

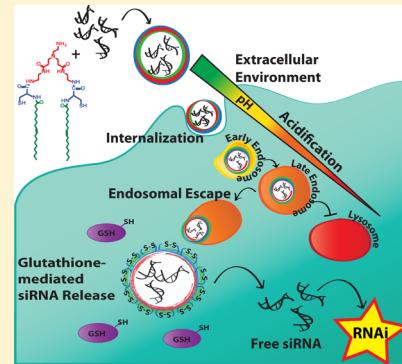
Multifunctional Cationic Lipid-Based Nanoparticles Facilitate Endosomal Escape and Reduction-Triggered Cytosolic siRNA Release

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ABSTRACT: Small interfering RNA (siRNA) has garnered much attention in recent years as a promising avenue for cancer gene therapy due to its ability to silence disease-related genes. Effective gene silencing is contingent upon the delivery of siRNA into the cytosol of target cells and requires the implementation of delivery systems possessing multiple functionalities to overcome delivery barriers. The present work explores the multifunctional properties and biological activity of a recently developed cationic lipid carrier, (1-aminoethyl)iminobis[N-(oleylcysteinyl-1-amino-ethyl)-propionamide] (ECO). The physicochemical properties and biological activity of ECO/siRNA nanoparticles were assessed over a range of N/P ratios to optimize the formulation. Potent and sustained luciferase silencing in a U87 glioblastoma cell line was observed, even in the presence of serum proteins. ECO/siRNA nanoparticles exhibited pH-dependent membrane disruption at pH levels corresponding to various stages of the intracellular trafficking pathway. It was found that disulfide linkages created during nanoparticle formation enhanced the protection of siRNA from degradation and facilitated site-specific siRNA release in the cytosol by glutathione-mediated reduction. Confocal microscopy confirmed that ECO/siRNA nanoparticles readily escaped from late endosomes prior to cytosolic release of the siRNA cargo. These results demonstrate that the rationally designed multifunctionality of ECO/siRNA nanoparticles is critical for intracellular siRNA delivery and the continuing development of safe and effective delivery systems.

KEYWORDS: *endosomal escape, gene therapy, nanoparticle, RNA interference, siRNA*



INTRODUCTION

Over the past decade, small interfering RNA (siRNA) has been explored intensely as a promising therapeutic candidate for gene therapy due to its ability to regulate gene expression through RNA interference (RNAi). RNAi is an endogenous regulatory mechanism reliant upon siRNA molecules to specifically target and regulate the expression of a gene through the posttranscriptional cleavage of the corresponding mRNA.^{1–3} As siRNA does not interact with chromosomal DNA, the possibility of adverse gene alterations commonly encountered with DNA-based gene therapies is greatly reduced. Further, through the appropriate design of siRNA, it is possible to harness RNAi to silence nearly any gene, offering a significantly broader therapeutic potential than conventional small molecule-based therapies. The application of siRNA to exploit the RNAi regulatory mechanism has revealed a host of new opportunities for the development of novel therapeutics systems.⁴ Recently, numerous cancer-associated genes have been identified, ranging from oncogenes to those genes responsible for tumor–host interactions and tumor resistance against chemo- and radiotherapy, positioning cancer as a well-suited candidate for siRNA-mediated gene therapy.⁵

While RNAi holds great potential as a promising therapeutic modality, a number of extracellular and intracellular obstacles have restrained the development and translation of RNAi-based technologies into the clinic.^{6,7} These challenges include the degradation of naked siRNA within the bloodstream by

endogenous nucleases, low cellular uptake of siRNA due to its anionic nature, immune response to naked siRNA, and rapid clearance by the RES system, which occurs within minutes of intravenous administration.⁸ Initial efforts to improve siRNA delivery relied upon viral delivery systems, as viruses have been evolutionarily programmed to efficiently deliver their genetic payload into host cells.^{9,10} Despite achieving high transfection efficiencies, viral-based delivery systems often produce adverse immunogenic effects and are associated with a high cost of production.¹¹ To overcome such challenges, various nonviral delivery vehicles, including liposomes, polycationic polymers, conjugates, and cationic lipid-based nanoparticles, have been developed.^{12–14} These formulations, while achieving varying degrees of success in terms of transfection efficiency, have encountered additional issues arising from cytotoxicity, hemotoxicity, nanoparticle aggregation in serum, and poor intracellular siRNA release from the delivery vehicles, thereby deeming them unsatisfactory for clinical use.^{15,16}

As the barriers for siRNA delivery are many, it is important for a delivery system to be multifunctional and address each of the major challenges. An ideal siRNA delivery system will incorporate the siRNA payload and protect it from degradation

