Efficient expression of stabilized mRNA PEG-peptide polyplexes in liver

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The expression efficiency in liver following hydrodynamic delivery of in vitro transcribed mRNA was improved 2000-fold using a codon-optimized mRNA luciferase construct with flanking 3′ and 5′ human β-globin untranslated regions (UTR mRNA) over an unoptimized mRNA without β-globin UTRs. Nanoparticle UTR mRNA polyplexes were formed using a novel polyacrylidene polyethylene glycol (PEG) peptide, resulting in an additional 15-fold increase in expression efficiency in the liver. The combined increase in expression for UTR mRNA PEG-peptide polyplexes was 3500-fold over mRNA lacking UTRs and PEG-peptide. The expression efficiency of UTR mRNA polyplex was 10-fold greater than the expression from an equivalent 1 μg dose of pGL3. Maximal expression was maintained from 4 to 24 h. Serum incubation established the unique ability of the polyacrylidene PEG-peptide to protect UTR mRNA polyplexes from RNase metabolism by binding to double-stranded regions. UTR mRNA PEG-peptide polyplexes are efficient nonviral vectors that circumvent the need for a nuclear uptake, representing an advancement toward the development of a targeted gene delivery system to transfect liver hepatocytes.

INTRODUCTION

The development of a nonviral gene delivery system that efficiently expresses proteins in the liver has been a long-sought goal for over 25 years. Preclinical studies have demonstrated that protein expression in hepatocytes could lead to curative treatments for liver metabolic diseases as well as diseases in other organs. Much of the effort in developing a nonviral gene delivery system for the liver has focused on packaging and targeting plasmid DNA. Despite much effort, systemic delivery of DNA formulations resulted in either negligible or very-low gene transfer efficiency in liver hepatocytes. In contrast, hydrodynamic (HD) delivery of naked plasmid DNA to liver achieves expression efficiency equivalent to adenovirus or AAV. Although HD delivery is highly efficient because it overcomes the rate limiting step of delivery of DNA to the nucleus, it is also an invasive delivery method requiring high volume and pressure. Alternatively, the delivery of mRNA to the cytosol leading to translation, circumvents the need for delivery to the nucleus. Despite this major advantage, the rapid metabolism of mRNA by ubiquitous RNase remains a significant hurdle in achieving an efficient expression of systemically delivered mRNA gene delivery systems.

Since the earliest report demonstrating in vivo expression following i.m. dosed naked mRNA, numerous studies have attempted to increase the stability and expression efficiency of mRNA formulations using cationic lipids. Intratracheal high pressure spraying of an mRNA Megafectin lipoplex resulted in transfection of the lung, whereas regeneration following myocardial infarction was achieved by intracardial injection of RNAI-MAX mRNA. Stemfect mRNA delivered nasally resulted in tumor vaccination. Alternatively, systemically delivered Stemfect mRNA produced low level expression in the spleen. A mannansylated histidinylated lipoplex dosed systemically resulted in expression in spleen macrophages which primed a tumor vaccine response. Although these studies demonstrate that mRNA lipoplexes possess improved in vivo gene transfer over naked mRNA, their efficiency in vivo is still very low due to relatively weak ionic binding of cationic lipids to mRNA.

In an attempt to further improve mRNA stability, nanoparticle delivery systems have been developed and tested in vitro and in vivo. Systemic delivery of targeted stealth mRNA lipoplexes in vivo led to transfection efficiency similar to DNA formulations in solid tumor. Intrathecally dosed mRNA polyplex nanomicelles produced measurable expression in the cerebrospinal fluid. Notably, none of the mRNA cationic lipid or nanoparticle formulations reported to date were able to transfect liver.

