Sequence engineering to de-immunize mRNA to expand its therapeutic window

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Background: In vitro transcribed (IVT) mRNA is an emerging therapeutic modality. Although similar to endogenous mRNA, IVT mRNA can be immunostimulatory unless mitigation strategies are applied. Immune activation can inhibit protein synthesis, induce toxicity, and trigger pro-inflammatory cytokine release. Previous studies showed that reducing cytidine (C) and uridine (U) content decreases immunogenicity and enhances translation. However, biased nucleotide usage may impair synthesis or translation through effects on secondary structure. We aimed to develop a sequence-engineering strategy that reduces C and/or U content while considering codon usage and secondary structure, to improve mRNA safety, protein yield, and widening the therapeutic window. Method: We developed an optimization strategy in which at least 10% of C nucleotides, and optionally U nucleotides, in wild-type mRNA are replaced by canonical non-C and/or non-U nucleotides, generating C-, U-, or UC-depleted sequences. This was applied to secNLuc, eGFP, and murine EPO mRNAs. Original codons were replaced with synonymous codons encoding the same amino acids but containing fewer C and/or U nucleotides. Alternative codons maintained frequencies close to the originals in the relevant exome; where no equivalent existed, higher-frequency alternatives were used. Expression of wild-type and modified mRNAs was assessed in HeLa cells 24 hours after transfection and in mice 6 hours after injection. Results: UC- and C-depleted mRNAs increased protein expression by over 2-fold to 15-fold versus wild-type mRNA, respectively, whereas U-depletion had a less significant effect. In vivo, U- and UC-depleted mEPO mRNAs induced 14-fold and 18-fold higher mEPO levels than wild-type mRNA, respectively. Conclusion: This canonical nucleotide-based optimization strategy reduces mRNA immunogenicity by lowering C and/or U content. Depending on sequence context, C- or UC-depletion maximized protein expression. In vivo, wild-type mEPO expression was negligible, highlighting the potential of this approach to increase mRNA therapeutic efficacy as an alternative to chemically modified nucleotides