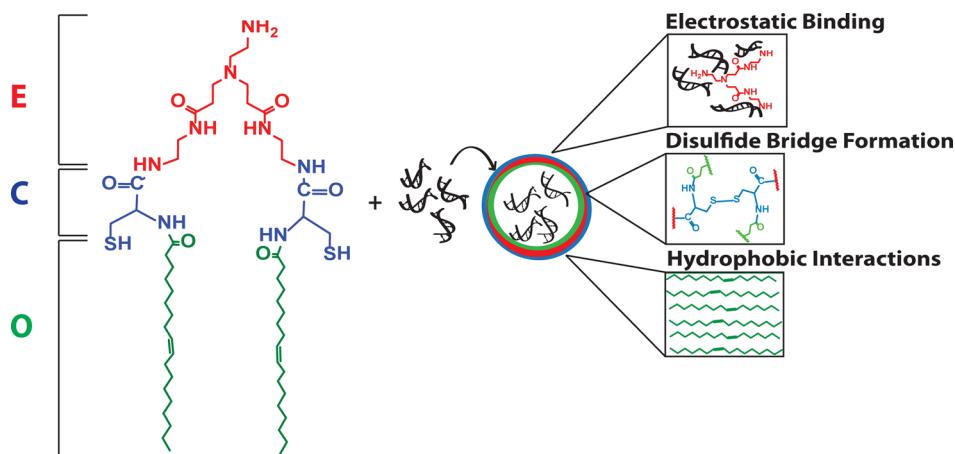
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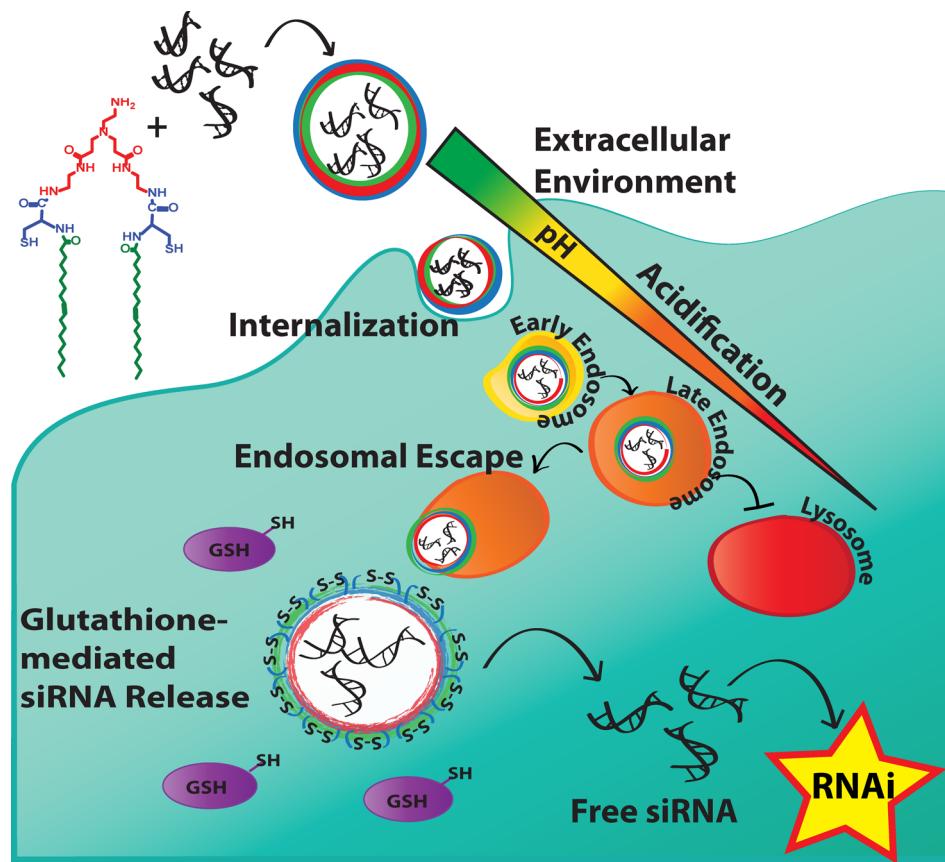
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Scheme 1. Formation of ECO/siRNA Nanoparticles via Electrostatic Interactions between the Cationic Head Group and Anionic siRNA, Autooxidation of Free Thiol Groups within the Cysteine Residues to Form Disulfide Crosslinks, and Hydrophobic Condensation of Lipid Tail Groups



Scheme 2. ECO/siRNA Nanoparticles Facilitate Cellular Internalization Resulting in Trafficking of the Nanoparticles into the Late Endosomes^a



^aWithin the late endosomes, the pH-sensitive nature of ECO promotes endosomal escape. Once released into the cytosol, endogenous glutathione (GSH) mediates reduction of disulfide bonds formed within ECO/siRNA nanoparticles to release the siRNA cargo. Upon release, free siRNA is able to initiate RNAi-induced gene silencing.

and clearance, allow for functionalization with targeting ligands to improve delivery specificity, promote cellular uptake, provide a mechanism to escape the fate of endosomal–lysosomal degradation, and ultimately facilitate the cytosolic release of the siRNA cargo into the target cells. To further improve the siRNA delivery capability with these design requirements taken into consideration, we recently developed a library of pH-

sensitive amphiphilic lipid carriers through solid-phase chemistry synthesis based on structural modifications of a previously validated multifunctional carrier.^{17–19} Each of the carrier designs was constructed to have three distinct regions of varying composition: (1) a cationic headgroup; (2) cysteine-based functionalizable linkers; and (3) a lipophilic region consisting of geminal lipid tails. We have shown that the

number of amino groups within the headgroup, the degree of unsaturation of the lipid tail groups, and the structure and composition of the linker group have a significant effect on various aspects of the delivery process, including cellular uptake and gene silencing efficiency.^{19,20} Among these carriers, ECO ((1-aminoethyl)iminobis[N-(oleylcysteinyl-1-amino-ethyl)-propionamide]) emerged as a lead multifunctional carrier for further development because of its effectiveness for mediating potent gene silencing in both cancerous and noncancerous cells.¹⁹