There have been only two reports of successful liver transfection with mRNA. The expression of mRNA in the liver was first observed by McCaffrey et al. in 2002 who measured luciferase expression by bioluminescence imaging (BLI) in mice following HD-dosing of 50 μg of naked mRNA to detect low level expression (10^6 photons s^-1 cm^-2 per steradian). The transient expression in the liver was only detectable at 3 h and required the co-administration of 30 μg of decoy RNA and 400 units of RNase inhibitor. In an attempt to improve transfection efficiency, in 2006 Wilber et al. refined the mRNA construct by inserting 5′ and 3′ Xenopus laevis β-globin untranslated regions (UTRs) flanking luciferase to increase mRNA cellular half-life. HD dosing of 50 μg of UTR mRNA resulted in a 15-fold increase in the expression efficiency at 3 h relative to mRNA lacking UTRs, but failed to significantly extend the expression. Co-administration of decoy mRNA and RNase inhibitors significantly improved efficiency but failed to extend peak expression past 12 h. Although these reports demonstrate the feasibility of expressing proteins in the liver when HD dosing mRNA, the efficiencies reported are far below
that are achievable with plasmid DNA due to mRNA's susceptibility to metabolism during delivery.

In the present study, mRNA was packaged into polyplexes using a novel polyethylene glycolylated (PEG) polycaridic peptide to overcome mRNA instability. This unique peptide has been shown to bind with high affinity to double-stranded DNA through polynucleotide, affording protection from metabolism in the circulation of mice for up to 12 h. mRNA is single stranded, but adopts a complex secondary structure that includes extensive double helices. We therefore hypothesized that a PEGylated polycaridic peptide would protect mRNA from RNase metabolism relative to PEG-peptides that bind mRNA through ionic interaction. The results demonstrate synergistic improvements in gene transfer efficiency when dosing UTR mRNA PEGylated polycaridic peptide polyplexes allowing a 1 μg HD-dose to achieve even greater expression than plasmid DNA at 24 h post injection.

RESULTS

Two earlier studies established the ability to hydrodynamically dose 50 ng of mRNA resulting in transient low level (10^6 photons cm^-2 steradian^-1 per second) luciferase expression in the liver at 3 h post mRNA delivery. A major goal of the present study was to significantly improve the efficiency of mRNA expression as a first step toward further development of a targeted mRNA delivery system for liver. To achieve this, a codon-optimized mRNA construct containing 3' and 5' β-globin UTRs was generated to extend mRNA half-life in the cytoplasm as first described by Malone et al. mRNA UTRs have been used to improve the translation of several proteins, including erythropoietin, urokinase receptor and β-globin, and may be generally applicable. In addition, mRNA was packaged into a polyplex by combining it with a PEG-peptide to improve its metabolic stability (Scheme 1).

We have previously shown that short polylysine PEG-peptides that possess multiple acidic residues incorporated onto Lys side chains (Acr) possess significantly higher binding affinity for double-stranded plasmid DNA affording PEG-peptide DNA polyplexes with much greater metabolic stability in the circulation. Consequently, the predicted folded stem-loop structures of mRNA should also provide significant double-stranded character to support high-affinity binding by peptide 1 through a combination of polynucleotide and ionic binding (Scheme 1). Conversely, the ionic binding of peptide 2 to mRNA was predicted to result in mRNA polyplexes that were less metabolically stable (Scheme 1).

Comparison of the binding of peptide 1 and peptide 2 to mRNA demonstrated that both peptides formed mRNA polyplexes that inhibited migration on gel electrophoresis at 0.1 nmol of PEG-peptide per μg mRNA (Figure 1). PEG-peptide polyplexes used for physical studies and in vivo experiments were formed at 0.8 nmol of peptide per μg of mRNA to fully protect the mRNA from RNase metabolism. Dynamic light scattering revealed that mRNA polyplexes possessed an average diameter of 104 nm and an average zeta potential of +15 mV. By comparison, pGL3 PEG-peptide polyplexes possessed a diameter of 170 nm and a zeta potential of +15 mV. The smaller size of an mRNA polyplex is consistent with the shorter length of mRNA (1.6 kb) versus plasmid DNA (5.3 kbp).

