Bioinspired Alkenyl Amino Alcohol Ionizable Lipid Materials for Highly Potent In Vivo mRNA Delivery

*Owen S. Fenton, Kevin J. Kauffman, Rebecca L. McClellan, Eric A. Appel, J. Robert Dorkin, Mark W. Tibbitt, Michael W. Heartlein, Frank DeRosa, Robert Langer, and Daniel G. Anderson**

Nucleic acid therapies could be leveraged to treat thousands of genetic disorders, many of which are difficult or impossible to manage with present day therapeutic approaches. For example, the successful delivery of short-interfering RNAs (siRNA) to cells in both rodents and nonhuman primates has been widely used for the treatment of hereditary diseases and cancer.[1] By contrast, the delivery of messenger RNA (mRNA) remains largely unexplored. Whereas siRNA sequences are employed to silence gene expression, mRNA therapeutics could be used to treat diseases caused by deficiencies in specific proteins.[2] This is because mRNA sequences can be translated into proteins once they are successfully transported into the cytoplasm of target cells. The implementation of mRNA therapeutics, therefore, could profoundly impact fields such as protein replacement therapy, vaccine development, and immune tolerization wherein the selective expression of proteins in vivo could treat disease.[3]

O. S. Fenton, K. J. Kauffman, R. L. McClellan, Dr. E. A. Appel, J. R. Dorkin, Dr. M. W. Tibbitt, Prof. R. Langer, Prof. D. G. Anderson
Koch Institute for Integrative Cancer Research
Massachusetts Institute of Technology
Cambridge, MA 02139, USA
E-mail: dgander@mit.edu

O. S. Fenton
Department of Chemistry
Massachusetts Institute of Technology
Cambridge, MA 02139, USA

K. J. Kauffman, Prof. R. Langer, Prof. D. G. Anderson
Department of Chemical Engineering
Massachusetts Institute of Technology
Cambridge, MA 02139, USA

J. R. Dorkin
Department of Biology
Massachusetts Institute of Technology
Cambridge, MA 02139, USA

Dr. M. W. Heartlein, Dr. F. DeRosa
Shire Pharmaceuticals
Lexington, MA 02421, USA

Prof. R. Langer, Prof. D. G. Anderson
Institute for Medical Engineering and Science
Massachusetts Institute of Technology
Cambridge, MA 02139, USA

Prof. R. Langer, Prof. D. G. Anderson
Harvard-MIT Division of Health Science and Technology
Massachusetts Institute of Technology
Cambridge, MA 02139, USA

DOI: 10.1002/adma.201505822

Before clinical translation can be realized, however, serious limitations with the in vivo delivery of mRNA must still be overcome. The high anionic charge density, size, and hydrophilicity of nucleic acids prevent meaningful levels of passive diffusion of mRNA across cell membranes.[4] To circumvent this barrier, our group and others have developed and implemented an array of lipid nanoparticles (LNPs) for the entrapment and subsequent delivery of nucleic acids in vivo.[1] Although these LNPs have been largely optimized for siRNA sequences, both Schlake and co-workers[5] and our research team[6] have recently employed LNPs derived from previously described components to deliver mRNA in vivo. Successful delivery was confirmed by quantifying serum protein levels, thereby establishing LNPs as viable delivery vehicles for mRNA.

Inspired by these results, we sought to design and synthesize novel LNP components capable of delivering mRNA with unprecedented levels of in vivo efficacy. In practice, LNPs are composed of cholesterol (aids in stability),[7] a phospholipid (modifies bilayer structure),[1a,8] a polyethylene glycol (PEG) derivative (decreases aggregation and nonspecific uptake),[9] and an ionizable lipid (complexes negatively charged RNA and enhances endosomal escape).[10] Evidence within the siRNA delivery community has implicated the chemical structure and identity of the ionizable lipid as the most pivotal component for efficacy. Accordingly, several rationally designed[11] and combinatorial chemistry[10b,12] methodologies have been explored to discover novel series of ionizable lipid materials capable of maximizing gene silencing at the lowest possible dose. This strategy both conserves precious therapeutic nucleic acid cargo and also serves to mitigate any possible issues with the toxicity of the LNPs themselves.