ECO is a cationic lipid containing three structural components hypothesized to play a significant role and function: a protonable ethylenediamine headgroup, two cysteine-based linker groups, and two oleic acid lipid tails (Scheme 1). The ethylenediamine headgroup allows for the electrostatic condensation of siRNA. The geminal oleic acid tails hydrophobically aggregate in an aqueous environment to stabilize the nanoparticles. The free thiol groups of the cysteine residues can be autoxidized into reducible disulfide linkages to further stabilize the nanoparticles. Additionally, the cysteine residues can also provide a means to functionalize the carrier with targeting moieties and/or biocompatible polymers, e.g., polyethylene glycol (PEG), to improve biocompatibility and target-specific delivery.¹⁸ Finally, the structure possesses pH-sensitive amphiphilicity, an essential ability for disrupting the membrane of endosomal and lysosomal compartments to promote escape and avoid degradation of the siRNA cargo within the acidic environment. Upon successful escape, the disulfide bonds within the nanoparticle backbone are designed to facilitate the release of siRNA in the reductive environment of the cytosol (Scheme 2).

Herein, we report a comprehensive evaluation of the multifunctional properties of ECO as a carrier for effective intracellular siRNA delivery. ECO/siRNA nanoparticles were formed and characterized over a range of N/P ratios. The physicochemical properties of the ECO/siRNA nanoparticles, including serum stability, pH-sensitivity, and bioreducibility, were determined in correlation with intracellular siRNA delivery and gene silencing efficiency. Further, the process of endosomal escape and mechanism of intracellular siRNA release following cellular uptake of ECO/siRNA nanoparticles was investigated in order to understand their gene silencing ability in U87 glioblastoma cancer cells. The response to environmental stimulus, coupled with the superior gene silencing and serum stability, is of particular interest and utility in overcoming the delivery barriers against nanoparticle-mediated gene therapy.

■ EXPERIMENTAL SECTION

Preparation of ECO/siRNA Nanoparticles. The ECO lipid carrier was synthesized as previously reported.¹⁹ ECO/siRNA nanoparticles were prepared at N/P ratios between 6 and 20. ECO and siRNA were diluted into equal volumes in nuclease-free water from stock solutions of 2.5 mM in ethanol and 18.8 μ M in nuclease-free water, respectively. The equal volumes of ECO and siRNA were mixed followed by a 30 min incubation period at room temperature under gentle agitation.

Nanoparticle Characterization. The size and zeta potential of the ECO/siRNA nanoparticles at different N/P ratios in PBS was determined by dynamic light scattering with a Brookhaven ZetaPALS Particle Size and Zeta Potential Analyzer (Brookhaven Instruments). Zeta potential measurements were repeated for nanoparticles incubated for 30 min in

serum-free and 10% and 50% serum media. To determine the pH-sensitivity of ECO, ECO/siRNA nanoparticles were formulated and incubated in PBS solutions at pH 7.4, 6.5, or 5.4 for 30 min prior to zeta potential measurement.

Entrapment Efficiency. A Ribogreen assay (Molecular Probes) was used to quantify the entrapment efficiency of siRNA within the ECO/siRNA nanoparticles.²¹ ECO/siRNA nanoparticles were prepared at various N/P ratios at a final siRNA concentration of 120 nM. Free siRNA following particle formation was detected using a SpectraMax microplate reader (Molecular Devices) with an excitation of 500 nm and emission of 525 nm. The entrapment efficiency of ECO/siRNA nanoparticles was calculated in reference to a linear standard curve by dividing the complexed siRNA concentration by the initial siRNA concentration and multiplying by 100%.

Heparin Displacement Assay. ECO/siRNA nanoparticles were prepared at an N/P ratio of 20 at a final siRNA concentration of 120 nM and incubated for 30 min at 37 °C with heparin solutions of varying concentrations based on heparin/siRNA (w/w) ratio, i.e., 0, 1, 2.5, and 5. Following the incubation period, each sample, after the addition of loading dye, was run on a 1% agarose gel containing ethidium bromide at 100 V for 25 min.

Gel Electrophoresis for siRNA Loading, Serum Protection, and Glutathione-Mediated Nanoparticle Reduction. The ability of ECO to complex and condense siRNA was assessed by gel electrophoresis. ECO/siRNA nanoparticles were prepared, and 15 μ L aliquots mixed with 3 μ L of loading dye (Promega) were loaded onto a 1% agarose gel containing ethidium bromide. The gel was submerged in 0.5X Tris/Borate/EDTA (TBE) buffer and run at 100 V for 25 min. Free siRNA was run as the control. SiRNA bands were visualized using an AlphaImager ultraviolet imaging system (Biosciences). For siRNA loading, ECO/siRNA complexes were prepared at N/P ratios between 6 and 20 and run on the gel as described above. For the assessment of glutathione-mediated nanoparticle reduction, ECO/siRNA nanoparticles were incubated with 1 h at 37 °C in the presence of 5 mM glutathione (GSH) (Sigma-Aldrich). Following incubation, samples were loaded onto a 1% agarose gel containing ethidium bromide and run in the same manner as described. Serum protection of siRNA by the complexes was assessed by incubation of ECO/siRNA complexes in 50% serum at 37 °C for 0.5, 1, 6, or 24 h. At each intermittent time point, aliquots were taken and stored at -80 °C. After the final aliquot was taken at 24 h, samples were incubated for 30 min with heparin at a heparin/siRNA (w/w) ratio of 5 to release the complexed siRNA cargo, and each sample was loaded on the 1% agarose gel and run as described above. Free siRNA was also incubated in 50% serum for 0.5, 1, 6, or 24 h and stored and run on the gel in a similar manner.

