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Integrating Combinatorial Lipid Nanoparticle and Chemically Modified Protein for Intracellular Delivery and Genome Editing

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CONSPECTUS

The use of protein to precisely manipulate cell signaling is an effective approach for controlling cell fate and developing precision medicine. More recently, programmable nucleases, such as CRISPR/Cas9, have shown extremely high potency for editing genetic flow of mammalian cells, and for treating genetic disorders. The therapeutic potential of proteins with an intracellular target, however, is mostly challenged by their low cell impermeability. Therefore, a developing delivery system to transport protein to the site of action in a spatiotemporal controlled manner is of great importance to expand the therapeutic index of the protein.

In this Account, we first summarize our most recent advances in designing combinatorial lipid nanoparticles with diverse chemical structures for intracellular protein delivery. By designing parallel Michael addition or ring-opening reaction of aliphatic amines, we have generated a combinatorial library of cationic lipids, and identified several leading nanoparticle formulations for intracellular protein delivery both in vitro and in vivo. Moreover, we optimized the chemical structure of lipids to control lipid degradation and protein release inside cells for CRISPR/Cas9 genome-editing protein delivery.

In the second part of this Account, we survey our recent endeavor in developing a chemical approach to modify protein, in particular, coupled with the nanoparticle delivery platform, to improve protein delivery for targeted diseases treatment and genome editing. Chemical modification of protein is a useful tool to modulate protein function and to improve the therapeutic index of protein drugs. Herein, we mostly summarize our recent advances on designing chemical approaches to modify protein with following unique findings: (1) chemically modified protein shows selective turn-on activity based on the specific intracellular microenvironment, with which

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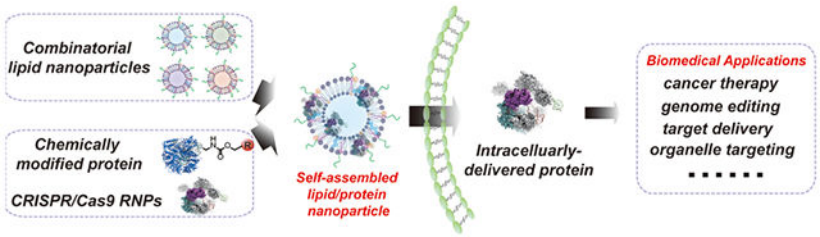
we were able to protein-based targeted cancer therapy; (2) the conjugation of hyaluronic acid (HA) to protein allows cancer cell surface receptor-targeted delivery of protein; (3) the introduction of nonpeptidic boronic acid into protein enabled cell nucleus targeted delivery; this is the first report that a nonpeptidic signal can direct protein to subcellular compartment; and (4) the fusion of protein with negatively supercharged green fluorescent protein (GFP) facilitates the self-assembly of protein with lipid nanoparticle for genome-editing protein delivery.

At the end of the Account, we give a perspective of expanding the chemistry that could be integrated to design biocompatible lipid nanocarriers for protein delivery and genome editing in vitro and in vivo, as well as the chemical approaches that we can harness to modulate protein activity in live cells for targeted diseases treatment.

Graphical Abstract



1. INTRODUCTION

Proteins are key regulators of signal transduction and physiological functions of cells.[1] The dysfunction or abnormality of protein is associated with various diseases, including cancers. Protein therapy, either to replace malfunctioning proteins inside cells or to modulate endogenous cell signaling using protein, offers a highly specific methodology for disease treatment.[2] More recently, the use of clustered regularly interspaced short palindromic repeat (CRISPR) associated protein 9 (CRISPR/Cas9) to edit genetic information on mammalian cells has shown great potency for developing new generations of protein therapeutics for treating genetic disorders.[3] To fully realize the therapeutic potential of proteins, however, one of the most challenging issues is to deliver active protein to tissues and cells,[4] and even subcellular compartments,[5] due to the intrinsic cell impermeability of most proteins. Additionally, proteins are an extremely diverse class of biomacromolecule, varying in terms of size, surface charge, and hydrophobicity and hydrophilicity from each other, which all pose unique challenges for delivering them into cells.[1] In this regard, the biomedical and therapeutic application of protein is greatly dependent on the method, efficiency, and targeting capability of the approaches to deliver protein into cells.

Over the past years, a number of approaches have been developed to facilitate protein delivery.[4] For instance, viral nanoparticles that display surface-binding ligands have been designed for targeted delivery of proteins.[6] Their therapeutic potential, however, is limited by the safety concerns and immunogenicity of viral nanoparticles. Alternatively, conjugating proteins with a cell penetrating TAT peptide efficiently delivers protein in vitro and in vivo.[7] These protein conjugates, however, usually suffer from low efficiency of endosome escape after entering into cells or low serum stability in vivo. Therefore, new drug delivery systems,

in particular nonviral nanocarriers to transport protein to cytosol or even specific subcellular organelles,[5] such as nucleus or mitochondria, are still highly desired.[4] Lipid nanoparticles and liposomes are traditional nanocarriers for gene delivery;[8] however, their potential for protein delivery was less studied, mostly due to the complicated and diverse chemical structures of proteins necessitating the customized design of lipid nanoparticles for each individual protein to be delivered.[9] To overcome these limitations and develop lipid nanoparticles for protein delivery, we have recently used a combinatorial library strategy to synthesize cationic lipids and conducted library screening to identify an efficient lipid-based protein delivery system.[10–15] In this Account, we will first summarize our recent advances on designing combinatorial lipid nanoparticles for intracellular protein delivery, and exploring the structure—activity relationships of these lipid nanoparticles for protein delivery. In addition, we will discuss the most recent advances of developing lipid nanoparticles for genome-editing protein delivery in vitro and in vivo.

In the second part of this Account, we will summarize our recent studies of chemical modification of protein, specifically coupled with a nanoparticle delivery platform, to advance protein delivery for targeted disease treatment and genome editing. Chemical modification of protein is a very meaningful technique that modulates protein structure, physiochemical property, and function to improve their therapeutic index.[16] We have demonstrated that the chemical modification of proteins can not only enhance the encapsulation of protein into lipid nanocarriers, but also modulate protein activity in response to intracellular microenvironment for targeted cancer therapy, as well as directing protein to target subcellular organelles.

2. DESIGNING COMBINATORIAL LIPID NANOPARTICLES AS DELIVERY VEHICLES

The past decades have witnessed a great success of designing cationic lipid nanoparticles for gene delivery and therapy.[17] A cationic lipid-siRNA formulation, called Patisiran was recently approved for TTR-mediated amyloidosis treatment.[18] When adapting the gene delivery strategy for protein delivery, there are several challenges of limiting an efficient protein delivery. First, protein shows varied net charges and hydrophobicity/hydrophilicity, resulting in difficulties in designing universal lipid-based protein delivery vehicles. Second, the three-dimensional structure of protein has to be maintained after lipid nanoparticle encapsulation and intracellular delivery for an effective protein therapy. Therefore, new approaches that enabling cationic lipids with diverse chemical structures to accommodate the chemistry diversity of protein are essential for protein delivery.

In recent years, Langer et al. have pioneered the use of a combinatorial library of lipid-like materials for gene and small interfering RNA (siRNA) delivery.[19] These lipids were synthesized through the parallel reaction between aliphatic amines and epoxides, or acrylamides and acrylates. The capability of selecting the amine and hydrophobic reactants from a large library allows one to easily diversify lipid structure and to improve gene delivery efficiency. The combinatorial strategy offers an additional advantage of studying the

chemical structure—activity relationships of lipid nanoparticles by simply tuning the chemical properties of the reactants for lipid synthesis.[20]

Inspired by above-mentioned pioneering work, we have expanded such a strategy to design synthetic lipids for gene and protein delivery, particularly by modulating the chemical structure of lipid tails.[21,22] For example, by integrating an unsaturated hydrophobic tail into the lipids (Figure 1A), we have found these lipids are very efficient for plasmid DNA and mRNA (mRNA) delivery by improving intracellular DNA and mRNA release (Figure 1B).[21] Recently, we have designed bioreducible lipids that incorporated a thiol-responsive disulfide bond into the hydrophobic tail of lipids (Figure 1C). These lipids can efficiently deliver siRNA into cells, where the intracellular glutathione (GSH) could trigger the disulfide bond degradation to facilitate endosome escape of siRNA, further enhancing the gene silencing effect efficiency.[22] Further, the treatment of MDA-MB-231 breast cancer cells with lipid/siPLK-1 complexes effectively knocked down PLK-1 and prohibited tumor cell growth (Figure 1D). More recently, we expanded the lipid library by designing chalcogen-containing lipid, which were synthesized by reacting lipophilic tails containing O, S and Se ethers (O17O, O17S, and O17Se) with aliphatic amines (Figure 1E) for genome-editing protein delivery,[23] as to be discussed later in this Account.