The ability of mRNA polyplexes to resist direct digestion with RNase was evaluated using gel electrophoresis (Figure 2). Both PEG-peptides were found to stabilize mRNA to RNase digestion, as demonstrated by the recovery of intact mRNA when PEG-peptide polyplexes were digested with up to 30 ng ml^-1 RNase A (Figure 2). By comparison, naked mRNA was completely digested by 3 ng ml^-1 of RNase. However, incubation of mRNA polyplexes with 100 ng ml^-1 RNase or higher resulted in either partial or complete digestion of mRNA. mRNA polyplexes prepared with PEG-peptides 1 and 2 were equally stabilized to direct RNase digestion (Figure 2).

Direct HD dosing of 20 μg of an unoptimized mRNA encoding luciferase (Supplementary Figure 1) resulted in transient expression as determined by BLI detection of 10^6 photons cm^-2 steradian^-1 per second in the liver at 4 h, returning to background signal at 24 h (Figure 3), consistent with the results from McCaffrey et al. To improve the expression efficiency of mRNA, a codon-optimized luciferase gene was synthesized possessing 3' and 5' human β-globin UTRs flanking the luciferase gene (Supplementary Figure 2). Direct HD dosing of 1 μg of UTR mRNA resulted in BLI detection of 3.7 x 10^7 photons cm^-2 steradian^-1 per second in the liver at 24 h post mRNA delivery (Figure 3). The expression efficiency with UTR mRNA was approximately 200-fold greater than mRNA lacking UTRs, and was only 10-fold lower than the efficiency afforded from HD delivery of 1 μg of pGL3 (Figure 3). Control experiments suggested that the HD delivery of UTR mRNA to the cytosol of hepatocytes was sufficient to mediate efficient expression. HD dosing of tailed, but uncapped UTR mRNA, or uncapped anduntailed UTR mRNA produced background BLI signal (Figure 3). However, HD dosing of capped, but untailed UTR mRNA, produced ~1.8 x 10^8 photons cm^-2 steradian^-1 per second in the liver at 24 h, which was only 10-fold less than fully capped and tailed UTR mRNA (Figure 3). This result suggests that HD dosing delivers capped UTR mRNA to the nucleus where some tailing occurs, resulting in 10-fold less expression compared with fully capped and tailed UTR mRNA.

PEG-peptide - UTR mRNA polyplexes were similarly protected from the action of RNase (Figure 2). Consequently, HD dosing of peptide 1 UTR mRNA polyplex produced 1.4 x 10^8 photons cm^-2 steradian^-1 per second in the liver at 24 h, which is significantly higher than naked UTR mRNA and peptide 2 UTR mRNA polyplex, supporting the hypothesis that peptide 1 polyintercalation affords greater mRNA polyplex stability in vivo (Figure 4). Additionally, the peptide 1 UTR mRNA polyplex produced significantly higher expression than pGL3 plasmid DNA.

Further evidence of the enhanced stability of peptide 1 UTR mRNA polyplex resulted from incubation in mouse serum (Figure 5). Naked UTR mRNA and peptide 2 UTR mRNA polyplex were unable to protect mRNA from metabolism, resulting in the complete loss of luciferase expression. However, incubation of peptide 1 UTR mRNA polyplex in mouse serum followed by HD dosing produce 1 x 10^8 photons cm^-2 steradian^-1 per second in the liver at 24 h post mRNA delivery, indicating increased RNase protection resulting from PEG-peptide binding to double-stranded regions of mRNA (Figure 5). Serial BLI analysis was used to examine the time course of luciferase expression mediated by HD delivery of a 1 μg dose of PEG-peptide UTR mRNA polyplex. The expression determined at 4 h remained constant for 24 h, after which it declined 10-fold per 24 h to reach background in 84 h (Figure 6).