Cell Culture. Human glioblastoma U87 cells expressing a luciferase reporter enzyme (U87-Luc) were obtained from ATCC (American Type Culture Collection) and cultured in Dulbecco's modified Eagle's media (Invitrogen) and supplemented with 10% fetal bovine serum (Invitrogen), 100 μ g/mL streptomycin, and 100 units/mL penicillin (Invitrogen). The cells were maintained in a humidified incubator at 37 °C and 5% CO₂.

In Vitro Transfection Efficiency. U87-Luc cells were seeded in 24-well plates at a density of 2×10^4 cells and allowed to grow for 24 h. Transfections were carried out in serum-containing (10% or 50% FBS) and serum-free media

with 40 nM antiluciferase siRNA concentration (Dharmacon: sense sequence: 5'-CUUACCGCUGAGUACUUUCGAdTdT-3', antisense sequence: 5'-UCGAAGUACUCAGCGUAAGdTdT-3'). Following a 4 h transfection period, the media was replaced with fresh serum-containing media, and the cells continued to grow for an additional 72 h. At 72 h, the cells were rinsed twice with PBS and lysed using the reporter lysis buffered provided in the Promega Luciferase Assay kit. Following lysis, the cells were centrifuged at 10,000g for 5 min, and 20 μ L cell lysate was transferred to a 96-well plate. To quantify luciferase expression, 100 μ L Luciferase Assay Reagent was added to each well, and the luminescence was read using a SpectraMax microplate reader (Molecular Devices). Luciferase activity was normalized to the total protein content measured from the cell lysate of each well using the BCA assay (Thermo Scientific). Data was presented relative to the control, which received no siRNA treatment. Lipofectamine RNAiMAX was used as a positive control and was prepared per the manufacturer's protocol (Life Technologies).

Cytotoxicity. U87 were transfected in 10% serum media with ECO/siRNA nanoparticles at an siRNA concentration of 40 nM in a 96-well plate with a seeding density of 1×10^4 cells. After 48 h, the MTT reagent (Invitrogen) was added to the cells for 4 h followed by the addition of SDS-HCl and further incubation for 4 h. The absorbance of each well was measured at 570 nm using a SpectraMax spectrophotometer (Molecular Devices). Cellular viability was calculated as the average of the set of triplicates for each N/P ratio and was normalized relative to the no treatment control.

Flow Cytometry for Nanoparticle Cellular Uptake and Uptake Kinetics Measurements. Cellular uptake and intracellular delivery of ECO/siRNA nanoparticles was evaluated quantitatively with flow cytometry. ECO/siRNA nanoparticles were prepared with 40 nM AlexaFluor488-labeled siRNA (Qiagen). Approximately 2.5×10^4 U87 cells were seeded onto 12-well plates and grown for an additional 24 h. The cells were transfected with ECO/siRNA nanoparticles in serum-free and 10% or 50% serum media. After 4 h, the transfection media was removed, and each well was washed twice with PBS. The cells were harvested by treatment with 0.25% trypsin containing 0.26 mM EDTA (Invitrogen), collected by centrifugation at 1000 rpm for 5 min, resuspended in 500 μ L of PBS containing 5% paraformaldehyde, and finally passed through a 35 μ m cell strainer (BD Biosciences). Cellular internalization of ECO/siRNA nanoparticles was quantified by the fluorescence intensity measurement of Alexa Fluor 488 fluorescence for a total of 10,000 cells per each sample using a BD FACSCalibur flow cytometer. Each N/P ratio was conducted in triplicate, and the data presented represents the mean fluorescence intensity and standard deviation.

Nanoparticle uptake kinetics was measured in a similar setup as described above. ECO/siRNA nanoparticles were formulated with 40 nM Alexa Fluor 488-labeled siRNA at an N/P ratio of 10. U87 cells were seeded in 24-well plates at a density of 2×10^4 cells and allowed to grow for 24 h. Nanoparticles were administered in serum-free and 10% or 50% serum media. Nanoparticle uptake was measured at various time points up to 4 h post-transfection. At each time point, the cells were washed twice with PBS, trypsinized, collected, and fixed with 5% paraformaldehyde in PBS before quantification of Alexa Fluor 488 fluorescence using a BD FACSCalibur flow cytometer. The mean fluorescence of 10,000 cells was quantified for each

replica. Data presented represents the mean and standard deviation of three replicas for each time point.

Protein Adsorption. ECO/siRNA nanoparticles were formulated at an N/P ratio of 10. To quantify BSA protein adsorption, 500 μ L of nanoparticle solution and 500 μ L of BSA solution at varying concentrations were added together, stirred, and incubated for 1 h at 37 °C. Nanoparticles were prepared such that the final amine concentration for each condition was 150 μ M. Serial dilutions of a stock BSA solution (4 mg/mL) were carried out to achieve the various protein concentrations: 2, 1, 0.5, 0.25, and 0.125 mg/mL. Following incubation, the ECO/siRNA nanoparticles were centrifuged at 10,000g for 20 min. The concentration of BSA was determined from the supernatant using UV-vis spectroscopy on a SpectraMax spectrophotometer (Molecular Devices) at 280 nm. A linear calibration curve from predetermined BSA concentrations was used. Relative BSA adsorption was calculated by dividing the amount of protein adsorbed for each BSA incubation concentration by the amount of protein adsorbed for 0.125 mg/mL BSA.

pH-Dependent Membrane Disruption Hemolysis Measurement.

