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Fast and Efficient CRISPR/Cas9 Genome Editing In Vivo Enabled by Bioreducible Lipid and Messenger RNA Nanoparticles

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Supporting Information

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

Conflict of Interest

The authors declare no conflict of interest.

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Abstract

A main challenge to broaden the biomedical application of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat (CRISPR) associated protein 9) genome editing technique is the delivery of Cas9 nuclease and single-guide RNA (sgRNA) into the specific cell and organ. An effective and very fast CRISPR/Cas9 genome editing in vitro and in vivo enabled by bioreducible lipid/Cas9 messenger RNA (mRNA) nanoparticle is reported. BAMEA-O16B, a lipid nanoparticle integrated with disulfide bonds, can efficiently deliver Cas9 mRNA and sgRNA into cells while releasing RNA in response to the reductive intracellular environment for genome editing as fast as 24 h post mRNA delivery. It is demonstrated that the simultaneous delivery of Cas9 mRNA and sgRNA using BAMEA-O16B knocks out green fluorescent protein (GFP) expression of human embryonic kidney cells with efficiency up to 90%. Moreover, the intravenous injection of BAMEA-O16B/Cas9 mRNA/sgRNA nanoparticle effectively accumulates in hepatocytes, and knocks down proprotein convertase subtilisin/kexin type 9 level in mouse serum down to 20% of nontreatment. The leading lipid nanoparticle, BAMEA-O16B, represents one of the most efficient CRISPR/Cas9 delivery nanocarriers reported so far, and it can broaden the therapeutic promise of mRNA and CRISPR/Cas9 technique further.

Keywords

CRISPR/Cas9; genome editing; lipid nanoparticles; messenger RNA delivery

The CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeat (CRISPR) associated protein 9) is a powerful and prominent tool for manipulating the genetic information of mammalian cells.[1–3] It has dramatically simplified genome editing procedure by using a single-guide RNA (sgRNA) to recognize target DNA, and the nuclease activity of Cas9 to introduce site-specific double-strand breaks (DSBs) at target gene loci.[4] Nevertheless, a main challenge that limits an effective CRISPR/Cas9 genome editing is the delivery of Cas9 nuclease and sgRNA into targeted cells, because neither Cas9 protein nor sgRNA is naturally present in mammalians and cell permeable.[5–7] Until now, viral vectors are the mostly used carriers for CRISPR/Cas9 genome editing delivery but are limited by the restricted packing capacity and side effects of virus.[8] In comparison, nonviral nanocarriers, including those of lipid nanoparticle,[9] polymers,[10–12] and inorganic nanoparticles,[13–15] have recently been developed to deliver CRISPR/Cas9 in the form of DNA plasmid,[14] messenger RNA (mRNA),[16,17] or Cas9/sgRNA ribonucleoproteins (RNPs)[9] to address many limitations of viral delivery. However, the low CRISPR/Cas9 genome editing efficiency using these nanocarriers, particularly their limited use for in vivo genome editing still challenges the effectiveness and potency of harnessing CRISPR/Cas9 technique for developing new gene therapy.

In this study, we report a new approach for systematic delivery of CRISPR/Cas9 by encapsulating Cas9 mRNA and sgRNA simultaneously into bioreducible lipid nanoparticle for efficient and very fast genome editing in vitro and in vivo. mRNA represents a promising class of nucleic acid therapeutics that complements to DNA plasmid, in terms of decreased risk of mutagenesis, transient effects, and reduced complexity.[18] It has also been reported that CRISPR/Cas9 genome editing delivered in the form of mRNA showed lower off-target effects than using Cas9 plasmid.[19] However, exogenous mRNA is cell impermeable, and this remains as a great barrier to the therapeutic application of mRNA, including genome editing.[20] Moreover, mRNA is different from DNA plasmid in terms of structure and stability, which all pose additional challenges to deliver mRNA into cells.[21] Herein, we designed bioreducible lipid nanoparticles composed of disulfide bond-containing hydrophobic tails for mRNA delivery and CRISPR/Cas9 genome editing (Scheme 1). We have recently demonstrated the use of these lipid nanoparticles as nanocarriers for Cas9/sgRNA RNPs delivery,[9,22,23] while their mRNA delivery and in vivo genome editing efficacy remains unknown. In this study, we identified a leading lipid, BAMEA-O16B, to deliver reporter mRNA and Cas9 mRNA/sgRNA complex both in vitro and in vivo via a screening and optimization approach.[9] BAMEA-O16B can encapsulate mRNA via electrostatic interaction to assemble nanoparticle, while releasing mRNA intracellularly in response to the reductive chemical signals through a disulfide bond exchange mechanism (Scheme 1a). It is noticeable that the simultaneous delivery of Cas9 mRNA/sgRNA knocks out GFP expression of human embryonic kidney (HEK) cells with efficiency up to 90%, and an effective gene knockout was observed even as fast as 24 h post Cas9 mRNA delivery, which is a significant enhancement compared to Cas9/sgRNA RNPs delivery in terms of in vitro genome editing efficiency.[9] Moreover, the intravenous injection of BAMEA-O16B/Cas9 mRNA/sgRNA nanoparticle effectively knocked mouse serum proprotein convertase subtilisin/kexin type 9 (PCSK9) level down to 20% of nontreated mouse. The bioreducible lipid BMEA-O16B represents one of the most efficient nonviral CRISPR/Cas9 genome editing delivery reported so far, and it could broaden the therapeutic promise of mRNA therapeutics and CRIS PR/Cas9 technique further.