3. INTRACELLULAR DELIVERY OF NATIVE PROTEINS USING COMBINATORIAL LIBRARY OF SYNTHETIC LIPIDS

We demonstrated that the library of synthetic lipids described above are very efficient for intracellular protein delivery. We selected cytotoxic RNase A and saporin as model proteins to screen effective lipid nanoparticles for protein delivery from the library of lipids. RNase A is an enzyme that degrades RNA, while saporin is a ribosome inactivator to prohibit protein synthesis. Once RNase A or saporin enters into cytosol, it can lead to cell apoptosis.[24,25] In a published study,[10] we investigated the delivery of RNase A and saporin using a library of lipids synthesized through the ring-opening reaction between 1,2-expoxyhexadecane and amines (Figure 2A). We screened 14 lipids by delivering RNase A and saporin to B16F10 melanoma cells, using cell viability as an index to evaluate the protein delivery efficiency (Figure 2B). We have found that each of these 14 lipids show widely different ability to deliver protein into cells depending on the head amine structure. B16F10 cells treated with RNase A/lipids all showed high cell viability, indicating none of the lipids can deliver RNase A. Our confocal microscopic study showed that the protein/lipid nanocomplex were internalized through endocytosis pathway, not through the lipid-assisted cell membrane permeation.[10] The fact that none of the lipids/RNase A complex can induce significant cell toxicity further excludes the enhanced membrane permeation theory. In a sharp comparison, cells treated with saporin complexed with lipids (EC16-1, EC16-12, and EC16-14) showed significantly decreased cell viability, indicating an efficient saporin delivery. Both RNase A and saporin have high pI (9.6 and 9.5, respectively) and are positively charged at the experimental condition (pH = 7), while saporin has more hydrophobic residues in its native state than RNase A, indicating that not only the electrostatic interaction between lipid and protein but also their hydrophobic interaction is critical for protein delivery. This was further confirmed by changing lipid tail length and studying its protein delivery efficiency. Lipid

EC14-1, which has a shorter tail length than EC16-1 does but has the same amine head, showed a lower efficiency to deliver saporin. This finding also highlights the advantage of using a combinatorial screening strategy to study the chemical structure–activity relationship of lipid-based protein delivery. Moreover, the leading cationic lipid, EC16-1, was able to deliver cytotoxic saporin to a broad panel of cancer cells to inhibit tumor cell growth in the presence serum proteins, highlighting the stability and compatibility of the synthetic lipids as a general protein delivery platform (Figure 2C).

We further studied the potential of EC16-1 for in vivo protein delivery by formulating EC16-1 with saporin, alone with 1,2-dioleoyl-snglycero-3-phosphoethanolamine (DOPE), cholesterol, and *N*-(palmitoyl)sphingosine [succinyl{methoxy-(polyethylene glycol)2000}] (C16-mPEG-ceramide). The formulated nanoparticles are around 200 nm in size, as characterized by transmission electron microscopy (TEM) (Figure 2D). Using 4T1 breast cancer tumor model, the EC16-1/saporin nanoparticles were effectively accumulated at the tumor site after an intravenous injection (Figure 2E). A tumor growth study indicated that the EC16-1/saporin nanoparticle was much more effective at suppressing tumor growth than free saporin protein or EC16-1 lipid nanoparticle alone (Figure 2F). Moreover, mouse body weight and cytokine secretion monitoring following EC16-1/saporin nanoparticle injection revealed minimal changes during the treatment, indicating the formulation was specifically toxic to the tumor, while remaining relatively safe for the mouse. The effective in vivo protein delivery using the synthetic lipid nanoparticles highlights its great biocompatibility and clinical translation potency for developing protein therapy.

In another study, we demonstrated that EC16-1 could deliver tumor suppressor protein, phosphatase and tensin homologue (PTEN) for targeted cancer therapy. PTEN is an antagonist that attenuates phosphatidylinositol-3,4,5-triphosphate (PIP3) signaling through AKT dephosphorylation. It has been previously found that a mutation or deficiency of PTEN gene is associated with a large percentage of cancers.[26] The potential of using PTEN for cancer therapy; however, is challenged by the introduction or restoration of PTEN protein in PTEN-deficient cancer cells. We have found that lipid EC16-1 could efficiently encapsulate recombinant PTEN to form nanocomplexes (Figure 3A, C), and deliver PTEN protein to PTEN-deficient PC-3 and LNCaP prostate cancer cell lines, resulting in phosphorylation of AKT and a significant increase of cell apoptosis (Figure 3B, D). In comparison, the delivery of PTEN to the MCF-7 cells, which are not PTEN-deficient, showed a much lower effect on prohibiting cell growth, demonstrating the appealing potency of PETN delivery for targeted cancer treatment (Figure 3E).

4. CHARGE REVERSAL MODIFICATION OF PROTEIN FOR INTRACELLULAR DELIVERY

Proteins have diverse physical properties and not all proteins can effectively form complex with lipids for intracellular delivery, as we observed in the case of RNase A (Figure 2B). Hence, it is intriguing whether one can develop a universal strategy that delivers a collection of proteins regardless of their charge and hydrophobicity. In this regard, chemical modification of proteins is an appealing approach to modulate protein structure, adding non-

natural and tunable physiochemical properties, for instance, the charge density or hydrophobicity/hydrophilicity to native protein to strengthen its interactions with lipid nanoparticles, and therefore to enhance delivery efficiency.[16]

In order to enhance the electrostatic interaction between the positively charged lipid nanoparticles and protein, we modified RNase A and saporin using *cis*-aconitic anhydride,[10] which converts the positively charged *ε*-amine of protein lysine into a negatively charged carboxylate (Figure 4A). Such a charge conversion process increases the negative charge density of protein, and therefore enhances the charge–charge binding between lipids and protein. In addition, the resulting *cis*-aconitic amide is pH-responsive; it is cleaved and restored to native lysine at pH 5.5 or in the acidic endosome and subsequently released into cytoplasm with the cationic lipid assisted endosomal escape.[27] Meanwhile, because of the essential role of lysine residue on maintaining RNase A activity, the modified RNase A-Aco showed decreased ribonuclease activity, while it could be efficiently recovered under an acidic intracellular environment (Figure 4B). Using the combinatorial screening approach, we have found the leading lipid EC16-1 efficiently delivered RNase A-Aco to prohibit B16F10 cell growth (Figure 4C, D) in a much higher efficiency than that of nonmodified RNase A. Similarly, the delivery of negatively charged saporin-Aco showed enhanced cytotoxicity against B16F10 cells, confirming that the charge conversion modification of protein is general to enhance protein delivery efficiency (Figure 4D).

With the above findings, we have found that modifying RNase A with a negatively charged biopolymer, hyaluronic acid (HA), was not only effective to alter protein net charge density for enhanced delivery, but also was able to bind cell surface receptor for targeted cancer therapy (Figure 5A).[12] HA is a highly negatively charged biopolymer that specifically binds to CD44 receptor, which is overexpressed on many solid tumor cell surfaces. The conjugation of HA with RNase A imparts negative charge to RNase A with minor effect on RNase A activity, further enhancing its electrostatic binding with lipid nanoparticles. The delivery of EC16-80/RNase A-HA nanoparticles to CD44-overexpressing A549 cells efficiently prohibited cell growth, while the pretreatment of A549 cells with anti-CD44 antibody decreased the cytotoxicity of EC16-80/RNaseA-HA nanoparticles(Figure 5B), due to the decreased CD44-mediated cellular uptake of EC16-80/RNase A-HA nanoparticle(Figure 5C), highlighting the potential of HA conjugation of protein for targeted cancer therapy.

5. BORONIC ACID MODIFIED PROTEIN FOR TARGETED CANCER THERAPY AND ACTIVE TARGETING OF NUCLEUS

Inspired by the success of modifying protein with chemical tags to enhance protein encapsulation into lipid nanoparticles, and to impart the protein conjugates with expanded functionality, we recently designed the modification of RNase A with aryl boronic acid, and further studied its potential as cancer cell targeted therapeutics and nucleus targeting.[28] It was previously reported that aryl boronic acid conjugates are responsive to reactive oxygen species (ROS),[29] particularly hydrogen peroxide (H_2O_2) which is up-regulated in cancer cells. We have modified RNase A with 4-nitrophenyl 4-(4,4,5,5-tetramethyl-1,3,2-

dioxaborolan-2-yl)benzyl carbonate (NBC), which afforded aryl boronic acid conjugated RNase A-NBC via a covalent carbamate linker (Figure 6A). RNase A-NBC showed significantly reduced enzyme activity, due to the caging of essential lysine residue of RNase A. The pretreatment of RNase A-NBC with 5 mM H_2O_2, however, restored the enzyme activity of RNase A-NBC up to 95% of native RNase A (Figure 6B). In comparison, a modified RNase A-NC lacking aryl boronic acid did not show similar ROS-responsive properties, indicating the determinative role of boronic acid in enabling ROS-responsive protein conjugates. Interestingly, RNase A-NBC showed a very high selectivity toward H_2O_2 activation over other biologically relevant ROS, including *tert*-butyl hydroperoxide (TBHP), hypochlorite (ClO), and nitroxide radical (NO·) (Figure 6C).

