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A novel fluorescent surfactant enhances the delivery of the Cas9 ribonucleoprotein and enables the identification of edited cells†

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Abstract

In this report, we designed and synthesized a novel fluorescent single tailed surfactant (termed FEDS), which can disrupt endosomes, complex lipofectamine, and can also identify cells that have been transfected. FEDS was able to increase the gene editing efficiency of lipofectamine/Cas9 ribonucleoprotein by 300% via a combination of fluorescent based enrichment and endosomal disruption.

Therapeutics based on proteins, RNA and DNA have tremendous therapeutic potential but have been challenging to develop due to delivery problems.^{1–3} Delivering such biomacromolecules is challenging because it requires developing multifunctional drug delivery vehicles that can perform multiple functions, in particular, biomolecule complexation, endosomal disruption, and also imaging of cells that have been transfected.^{4–6} Although a wide number of peptides, proteins and polymers have been developed that can disrupt endosomes,^{7–11} and a wide number of dyes, proteins and quantum dots have been developed that can image transfected cells,^{12–14} combining imaging agents with endosomal disruptive agents together into a single delivery vehicle remains a challenge because of their large size and molecular complexity.⁶ There is therefore a great need for the development of molecular scaffolds with simple and well defined chemical structures that can efficiently incorporate multiple functions into a single delivery vehicle.

Single tailed surfactants based on Triton-X have great potential to serve as scaffolds for developing multifunctional drug delivery vehicles. Triton-X based surfactants have a low molecular weight (<1000),¹⁵ yet have a membrane disruptive efficiency comparable to peptide based endosomal disruptive agents,¹⁶ which are 5–10 times larger in molecular weight. In addition, Triton-X based surfactants are straightforward to synthesize and non-antigenic, as they are composed of PEG chains conjugated to alkyl chains. More

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Conflicts of interest

There is no conflict of interests to declare.

importantly, Triton-X based surfactants can potentially be engineered to incorporate other functional moieties, such as a fluorophore, without losing their membrane disruptive activity. This is because Triton-X based surfactants disrupt membranes due to their cone shaped molecular structure, and will tolerate modifications as long as their cone shaped structure is maintained.¹⁷ However, the ability of Triton X based surfactants to act as multifunctional delivery scaffolds has never been investigated.

In this report, we designed and synthesized a fluorescent endosomal disruptive surfactant termed FEDS, which can disrupt endosomes, complex cationic lipid based delivery vehicles, and can also identify cells that have been transfected with cationic lipids. FEDS is composed of a methyl-amino-benzyl amide fluorophore, connected to a 10 carbon alkyl chain, and also has hydrophilic PEG and carboxyl groups at its termini (see Fig. 1a). FEDS has an amphiphilic structure similar to Triton X, and consequently should be membrane disruptive. In addition, FEDS has its hydrophobic alkyl group terminated with a hydrophilic carboxyl group, which is designed to prevent it from being membrane disruptive at pH 7.4. However, at the acidic pH of the endosome FEDS should recover its membrane disruptive ability because of protonation of its carboxyl group (see Fig. 1b). In addition, the negatively charged carboxyl group of FEDS should allow it to assemble with cationic lipid/biomolecule complexes to form FEDEX. Finally, the methyl-amino-benzyl amide fluorophore in FEDS will identify cells that have internalized FEDEX, and this can be used to enrich for cells that have been transfected. For example, in this report we used the fluorescence of FEDS to enrich for cells that were gene edited, after transfection with FEDEX (see Fig. 1b).

FEDS was synthesized according to the scheme shown in Fig. 2a and was synthesized in 6 steps. Firstly, the hydrophobic domain of FEDS was constructed by alkylating isatoic anhydride with 1-bromo-8-tritylmercaptooctane (**1**), using sodium hydride as a base. The hydrophilic portion of FEDS was formed by performing a ring-opening reaction on the anhydride ring (**2**) with amino-PEG (1000 M_w). The thiol group of FEDS was then deprotected, and the final compound was synthesized by performing a disulfide exchange reaction between compound **4** and 3-(pyridin-2-yl)disulfaneyl)propanoic acid. FEDS has an excitation maximum at 350 nm and an emission maximum at 460 nm (Fig. 2b). The quantum yield of FEDS was also measured,^{18,19} using quinine sulfate in 0.05 M H_2SO_4 as a standard, and had a quantum yield of 0.15 (Fig. S1, ESI[†]).

