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Achieving Efficient Delivery of Morpholino Oligos in Cultured Cells

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Summary: One of the many features that make morpholino oligos unique among the antisense structural types is an uncharged backbone. While this feature eliminates the nonspecific interactions of traditional S-oligos, it also renders the morpholino undeliverable via the traditional lipid-based delivery systems. This article describes a highly efficient method of delivering morpholino oligos into adherent and nonadherent cultured cells. In this system, a nonionic morpholino oligo is paired to a complementary DNA "carrier." The DNA is then bound electrostatically to a partially ionized, weakly-basic ethoxylated polyethylenimine (EPEI). This morpholino/DNA/EPEI complex is efficiently endocytosed, and when the pH drops within the endosome, the EPEI more fully ionizes, resulting in permeabilization of the endosomal membrane and release of the morpholino into the cytosol. This article describes optimization of delivery in HeLa cells and provides the basis for delivery in any cultured endocytic cell type. genesis 30:94–102, 2001.

Key words: morpholino; antisense oligos

INTRODUCTION

The microinjection techniques used for delivery of oligos into the developmental systems described in this journal have allowed many researchers to utilize the outstanding properties of morpholino oligos to generate Morphants. Unfortunately these techniques are not applicable to cultured mammalian cells, and until now delivery of morpholino oligos into cultured mammalian cells has been sporadic.

When antisense oligos are added to cultured animal cells, the oligos enter the cells predominantly or solely via endocytosis (Neckers, 1993). Subsequently, the new endosomes acquire degradative enzymes to become lysosomes. Since antisense oligos are generally too large and polar to diffuse across the endosome/lysosome membrane, the oligos do not gain entry into the cytosol/nuclear compartment where their target sequences reside, but instead the oligos are sequestered in the endosome/lysosome compartment where they are degraded or are exocytosed back to the extracellular medium.

Thus, a key challenge in the antisense field is to avoid this unproductive endocytosis/degradation/exocytosis process, and instead achieve delivery into the cytosol/nuclear compartment of cultured cells in a manner that does not unduly reduce cell viability or perturb cell metabolism. While much effort has been expended in developing effective methods for delivering phosphorothioate and other polyanionic antisense types (Thierry *et al.,* 1992; Lewis *et al.,* 1996), considerably less effort has been devoted to developing methods for delivering advanced nonionic antisense types such as PNAs (Nielsen *et al.,* 1993) and morpholinos (Summerton and Weller, 1997), which are free of many of the problems and limitations plaguing ionic types (Summerton, 1999).

Here I describe a nuclear/cytosolic delivery method for nonionic morpholino oligos that are paired to partially complementary DNA oligos. The morpholino/DNA duplex is then complexed with the weakly-basic polyamine, ethoxylated polyethylenimine (EPEI), and the composite complex added to cultured animal cells. In this delivery scheme, the EPEI delivery component serves three purposes: (1) It electrostatically binds the anionic DNA component of the morpholino/DNA duplex; (2) It electrostatically binds to the anionic plasma membrane of cells and thereby enhances endocytosis; (3) I speculate that acidification of the endosome further ionizes the endocytosed EPEI, and this more highly ionized EPEI acts to permeabilize the endosomal membrane and thereby allow passage of morpholino oligo from the endosome into the cytosol of the cell. It should be noted that weakly endocytic cell types are not expected to be efficient delivery targets for this method.

A VIABLE SOLUTION TO DELIVERING NONIONIC ANTISENSE OLIGOS AT LOW CONCENTRATION

Until now morpholino oligos were best delivered into cultured cells by scrape-loading (Partridge *et al.,* 1996) or osmotic delivery (Morcos, 2000), both of which suffer from significant cell-type limitations and effectiveness. One group (Kang *et al.,* 1999) compared a variety of delivery reagents for their ability to deliver DNA-based oligonucleotides. Of the 9 reagents tested, Exgen 500

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(MBI Fermentas), a weak-base polyamine, achieved the highest ratio of delivery versus cytotoxicty. Previously, a similar weak-base polyamine had been shown to enhance delivery of liposomal formulations (Bandyopadhyay *et al.,* 1998). We tested a variety of similar polyamines in an attempt to achieve the greatest delivery of morpholino oligos with little or no toxicity. We chose polyamines that have a pka such that they would be partially ionized in culture media to interact with DNA and the cell surface and become more ionized in the endosome/lysosome, permeabilize the endosome/lysosome membrane, and deliver oligo into the cytosol. We settled on a reagent similar to ExGen 500, an ethoxylated polyethylenimine (EPEI) to deliver morpholino oligos paired to partially complementary single-stranded DNA oligonucleotides. The charge density of the 50-kDa EPEI is equivalent to that of the linear 25-kDa polyamine, Exgen 500, however, the pKa of EPEI is slightly lower, which reduces ionization at the pH of extracellular media, making it less likely to permeabilize the plasma membrane.