The bioreducible lipids were synthesized by heating amine and acrylates or acrylamides featuring a disulfide bond according to our previous reports (Scheme 1b).[9,24–26] The lipids were named by amine number or name followed by O16B or N16B to discriminate the use of acrylate or acrylamide, respectively (Scheme 1b). The as-purified lipids were formulated with cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and DSPE-PEG_{2000} for all cellular delivery experiments in this study (see “Materials and Methods” in the Supporting Information for details). To facilitate the screening of effective lipids for mRNA delivery, luciferase-encoding mRNA was assembled with different lipids at weight ratio of 1:15 to transfect A375 human melanoma cells. The luciferase expression of transfected A375 cells was measured and compared to cells treated with luciferase mRNA alone or luciferase mRNA complexed with a commercial transfection lipid, Lipofectamine 2000 (LPF2K). As shown in Figure 1a, cells treated with mRNA alone did not show detectable luciferase expression, indicating that mRNA alone is not able to enter cells, while cells treated with the complexes of mRNA and lipids showed varied luciferase expression that depends on the amine and tail structure of the lipids. Two bioreducible lipids, BAMEA-

O16B (amine 11) and PPPDA-N16B (amine 13), delivered luciferase mRNA in a comparable efficiency to that of LPF2K. A further study of the biocompatibility of the two lipids indicated that the mRNA lipoplexes of BAMEA-O16B and PPPDA-N16B showed a lower cytotoxicity than LPF2K (Figure S1, Supporting Information), highlighting the advantage and necessity of designing combinatorial lipid nanoparticle for discovering efficient yet biocompatible nanocarriers for mRNA delivery. In the rest of this study, we selected one of the leading lipids, BAMEA-O16B, for detailed mRNA delivery and CRISPR/Cas9 genome editing studies.

The leading lipid BAMEA-O16B is a general nanocarrier for mRNA delivery, as evidenced by its capability and high efficiency to deliver red fluorescent protein (RFP)-encoding mRNA into HeLa cells. The treatment of human cervical cancer cells (HeLa) with BAMEA-O16B/RFP mRNA nanoparticles resulted in efficient RFP expression (Figure 1b). Flow cytometry analysis (Figure 1c) indicated that the RFP-positive cells are dependent on mRNA dose, and the transfection efficiency could be as high as 90% when 160 ng mL^{-1} of RFP mRNA was delivered into HeLa cells. Altogether, both luciferase and RFP mRNA delivery demonstrated the effectiveness of BAMEA-O16B nanoparticle for efficient yet safe mRNA delivery.

To further elaborate that the integration of disulfide bonds into BAMEA-O16B promotes intracellular mRNA release to enhance mRNA transfection efficiency, we synthesized a control lipid with a chemical structure similar to BAMEA-O16B, while lacking the disulfide bond, named as BAMEA-O16 (Figure 2a). Agarose gel electrophoresis study indicated that BAMEA-O16B and BAMEA-O16 showed comparable mRNA encapsulation efficiency (Figure S2, Supporting Information). Interestingly, glutathione (GSH) treatment (5×10^{-3} M) resulted in the efficient release of mRNA from BAMEA-O16B/mRNA complex, but not from the BAMEA-O16/mRNA complexes. The similar mRNA encapsulation capability was further confirmed by the comparable size and zeta potential of the two mRNA complexes, as revealed by the dynamic light scattering (DLS) analysis (Table S1, Supporting Information). It is also of note that BAMEA-O16B or BAMEA-O16 showed a comparable RNA delivery efficiency. The treatment of HeLa cells with fluorescent-labeled RNA complexes (10×10^{-9} M) of BAMEA-O16B or BAMEA-O16 resulted in RNA internalization efficiency higher than 90% at all different lipid and RNA ratios (Figure 2b). Confocal laser scanning microscopy (CLSM) imaging of HeLa cells, however, indicated that the BAMEA-O16B/RNA treated cells showed a higher endosome escape efficiency than that of BAMEA-O16/RNA treatment (Figure 2c); this is mostly due to the bioreducible nature of BAMEA-O16B that facilitates the release of RNA more efficiently in response to the reductive intracellular environment. Moreover, RFP mRNA delivery using BAMEA-O16B nanoparticles resulted in a higher RFP expression in HeLa cells than that of BAMEA-O16 facilitated mRNA delivery. For example, the delivery of 160 ng mL^{-1} RFP mRNA using BAMEA-O16B resulted in fourfold enhancement of RFP expression than using BAMEA-O16 nanoparticles (Figure 2d).