FEDS is designed to enhance the transfection ability of lipofectamine by disrupting endosomes. For this to be possible, FEDS has to selectively disrupt endosomal membranes over the cellular plasma membrane. To probe for this ability, a hemolysis assay was used to determine if FEDS has pH sensitive membrane disruptive properties. The hemolysis assay was performed at pH 5.5, which mimics the pH of the endosome, and at pH 7.4, which mimics the extracellular environment. FEDS was incubated with rabbit erythrocytes for 30 minutes at 37 °C, and the samples were centrifuged and the hemoglobin release, triggered by membrane disruption, was determined by measuring the absorbance at 541 nm. The result, shown in Fig. 3, illustrates that FEDS caused hemolysis of rabbit RBCs at pH 5.5 but not at pH 7.4. We performed a hemolysis study with FEDS as well as its reduced precursor **4**. The

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reduced form of FEDS should be membrane disruptive at both pH 5.5 and 7.4, because of the lack of a carboxylic acid group. Fig. 3b demonstrates that reduced FEDS is membrane disruptive at both pH 5.5 and 7.4. This suggests that the pH-dependent membrane disruptive nature of FEDS is primarily caused by its carboxylic acid group. FEDS therefore has the pH dependency needed to function as an endosomal disruptive agent.

FEDS is designed to enhance the intracellular delivery of biomolecules by disrupting endosomes. We investigated if FEDS could enhance the ability of lipofectamine to deliver Cas9 RNP by forming FEDEX. GFP-expressing RT HEK cells, under the control of a doxycycline (Dox) promoter, and Cas9 RNP designed to knock out the GFP gene *via* indel mutations were used for these experiments. Cas9 RNP was formed by first annealing crRNA with tracrRNA at a 1: 1 molar ratio at 25 μ M and was further assembled with Cas9 protein at a 1.1/1 (RNA/ protein) molar ratio on ice for 10 min. FEDEX was formed by first assembling Cas9 RNP and lipofectamine 2000 at a 5 μ L (Cas9 protein 1 μ g μ L⁻¹) to 1 μ L ratio, this complex was then mixed with a 100 μ g of FEDS in 150 μ L of Opti-MEM medium. RT HEK cells were transfected with FEDEX for 24 hours and further activated by Dox to determine the percentage of GFP negative cells, via flow cytometry. Gene editing efficiency was calculated by subtracting the percentage of GFP negative cells in the untreated cells from the percentage of GFP negative cells in the treated cells (see Fig. S3, ESI[†]). Fig. 4 demonstrates that FEDS dramatically improved the ability of lipofectamine 2000 to deliver the Cas9 RNP. RT HEK cells treated with lipofectamine 2000 were 13.2% GFP negative, whereas RT HEK cells treated with FEDEX were 24.2% GFP negative. In addition, this improvement in transfection activity was further verified *via* the T7E1 endonuclease assay (Fig. S7, ESI[†]).

We further tested if FEDEX could enhance gene editing in primary Ai9 myoblasts, which are a challenging to transfect cell preparation. Ai9 myoblasts have the tdTomato gene containing a STOP cassette integrated into their genome.²⁰ If the STOP sequence in Ai9 myoblasts is knocked out by gene editing, then Ai9 myoblasts express tdTomato, which can be detected by flow cytometry. We found that Ai9 myoblasts treated with lipofectamine 2000+ Cas9 RNP were 1.41% tdTomato positive, whereas FEDEX treated cells were 2.47% tdTomato positive, which represents a 75% increase in editing efficiency (Fig. S4, ESI[†]). These results show that FEDS can enhance the intracellular delivery of biomolecules.