Interestingly, however, there are no reports yet detailing the creation of a new series of ionizable lipids for the expressed purpose of improving mRNA LNP delivery in vivo. We hypothesized that ionizable lipids based upon alkenyl amino alcohols (AAA), a functional group combination found in sphingosine and other bioactive molecules, could promote high levels of in vivo protein expression when formulated into mRNA LNPs (**Figure 1**).[13] We envisioned that we could furnish AAA ionizable lipids via a ring opening reaction between alkenyl epoxides (AE) with a polyamine core (**Figure 2**a).[12] It is important to note, however, that no AEs of suitable tail length are commercially available, nor are they trivial to synthesize on account of the difficulty of selectively oxidizing singular alkenes in the presence of electronically similar carbon–carbon double bonds. As such, we report the first detailed procedures for AE synthesis and characterization beginning from biologically relevant fatty

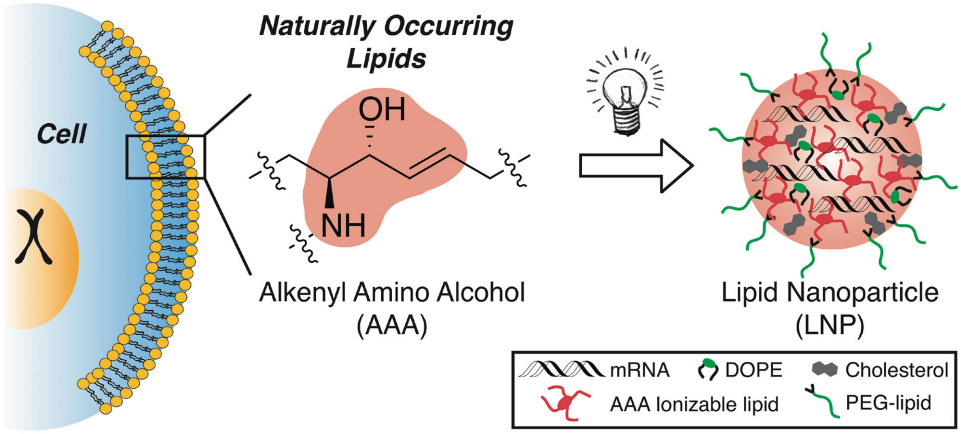

Figure 1. Naturally occurring components of the cell membrane contain alkenyl amino alcohol (AAA) functionality serving as the inspiration for the development of AAA ionizable lipids. These lipids form the basis of lipid nanoparticles exhibiting highly efficient in vivo delivery of mRNA.

acid starting materials (Figure S1, Supporting Information). **AE-00** through **AE-03** were then each reacted in turn with polyamine **1** to afford AAA ionizable lipids **OF-00** through **OF-03**. While **OF-00** through **OF-03** represent the first four members of this series of materials, we hope that the chemical versatility of **AE-00** through **AE-03** will serve as inspiration for future generations of AAA ionizable lipids for nucleic acid delivery.

Lipids **OF-00** through **OF-03** were then formulated with cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, C14-PEG-2000, and unmodified mRNA coding for human erythropoietin