Having demonstrated the effectiveness of using BAMEA-O16B for reporter mRNA delivery, we next examined the capability of BAMEA-O16B to simultaneously deliver Cas9 mRNA and sgRNA for genome editing. Cas9 mRNA is around 4500 nucleotides in size,[27] and it is

much longer than luciferase or RFP mRNA (≈1000 nt). To further evaluate the efficacy of the library of bioreducible for Cas9 mRNA delivery and genome editing, we treated GFP stably expressing HEK cells with the lipid nanoparticle encapsulating Cas9 mRNA and GFP-targeting sgRNA, and monitored the GFP expression change before and after the mRNA delivery. The on-target GFP genome editing could induce the shift of reading frame of GFP gene, and thereby preventing GFP expression. As shown in Figure S3 in the Supporting Information, 7 of the 32 lipids can efficiently deliver Cas9 mRNA and sgRNA to knock down GFP expression of HEK cells, among which lipid BAMEA-O16B showed the highest GFP knockout efficiency. We found that the electrostatic interaction of BAMEA-O16B and Cas9 mRNA/sgRNA assembled well-dispersed nanoparticles around 230 nm in size (Table S1 and Figure S4, Supporting Information). Meanwhile, the BAMEA-O16B/Cas9 mRNA nanocomplex is highly biocompatible for genome editing delivery. HEK cells treated with different concentrations of BAMEA-O16B/Cas9 mRNA nanocomplex all retained viability greater than 90%, which is also higher than HEK cells treated with LPF2K/Cas9 mRNA/sgRNA nanocomplex (Figure S5, Supporting Information).

CLSM imaging indicated that BAMEA-O16B/Cas9 mRNA/sgGFP nanoparticle treatment resulted in a complete loss of the GFP fluorescence of HEK-GFP cells, while free Cas9 mRNA and sgRNA treatment did not show similar GFP knockout effect (Figure 3a). Quantitative analysis of GFP expression of HEK-GFP cells showed that when the concentration of Cas9 mRNA delivered to cells increased from 20 to 160 ng mL^{-1}, the GFP knockout efficiency increased from 35% to higher than 90% accordingly (Figure 3b). Meanwhile, when the sgGFP was replaced with a scramble sgRNA, we did not observe an effective gene editing and cellular GFP loss (Figure 3c). It is noticeable that BAMEA-O16B/Cas9 mRNA/sgRNA treatment knocked out GFP expression at protein level very fast (Figure 3b). 40% GFP knockout was observed as early as 24 h post Cas9 mRNA delivery, and this ratio was increased up to 90% 36 h post Cas9 mRNA delivery, though a further extension of delivery time did not increase the gene edit efficiency, we attributed the high yet very fast gene editing efficiency to the high mRNA delivery efficiency using lipid nanoparticles.

Further, we showed that BAMEA-O16B mediated Cas9 mRNA delivery is able to regulate endogenous gene expression, and therefore is of great potential for developing new gene therapy. Human papillomavirus type 18 (HPV18), an essential gene that promotes human cervical cancer progression was selected as a target to study.[28] SgRNA that targets HPV18 was delivered into HeLa cells along with Cas9 mRNA using BAMEA-O16B, the viability of HeLa cells following the treatment was measured and compared to that with scramble sgRNA and Cas9 mRNA treatment. As shown in Figure 3d, BAMEA-O16B/Cas9 mRNA/sgHPV18 treatment significantly prohibited HeLa growth compared to that of a scramble sgRNA and Cas9 mRNA delivery (Figure 3d). For example, the delivery of 320 ng mL^{-1} Cas9 mRNA and 26×10^{-9} M sgHPV18 reduced HeLa cell viability down to 30%, while the replacement of sgHPV18 with a scramble sgRNA did not show similar effect on inhibiting HeLa cell growth.

Despite the previous reports of designing nonviral nanoparticle for CRISPR/Cas9 delivery, its potency for in vivo genome editing remains very limited, mostly due to the low gene

editing efficiency using these nanocarriers, and the requirement of delivering Cas9 and sgRNA separately and locally.[29] To further demonstrate the potential of BAMEA-O16B nanoparticle for in vivo mRNA delivery and CRISPR/Cas9 genome editing, we selected proprotein convertase subtilisin/kexin type 9, an enzyme secreted from hepatocytes and involved cholesterol homeostasis as a target to study.[30] PCSK9 plays an important role in lipid metabolism by modulating the density of low-density lipoprotein cholesterol receptors (LDL-R) in liver, genetic studies reveal that the loss of PCSK9 is associated with a reduced risk of cardiovascular disease.[31] Therefore, CRISPR/Cas9 genome editing mediated PCSK9 knockdown, in particularly using nonviral delivery system, would be of great potency for developing new treatment for cardiovascular disease.[29,31]