The hemolytic activity was measured to determine the membrane-disruptive ability of ECO/siRNA nanoparticles at pH levels corresponding to various stages of intracellular trafficking. Red blood cells (RBCs) isolated from rats (Innovative Research Inc.) were diluted 1:50 in PBS solutions at pH 7.4, 6.5, and 5.4. ECO/siRNA nanoparticles were prepared at a volume of 100 μ L and incubated with an equal volume of the various RBC solutions in a 96-well plate at 37 °C for 2 h. Nanoparticles were prepared such that the final amine concentration for each pH condition was 150 μ M. Following incubation, samples were centrifuged and the absorbance of the supernatants was determined at 540 nm. Hemolytic activity was calculated relative to the hemolytic activity of 1% Triton X-100 (Sigma-Aldrich), a nonionic surfactant. Each pH was conducted in triplicate, and the data presented represents the mean and standard deviation.

Inhibition of Glutathione-Dependent Reduction with BSO. Intracellular glutathione (GSH) was depleted in order to establish the role of cytosolic reduction of ECO/siRNA nanoparticles on gene silencing. U87 cells were plated and prepared in the same manner as during transfection studies. The cells were incubated overnight with 200 μ M bathionine-sulfoximine (BSO) obtained from Sigma-Aldrich prior to transfection, which was carried out as described earlier with an N/P ratio of 10 and an antiluciferase siRNA concentration of 40 nM. Luciferase expression was quantified with a luciferase assay and normalized with a BCA assay 48 h post-transfection as described above.

Confocal Microscopy of Cellular Uptake of ECO/siRNA Nanoparticles and Intracellular Release of siRNA. Live cell confocal microscopy was used to assess the cellular uptake and intracellular release of siRNA. Approximately 1×10^5 U87 cells were seeded onto glass-bottom microwell dishes. After 24 h, the cells were stained with 5 μ g/mL Hoechst 33342 (Invitrogen) and treated with ECO/siRNA nanoparticles in 10% serum media. Nanoparticles were formed at an N/P ratio of 10 and a 20 nM siRNA concentration with an Alexa Fluor 488-labeled siRNA. Images were taken using an Olympus FV1000 confocal microscope for up to 72 h, while the cells were housed in a humidified weather station under 5% CO₂.

Immunofluorescence of Intracellular Trafficking of ECO/siRNA Nanoparticles. Following transfection with

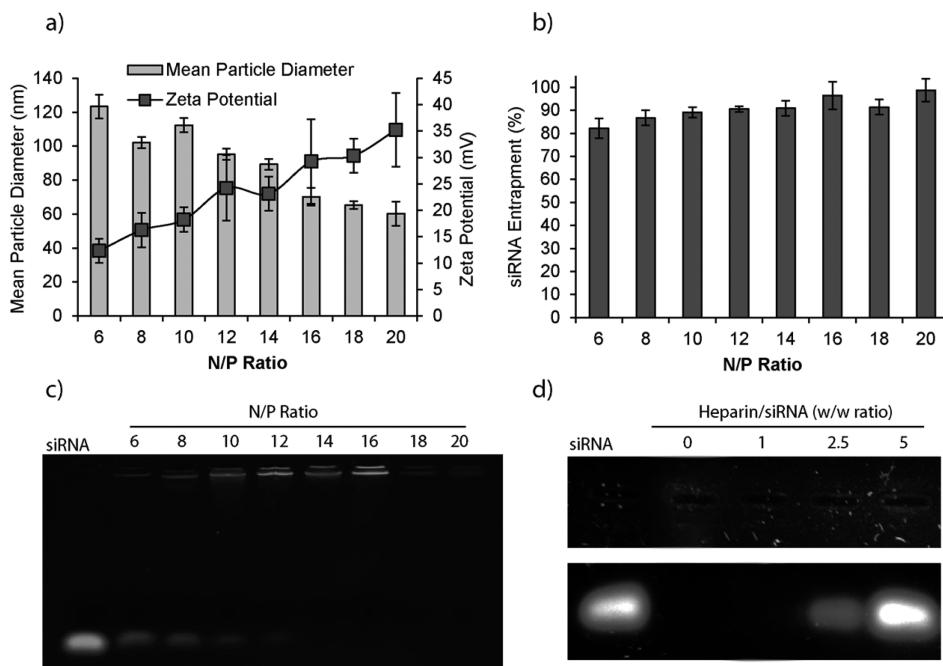


Figure 1. Physicochemical evaluation of ECO/siRNA nanoparticles. (a) Effect of N/P ratio on mean particle diameter and surface charge. (b) siRNA entrapment within nanoparticles determined by RiboGreen RNA quantitation assay over a range of N/P ratios. (c) Agarose gel retardation of ECO/siRNA nanoparticles compared to free siRNA over a range of N/P ratios. (d) Heparin displacement assay. ECO/siRNA nanoparticles were prepared at N/P ratio of 20 and incubated for 30 min at 37 °C with varying amounts of heparin, based on heparin/siRNA (w/w) ratio.