We compared delivery of morpholino oligos by EPEI delivery to that of scrape-loading (GENE TOOLS; adhesion plaque removal), osmotic delivery (GENE TOOLS; osmotic shock), and Exgen 500 delivery (MBI Fermentas) as described in each respective protocol using a previously described positive test system for antisense activity in HeLa cells. In the test system, luciferase expression is achieved only when an antisense oligo blocks a dominant mutant splice site, which restores normal splicing and read-through of an otherwise truncated luciferase-encoding transcript (Kang *et al.,* 1998). We find that delivery of the prepaired morpholino/DNA oligos at 1 μmol/L with EPEI at 0.56 μmol/L yields 10 times the luciferase activity achieved with scrape-load delivery at the same concentration, while osmotic delivery at 1 μmol/L morpholino oligo is unable to generate measurable luciferase activity (Fig. 1). Interestingly, the Exgen 500 fairs only slightly better than osmotic delivery at 1 μmol/L morpholino/DNA. We postulate this reduction in delivery for Exgen 500 is due to the suboptimal pka of Exgen 500 compared to EPEI. The addition of 10% serum to the delivery media reduced luciferase activity more than four-fold, indicating that serum components interfere with delivery. It should be noted that the splice-correcting ability seen with EPEI at 1 μmol/L morpholino is greater than the highest level of luciferase activity seen for scrape-loading at 25 μmol/L on a per cell basis, and that a 4-mispair and an invert control oligo are unable to restore luciferase activity in this system (data not shown).

In defining the best conditions for EPEI delivery of morpholino oligos, there are several factors that need to be addressed, including the structure of the DNA component, the concentration of the DNA component, the concentration of the EPEI reagent, the time of complex formation, and the incubation time for cells with the delivery complex.

DEFINING THE DNA COMPONENT OF DELIVERY

The DNA oligos act as the "adaptor" to couple the EPEI and morpholino oligo. The single-stranded DNA oligo interacts ionically with EPEI and hydrogen bonds to the morpholino oligo through the sequence partially complementary to the morpholino oligo. We hypothesized that DNA oligos paired along the entire length of a morpholino oligo would be unfavorable due to a highly stable interaction. Such stability might not only interfere with morpholino pairing to the mRNA target sequence but also protect the DNA from nucleases in the cytosol. To assure that the DNA remains paired to the morpholino through the delivery step, a 16-base complementary overlap was chosen to maintain a Tm greater than 37°C. We expected that such an overlap, while fairly strong, would not adversely affect the morpholino oligo once in the cytosol due do the instability of the DNA component, as well as the higher affinity of morpholino oligos for the RNA target. To increase the interaction with EPEI and expose the DNA component to exonucleases, we added an additional 10 noncomplementary bases to the DNA oligos. We tested the effect of a 5′ overhang versus a 3′ overhang as well as composition of the 10-base overhang on activity. Altering the composition of the 10-base overhang has no significant impact on activity, however, placement of the extra 10 bases on the 5′ end of the DNA oligo increases luciferase expression three-fold over a 3′ overhang in our test system (Fig. 2). The result was unexpected as the 3′ end of the oligo is initially encountered by the ribosomal scanning machinery. We speculate that the 5′ overhang may provide a better target for nucleases.

DEFINING AN OPTIMAL EPEI CONCENTRATION FOR DELIVERY

The relative concentrations of EPEI reagent and DNA are the critical variables in achieving delivery. Delivery with this method relies on the charge interaction with the cell and subsequent endocytosis. High EPEI concentrations relative to DNA concentrations can overwhelm the plasma membranes with charge, while insufficient concentrations relative to DNA will result in the DNA blocking the positive charges necessary for EPEI interaction with the cell. In theory, a specific ratio of DNA to EPEI concentration should achieve delivery without toxicity. As our design constraints fix the DNA component to 26-mer lengths, results should be reproducible irrespective of the morpholino oligo to be delivered with the DNA-EPEI interaction based entirely on charge and concentration. Once defined, maintaining the ratio of DNA to EPEI concentrations is critical in achieving reproducible delivery.

To define the optimal EPEI to DNA ratio, we fixed the concentration of the prepaired morpholino:DNA duplex at 1 μmol/L and then varied the EPEI concentration in a range from 0.3 μmol/L to 1.0 μmol/L in a final delivery mixture. Nearly confluent cultured HeLa cells were ex-