DISCUSSION

The ease of plasmid DNA production along with its successful packaging into polyplexes and lipoplexes has led to the development of efficient in vitro transfection agents. More sophisticated blood compatible polyplexes and liposomes possessing targeting ligands have been delivered systemically to tumors in vivo. However, the systemic delivery of plasmid DNA to nondividing quiescent liver hepatocytes remains a significant challenge. Once inside hepatocytes, the endosomal escape of DNA nanoparticles remains inefficient and nuclear delivery is yet to be convincingly demonstrated. The nuclear envelope presents a major barrier to DNA delivery. Although the membrane is perforated by many nuclear pore complexes, the maximum diameter allowed through the pore is approximately 39 nm, smaller than most DNA nanoparticles. When plasmid DNA was microinjected into nondividing cells, only 0.1% of plasmids were detected in the nucleus.
The successful delivery of siRNA to the cytosol of hepatocytes in animals has prompted this and other investigations into the delivery of mRNA to the cytosol of hepatocytes, as a first step toward bypassing nuclear uptake as the major impediment to the development of i.v. dosed targeted gene delivery systems. As with the development of siRNA delivery systems, optimization of the oligonucleotide construct for potency is a prerequisite to packaging and delivery. HD dosing lends itself well to the optimization of DNA constructs that are designed for hepatocyte expression. The rapid delivery in a large volume of saline displaces serum nucleases and results in the nuclear delivery of naked plasmid DNA. However, prior HD dosing studies demonstrated that luciferase-expressing mRNA was not efficiently expressed, pointing to the need to improve the mRNA construct and further stabilize mRNA to RNase.

Compared with an unoptimized mRNA construct, codon optimization combined with installing a 5' and 3' human β-globin UTR into the mRNA produced a 2000-fold improvement in luciferase expression efficiency following HD dosing (Figure 3). These changes increased the expression, so that a 1 μg dose of UTR mRNA was equivalent to 1 μg of plasmid DNA (pGL3) when assayed at 24 h following HD delivery (Figure 3) in addition to producing a transient expression profile (Figure 6) equivalent to pGL3. The 2000-fold enhancement is significantly greater than the 15-fold increase reported by Wilber et al. who included 5' and 3'-UTRs from X. laevis. While many elements of an mRNA's design impact stability and expression efficiency, such as configuration of 3' UTRs and the length of the poly-A tail, the improvement in efficiency reported here most likely results from individual or combined contributions from the use of a β-globin 3' UTR.
and 5’ UTRs from human origin instead of X. laevis, codon optimization of luciferase, and the length of the poly-A resulting from tailing pre-mRNA using polyadenosine polymerase. One of the greatest challenges in developing an i.v. dosed mRNA delivery system is protecting the oligonucleotide from metabolism by endogenous RNase. Nonviral gene delivery systems that rely solely upon ionic binding with oligonucleotides either possess insufficient affinity to remain stable in the circulation or are highly charged making them incompatible with blood. The present study used a polyacridine PEG-peptide with proven ability to protect plasmid DNA in the circulation to stabilize mRNA through intercalative binding to double-stranded regions of the stem-loop structures (Scheme 1). Two experimental results establish that a PEGylated polyacridine peptide provides superior protection to mRNA relative to a comparable polylysine PEG-peptide that lacks the ability to intercalate mRNA. Polyacridine PEG-peptide mRNA polyplexes provided a significant 15-fold enhanced luciferase expression upon HD dosing relative to mRNA polyplexes prepared with polylysine PEG-peptide (Figure 4). Furthermore, the transfection of an mRNA polyplex is preserved following pre-incubation in mouse serum only when protected by a polyacridine PEG-peptide (Figure 5).