The intracellular delivery of RNase A-NBC using EC16-80 lipid nanoparticle efficiently prohibited B16F10 cell growth, while the delivery of non-ROS responsive RNase A-NC showed lower cytotoxicity, mostly due to the inhibited activity of RNase A-NC that could not be effectively restored inside cells (Figure 6D). To confirm whether intracellular ROS plays critical roles in activating RNase A-NBC, B16F10 cells were treated with phorbol 12-myristate 13-acetate (PMA) that potentiates intracellular ROS before EC16-80/RNase A-NBC delivery. It was found that a higher cytotoxicity of RNase A-NBC with PMA pretreatment was observed than that without PMA treatment, indicating the in situ generated ROS in PMA-treated cells can activate RNase A-NBC to enhance its cytotoxicity (Figure 6E). The in situ activation of RNase A-NBC by ROS in live cells further enabled the use of RNase A-NBC for targeted cancer therapy by making use of different ROS level between cancer cells and noncancerous cells. To study this potential, three noncancerous cell lines, including NIH-3T3, Raw264.7, and NCF (neonatal rat cardiac fibroblast), along with four cancer cell lines (HeLa, B16F10, PC-3, and MDA-MB-231) were selected and treat with EC16-80/RNase A-NBC nanoparticles under the same conditions. Interestingly, EC16-80/RNase A-NBC showed comparable cellular uptake efficiency with all seven cell lines, however EC16-80/RNase A-NBC prohibited cancer cell growth much more efficiently than that of noncancerous cells (Figure 6F), highlighting the potency of using RNase A-NBC for targeted cancer treatment.

We further found that the conjugation of boronic acid to protein also enabled cell nucleus targeted protein delivery via an active transportation pathway. Intracellular targeting of proteins to specific organelles, such as nucleus and mitochondria, has the same potential as cellular targeting to improve the therapeutic index of protein. Interestingly, we have found that boronic acid could serve as a general nonpeptide nuclear localizing signal (NLS) to direct protein with different size and charge, including green fluorescent protein (GFP), RNase A, chymotrypsin, and DsRed for nuclear transportation, with the aid of a cytosol protein delivery using gold nanoparticles stabilized capsule (NPSC) (Figure 7A).[30] The aryl boronic acid conjugated GFP (GFP-BB) showed a high efficiency of nuclear accumulation after an intercellular delivery (Figure 7B). Control experiments of delivering benzyl-modified GFP or native GFP did not show any effective nucleus targeting, indicating that both boronic acid and benzyl group of NBC facilitate the nucleus targeting. A detailed mechanism study revealed that GFP-BB accumulated in the nucleus through active transportation via an importin α/β pathway, due to the binding between GFP-BB and importin. Therefore, the benzyl boronic acid conjugation expands the chemical tools that

could be used for subcellular organelle targeted delivery, decreasing the off-target effects of protein therapeutics.

6. DEVELOPING COMBINATORIAL LIPID NANOPARTICLES FOR GENOME-EDITING PROTEIN DELIVERY

Genome editing is a powerful molecular tool to precisely manipulate the genetic information on mammalian cells; it holds a great potential for developing new generation of gene therapy.[31] CRISPR/Cas9 genome editing is the most recently developed technique that uses complementary base pair binding between genome loci and a single-guide RNA (sgRNA) to direct Cas9 nuclease to create a double-strand break (DSB) at a gene locus for genome editing.[32] The application of CRISPR/Cas9 genome editing for treating diseases and genetic disorders, however, necessitates the delivery of reprogrammable Cas9 nuclease and sgRNA to cell nucleus, as neither of these molecules are naturally present in mammalian cells.[33] Direct delivery of Cas9/sgRNA remains a challenge due to the large size of Cas9 protein (~160 kDa) and the negatively charged nature of sgRNA. Inspired by our findings of using combinatorial lipid nanoparticles for protein delivery, we have recently extended the combinatorial strategy into developing lipid nanoparticles for delivery of gene editing proteins, including Cre recombinase and Cas9/sgRNA ribonucleoproteins (RNPs).

We have demonstrated that by designing the lipid to be specifically degraded inside cells, we were able to control protein release from lipid/protein nanoparticle for genome-editing protein delivery (Figure 8A). A new set of bioreducible lipids synthesized via the Michael addition between amine and acrylate bearing disulfide bond showed high efficiency to deliver genome-editing proteins (Figure 8B).[11] We screened 12 bioreducible lipids to assess their ability to deliver a modified Cre recombinase, fused with a negatively supercharged green fluorescent protein, (−27)GFP. We found that the leading lipid 8-O14B could encapsulate (−27)GFP-Cre with an efficiency higher than 90%. The 8-O14B/(−27)GFP-Cre nanocomplex can facilitate the intracellular delivery and protein release into the cytosol for nucleus targeting, which is required for effective genome editing (Figure 8C). The delivery of 25 nM (−27)GFP-Cre into HeLa-DsRed cells, which has a genetically integrated loxP-flanked STOP cassette to prohibit DsRed expression, resulted in Cre-medicated gene recombination (removal of the loxP stopper and induction of DsRed exprerssion) with efficiency up to 80% (Figure 8D).

Additionally, the library screening strategy allows us to conduct a detailed structure–activity relationship study of protein delivery and genome editing. For example, four bioreducible lipids synthesized from amine 8 and acrylates featuring different tail length showed varied protein delivery and gene recombination efficiency (Figure 9A). Lipid nanoparticles with tails containing 14-, 16-, and 18-carbon were much better for protein delivery than those with 12-carbon lipids.[11] Also, the charge density of negative supercharged GFP determined lipid/protein interaction and protein delivery efficiency. The fusion of (−20)GFP and (−7)GFP to the Cre protein decreased protein delivery efficiency when compared to (−27)GFP-Cre and (−30)GFP-Cre using the 8-O14B lipid nanoparticle (Figure 9B), while all negatively supercharged GFP fused Cre variants showed higher gene recombination

efficiency than that of native Cre protein. Moreover, the pK_a value of the head amine and the membrane disruption ability of chalcogen-containing lipid nanoparticles affected protein delivery and genome editing efficiency.[14] Lipids generated from amines with apparent pK_a > 5.1 showed a higher likelihood of displaying high protein delivery efficiency (Figure 9C). Meanwhile, a higher membrane disruption ability of lipid (OD_{405} > 0.2) is a critical factor for achieving high protein delivery efficiency (Figure 9D). Lipid nanoparticles possess both properties (i.e., pK_a > 5.1 and OD_{405} > 0.2), and the relative hit rate to mediate high protein transfection efficiency was 77% (Figure 9E). Moreover, the membrane disruption ability of lipids appears to be the more influential factor in determining in vitro (−30)GFP-Cre delivery and gene recombination efficiency (Figure 9F).

The leading bioreducible lipid 8-O14B can deliver (−27)GFP-Cre into mouse brain for effective gene recombination in vivo. The injection of (−27)GFP-Cre/8-O14B nanoparticle to different brain regions of $Rosa26^{tdTomato}$ mouse that genetically integrated loxP-flanked STOP cassette prevents the transcription of fluorescent tdTomato: dorsomedial hypothalamic nucleus (DM), dentate gyrus (DG), mediodorsal thalamic nucleus (MD), cortex, bed nucleus of the stria terminalis (BNST), ventral lateral septal nucleus (LSV), paraventricular nucleus of hypothalamus (PVN), and latera hypothalamus (LH) resulted in efficient gene recombination and tdTomato expression (Figure 10A). The tail-vein injection of chalcogen-containing lipid/(−30)GFP-Cre nanoparticles to an Ai14 mouse model bearing similar loxP-flanked STOP cassette resulted in the deletion of STOP cassette and the expression of the red fluorescent tdTomato in lung (Figure 10B),[14] highlighting the potency of using these nanoparticles for in vivo genome editing and targeted diseases treatment.

By directly delivering Cas9/sgRNA ribonucleoprotein and targeting the EGFP reporter of GFP-HEK cells, we have identified several bioreducible lipids for Cas9/sgRNA RNP delivery to knock down GFP expression with efficiency greater than 70% (Figure 11),[11] which is comparable to that of commercial transfection reagent, Lipofectamine 2000, highlighting the potential of the combinatorial strategy to develop lipid nanoparticles for CRISPR/Cas9 delivery and genome editing.