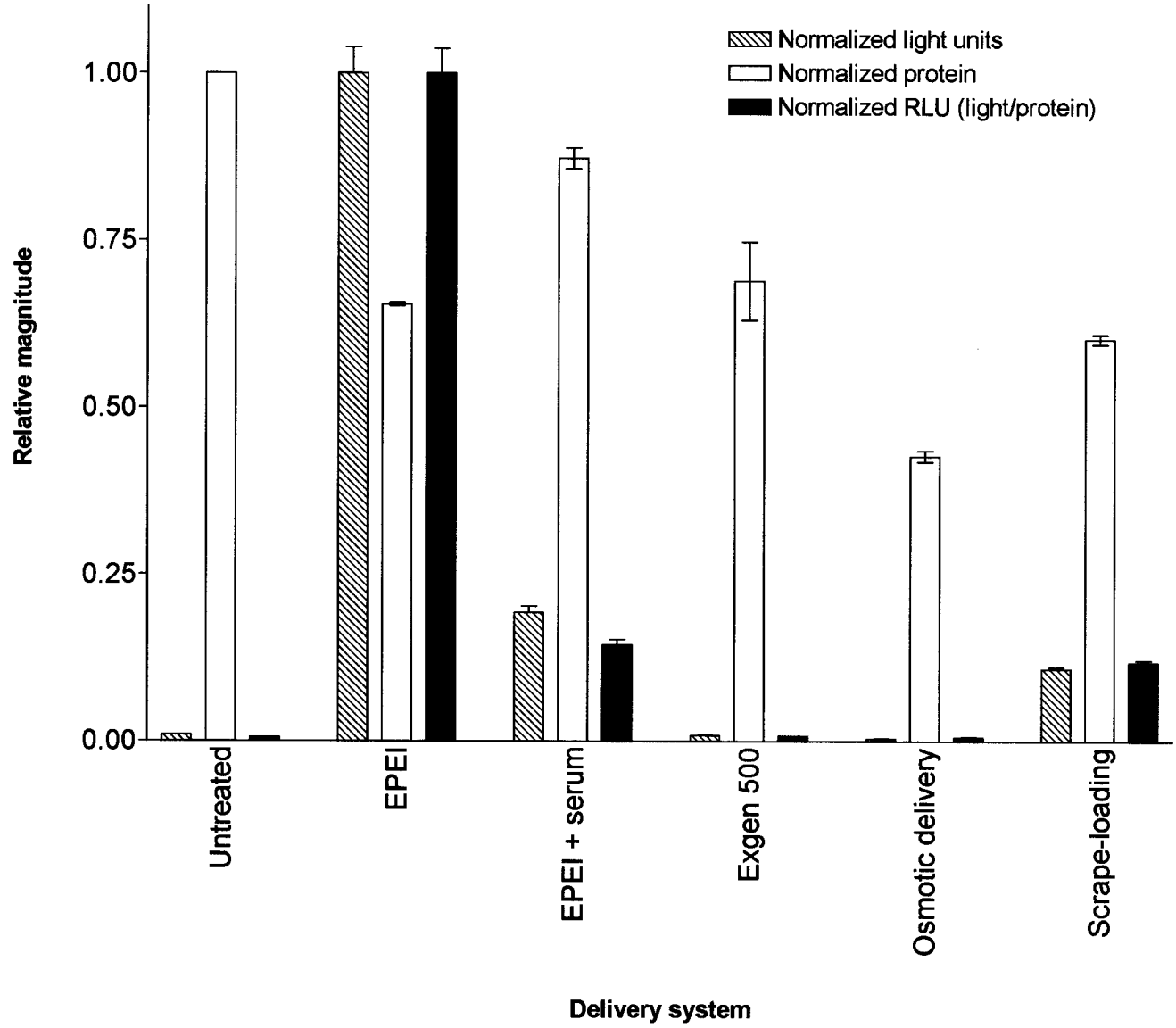
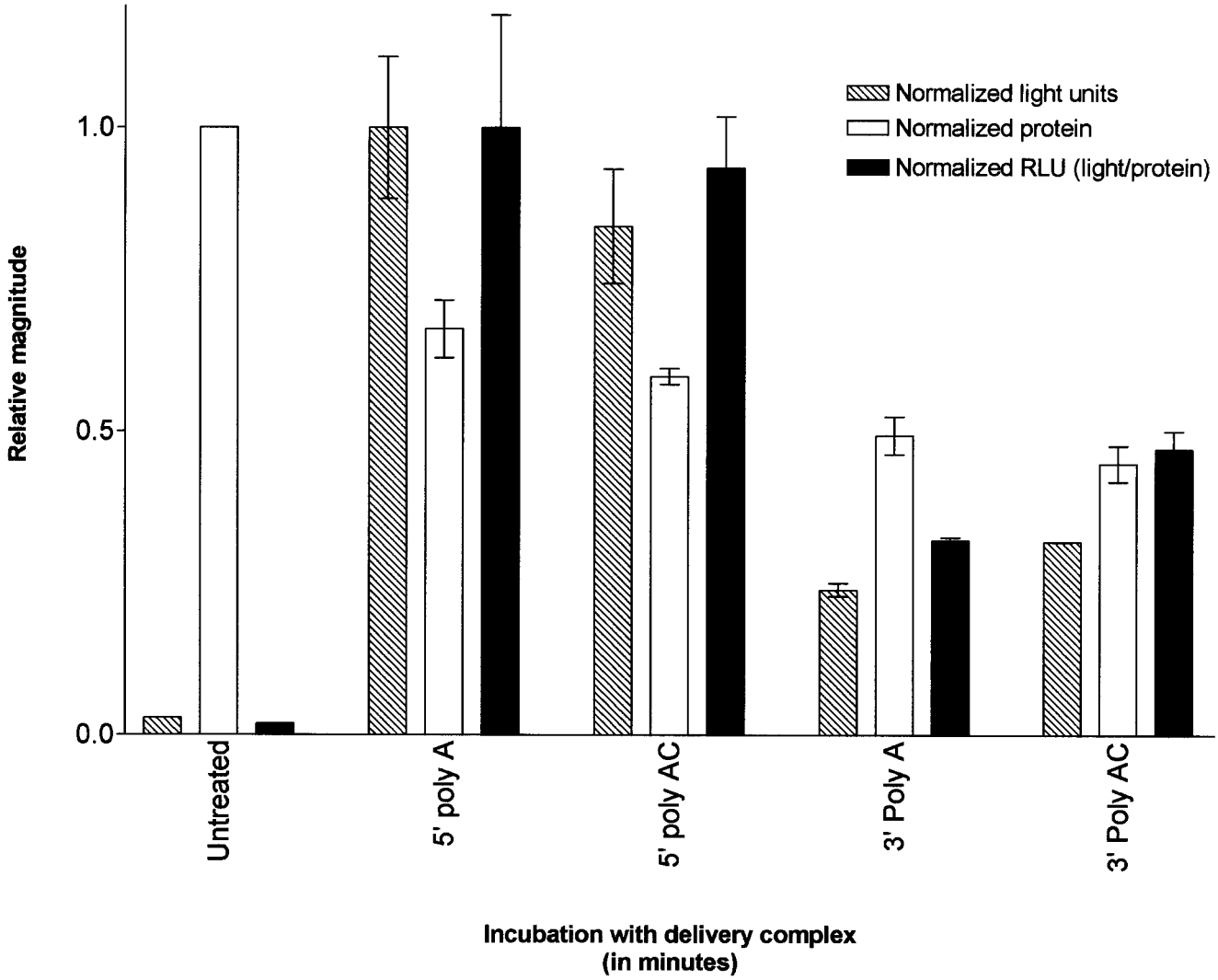