ECO/siRNA particles containing Alexa Fluor 647-labeled siRNA (Qiagen), U87 cells were fixed at various time points with 4% formaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton-X 100 (in PBS) for 5 min at room temperature. Cells were then incubated in blocking buffer (2% BSA in PBS) for 1 h. The primary antibody, rabbit antilysosomal-associated membrane protein 1 (LAMP1) (Abcam), was added at 1 µg/mL in blocking buffer and incubated at room temperature for 1 h. The secondary antibody, Alexa Fluor 488 goat antirabbit IgG (Life Technologies), was used at a 1:1000 dilution for 1 h. Samples were thoroughly washed with PBS and imaged using an Olympus FV1000 confocal microscope.

Statistical Analysis. Experiments were performed in triplicate and presented as the mean and standard deviation. Statistical analysis was conducted with ANOVA and two-tailed Student's *t* tests using a 95% confidence interval. Statistical significance was established only when *p* < 0.05.

RESULTS AND DISCUSSION

Effect of N/P Ratio on the Physicochemical Properties of ECO/siRNA Nanoparticles. The physicochemical properties of siRNA nanoparticles can have a direct impact on the efficacy of intracellular siRNA delivery and gene silencing of the delivery system.^{22–24} The understanding of these physicochemical properties, including particle size, surface zeta potential, siRNA entrapment, and particle stability, in correlation with the intracellular siRNA delivery and gene silencing efficiency is crucial for formulating a safe and effective siRNA delivery system suitable for clinical development.²⁵ The physicochemical properties can be tailored based on the ratio of cationic and anionic charge (N/P ratio) within the ECO/siRNA nanoparticles. The impact of N/P ratio on these parameters was investigated for ECO/siRNA nanoparticles between an N/P ratio of 6 and 20.

The particle size of ECO/siRNA nanoparticles decreased while their zeta potential increased as the N/P ratio increased (Figure 1a). The ability of ECO to complex and entrap siRNA increased as a function of N/P ratio, from 82.1 ± 4.3% at N/P = 6 to 98.7 ± 5.0% at N/P = 20, as demonstrated by a RiboGreen fluorescence-based assay (Figure 1b). The complexation of ECO with siRNA was further validated through an agarose gel retardation assay (Figure 1c). Compared to naked siRNA, a decrease in particle-bound siRNA migration as the N/P ratio increased was observed. At an N/P ratio ≥ 14, the complexed siRNA was completely prevented from migrating through the gel indicating that the interactions between ECO and siRNA were strong enough to resist dissociation during electrophoresis. Interestingly, at an N/P ratio ≥ 18, no siRNA signal was observed in the loading well, suggesting that the negatively charged siRNA was completely neutralized as ethidium bromide was not able to intercalate.²⁶ Some cationic polymers with high charge density, such as PEI, can form inseparable complexes with siRNA such that the siRNA cargo cannot be released once internalized into the cytosol.²⁷ Therefore, it is important that the interactions between the siRNA and carrier be stable during cellular uptake but do not impede the cytosolic release of the siRNA. To study the electrostatic interaction of the siRNA with ECO, ECO/siRNA nanoparticles were subject to heparin displacement. Heparin is an anionic polysaccharide and a major component of extracellular matrix that can compete with siRNA for binding to disrupt ECO/siRNA complex stability.²⁸ No decomplexation of siRNA from the nanoparticles occurred at heparin/siRNA (w/w) ratio of 1. Partial decomplexation of siRNA from the ECO/siRNA nanoparticles, as determined by siRNA release on an agarose gel, occurred at heparin/siRNA (w/w) ratio of 2.5, while full decomplexation was observed at a ratio of 5 (Figure 1d).

These results suggest that the N/P ratio plays an essential role in regulating size, charge, and ability of the ECO lipid carrier to complex siRNA into stable nanoparticles. While particle size decreases, increasing the N/P ratio will increase the zeta potential. Increased concentrations of amino groups enhance the ability of ECO to complex the siRNA cargo by facilitating stronger ionic interactions and compact particle formation.

Effect of N/P Ratio on the Biological Properties of ECO/siRNA Nanoparticles. The N/P ratio significantly influences the physicochemical parameters of ECO/siRNA nanoparticles, which can in turn influence the biological properties and activity of the nanoparticles. The effect of the N/P ratio on the cellular uptake, gene silencing, and cytotoxicity of the siRNA nanoparticles was investigated in vitro with U87 glioblastoma cells expressing a luciferase reporter gene (U87-Luc). Cellular uptake of ECO/siRNA nanoparticles was determined using an Alexa Fluor 488-labeled siRNA with flow cytometry in serum-free and 10% and 50% serum media (Figure 2a). Cellular uptake was found to increase in an N/P ratio-dependent manner for all transfection conditions. Under serum-free conditions, Lipofectamine RNAiMAX (Lipofect.) mediated higher cellular uptake than ECO for all N/P ratios. However, for 10% and 50% serum conditions, ECO/siRNA nanoparticles at an N/P of 20 had enhanced cellular uptake compared to Lipofectamine. A significant reduction in cellular uptake was observed in 10% and 50% serum media for N/P ratios ≤ 12 when compared to serum-free media ($p < 0.05$). At N/P ≥ 14 , cellular uptake in all three transfection conditions was not significantly different. As shown in the above study, high N/P ratios resulted in an increase in both surface zeta potential and stability of the nanoparticles (Figure 1). It is thought that the positive surface charge facilitated stronger interaction with the cells and, consequently, higher cellular uptake at these N/P ratios. Improved nanoparticle stability might also be responsible for competent cellular uptake at high N/P ratios in serum media.

