» Relative efficiency of mRNA synthesis by *in vitro* transcription using T7 RNA polymerase with N1-modified ΨTPs; 7.5 mM triphosphates, 37 °C for 3 h; 2100 Agilent Bioanalyzer with RNA nano chip

Figure 6: Sequence Engineering of FLuc mRNA (Ψ or MOM1Ψ Substitution, Bioanalyzer)

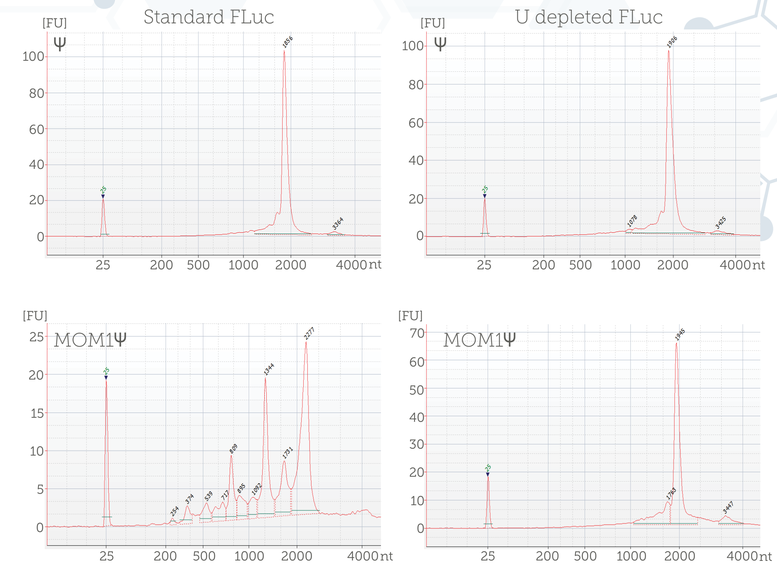
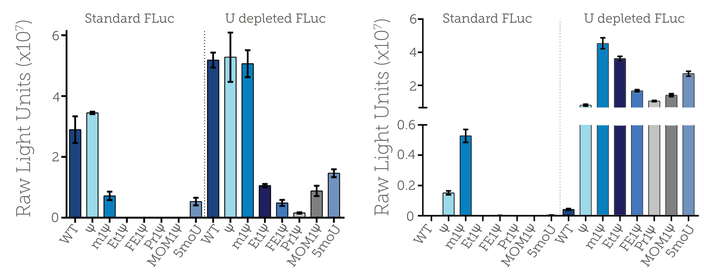


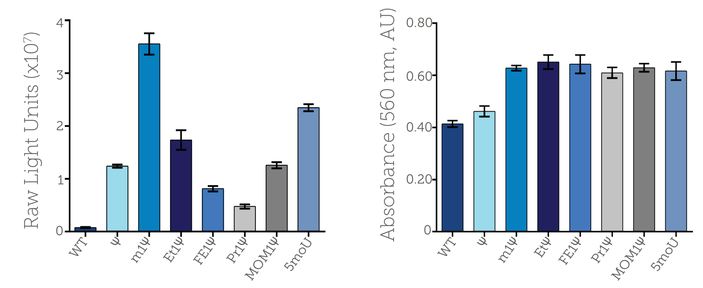
Figure 7: *In Vitro* Translation and Cell Activity of Modified Luciferase mRNAs

» U depleted mRNA sequences translated better in wheat germ extracts
» mRNA with bulkier pseudouridine modifications did not translate well
» U depleted mRNA sequences resulted in higher activity in THP-1 cells
» We therefore continued our studies using the U depleted mRNA sequence



Translation in wheat germ extracts | Activity in THP-1 cells

Figure 8: N1-Substituted Ψ Derivatives Resulted in Lower Toxicity Compared to WT and Ψ in FLuc mRNAs



Activity in THP-1 cells | Toxicity (MTT assay*) in THP-1 cells

*Lower absorbance reflects decreased cellular metabolism indicating toxicity

Conclusions

» We have synthesized a number of 5′-triphosphates of N1-modified pseudouridine derivatives
» These nucleoside 5′-triphosphates were used for the synthesis of modified mRNAs by *in vitro* transcription using WT and U-depleted templates
» Efficiency of transcription using U-depleted templates greatly improved quality and yield
» N1-substituted Ψ mRNAs show potential translational and immunological properties
» Translational activity of modified mRNAs in wheat germ extracts did not directly correlate with cell activity, which may indicate differences in immune stimulation by these mRNAs

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