FIG. 1. Comparison of current delivery methods at 1 μmol/L morpholino oligo. Using our positive test system for luciferase expression in HeLa cells, EPEI, Exgen 500, Osmotic, and Scrape-load delivery methods were tested at 1 μmol/L final morpholino oligo concentration as per the respective delivery protocols. For the EPEI delivery, either serum-free (labeled EPEI) or 10% serum-containing (EPEI + serum) media was used during the 3-h incubation with oligo/EPEI delivery complexes. Cellular extracts were assayed for luciferase activity in triplicate (error bars are indicated) from samples processed 24 h after initiation of delivery. RLUs (relative light units) in this and subsequent figures were determined by dividing light units by the protein concentration in the same sample. Untreated cells served as a control for background luciferase activity in this and in results displayed in subsequent figures.

posed to the delivery mixture for 3 hours in serum-free media. In our test system, luciferase activity per unit cell increases as the concentration approaches 0.7 μmol/L before falling off at 1.0 μmol/L (Fig 3). Interestingly, while 0.7 μmol/L EPEI appears to be optimal for delivery, microscopic examination suggests cellular deformity beginning at 0.7 μmol/L EPEI that increases substantially and is accompanied with cell death at 1.0 μmol/L (data not shown). The cellular deformities include cellular atrophy and spillage of cellular content consistent with toxicity.

DEFINING THE OPTIMAL DNA OLIGO CONCENTRATION TO ACHIEVE DELIVERY

The study of EPEI concentrations suggests that maintaining EPEI concentration at 0.56 μmol/L with morpholino:DNA at 1.0 μmol/L yields excellent results without visible toxicity. Can we achieve greater delivery by fixing the EPEI concentration at 0.56 μmol/L while varying the DNA concentration? To answer this question, we performed another set of delivery experiments in which the EPEI concentration was fixed at



5' Poly A DNA oligo 3′AGTCAATGTTAAATATAAAAAAAAAA-5′
Morpholino oligo 5′-CCTCTTACCTCAGTTACAATTTATA-3′

5' Poly AC DNA oligo 3′AGTCAATGTTAAATATCACACACACA-5′
Morpholino oligo 5′-CCTCTTACCTCAGTTACAATTTATA-3′

3' Poly A DNA oligo 3′AAAAAAAAAAGGAGAATGGAGTCAATG-5′
Morpholino oligo 5′-CCTCTTACCTCAGTTACAATTTATA-3′

3' Poly AC DNA oligo 3′CACACACACAGGAGAATGGAGTCAATG-5′
Morpholino oligo 5′-CCTCTTACCTCAGTTACAATTTATA-3′

FIG. 2. (A) Effect of 5′ versus 3′ overhang of the DNA component. DNA oligos (26 mer) were designed such that they maintain a 16-base consecutive complementarity with the morpholino oligo used in the positive test system. An additional 10 noncomplementary bases (poly A or poly AC) were added to the DNA oligo in order to create either a 5′ DNA overhang or a 3′ DNA overhang when paired to the morpholino oligo. Each of these 4 DNA oligos (5′ Poly A, 5′ Poly AC, 3′ Poly A, and 3′ Poly AC) were separately paired to the morpholino oligo and delivered as per the standard protocol described in the text. Luciferase activity was determined in triplicate (error bars indicated) from cellular extracts processed 24 h after initiation of delivery. **(B)** The sequences of the morpholino/DNA heteroduplexes. The same morpholino oligo was paired to each of 4 DNA oligos, each with 16 bases of complementary sequence plus 10 additional bases consisting of a 5′ poly A, 5′ poly AC, 3′ poly A, or a 3′ poly AC.