7. CONCLUSIONS AND PERSPECTIVES

In summary, we summarize here some of our strategies that integrate combinatorial lipid nanoparticles and chemical modification of proteins for enhancing protein delivery and genome editing. By designing mild and parallel synthetic chemical approaches to synthesize a library of cationic lipids, we have investigated how head amine, hydrophobic tail length and substitute number, and lipid biodegradation can be selected and controlled for advancing protein delivery. From the perspective of chemistry, the lipid nanoparticles with same amine heads and different hydrophobic tails could possess very different physicochemical properties, intracellular delivery and genome editing efficiencies, emphasizing the important role of chemistry principles played in the designing of effective and safe protein delivery vehicles. Furthermore, we have developed novel chemical modification approaches to modulate protein function, and to improve the therapeutic promise of proteins. The modification of protein with chemical tags that could be removed inside cells, particularly in response to pathological microenvironment, such as reactive oxygen species and pH,

enabled the modulation of protein activity for targeted cancer therapy. Moreover, the chemical modification of protein is an enabling technology for developing cell surface or subcellular organelle targeted protein delivery, expanding the chemical tools that we can use to design precise protein therapeutics. In terms of genome-editing protein delivery, our combinatorial strategy provides a very effective solution to the delivery of Cas9/gRNA ribonucleoprotein for precise genome editing.

For future perspectives, integrating combinatorial lipid nanoparticle and synthetic protein modification would offer advanced chemical approaches to improve protein delivery and therapy. Quantifying the amount of protein delivered into cells, such as using quantitative fluorescence imaging and electron microscopy analysis with the aid of appropriate labeling of protein with inorganic nanoparticle or fluorescence would be desirable to optimize lipid nanoparticle-based protein delivery.[34] The correlation of the lipid chemical structure and in vivo protein delivery, particularly CRISPR/Cas9 genome editing delivery, would be more relevant to the development of a new generation of protein therapy. The nonviral delivery of Cas9/gRNA RNP has been proved to be effective for genome editing in vitro, while its in vivo performance, on-target genome editing, and safety have not been carefully explored. The formulation of stable and targeted CRISPR/Cas9 nanoparticles for in vivo genome editing would be highly desired, and can be achieved by leveraging the expertise from combinatorial nanoparticle delivery of protein and gene products. In terms of developing chemical approaches for protein modification, coupling site-specific and residue-specific modification of proteins, such as expanding the genetic code to include non-natural amino acids, which may expand the types of chemistry available for protein modification, could be used to modulate protein function in situ. The combination of these chemical engineering approaches with the nanoparticle delivery system will initiate an evolutionary change of the strategy in developing precise protein therapy.

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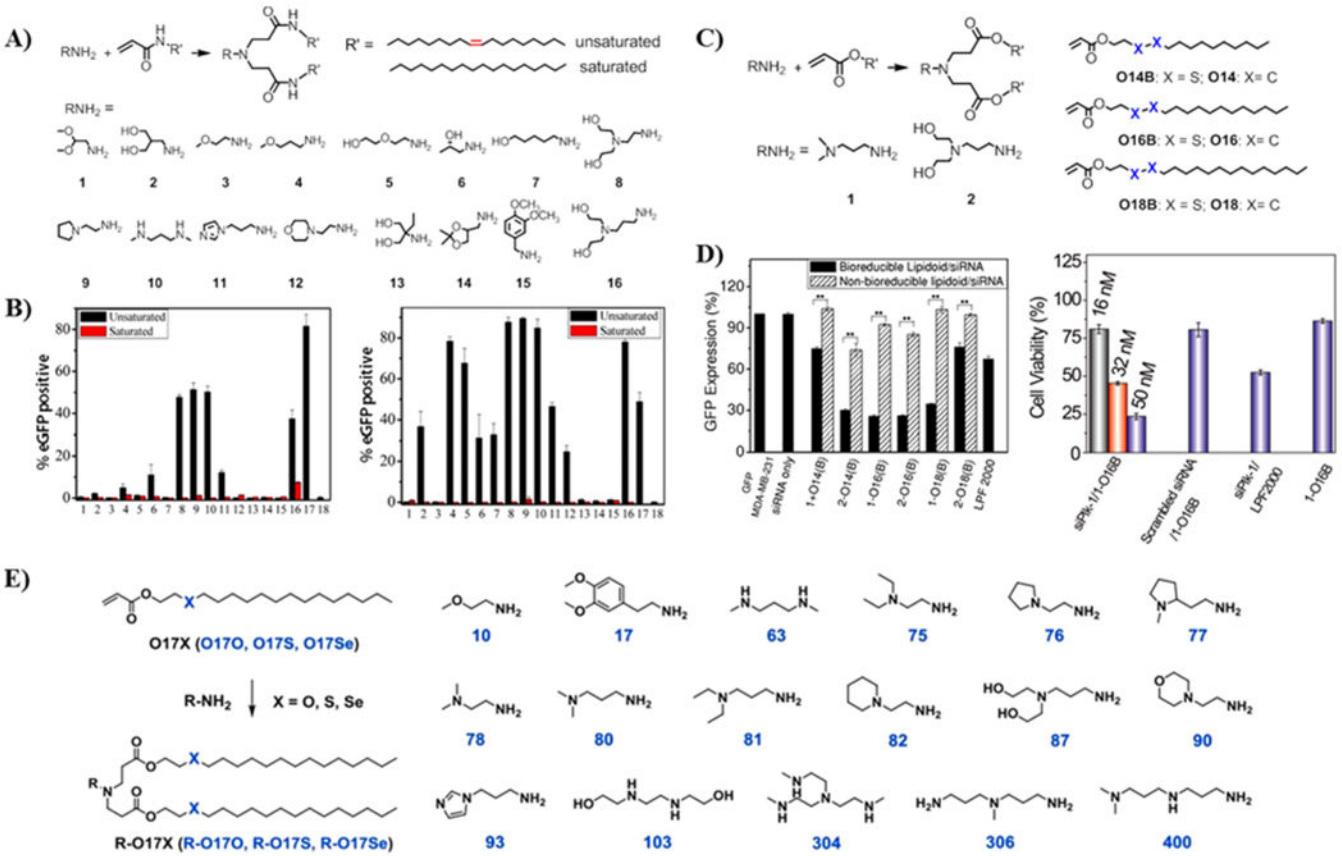

Figure 1.

(A) Synthesis of lipids bearing unsaturated tail for enhanced gene delivery. (B) eGFP-encoding DNA plasmid and mRNA (right) delivery into HeLa cells using both saturated and unsaturated lipids. (C) Synthesis of bioreducible lipids via the Michael addition between amine and acrylate. (D) GFP gene silencing (left) and PLK-1 silencing (right) of MDA-MB-231 cells treated with bioreducible siRNA lipoplexes. (E) Synthesis of chalcogen-containing lipids for protein delivery.