This study demonstrates that in vitro transcribed mRNA possessing natural nucleotides is of comparable efficiency as plasmid DNA (pGL3) when delivered by HD dosing. It is also possible that mRNA mediated expression may be further enhanced through the use of modified nucleotides such as pseudouridine and/or 5-methylcytidine, which have been shown to improve protein expression and reduce immune response. These findings show that mRNA has the potential to replace DNA in some systemically delivered gene delivery applications. The transient expression of mRNA may be desirable in certain applications such as genome editing using zinc finger nucleases, TALEN nucleases or the CRISPR/Cas9 system, or integration through transposases where short term mRNA expression is needed to avoid potential genotoxicity. PEGylated polycrindle peptide protects mRNA from RNase activity in vitro, and enhances luciferase expression in liver by approximately 15-fold, suggesting that mRNA stability may be improved even further by optimizing peptides for higher affinity binding to mRNA. Although HD dosing may not be directly applicable to delivery of mRNA to humans, it remains a useful research tool to allow optimization of mRNA constructs and polyplex design to increase transfection efficiency, which is prerequisite to the development of efficient targeted mRNA gene delivery systems.

MATERIALS AND METHODS
DNA and mRNA Preparation
pGL3 control vector (Promega, Madison, WI, USA), a 5.3 kbp plasmid encoding the firefly luciferase gene controlled by an SV40 promoter, was grown in DH5α Escherichia coli and purified by Qiagen Gigaprep kit (Qiagen, Germantown, MD, USA). Template DNA for in vitro transcription was prepared by synthesizing the firefly luciferase gene with flanking 5’ and 3’ UTRs derived from human β-globin and codon optimization for expression.
in mice (GenScript, Piscataway, NJ, USA). The synthetic gene (Luc-UTR) was inserted into the pcDNA3.1(-) vector (Life Technologies, Grand Island, NY, USA) between the XbaI and BamHI sites, downstream from the T7 promoter site. Luc-UTR pcDNA3.1(-) was grown in DH5α E. coli and isolated using a Qiagen Miniprep kit (Qiagen).

Purified Luc-UTR pcDNA3.1(-) plasmid was linearized with HindIII-HF (New England Biolabs, Ipswich, MA, USA) at 37 °C for 60 min. Residual RNase A from the miniprep was removed by digestion with 1.2 U of proteinase K (Thermo Fisher Scientific, Pittsburgh, PA, USA) in 0.5% SDS (Research Products International, Mt. Prospect, IL, USA). Linearized template DNA was purified by phenolchloroform:isoamyvl alcohol extraction and isopropanol precipitation. Pre-mRNA was produced by in vitro transcription using the Ambion MEGAscript T7 Kit (Life Technologies) according to manufacturer's instructions. Briefly, 1 μg of linearized template Luc-UTR pcDNA3.1(-) DNA was combined with 7.5 mM ATP, GTP, CTP, UTP and 10 × reaction buffer and T7 RNA Polymerase in a total volume of 20 μl, then reacted at 37 °C for 4 h. After transcription, two units of TURBO DNAse were added and allowed to digest template DNA at 37 °C for 15 min followed by the addition of 100 mM EDTA in 5 M ammonium acetate to terminate the digestion. The resulting pre-mRNA was purified by phenolchloroform:isoamyvl alcohol extraction and isopropanol precipitation, then quantified by absorbance.

A 3' Poly A tail was added to the pre-mRNA using the Ambion Poly A Tailing Kit (Life Technologies) adapted from the manufacturer's instructions. Briefly, 200 μg of pre-mRNA was prepared in 100 μl of 50 mM Tris-HCl pH 7.9 containing 1 mM ATP, 250 mM NaCl, 10 mM MgCl2 and 8 units of template DNA was purified by phenolchloroform:isoamyvl alcohol extraction and isopropanol precipitation. Pre-mRNA was produced by in vitro transcription using the Ambion MEGAscript T7 Kit (Life Technologies) according to manufacturer's instructions. Briefly, 1 μg of linearized template Luc-UTR pcDNA3.1(-) DNA was combined with 7.5 mM ATP, GTP, CTP, UTP and 10 × reaction buffer and T7 RNA Polymerase in a total volume of 20 μl, then reacted at 37 °C for 4 h. After transcription, two units of TURBO DNAse were added and allowed to digest template DNA at 37 °C for 15 min followed by the addition of 100 mM EDTA in 5 M ammonium acetate to terminate the digestion. The resulting pre-mRNA was purified by phenolchloroform:isoamyvl alcohol extraction and isopropanol precipitation, then quantified by absorbance.