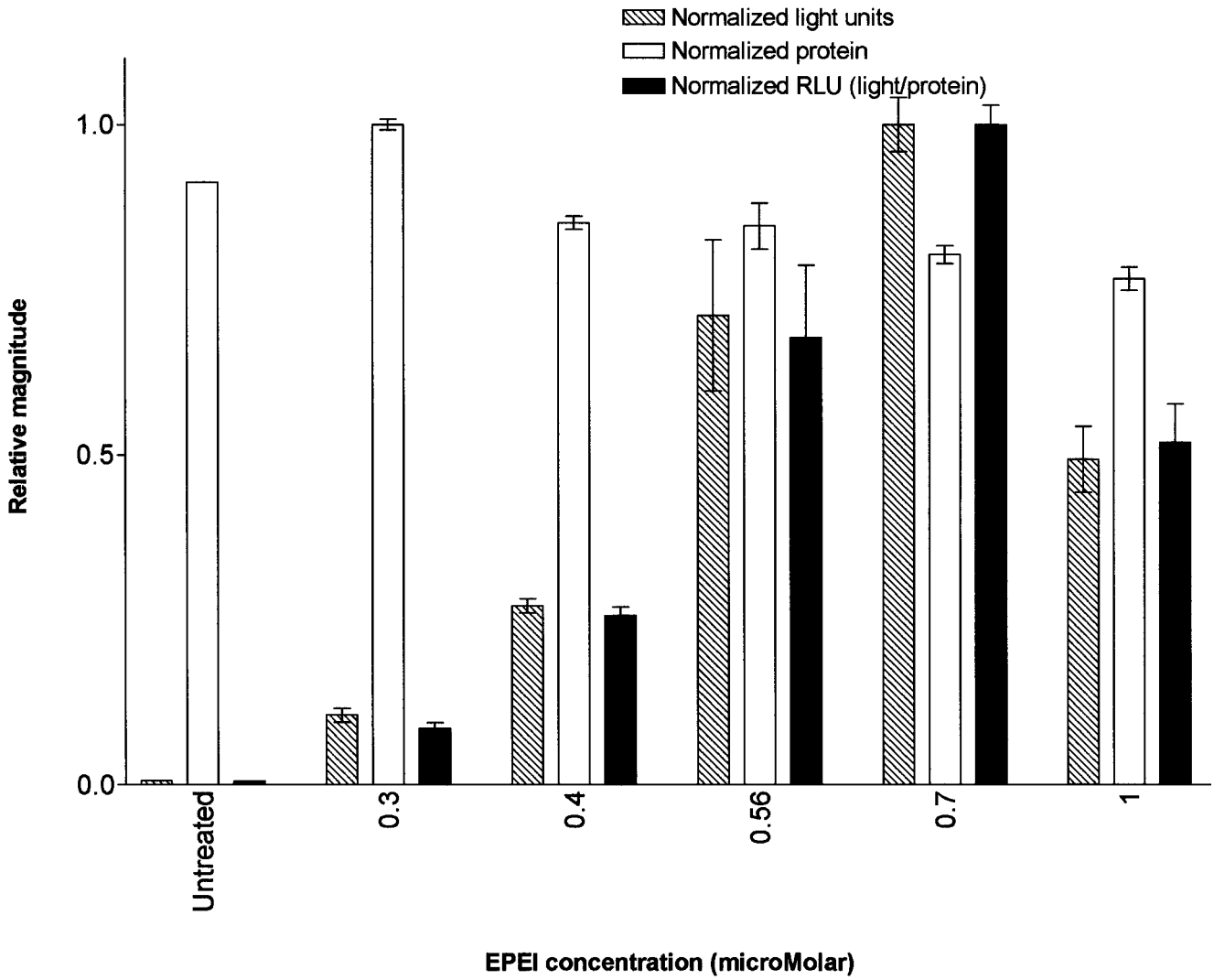


FIG. 3. Effect of varying EPEI concentration on delivery at 1 μmol/L heteroduplex oligo. Morpholino/DNA oligo duplexes were fixed at 1 μmol/L final concentration and complexes were allowed to form with increasing EPEI concentrations from 0.3 μmol/L to 1.0 μmol/L final. The resulting complexes that vary only in EPEI concentration were delivered into the positive test system for luciferase expression in HeLa cells, and cellular extracts were assayed for luciferase activity in triplicate (error bars are indicated) from samples processed 24 h after initiation of delivery.

0.56 μmol/L and the morpholino:DNA duplex concentration was varied from 0.75 μmol/L to 1.25 μmol/L and exposed to nearly confluent HeLa cells for 3 h in serum-free media. In our test system, luciferase activity peaks at a morpholino:DNA duplex concentration of 1.0 μmol/L (Fig. 4). We hypothesize that concentrations of the DNA component exceeding 1.0 μmol/L are impeding the EPEI component's ionic interaction with the cells.

OPTIMIZING THE DELIVERY COMPLEX

It has previously been suggested that delivery of DNA with a similar polyethelenimine to EPEI called ExGen 500 requires that complexes (i.e., precipitates) be allowed to form at room temperature for at least 10 min. In order to dilute serum-free media no more than 10% and avoid toxicity, complexes are allowed to form at 10× the concentration of each component in the final delivery mixture. We tested the effect of complex formation on delivery by varying the length of incubation of EPEI:morpholino/DNA complexes in a range from 10 min to 80 min prior to the addition of serum-free media. Incubation for 20 min results in optimal delivery in our test system with a substantial reduction in delivery as the time of incubation increases (Fig. 5). This sharp decline in effective delivery is likely due to the formation of larger EPEI-morpholino:DNA aggregates that fail to be endocytosed (as seen by microscopic examination). Diluting 10-fold and vortexing