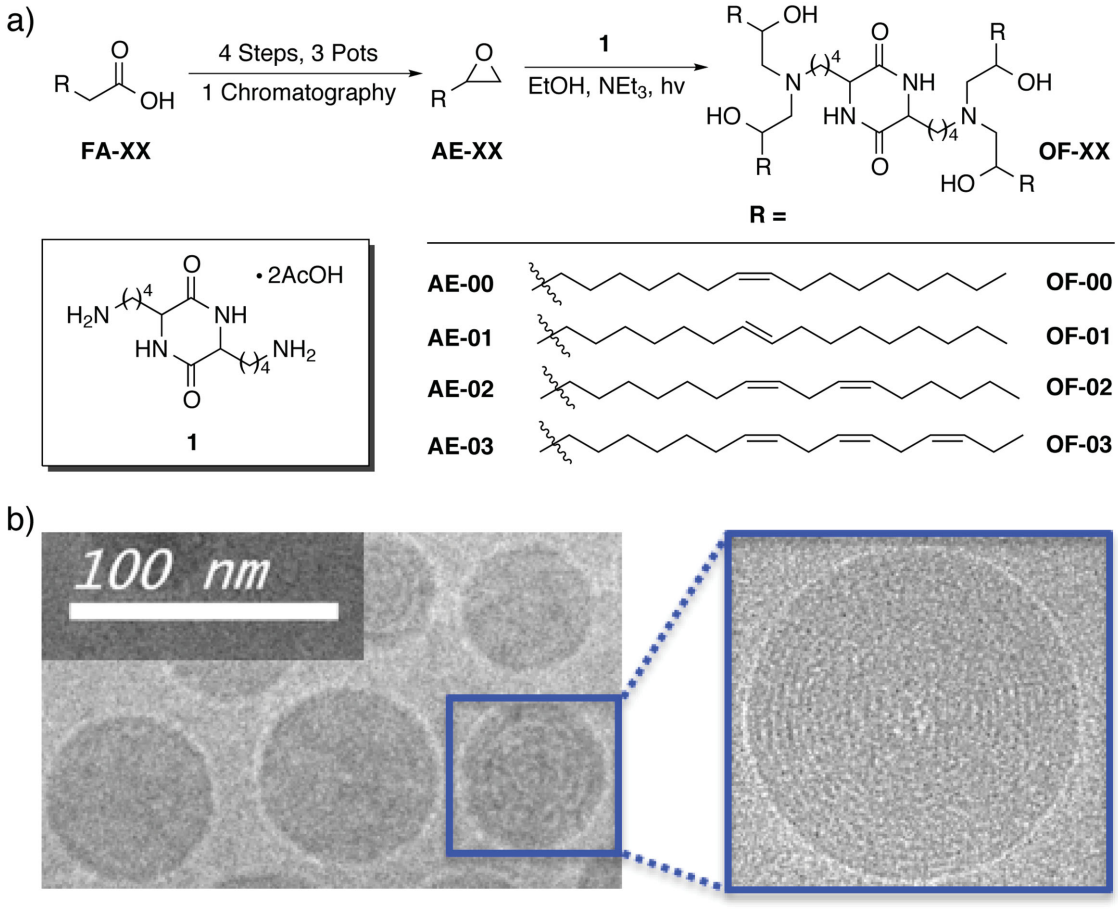

Figure 2. a) Synthesis of **OF-00** through **OF-03**, seminal members of the AAA class of ionizable lipids. b) Representative cryogenic transmission electron microscopy of **OF-02** LNPs.

(EPO) into mRNA LNPs.[6] EPO was selected as a model protein to evaluate the relationship between ionizable lipid identity and mRNA LNP efficacy for two reasons: (1) the associated protein is secreted directly into the bloodstream allowing for robust protein quantification, and (2) EPO has potential therapeutic applications in such areas as anemia.[4,14] Cryogenic transmission electron microscopy images of **OF-02** LNPs detail a spherical morphology and a multilamellar structure (Figure 2b). Additional physical properties include a narrow polydispersity index (0.130) and an average particle diameter around 100 nm.