The gene silencing efficiency of ECO/siRNA nanoparticles was determined in U87-Luc cells using an antiluciferase siRNA at 72 h post-transfection in serum-free and 10% and 50% serum transfection conditions. At a 40 nM siRNA concentration, gene silencing was dependent upon the N/P ratio, although this trend was more evident in the presence of serum (Figure 2b). High gene silencing efficiency was observed for the nanoparticles throughout the N/P ratio range in serum-free media: luciferase expression was inhibited to $7.2 \pm 3.4\%$ for N/P = 6 and $3.7 \pm 3.3\%$ for N/P = 20 at 72 h post-transfection. In the presence of 10% serum, luciferase silencing increased in an N/P dependent manner from $38.41 \pm 8.19\%$ luciferase expression for N/P = 6 to $1.91 \pm 0.97\%$ luciferase expression for N/P = 20 at 72 h. Similarly for 50% serum, luciferase silencing was less efficient for N/P ratios between 6 and 12 but was comparable to serum-free and 10% serum for N/P > 12 . At N/P ≥ 10 , ECO/siRNA nanoparticles matched or exceeded the performance of Lipofectamine RNAiMAX in their respective transfection conditions. It is interesting to note that in serum-free media ECO/siRNA nanoparticles were equally as efficient at silencing luciferase for N/P = 6 as they were for N/P = 20 despite a 4-fold difference in cellular uptake. One possible explanation may be that the RNAi machinery becomes saturated beyond a certain intracellular siRNA concentration.^{29,30} Alternatively, it has been suggested that the efficiency of siRNA delivery via lipid nanoparticles is limited by endocytic

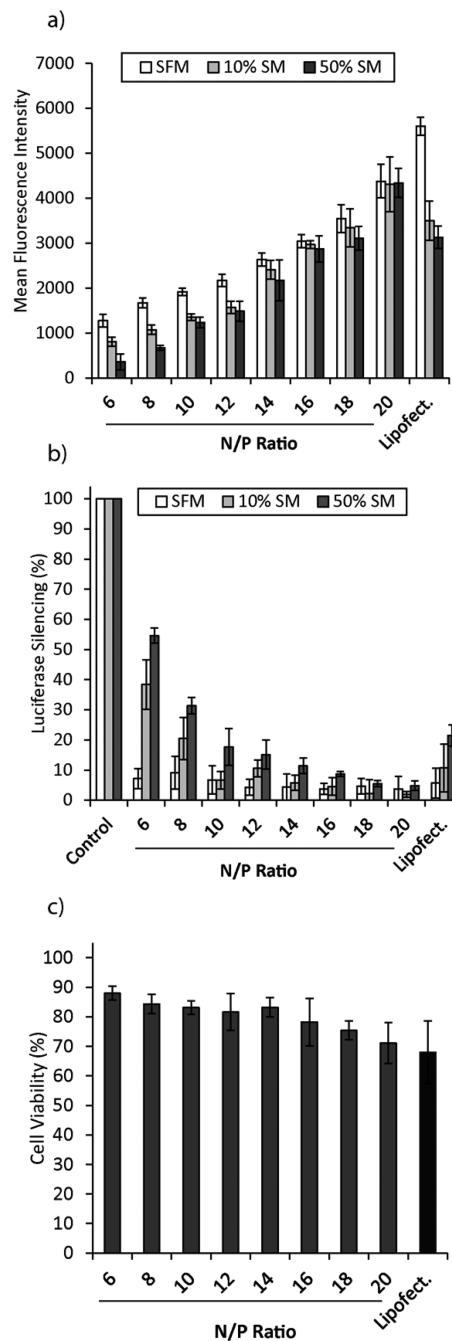


Figure 2. Biological activity of ECO/siRNA nanoparticles in U87 Glioblastoma cells. (a) Cellular uptake quantified by flow cytometry for ECO/siRNA nanoparticles containing an Alexa Fluor 488-labeled siRNA in serum-free media (SFM), 10% serum media (10% SM), and 50% serum media (50% SM). Cellular uptake was found to be significantly higher in SFM for N/P ratios ≤ 12 ($p < 0.05$). (b) Luciferase silencing efficiency of ECO/siRNA nanoparticles after 72 h in serum-free media and 10% and 50% serum media at 40 nM siRNA compared to Lipofectamine RNAiMAX (Lipofect.). Quantified using a luciferase assay and normalized with a BCA assay. (c) Cell viability assessed with an MTT assay in 10% serum media for ECO/siRNA nanoparticles.

recycling, in which the siRNA nanoparticles within the endocytic vesicles are expelled from the cytosol back into the extracellular environment.³¹ For transfection conditions containing serum, gene silencing efficiency correlated with cellular