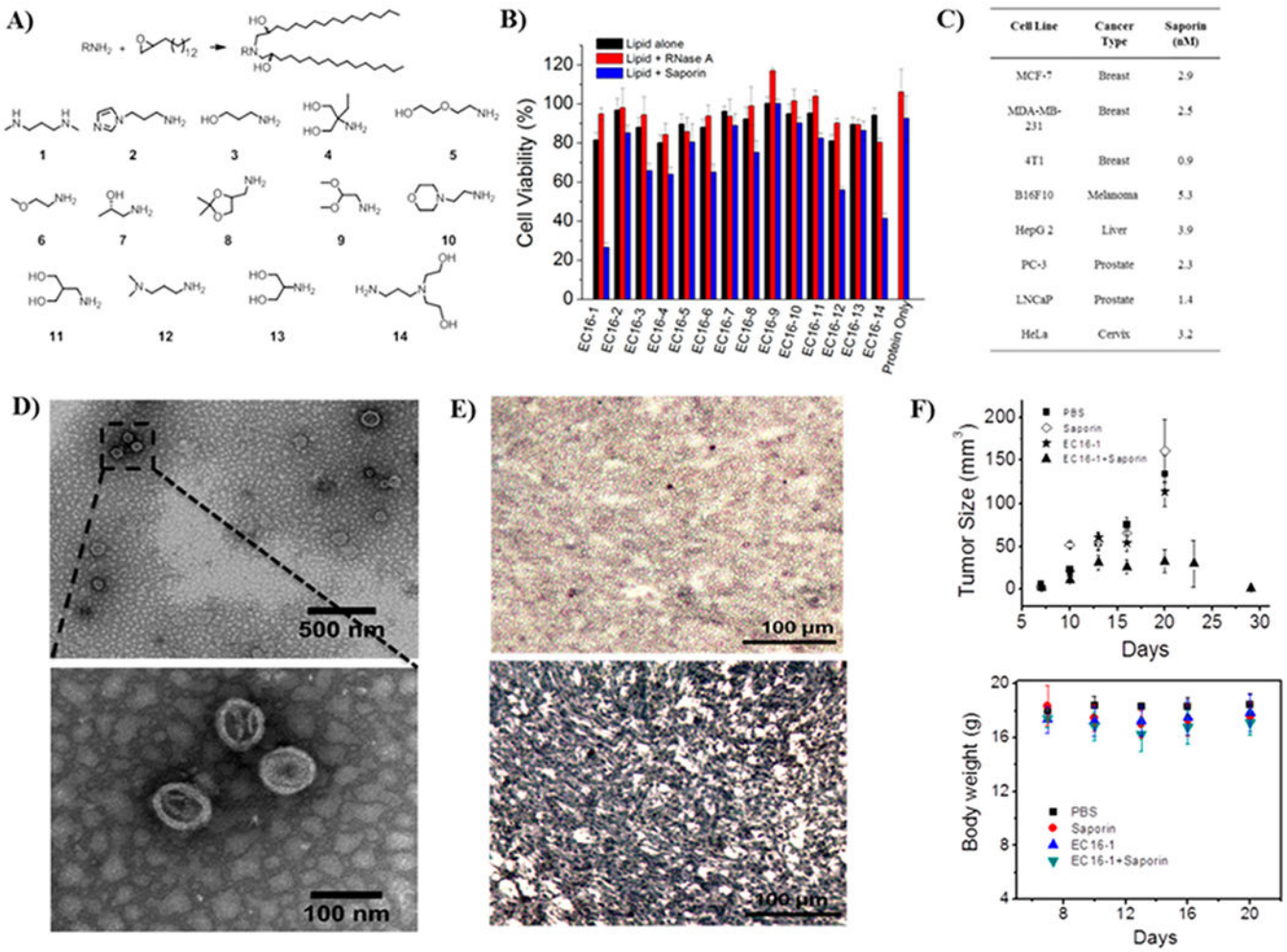

Figure 2.

(A) Synthesis of combinatorial library of lipid nanoparticle for protein delivery. (B) Cell viability of B16F10 cells treated with lipid alone, or lipid/protein nanocomplex. (C) Lipid EC16-1 can deliver saporin to a broad range of tumor cells. IC_{50} value of EC16-1/saporin nanocomplex against different cancer cells. (D) TEM image of EC16-1/saporin nanoparticle for in vivo protein delivery. (E) EC16-1/saporin nanoparticle was effectively accumulated at tumor site of 4T1 breast cancer tumor model; top: free protein treatment, bottom: EC16-1/saporin nanoparticle treatment; the dark color was generate by the immunohistochemistry staining of sapoin in tumor tissue slides. (F) Tumor growth cure (top) and mouse body weight change (bottom) of 4T1 tumor bearing mouse treated with EC16-1/saporin nanoparticle.

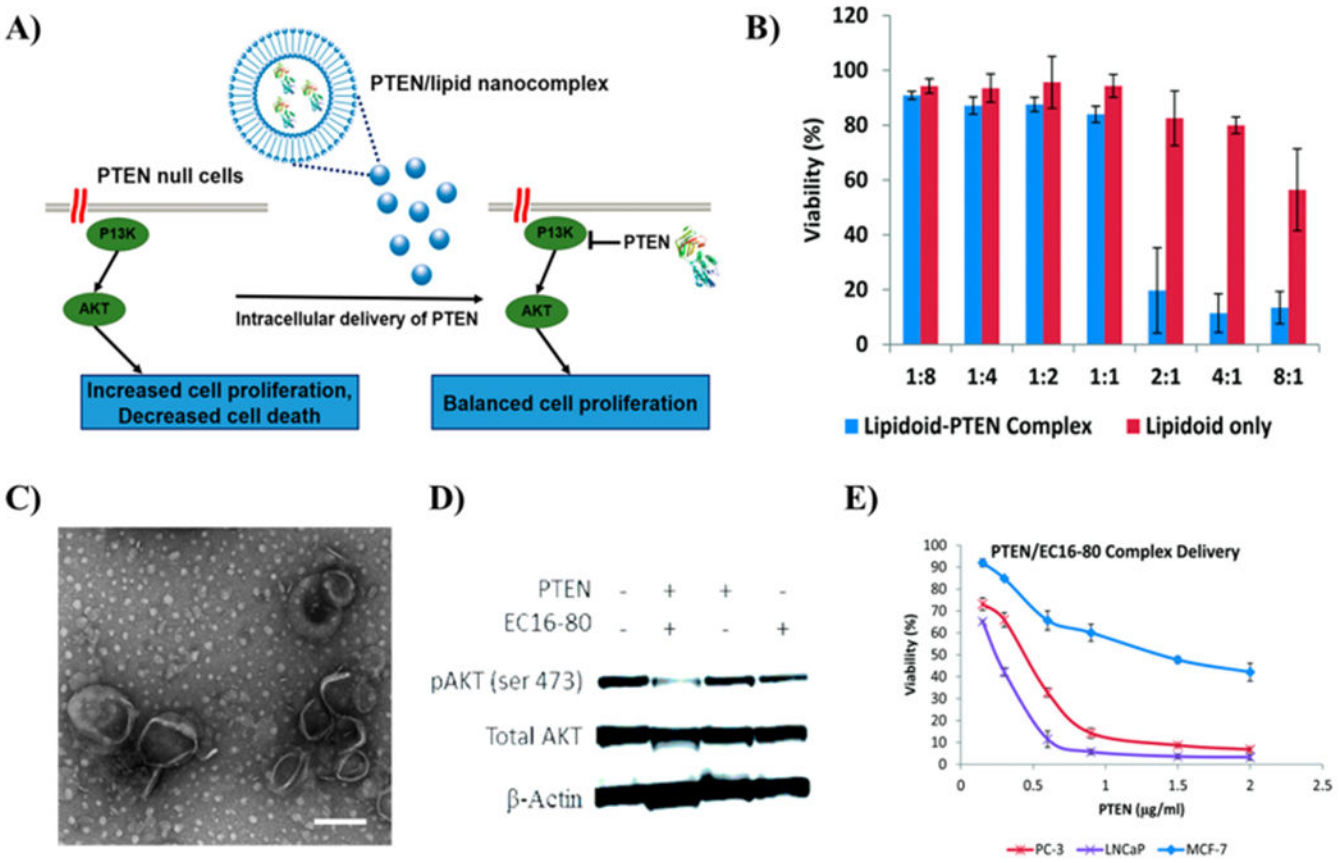

Figure 3.

(A) Intracellular delivery of PTEN to modulate AKT signaling. (B) Viability of PC-3 cells treated with different ratio of EC16-80/PTEN nanocomplex. (C) TEM image of PTEN/EC16-80 nanocomplexes. (D) Western blot detection of AKT phosphorylation of PC-3 cells with different PTEN treatments. (E) Viability of PTEN-deficient PC-3 and LNCaP cells and wild-type MCF-7 cells treated with EC16-80/PTEN nanoparticles.

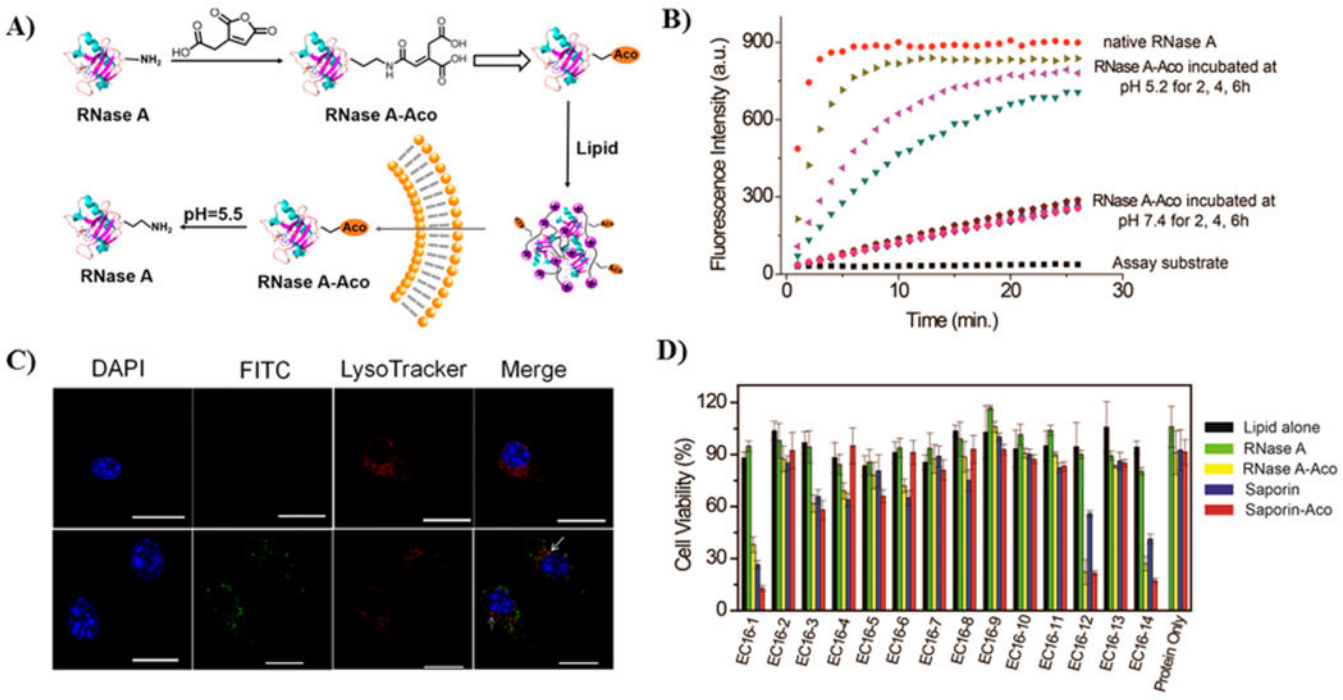

Figure 4.