The nanoparticle diameters, polydispersity indices, and encapsulation efficiencies for each **OF-00** through **OF-03** LNP formulation can be found in Table S1, Supplementary Information. Ionizable lipid **cKK-E12** was also formulated alongside these compounds to be used as a positive control in our study. **cKK-E12** was chosen because it is capable of silencing Factor VII expression in mice at siRNA doses as low as 0.002 mg kg^{-1}, and as such it represents a benchmark ionizable lipid in the field of nucleic acid delivery.[12] Each resultant mRNA loaded LNP was then injected intravenously at a 0.75 mg kg^{-1} dose in C57BL/6 mice alongside phosphate buffered saline (PBS) as a negative control. At 6 h, the serum EPO levels were quantified (**Figure 3**a). The PBS control imparted no significant EPO production in vivo, whereas positive control **cKK-E12** LNPs promoted a serum EPO concentration of 7100 ± 700 ng mL^{-1}. Excitingly, **OF-02** LNPs significantly outperformed benchmark lipid **cKK-E12** LNPs, promoting an approximate twofold increase in EPO concentration to 14 200 ± 1500 ng mL^{-1}. Additionally, **OF-02** outperformed two other benchmark ionizable lipids from the nucleic acid delivery field, namely **503013**[15] and **C12-200**,[10b] whose respective LNP promoted in vivo EPO concentrations were 2800 ± 200 and 7100 ± 500 ng mL^{-1} at an identical dose. To the best of our knowledge, therefore, **OF-02** LNPs represent the most potent mRNA delivery vehicle reported to date in the scientific literature.

The **OF-00**, **OF-01**, and **OF-03** LNPs also allow the deduction of structure/function relationships within this new series of AAA ionizable lipids. We note two general structure/function trends of interest. First, we note that only alkenes with a *cis* geometry promote in vivo efficacy—**OF-00** and **OF-01** exclusively differ in the *cis*/*trans* geometry of their alkenes, and only **OF-00** produces meaningful EPO concentrations. Second, the optimal number and placement of two *cis* alkenes per tail matches those observed in optimized siRNA LNPs.[11,16] While we are still discerning why these specific trends are observed, these empirical findings could potentially shape subsequent generations of AAA lipids. It is interesting to note, therefore, that only the linoleic acid derivative **OF-02** promotes significantly higher levels of EPO expression than the positive control, although oleic acid derivative **OF-00** also demonstrates