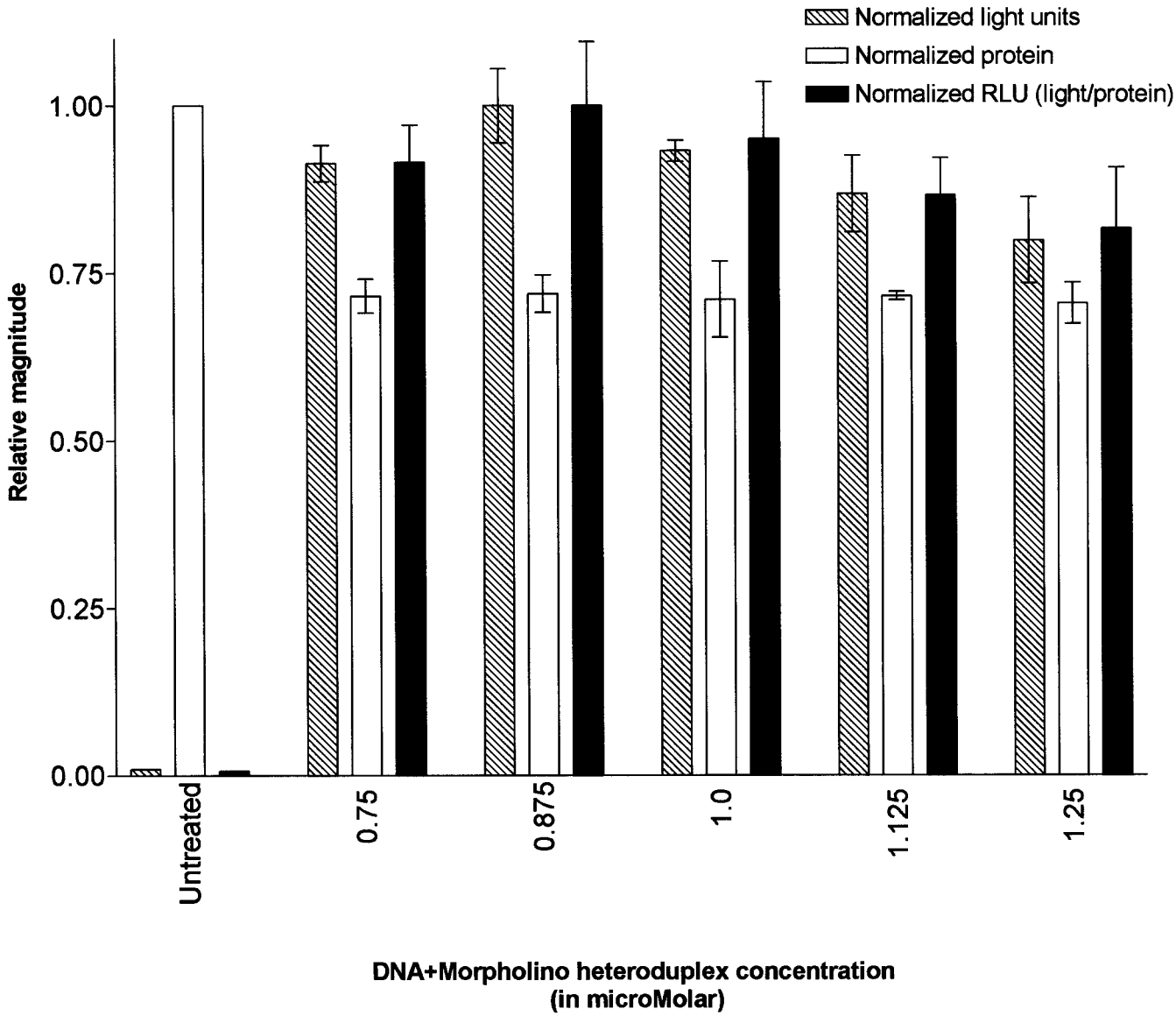


FIG. 4. Effect of varying morpholino:DNA concentrations on delivery at 0.56 μmol/L EPEI. EPEI concentration was fixed at 0.56 μmol/L final in the delivery mixture, and complexes were allowed to form with increasing morpholino/DNA duplex concentrations from 0.75 μmol/L to 1.25 μmol/L final. The resulting complexes that vary only in morpholino/DNA duplex concentration were delivered into the positive test system for luciferase expression in HeLa cells, and cellular extracts were assayed for luciferase activity in triplicate (error bars are indicated) from samples processed 24 h after initiation of delivery.

after a 20-min incubation appear to reduce the likelihood of larger complexes readily forming.

OPTIMIZING EXPOSURE TO THE DELIVERY MIXTURE

We suspected that increased exposure to the delivery solution could result in increased delivery proceeded by toxicity due to overwhelming exposure to the EPEI component. This hypothesis is based on the nature of the complexes settling onto cells and ultimately becoming endocytosed. Hypothetically, longer incubation times allow gravitational forces to settle increasing amounts of delivery complexes onto cells. We incubated nearly confluent HeLa cells with EPEI and morpholino:DNA at 0.56 μmol/L and 1.0 μmol/L, respectively, in serum-free media. The delivery mixture was replaced with serum-containing media in a range from 20 min to 7 h after exposure. In our test system, 20-min incubation with the delivery mixture yields little or no luciferase activity. By 80 min, delivery is substantial and peaks at 3 h with a noticeable but gradual decline up to 7 h exposure (Fig. 6). Additionally, by the 4:20 time point, microscopic observation of the cells reveal cellular