(A) pH-responsive chemical modification of RNase A for enhanced intracellular delivery. (B) Ribonuclease activity assay of RNase A-Aco under neutral and acidic conditions. (C) Confocal laser scan microscopy (CLSM) images of B16F10 cells treated with fluorescently labeled RNase A-Aco (FITC-RNase A-Aco) alone (top) or complexed with lipid EC16-1 (bottom); scale bar: 20 μm. (D) Viability of B16F10 cells treated with library of lipids, or their nanocomplexes with different protein as indicated.

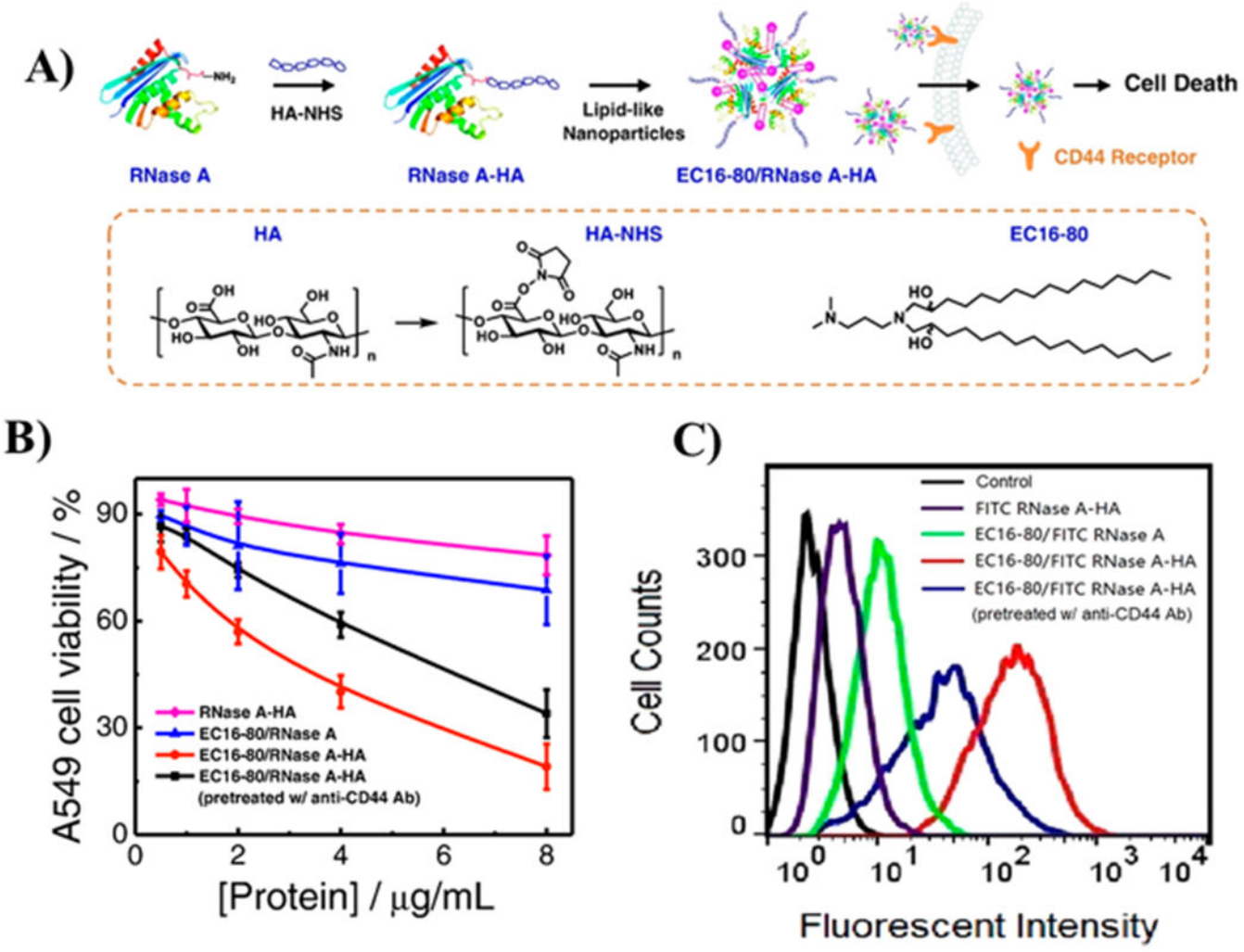

Figure 5.

(A) Hyaluronic acid modified HA-RNase A and its delivery to CD44-overexpressing cells using EC16-80 nanoparticles. (B) Cell viability of A549 cells treated with increased concentration of EC16-80/RNase A-HA nanoparticle in the presence and absence of anti-CD44 antibody. (C) Flow cytometry analysis of the cellular uptake of EC16-80/FITC-RNase A-HA nanoparticles by A549 cells with and without anti-CD44 antibody pretreatment.

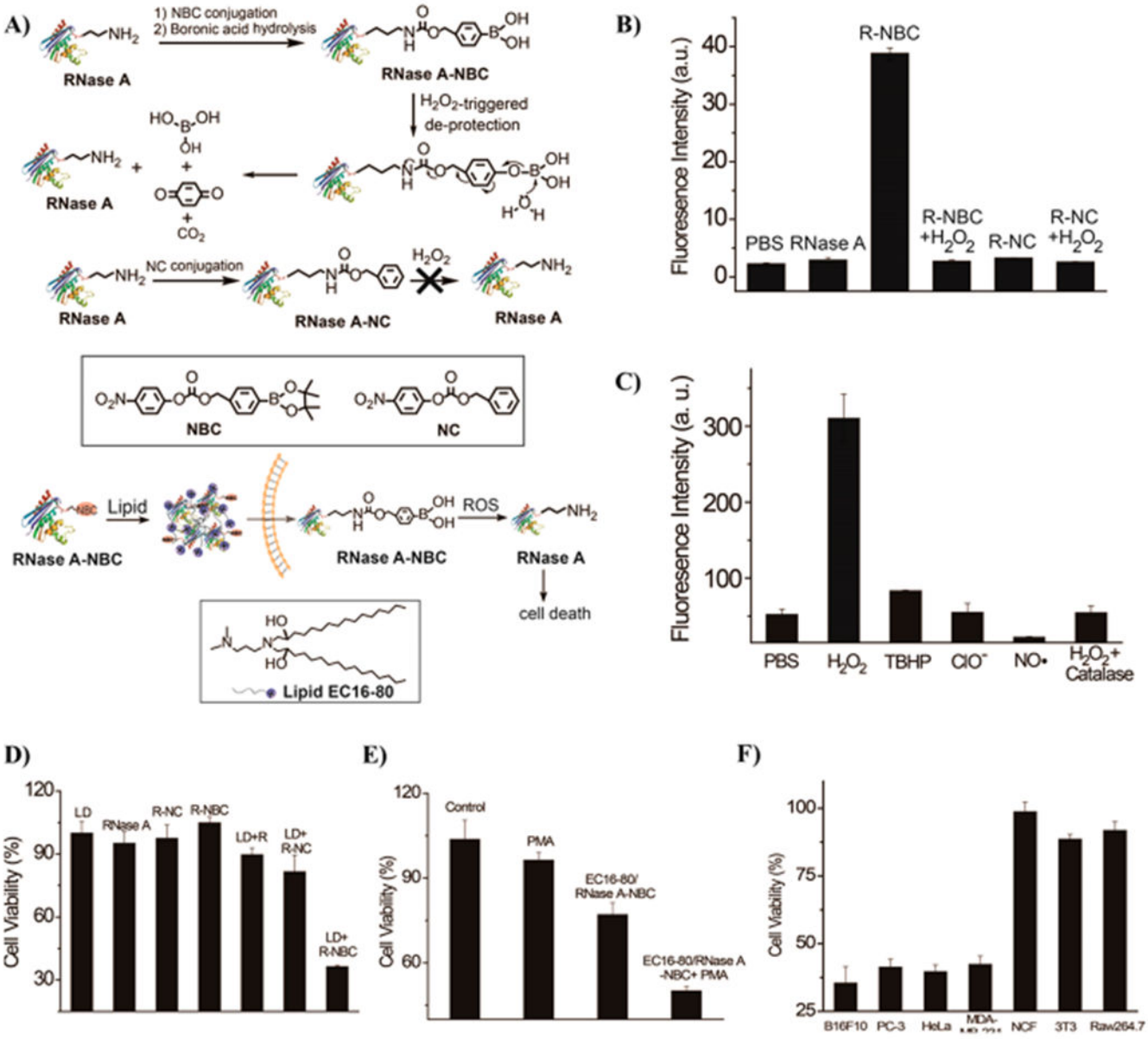

Figure 6.