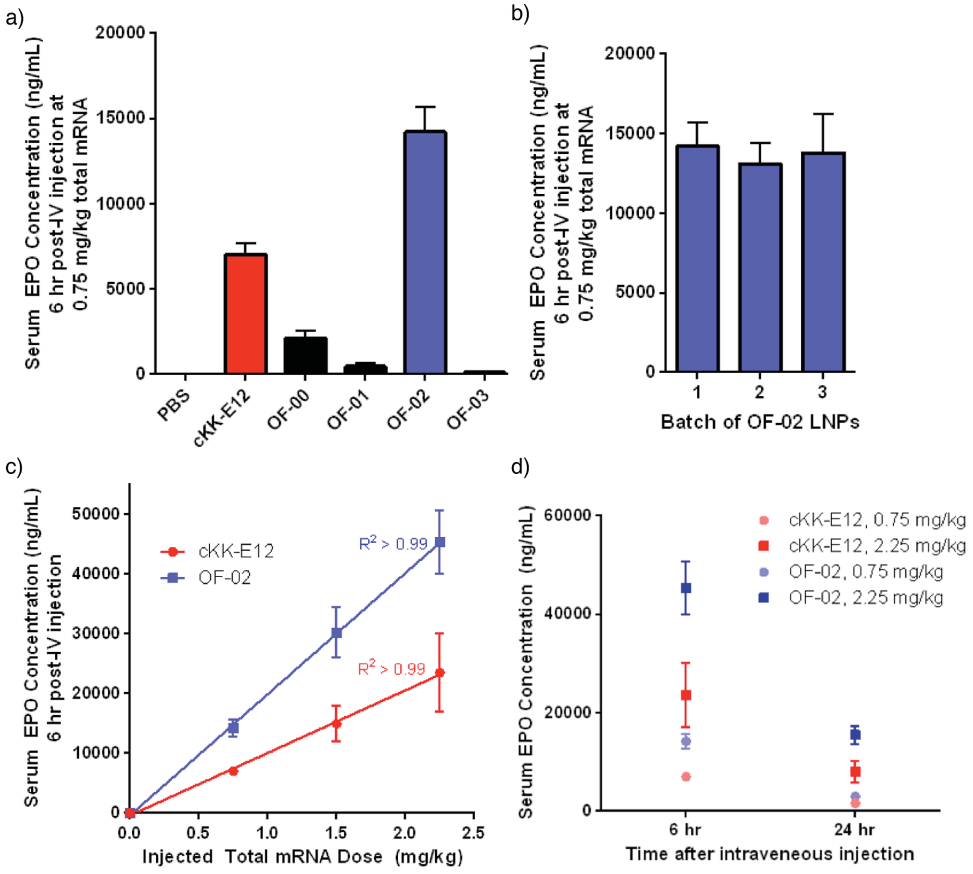

Figure 3. a) In vivo expression of EPO following administration of AAA LNPs for delivery of mRNA. b) Batch-to-batch variability of **OF-02** LNPs for EPO mRNA delivery in vivo. c) In vivo dose–response curves for **OF-02** and **cKK-E12** LNPs. d) EPO expression following administration of **OF-02** and **cKK-E12** LNPs at 6 and 24 h. Data presented as mean + standard deviation ($n = 3$).

modest activity promoting a serum EPO concentration of 2100 ± 500 ng mL^{-1} using the same dose.

With this information in hand, our attention then shifted from exploring the general properties of the new AAA series of ionizable lipids to further characterizing LNPs made from our lead material **OF-02.** The clinical translation of nucleic acid delivery vehicles is in part predicated on high reproducibility of the chemical constituents and formulation of LNPs. To test this, three independent batches of **OF-02** were synthesized and then formulated into LNPs. The average serum concentration among all batches was found to be 13 700 ± 1700 ng mL^{-1} and demonstrated minimal batch-to-batch variability (Figure 3b). Next, a dose–response curve was collected at 0.75, 1.5, and 2.25 mg kg^{-1} total EPO mRNA dose for both **OF-02** and **cKK-E12** LNPs (Figure 3c). **OF-02** LNPs outperformed their **cKK-E12** counterparts roughly twofold across all doses studied, reaching a maximum EPO concentration of 45 400 ± 5300 ng mL^{-1} at the 2.25 mg kg^{-1} dose. It is also interesting to note that both sets of LNPs promote EPO production in a linear fashion with respect to dose. This trend implies that we have not yet reached a saturation point for the intracellular translation machinery, suggesting protein production is currently only limited by the dose of mRNA. Moreover, it is important to note that no animal mortality was observed at all doses studied, and that mice treated with both **cKK-E12** and **OF-02** LNPs displayed similar weight loss profiles at identical doses (Figure S2, Supporting Information). **OF-02** LNPs therefore represent a tunable handle for in vivo EPO production readily capable of exceeding normal human EPO levels (40–250 pg mL^{-1}) in our chosen mouse model.[17] Finally, **OF-02** LNPs also outperformed their **cKK-E12** counterparts at 24 h, independent of dose (Figure 3d). The sharp decrease in EPO concentration as a function of time highlights one of the many exciting potential therapeutic advantages of mRNA delivery in vivo; in contrast to permanent gene replacement therapies, mRNA delivery offers transient, dose–response dependent protein expression in vivo, a property that could one day prove useful for a variety of genetic disorders.

Finally, we were interested to determine if the efficacy differences observed between **ckk-E12** and **OF-02** LNPs were due to variations in biodistribution. mRNA coding for luciferase was independently formulated with both **ckk-E12** and **OF-02** in the same fashion as for EPO delivery, and mouse organs were harvested 24 h post injection. The tissues were subsequently imaged ex vivo to measure the total luminescence per organ, demonstrating that mRNA from both **ckk-E12** and **OF-02** LNPs is predominantly translated in the liver with minimal translation in the spleen and negligible translation in other organs (Figure 4a,b). Quantification of these data also confirms nearly identical biodistribution profiles for the two formulations, suggesting that the increased efficacy of **OF-02** LNPs is not due to a difference in tissue targeting (**Figure 4**c). Since more than 4000 human diseases are caused by liver genetic disorders such