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Isoamyl alcohol extraction and isopropanol precipitation, quantification was incubated at 37 °C for 1 h, then purified by phenolchloroformisoamyl alcohol extraction and isopropanol precipitation, quantified by absorbance.

The S’ cap was added to tail mRNA using Vaccinia Capping System and mRNA Cap 2’-O-methyltransferase (New England Biolabs) according to the manufacturer’s instructions. Tailed mRNA (150 μg) was heat denatured at 65 °C for 5 min, then chilled on ice for 5 min, before combining it with 0.5 mM GTP, 0.2 mM SAM, 150 units of Vaccinia capping enzyme and 750 units mRNA cap 2’-O-methyltransase in a total volume of 300 μl. The reaction was incubated at 37 °C for 1 h, then purified by phenolchloroform:isoamyl alcohol extraction and isopropanol precipitation, quantified by absorbance at 260 nm and stored at −20 °C. Typical yields for a 20 μl reaction were ~200 μg of pre-mRNA. After tailing and capping, the final yield ranged from 100 to 150 μg of pre-mRNA produced a 260/280 nm absorbance ratio of ~2.0, as did purified tailed and capped mRNA.

The size and purity of mRNA was determined using agarose native gel electrophoresis. Prior to electrophoresis, mRNA (1 μg) was denatured at 65 °C for 5 min and chilled on ice for 5 min then combined with 5 × 1B Loading Medium (Faster Better Media, Baltimore, MD, USA) and loaded onto a 1% agarose gel prepared with 50 ml of 0.5 × lithium boric acid Loading Medium (Faster Better Media, Baltimore, MD, USA) and loaded onto a 1% agarose gel prepared with 50 ml of 0.5 × lithium boric acid electrophoresis buffer containing 2 μl of 50 μg ml−1 ethidium bromide (Faster Better Media), then electrophoresed at 145 V for 30 min. Gels were imaged using a UVP BioSpectrum Imaging System and VisionWorks LS software (UVP, Upland, CA, USA), revealing two bands of equal intensity, with the slower migrating band resulting from mRNA secondary structure. Heat denaturation resulted in loss of the slower migrating native band, resulting in a single denatured band for mRNA. A clear upward band shift was observed by Abs 409 nm (each acridine 409 nm = 9266 M−1 cm−1), whereas Trp fluorescence (BLI) in an IVIS Imaging 200 Series (Xenogen, Hopkins, MA, USA), with 2.5% isoflurane, medium binnning. 24.6 cm field of view, and 10 s acquisition time.

The metabolic stability of mRNA polyplexes was determined by incubation in mouse serum. mRNA (1 μg) and PEG-peptide (1 nmol) were combined to form mRNA polyplexes in a total volume of 5 μl of 5 μl HEPES pH 7.0. Mouse serum (15 μl) was added and allowed to incubate at room temperature for 30 min. mRNA polyplexes in serum were diluted with 1.8 ml of normal saline then dosed hydrodynamically and imaged at 24 h by BLI as described above. The kinetics of luciferase expression in liver was determined by hydrodynamically dosing 1 μg of UTR mRNA polyplex in triplicate mice. Luciferase expression was measured by BLI at 4, 24, 36, 48, 60, 72, 84 and 96 h.

Statistical Analysis

BLI results were analyzed for statistical significance by calculating the base 10 logarithm of each luminescence measurement. The log transformed data was analyzed by ordinary one-way analysis of variance using GraphPad Prism version 6.02 (GraphPad Software, La Jolla, CA, USA), with Dunnett’s Multiple Comparisons Test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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