(A) ROS-responsive chemical modification of RNase A and its intracellular delivery using EC16-80 lipid nanoparticles. (B) H_2O_2 treatment efficiently restored RNase A-NBC activity. (C) Selective activation of RNase A-NBC by H_2O_2 in the presence of various endogenous ROS. (D) Viability of B16F10 cells treated with different EC16-80/protein complexes as indicated. (E) PMA pretreatment enhanced the cytotoxicity of EC16-80/RNase A-NBC against B16F10 cells. (F) EC16-80/RNase A-NBC nanoparticle delivery selectively prohibited tumor cell proliferation.

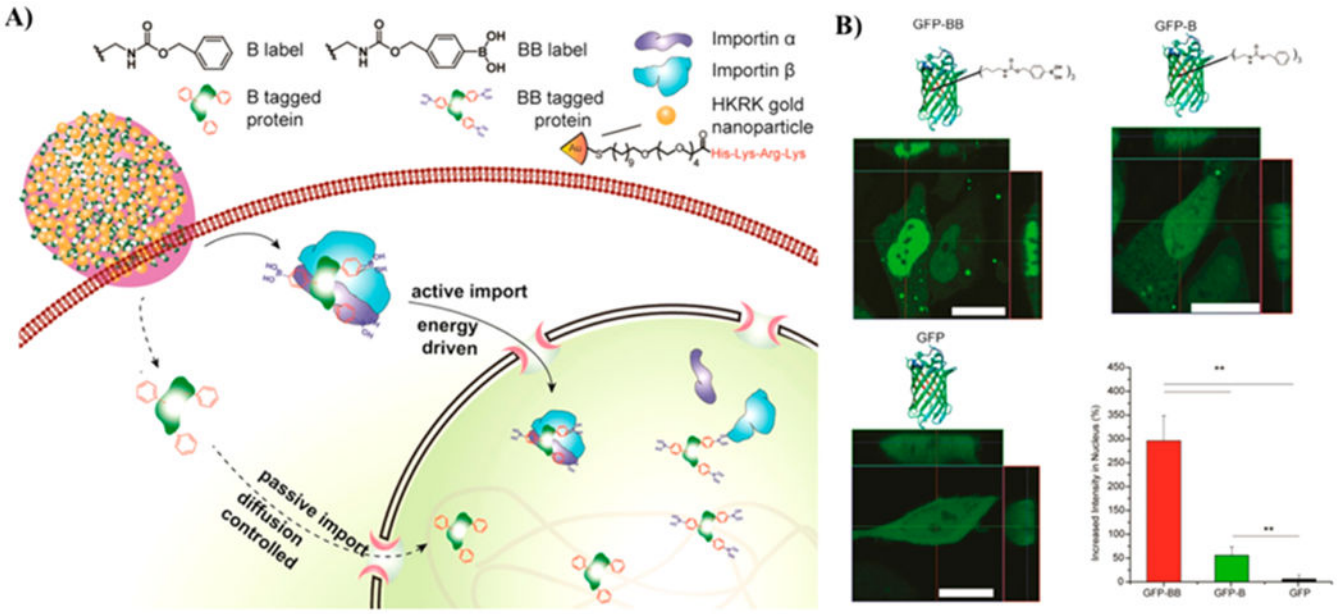

Figure 7.

(A) Active targeting of cell nucleus via conjugating benzyl boronate (BB) to proteins. (B) CLSM images of HeLa cell treated with GFP-BB, GFP-B, and native GFP complexed with NPSCs; quantitative analysis of the increased fluorescence intensity of GFP in the nucleus treated with different proteins.

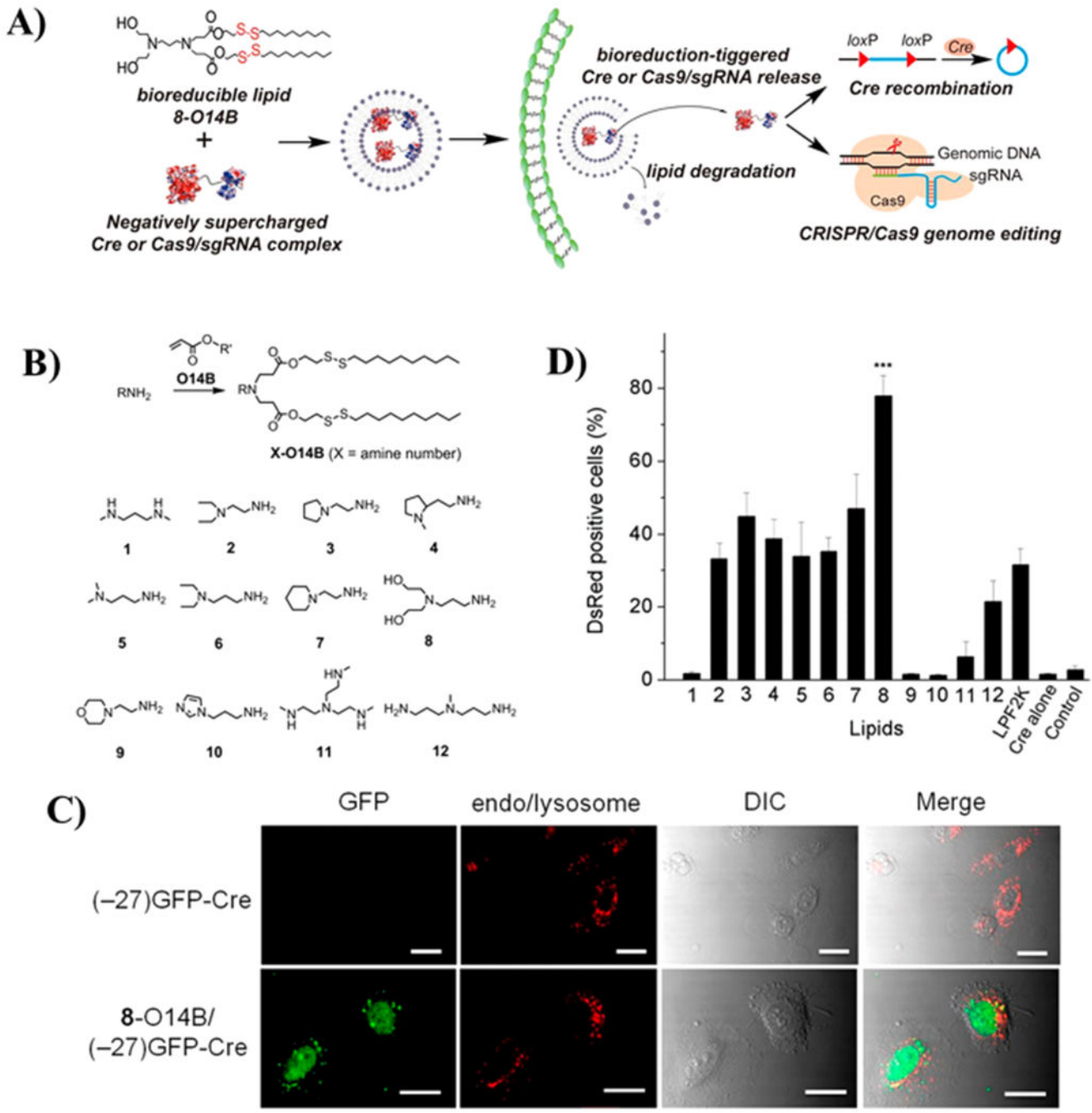

Figure 8.