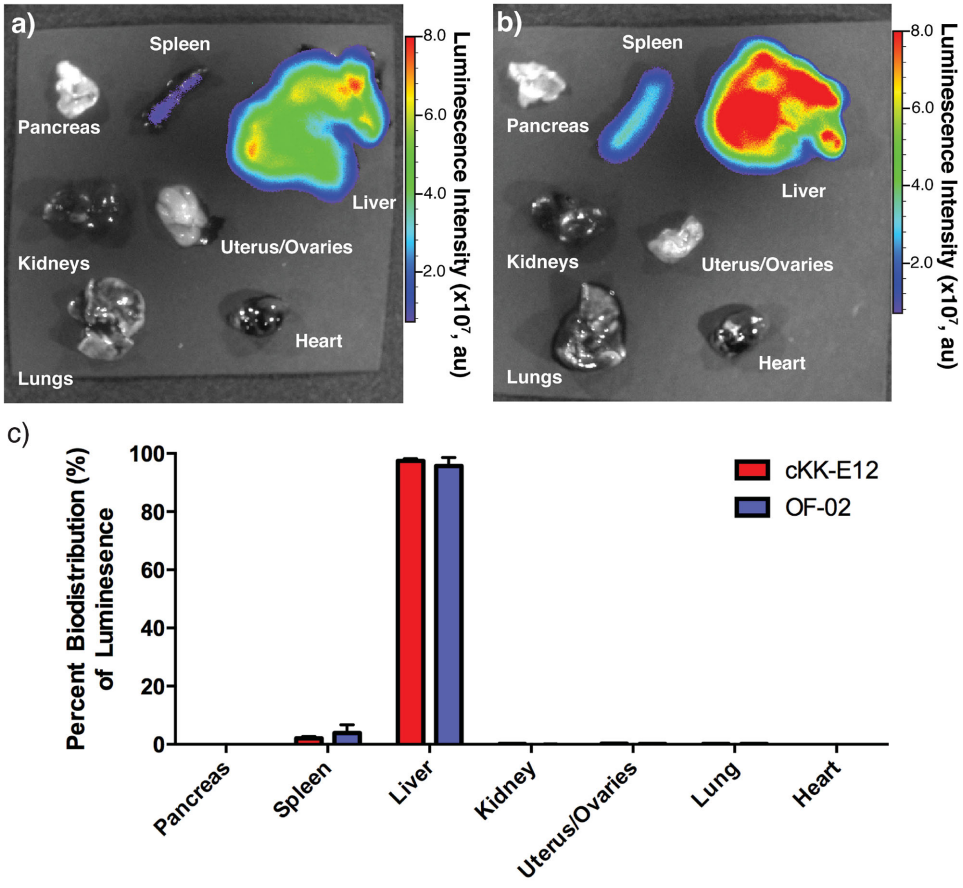

Figure 4. Representative luminescence biodistribution of a) **cKK-E12** and b) **OF-02** LNPs with luciferase mRNA ex vivo and c) associated quantification. Data presented as mean + standard deviation (n = 3).

as hemophilias A and B, **OF-02** LNPs represent a promising delivery vehicle for therapeutic mRNA delivery to the liver.[18]

In summary, we have synthesized a new series of AAA ionizable lipid materials for mRNA LNP delivery. To the best of our knowledge, compounds **OF-00** through **OF-03** represent the first examples of AAA ionizable lipids in the scientific literature, and we hope that their alkene-epoxide precursors **AE-00** through **AE-03** can serve as versatile scaffolds for the synthesis of future generations of these ionizable lipids. **OF-02** LNPs yielded a twofold increase in EPO production in vivo as compared to benchmark LNPs in the literature across a broad linear dose–response window. This illustrates that **OF-02** presents a tunable handle over in vivo protein expression, which is important in protein replacement therapies.[2] Batch-to-batch variability, dose–response curves, and time course studies were coupled with biodistribution data, highlighting the exceptional potency with which these LNPs can deliver mRNA to the liver. Future work will study the potential of **OF-02** LNPs for therapeutic applications and establish further groundwork necessary for translating this novel mRNA delivery vehicle to the clinic. In total, this study demonstrates efficient mRNA delivery with **OF-02** as well as the importance of utilizing synthetic chemistry in tandem with biological inspirations to further improve and understand nucleic acid delivery in vivo.