FEDS has a fluorophore embedded in it, and hence can be used to identify cells that have internalized high amounts of it. The level of FEDEX internalization was hypothesized to correlate with the rate of gene editing, because cells that have internalized high amounts of FEDEX should also have internalized high amounts of Cas9 RNP. Enriching for cells that have a high rate of gene editing has numerous potential applications in biology (Fig. 5a). At present, for most cell lines, gene editing can only be determined by isolating cell clones and performing deep sequencing. This requires a significant amount of time and investment.^{21,22}

We therefore performed experiments to determine if FEDS can enrich for cells that have been gene edited. RT HEK cells were incubated with FEDEX, and the cells were then sorted by FACs to identify cells that had internalized high and low amounts of FEDEX, and their levels of gene editing were compared. As a control, cells were incubated with Cas9 RNP and

lipofectamine 2000. Overall, cells treated with Lipo2000/ RNP had a moderate GFP knockdown rate of 16.7% (Fig. 5d and Fig. S8a, ESI†) and cells with low levels of FEDEX internalized had a GFP knockdown rate of 20.6% (Fig. 5a and Fig. S8b, ESI†). In contrast, cells with high levels of FEDEX had a striking increase in gene editing, and had a GFP knockdown rate of 50.9% (Fig. 5b and Fig. S8b, ESI†). These results demonstrate that FEDEX can enrich for cells that have been gene edited. The fluorescence of FEDEX can also be visualized by fluorescence microscopy, and fluorescent microscopy can also be potentially be used to enrich for gene edited cells (see Fig. S6, ESI†).

In conclusion, we present here a new fluorescent surfactant FEDS, which enhances the ability of lipofectamine to deliver the Cas9 RNP. In addition, FEDEX is fluorescent and cells that have internalized high amount of FEDEX can be identified *via* flow cytometry, and this enables the enrichment of cells that have been gene edited. We anticipate numerous applications of FEDS, given its unique combination of fluorescence and endo-somal disruptive ability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Fluorescent endosomal disruptive complex (FEDEX) enhances the delivery of the Cas9 ribonucleoprotein in cells and enables the identification of edited cells. (a) Structure of FEDS, FEDS contains a PEG chain, a fluorophore, an aliphatic chain, and a carboxylate connected by a disulfide linker. (b) FEDS is incorporated into lipofectamine based liposomes that contain Cas9 RNP to form FEDEX. Cells that internalize FEDEX can be identified by flow cytometry due to the fluorescence of FEDS, and this enriches for cells that

have been edited. In addition, FEDS can disrupt endosomes, and this further increases the gene editing efficiency of the Cas9 RNP/lipofectamine complex.

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Fig. 2.

Synthesis of FEDS and its fluorescent properties. (a) Six step synthetic route used to generate FEDS. Briefly, isatoic anhydride was alkylated with 1-bromo-8-tritylmercaptooctane, a ring-opening reaction was performed with the anhydride ring with amino-PEG to form compound **3**. The final FEDS was synthesized by performing a disulfide exchange reaction between compound **3** and compound **4**. (b) Fluorescent spectrum of FEDS with excitation at 350 nm.

Fig. 3. Hemolysis efficiency of FEDS at pH 5.5 ($\square$) and pH 7.4. (o) at different concentrations. (a) FEDS caused strong hemolysis at pH 5.5, but no hemolysis at pH 7.4. (b) The reduced FEDS is membrane disruptive at both pH 5.5 and pH 7.4. The absorbance at 541 nm of rabbit RBCs incubated in water followed by three freeze-thaw cycles was set as the 100% hemolysis standard.

Fig. 4.

FEDS enhances the ability of lipofectamine to deliver the Cas9 RNP. Knockdown of GFP in RT-HEK cells treated with different Cas9 RNP formulations. FEDS dramatically improved the ability of lipofectamine 2000 (FEDEX vs. Lipo 2000/RNP) to deliver the Cas9 RNP.

Fig. 5.

FEDEX can enrich for cells that have been gene edited. (a) Schematic illustration of FACS sorting and enrichment of edited cells. Cells that have internalized large amounts of FEDEX can be identified by flow cytometry. These cells have a higher rate of gene editing, and FEDEX enables the enrichment of gene edited cells. (b) GFP expression of RT HEK cells sorted from FEDEX negative cells after Dox activation. (c) GFP expression of FEDEX positive RT HEK cells after Dox activation. (d) GFP expression of RT HEK cells transfected

by lipo2000/RNP after Dox activation. (e) GFP expression of RT HEK cells after Dox activation.

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