We first studied the biodistribution of BAMEA-O16B nanoparticle for in vivo mRNA delivery. To this end, BAMEA-O16B/luciferase mRNA nanoparticles were formulated and intravenously injected to mice via tail vein at an mRNA dosage of 0.6 mg kg^{-1}. We studied the biodistribution of the mRNA expression using the bioluminescence imaging of organs. The injection of BAMEA-O16B/luciferase mRNA nanoparticle resulted in effective expression of luciferase in mouse liver (Figure 4a). A detailed cellular localization study by delivering BAMEA-O16B/RFP mRNA nanoparticles revealed that the BAMEA-O16B/RFP mRNA nanoparticle was mostly delivered in hepatocytes (Figure 4b). There is only one cargo when delivering luciferase mRNA and RFP mRNA, while there are both mRNA and gRNA to be delivered for CRISPR-mediated gene editing. The biodistribution of the BAMEA-O16B/Cas9 mRNA/sgRNA might be different than the lipid nanoparticle complexed with luciferase mRNA or RFP mRNA. In order to evaluate this, we delivered a Cy3-labeld RNA together with Cas9 mRNA using BAMEA-O16B at an mRNA dosage of 0.6 or 0.8 mg kg^{-1} sgRNA, and evaluated the biodistribution using fluorescence imaging. The results showed that the Cas9 mRNA and sgRNA mainly accumulated in the liver (Figure S6, Supporting Information). To this end, we subsequently studied whether the BAMEA-O16B can deliver Cas9 mRNA and sgRNA (for PCSK9) into hepatocytes to facilitate PCSK9 knockdown.

The potential and efficacy of BAMEA-O16B nanoparticle for in vivo genome editing was studied by injecting BAMEA-O16B/Cas9 mRNA/sgPCSK9 nanopartides into C57BL/6 mice, followed by serum PCSK9 level quantification to evaluate the in vivo genome editing efficacy. As shown in Figure 4c, the intravenous injection of BAMEA-O16B/Cas9 mRNA/sgPCSK9 nanoparticle reduced mouse serum PCSK9 down to 20% of that with DPBS injection or BAMEA-O16B/Cas9 mRNA/scramble sgRNA nanoparticle injections. Meanwhile, histological examination of the mouse liver after BAMEA-O16B/Cas9 mRNA/sgRNA nanoparticle treatment using haematoxylin and eosin (H&E) staining did not show signs of inflammation (Figure S7, Supporting Information). Moreover, the nanoparticle injections did not induce obvious hepatocellular injury, as revealed by the minimal change of serum aspartate transaminase (AST), alanine aminotransferase (ALT), and total bilirubin of mice with all injections (Figure S8, Supporting Information). Altogether, the results clearly demonstrated the high efficacy and biocompatibility of BAMEA-O16B nanoparticle for in vivo genome editing.

In summary, we report here a fast and very potent CRISPR/Cas9 genome editing in vitro and in vivo enabled by simultaneous delivery of Cas9 mRNA and sgRNA using bioreducible lipid nanoparticles. The leading lipid, BAMEA-O16B, can deliver Cas9 mRNA and sgRNA while efficiently release them in response to the reductive intracellular environment. The BAMEA-O16B/Cas9 mRNA/sgRNA nanoparticle delivery can knock out cellular GFP expression with efficiency as high as 90% in cultured cells, and knock down mouse PCSK9 by 80% in vivo. Our findings established BMEA-O16B as a general yet efficient nanoparticle platform for mRNA delivery and CRISPR/Cas9 genome editing, it represents one of the most efficient CRISPR/Cas9 delivery nanocarriers reported so far, and it will broaden the therapeutic promise of mRNA therapeutics and CRISPR/Cas9 technique further. PCSK9 is a valid therapeutic target and the antibody against PCSK9 is already approved by FDA for reducing risk of cardiovascular diseases.[32] We envision the efficient knockdown of PCSK9 gene using CRISPR/Cas9 based technology together with nonviral nanoparticle delivery system can have the potential for clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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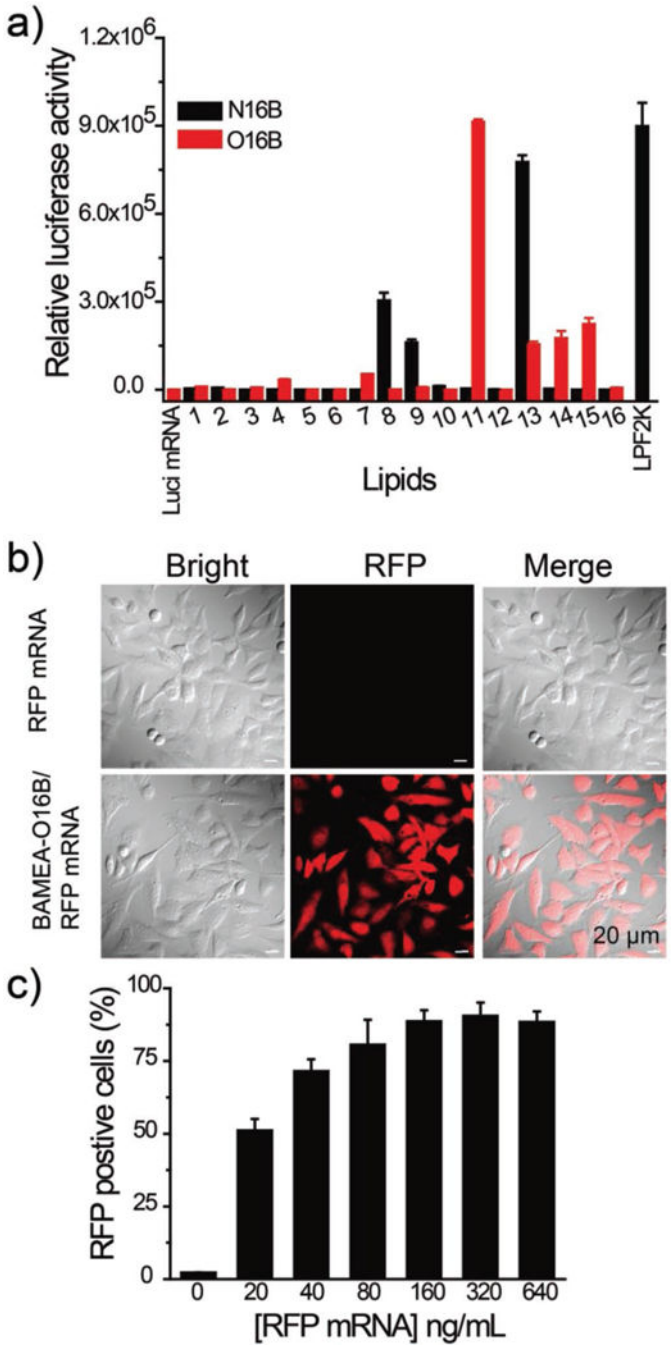