Experimental Section

Animal Experiments: All animal studies were approved by the M.I.T. Institutional Animal Care and Use Committee and were consistent with local, state, and federal regulations as applicable. LNPs were intravenously injected in female C57BL/6 mice (Charles River Labs, 18–22 g) via the tail vein. After 6 or 24 h, blood was collected via the tail vein and serum was isolated by centrifugation in serum separation tubes. Serum EPO levels were quantified with an ELISA assay (Human Erythropoietin Quantikine IVD ELISA Kit, R&D Systems, Minneapolis, MD). 24 h after injection of Luc-mRNA LNPs, mice were injected intraperitoneally with 130 µL of D-luciferin (30 mg mL^{-1} in PBS). After 15 min, mice were sacrificed and the organs were isolated (pancreas, spleen, liver, kidneys, lungs, heart, uterus, and ovaries) and imaged with an in-vivo imaging system (IVIS) (Perkin Elmer, Waltham, MA). Luminescence was quantified using LivingImage software (Perkin Elmer).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This material is based upon work supported by the National Science Foundation Graduate Research Fellowship (Owen S. Fenton). This work was also supported in part by Shire Pharmaceuticals (Lexington, MA). E.A.A. is grateful for financial support through a Wellcome Trust-MIT postdoctoral fellowship and a Margaret A. Cunningham Immune Mechanisms In Cancer Research Fellowship Award. The authors also thank the Nanotechnology Materials Core Facility at the Koch Institute at MIT.

Received: November 24, 2015
Revised: January 8, 2016
Published online: February 18, 2016