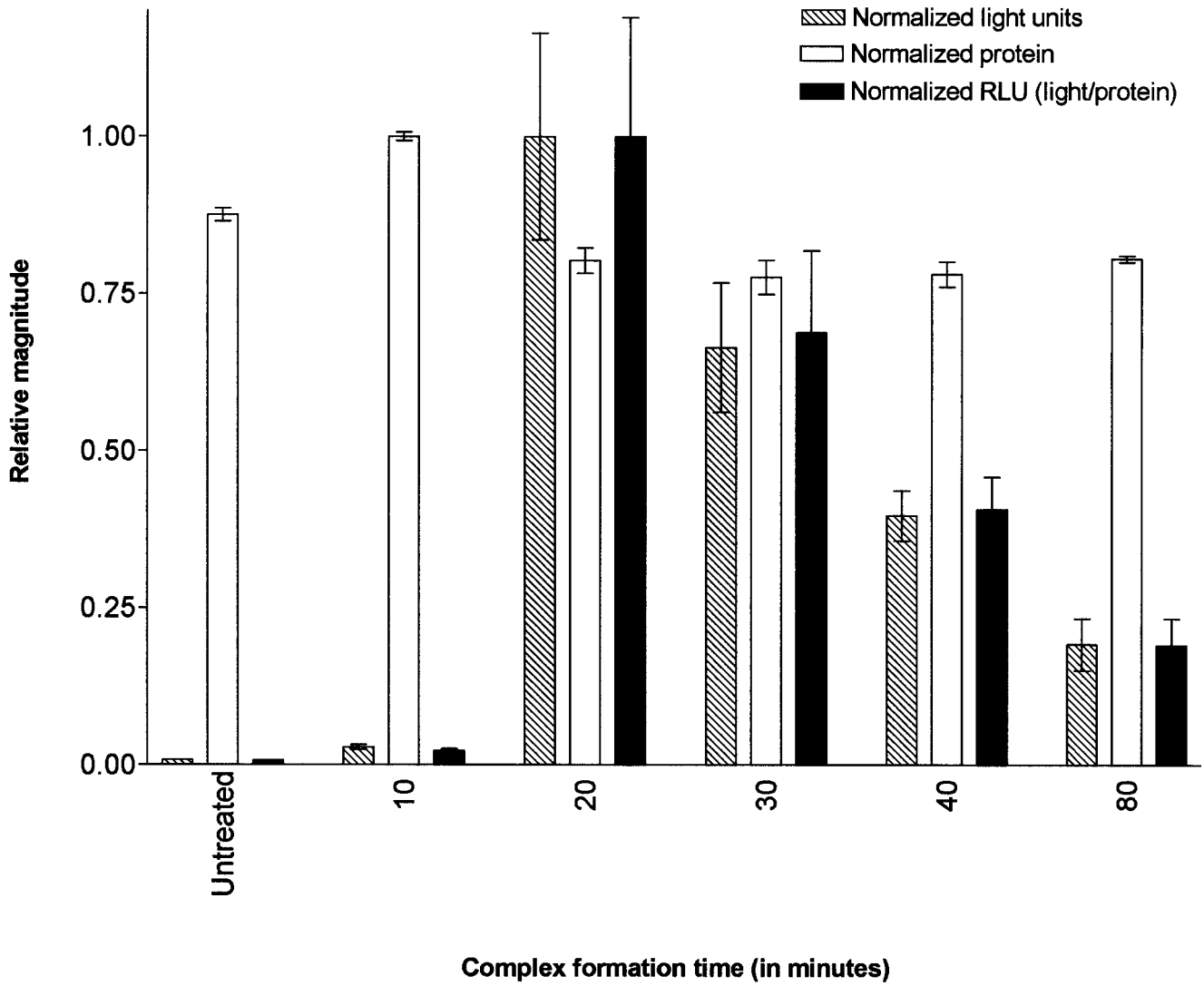


FIG. 5. Effect of complex formation time of EPEI with DNA+morpholino on delivery. Delivery complexes with a fixed morpholino/DNA duplex concentration of 1.0 μmol/L and a fixed EPEI concentration of 0.56 μmol/L were mixed and allowed to form from 10 to 80 min prior to a 10-fold dilution with serum-free media. The resulting complexes were delivered into the positive test system for luciferase expression in HeLa cells, and cellular extracts were assayed for luciferase activity in triplicate (error bars are indicated) from samples processed 24 h after initiation of delivery.

deformities including cellular atrophy and spillage of cellular contents consistent with toxicity (data not shown).

THE STANDARD PROTOCOL

Based on the data presented in this article, we have defined the following protocol that should work for most endocytic cell types. We have reduced the EPEI amount slightly from what achieves optimal delivery in HeLa cells to reduce the chance of toxicity in cells sensitive to the partially ionized EPEI.

From a stock morpholino:DNA mixture (500 μmol/L: 357 μmol/L), take 5.6 μl and add it to 188.8 μl of water. Vortex and add 5.6 μl of the 200 μmol/L EPEI reagent. Vortex and let stand 20 min at room temperature, after which add 1.8 μl of serum-free media and vortex briefly. Remove the media from nearly confluent cells and add a volume of the delivery mixture appropriate to cover the cells (i.e., 500 μl to a well of a 24-well plate) and briefly swirl. After a 3-h incubation, remove the delivery mixture and replace with serum-containing media. Results can be assayed 24 h to 96 h later.