Figure 1.

Intracellular delivery of luciferase and RFP mRNA. a) Luciferase expression of A375 cells treated with luciferase mRNA alone (160 ng mL^{-1}) or nanocomplexes of different lipid nanoparticles; b) CLSM images of HeLa cells transfected with RFP mRNA alone (320 ng mL^{-1}) or in the form of BAMEA-O16B/RFP mRNA nanoparticles. Scale bars: 20 μm; c) mRNA dose dependent RFP expression of HeLa cells treated with increased concentration of BAMEA-O16B/RFP mRNA. RFP expression profile was quantified 24 h after mRNA delivery.

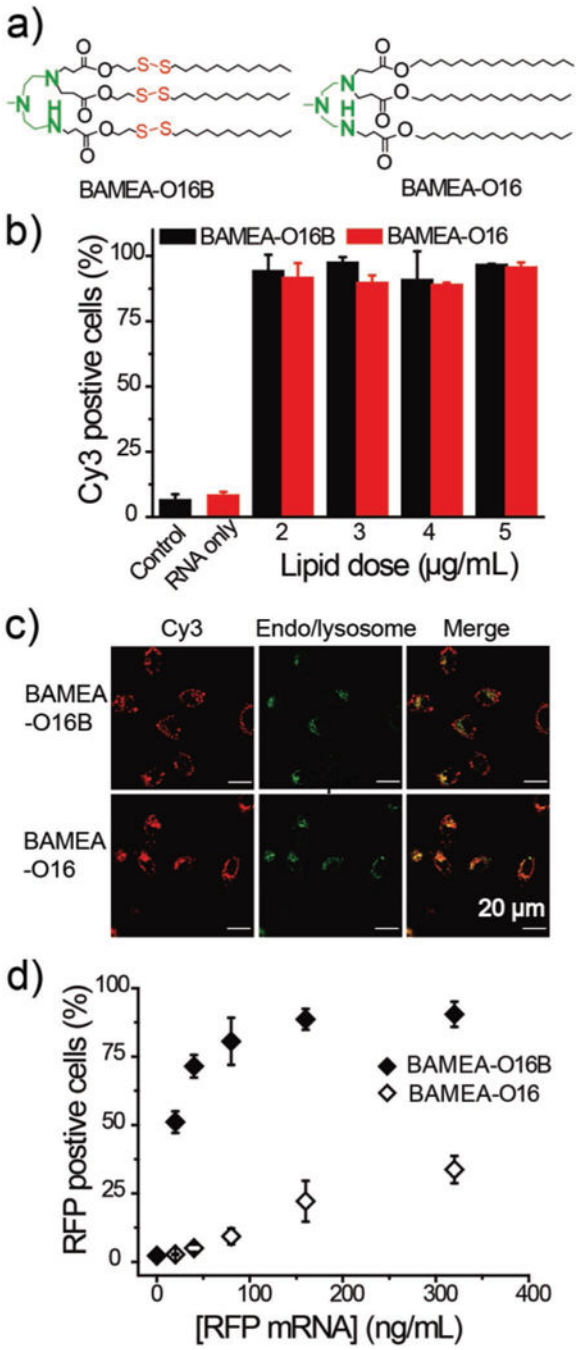


Figure 2.