(A) Designing bioreducible lipid nanoparticle for genome-editing protein delivery. (B) Synthesis route of bioreducible lipids and the chemical structures of amines used for lipid synthesis. (C) CLSM images of HeLa-DsRed cells treated with (−27)GFP-Cre alone or 8-O14B nanocomplexes. (D) DsRed expression profile of HeLa-DsRed cells treated with (−27)GFP-Cre alone and different lipid nanocomplexes.

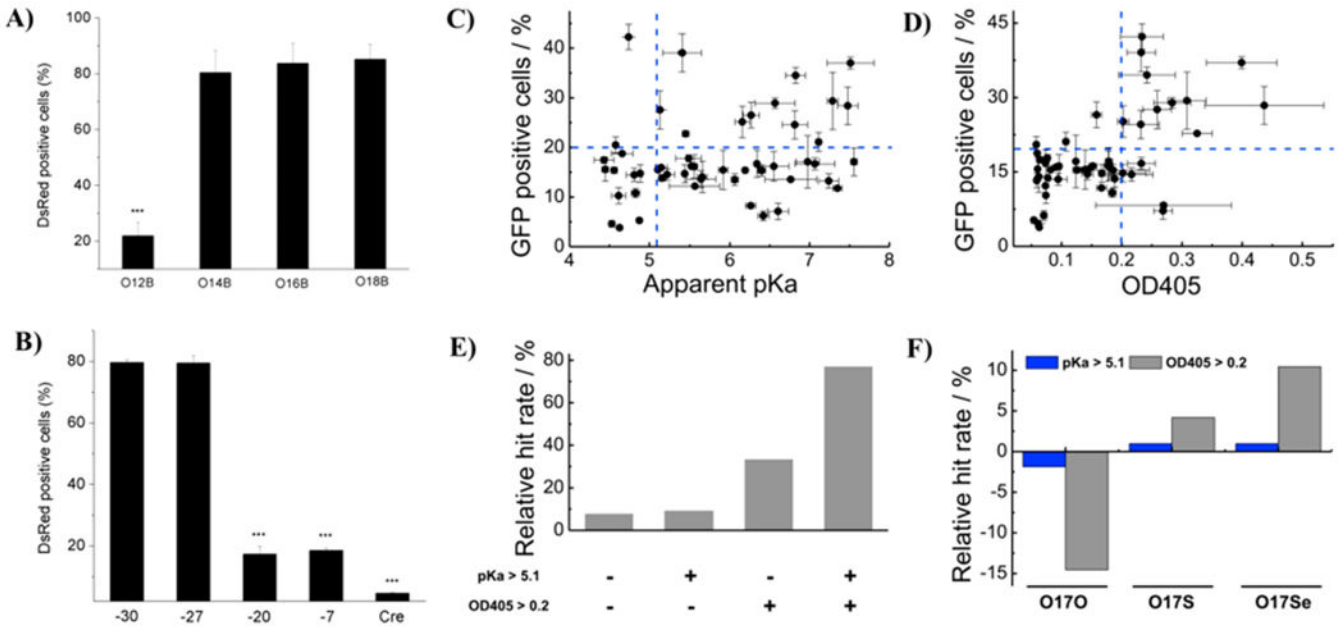

Figure 9.

(A) Tail length effect of bioreducible lipid and charge density of supercharged Cre recombinase. (B) determined gene recombination efficiency. Apparent pK_a values (C) and phospholipid bilayer membrane disruption ability (D) influenced (−30)GFP-Cre protein delivery efficiency. Relative hit rates of efficacious lipid with (E) two, one, or no properties possessed. (F) Membrane disruption ability of lipid is a more influential factor for protein delivery.

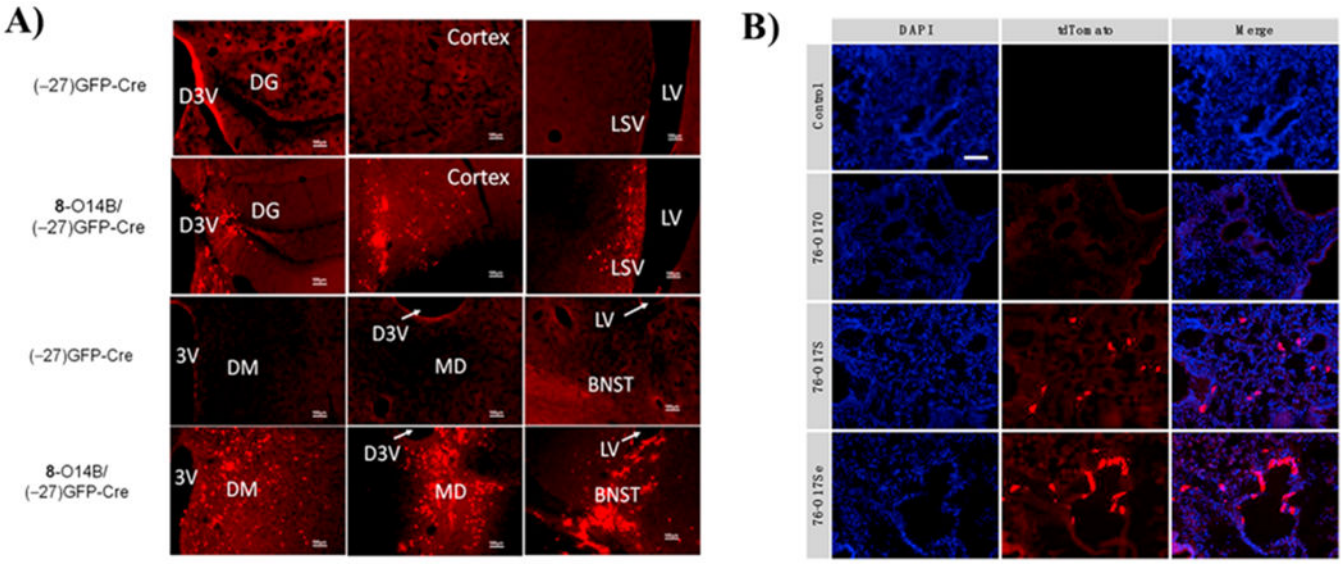

Figure 10.

(A) In vivo gene recombination in mouse brain via the injection of (−27)GFP-Cre/8-O14B nanoparticle. (B) Lung-targeted gene recombination of Ai14 mice following the tail-vein injection of chalcogen-containing lipid/(−30)GFP-Cre nanoparticles.

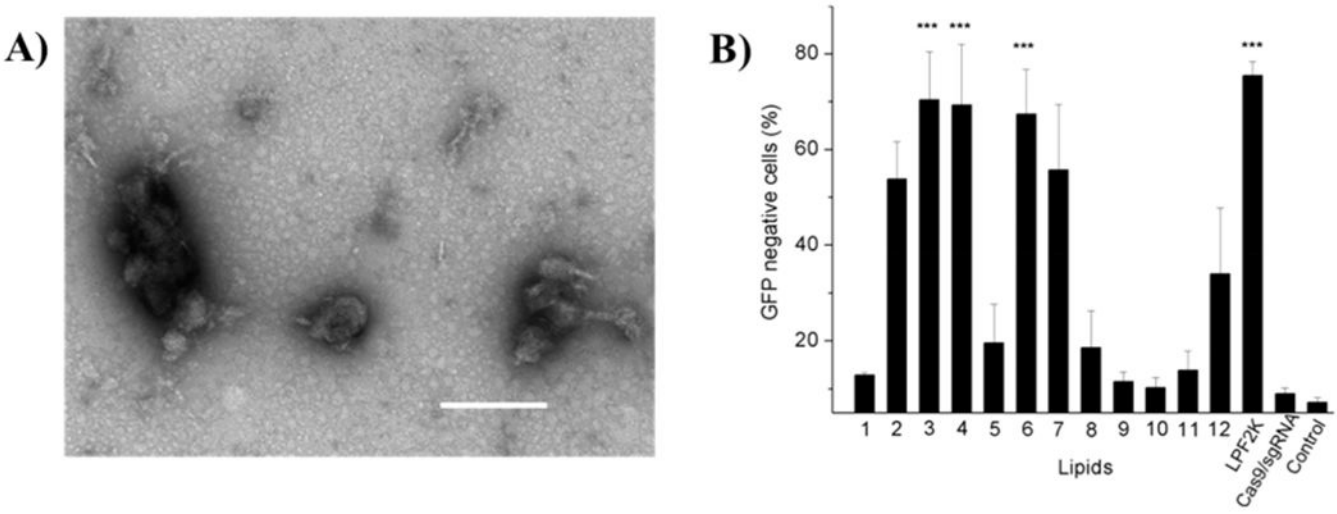

Figure 11.

(A) Nanoparticle structure of bioreducible lipid/Cas9/sgRNA complex. (B) GFP knockout of GFP-HEK cells treated with Cas9/sgRNA RNP alone or different Cas9/sgRNA and bioreducible lipd nanocomplexes.