[1] a) R. Kanasty, J. R. Dorkin, A. Vegas, D. Anderson, *Nat. Mater.* **2013**, *12*, 967; b) K. A. Whitehead, R. Langer, D. G. Anderson, *Nat. Rev. Drug Discov.* **2009**, *8*, 516.
[2] U. Sahin, K. Kariko, O. Tureci, *Nat. Rev. Drug Discov.* **2014**, *13*, 759.
[3] B. Leader, Q. J. Baca, D. E. Golan, *Nat. Rev. Drug Discov.* **2008**, *7*, 21.
[4] M. S. D. Kormann, G. Hasenpusch, M. K. Aneja, G. Nica, A. W. Flemmer, S. Herber-Jonat, M. Huppmann, L. E. Mays, M. Illenyi, A. Schams, M. Griese, I. Bittmann, R. Handgretinger, D. Hartl, J. Rosenecker, C. Rudolph, *Nat. Biotechnol.* **2011**, *29*, 154.
[5] A. Thess, S. Grund, B. L. Mui, M. J. Hope, P. Baumhof, M. Fotin-Mleczek, T. Schlake, *Mol. Ther.* **2015**, *23*, S55.
[6] K. J. Kauffman, J. R. Dorkin, J. H. Yang, M. W. Heartlein, F. DeRosa, F. F. Mir, O. S. Fenton, D. G. Anderson, *Nano Lett.* **2015**, *15*, 7300.
[7] a) J. J. Lu, R. Langer, J. Z. Chen, *Mol. Pharmaceut.* **2009**, *6*, 763; b) T. M. Allen, P. R. Cullis, *Adv. Drug Deliver. Rev.* **2013**, *65*, 36.
[8] I. S. Zuhorn, U. Bakowsky, E. Polushkin, W. H. Visser, M. C. A. Stuart, J. B. F. N. Engberts, D. Hoekstra, *Mol. Ther.* **2005**, *11*, 801.
[9] B. L. Mui, Y. K. Tam, M. Jayaraman, S. M. Ansell, X. Y. Du, Y. Y. C. Tam, P. J. C. Lin, S. Chen, J. K. Narayanannair, K. G. Rajeev, M. Manoharan, A. Akinc, M. A. Maier, P. Cullis, T. D. Madden, M. J. Hope, *Mol. Ther. Nucleic Acids* **2013**, *2*, e139.
[10] a) G. Sahay, W. Querbes, C. Alabi, A. Eltoukhy, S. Sarkar, C. Zurenko, E. Karagiannis, K. Love, D. L. Chen, R. Zoncu, Y. Buganim, A. Schroeder, R. Langer, D. G. Anderson, *Nat. Biotechnol.* **2013**, *31*, 653; b) K. T. Love, K. P. Mahon, C. G. Levins, K. A. Whitehead, W. Querbes, J. R. Dorkin, J. Qin, W. Cantley, L. L. Qin, T. Racie, M. Frank-Kamenetsky, K. N. Yip, R. Alvarez, D. W. Y. Sah, A. de Fougerolles, K. Fitzgerald, V. Koteliansky, A. Akinc, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 9915.
[11] S. C. Semple, A. Akinc, J. X. Chen, A. P. Sandhu, B. L. Mui, C. K. Cho, D. W. Y. Sah, D. Stebbing, E. J. Crosley, E. Yaworski, I. M. Hafez, J. R. Dorkin, J. Qin, K. Lam, K. G. Rajeev, K. F. Wong, L. B. Jeffs, L. Nechev, M. L. Eisenhardt, M. Jayaraman, M. Kazem, M. A. Maier, M. Srinivasulu, M. J. Weinstein, Q. M. Chen, R. Alvarez, S. A. Barros, S. De, S. K. Klimuk, T. Borland, V. Kosovrasti, W. L. Cantley, Y. K. Tam, M. Manoharan, M. A. Ciufolini, M. A. Tracy, A. de Fougerolles, I. MacLachlan, P. R. Cullis, T. D. Madden, M. J. Hope, *Nat. Biotechnol.* **2010**, *28*, 172.
[12] Y. Z. Dong, K. T. Love, J. R. Dorkin, S. Sirirungruang, Y. L. Zhang, D. L. Chen, R. L. Bogorad, H. Yin, Y. Chen, A. J. Vegas, C. A. Alabi, G. Sahay, K. T. Olejnik, W. H. Wang, A. Schroeder, A. K. R. Lytton-Jean, D. J. Siegwart, A. Akinc, C. Barnes, S. A. Barros, M. Carioto, K. Fitzgerald, J. Hettinger, V. Kumar, T. I. Novobrantseva, J. N. Qin, W. Querbes, V. Koteliansky, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 5753.
[13] a) N. Bartke, Y. A. Hannun, *J. Lipid Res.* **2009**, *50*, S91; b) Y. A. Hannun, L. M. Obeid, *Nat. Rev. Mol. Cell. Biol.* **2008**, *9*, 139.
[14] a) K. Kariko, H. Muramatsu, J. M. Keller, D. Weissman, *Mol. Ther.* **2012**, *20*, 948; b) S. Liu, J. A. Ren, Z. W. Hong, D. S. Yan, G. S. Gu, G. Han, G. F. Wang, H. J. Ren, J. Chen, J. S. Li, *Nutr. Clin. Pract.* **2013**, *28*, 120.
[15] K. A. Whitehead, J. R. Dorkin, A. J. Vegas, P. H. Chang, O. Veiseh, J. Matthews, O. S. Fenton, Y. L. Zhang, K. T. Olejnik, V. Yesilyurt, D. L. Chen, S. Barros, B. Klebanov, T. Novobrantseva, R. Langer, D. G. Anderson, *Nat. Commun.* **2014**, *5*, 4277.
[16] J. Heyes, L. Palmer, K. Bremner, I. MacLachlan, *J. Control Release* **2005**, *107*, 276.
[17] M. Cazzola, F. Mercuriali, C. Brugnara, *Blood* **1997**, *89*, 4248.
[18] J. McClellan, M. C. King, *Cell* **2010**, *142*, 353.