Cellular uptake and endosome escape study of BAMEA-O16B/RNA nanoparticles. a) Chemical structure of BAMEA-O16B and BAMEA-O16. b) Cellular uptake efficiency and c) CLSM images of HeLa cells treated with BAMEA-O16B/Cy3-RNA or BAMEA-O16/Cy3-RNA nanoparticles. HeLa cells were delivered with 10×10^{-9} M Cy3-RNA complexed with the lipids at indicated ratios for delivery, and the endosome was costained using LysoTracker@Green (Scale bars: 20 μm); d) BAMEA-O16B/mRNA delivery showed an enhanced RFP expression in HeLa cells than that of BAMEA-O16 nanoparticles.

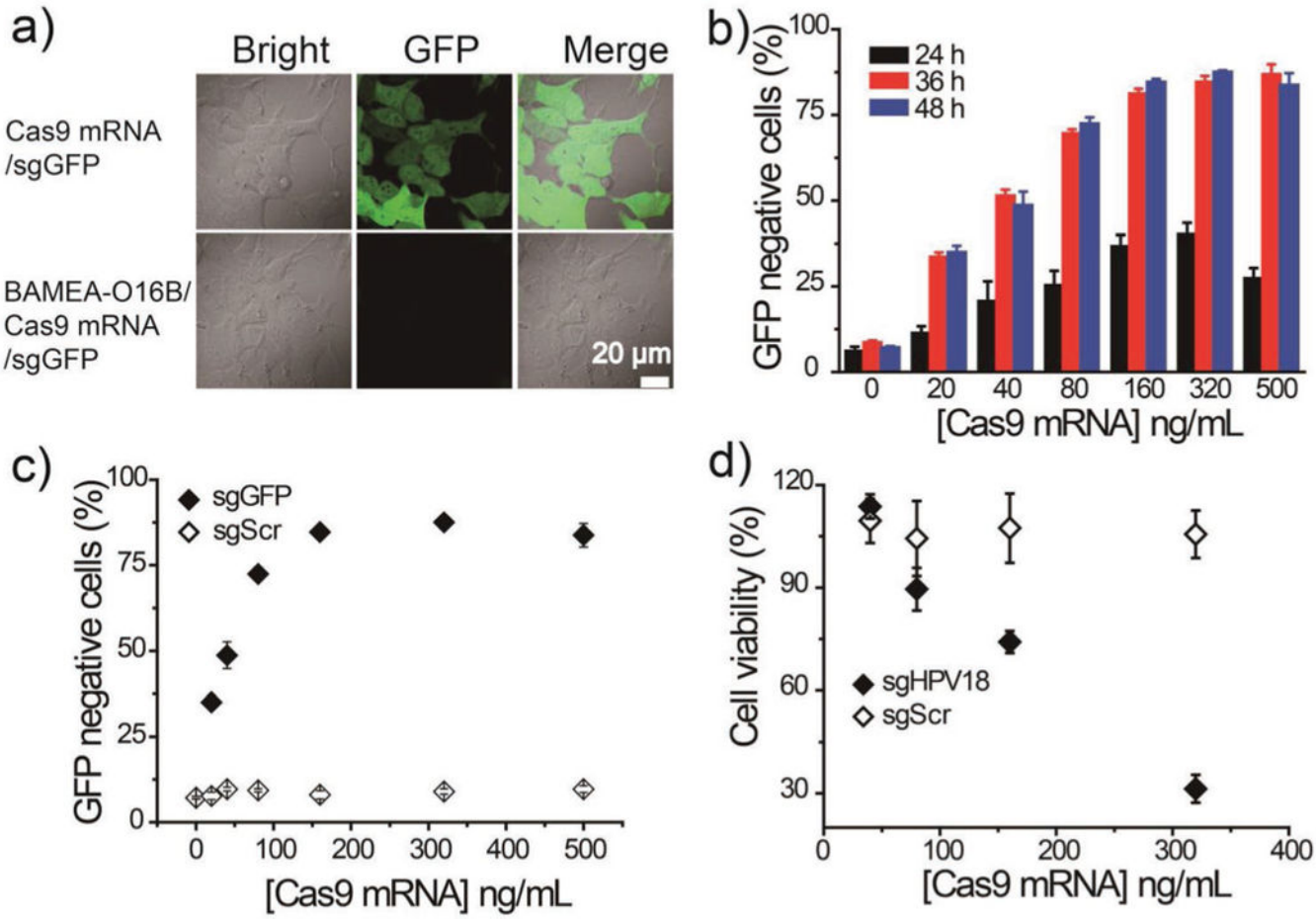


Figure 3.