THE EFFECTIVENESS OF EPEI DELIVERY

To achieve delivery of uncharged morpholino oligos, we set out to modify rather than reinvent current delivery methods. The reduced toxicity of delivery associated with weak-base polyamines like ExGen 500 and our

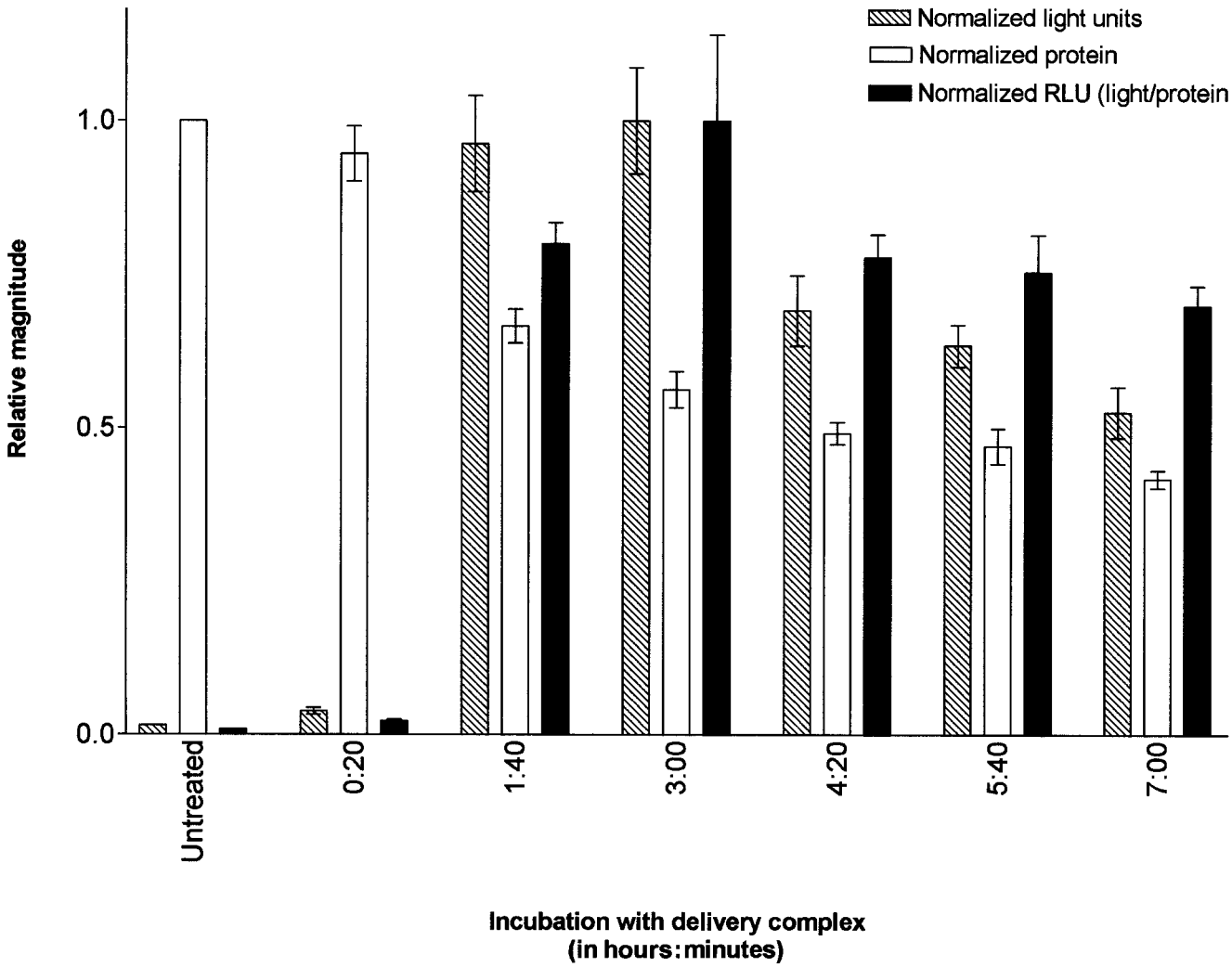


FIG. 6. Optimizing incubation time of complexes with cells. Delivery complexes with a fixed morpholino/DNA duplex concentration of 1.0 μmol/L and a fixed EPEI concentration of 0.56 μmol/L were mixed and allowed to form for 20 min prior to a 10-fold dilution with serum-free media. The resulting complexes were delivered into the positive test system for luciferase expression in HeLa cells, incubated from 20 min to 7 h prior to replacement of media, and cellular extracts were assayed for luciferase activity in triplicate (error bars are indicated) from samples processed 24 h after initiation of delivery.

discovery that partially complementary DNA/morpholino heteroduplexes could form complexes with EPEI allowed us to create an efficient delivery method for morpholinos. The EPEI special delivery reagent has been tested for delivery of morpholinos by many researchers in a variety of cell types with very positive results (unpublished observations). With the exception of T cells, reportedly more difficult cell lines have been delivered without difficulty, including the human leukemia cell line HL60 and primary hepatocytes. In most cases, following the concentrations and incubation times outlined above will work without need for modification. As you can see from the results presented here, there is likely potential for optimizing delivery for any given cell type. Delivery can typically be increased by increasing the EPEI concentration in small increments. However, if toxicity is observed, decreasing the EPEI concentration in increments and/or increasing the morpholino:DNA concentrations in increments can reduce toxicity while retaining measurable delivery.

ACKNOWLEDGMENTS

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