CRISPR/Cas9 mRNA delivery and genome editing in cultured cells. a) CLSM images of HEK-GFP cells treated with Cas9 mRNA/sgGFP alone or BAMEA/Cas9 mRNA/sgGFP nanoparticles. Scale bar: 20 μm. b) Delivery of BAMEA/Cas9 mRNA/sgGFP nanoparticles for fast and efficient genome editing. HEK-GFP cells were treated with different doses of Cas9 mRNA and sgGFP (13×10^{-9} M), the GFP expression was quantified at different time points after delivery, c) sgRNA sequence specific genome editing and GFP knockout using BAMEA/Cas9 mRNA/sgGFP nanoparticles. GFP-HEK cells were treated with BAMEA/Cas9 mRNA/sgGFP or BAMEA/Cas9 mRNA/scramble sgRNA nanoparticles, and the GFP knockout efficiency was determined 48 h post mRNA delivery. d) The delivery of BAMEA/Cas9 mRNA/sgHPV18 nanoparticles efficiently and selectively prohibited HeLa cell growth.

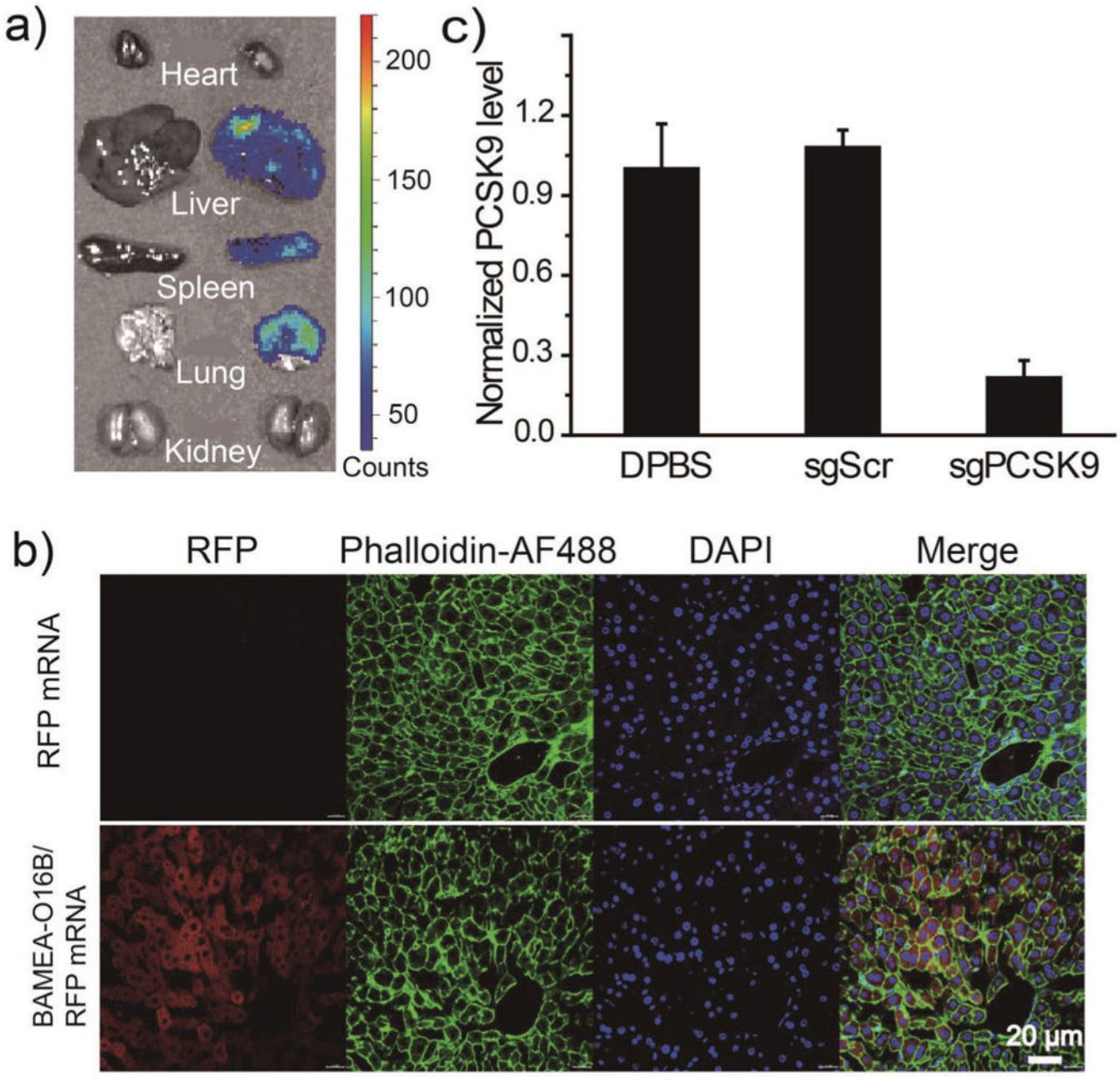
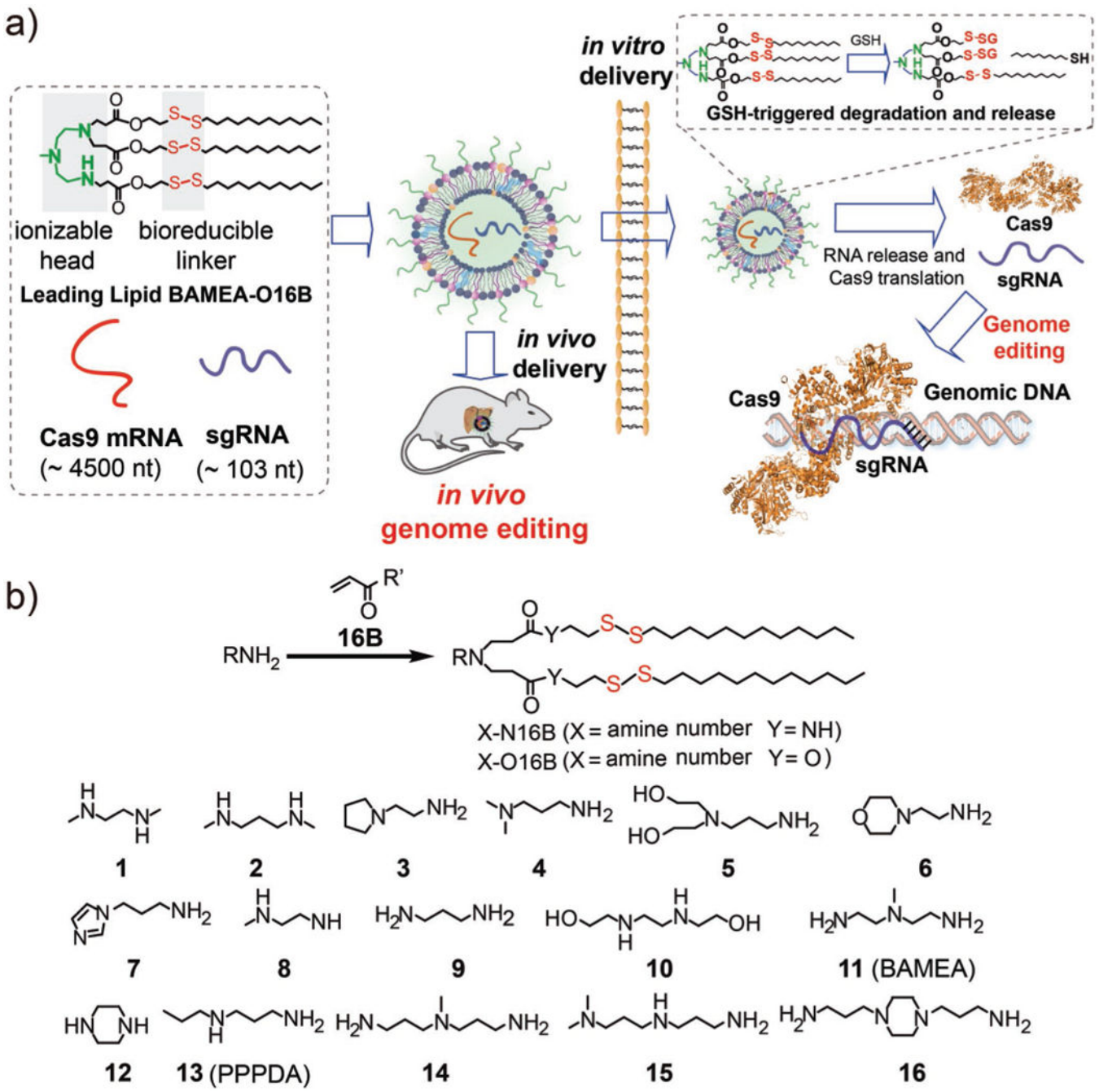


Figure 4.

Cas9 mRNA/sgRNA delivery and genome editing in vivo. a) Bioluminescence imaging of the tissues of mice injected with BAMEA-O16B/luciferase mRNA nanoparticles (right) or free mRNA (left). b) Fluorescent imaging of mouse liver injected with BAMEA-O16B/RFP mRNA nanoparticles indicated the efficient delivery of mRNA into hepatocytes. Scale bar: 20 μm. c) BAMEA-O16B/Cas9 mRNA/sgPCSK9 nanoparticles treatment effectively decreased mouse serum PCSK9. The serum PCSK9 level of Cas9 mRNA nanoparticle treatment was normalized to that injected with DPBS control.



Scheme 1.

Schematic diagram of Cas9 mRNA delivery in vitro and in vivo. a) Illustration of formulating bioreducible lipid/Cas9 mRNA/sgRNA nanoparticle for CRISPR/Cas9 genome editing delivery in vitro and in vivo. b) Synthesis route, lipid nomenclature, and chemical structure of amines used for lipid synthesis.