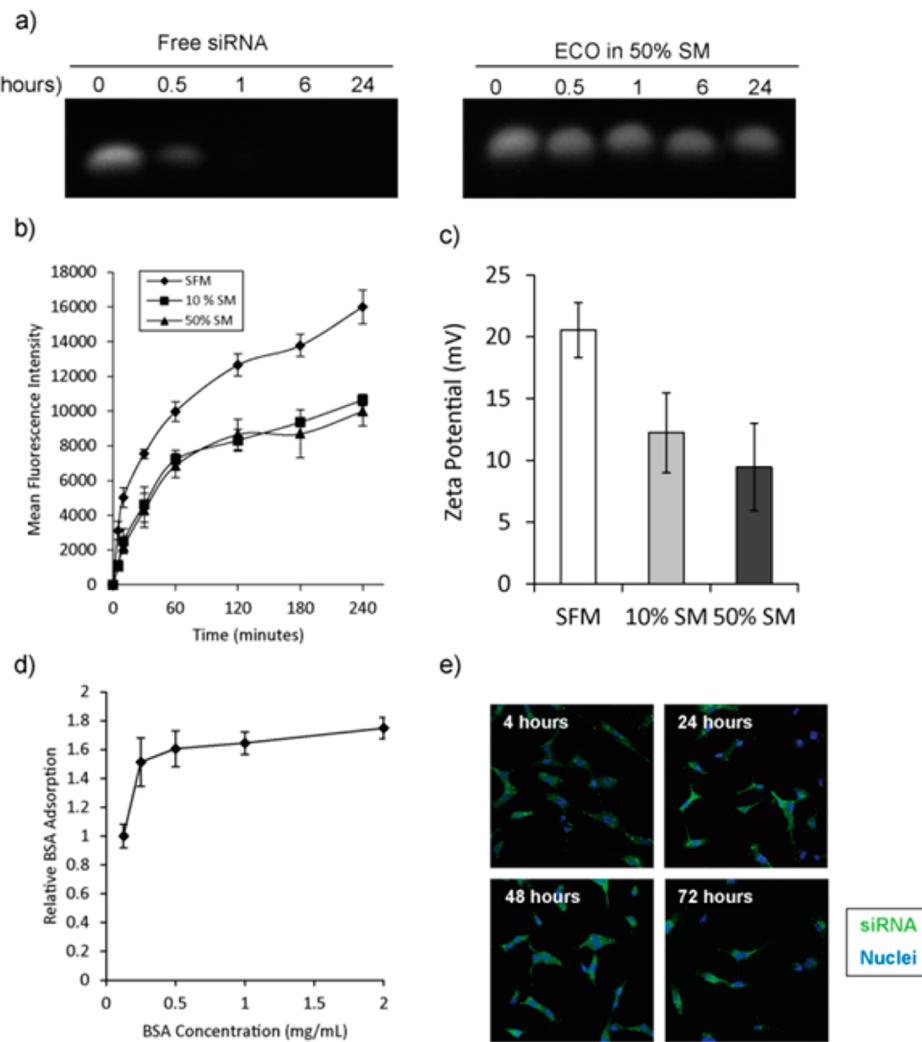


Figure 3. (a) Susceptibility to serum-degradation of free or complexed siRNA within ECO nanoparticles. Samples were incubated in 50% serum for 0.5, 1, 6, and 24 h. Glutathione (5 mM) was used to release complexed siRNA from ECO, and the integrity of siRNA cargo was evaluated with an agarose gel electrophoresis assay. (b) Kinetics of nanoparticle uptake with Alexa Fluor 488-labeled ECO/siRNA nanoparticles in U87 cells in serum-free media (SFM) and 10% and 50% serum media (10% SM and 50% SM). Levels of cellular uptake of nanoparticles in SFM were found to be significantly higher than those in 10% and 50% SM for all time points ($p < 0.05$). (c) ECO/siRNA nanoparticles were formulated at an N/P of 10 and the zeta potential was evaluated following incubation in serum-free media, 10% serum media, or 50% serum media. Zeta potential of nanoparticles was found to be significantly diminished by the presence of serum ($p < 0.05$). (d) Relative adsorption of bovine serum albumin (BSA) to the ECO/siRNA nanoparticles after 1 h incubation at 37 °C as a function of BSA incubation concentration. (e) Live-cell confocal imaging of cellular uptake of ECO/siRNA nanoparticles in U87 cells and cytosolic distribution of Alexa Fluor 488-labeled siRNA in 10% serum media. A dispersed siRNA-based fluorescent signal is present 4 h post-transfection and remains upward of 72 h.

uptake due to significantly lower cellular uptake at low N/P ratios compared to the serum-free transfection condition.

While a higher N/P ratio led to improved cellular uptake and gene silencing, unwanted cytotoxic effects may arise as a result and should therefore be monitored closely to ensure complete safety of a delivery system. The cytotoxicity of the ECO carrier was evaluated using an MTT assay (Figure 2c). Cell viability was evaluated 48 h post-transfection and was found to gradually decrease as the N/P ratio increased. Cell viability was especially compromised at N/P > 14, which may be a result of increased positive charge densities at high N/P ratios.³² Importantly, the overall viability of those cells treated with ECO/siRNA nanoparticles at all N/P ratios remained higher than those treated with Lipofectamine RNAiMAX.

Increased cellular uptake may be a direct consequence of the zeta potential promoting interactions with the negatively

charged cell membrane at high N/P ratios. However, increased zeta potential will negatively influence the biocompatibility of the delivery system. Additionally, low N/P ratios are not as efficient at inducing gene silencing in the presence of serum, which may be in part contributed to reduced cellular uptake when compared to higher N/P ratios and also to their lower siRNA entrapment efficiency and lower stabilities. In an effort to optimize transfection conditions to maximize gene silencing while minimizing cytotoxic effects in U87 glioblastoma cells, an N/P ratio of 10 appeared to be the best formulation of the ECO/siRNA nanoparticles and was chosen for further studies. At an N/P of 10, ECO/siRNA nanoparticles averaged 112 nm in diameter, had a zeta potential of +18.2 mV, and silenced luciferase to 6.6% at 72 h in 10% serum media while maintaining good cell viability.

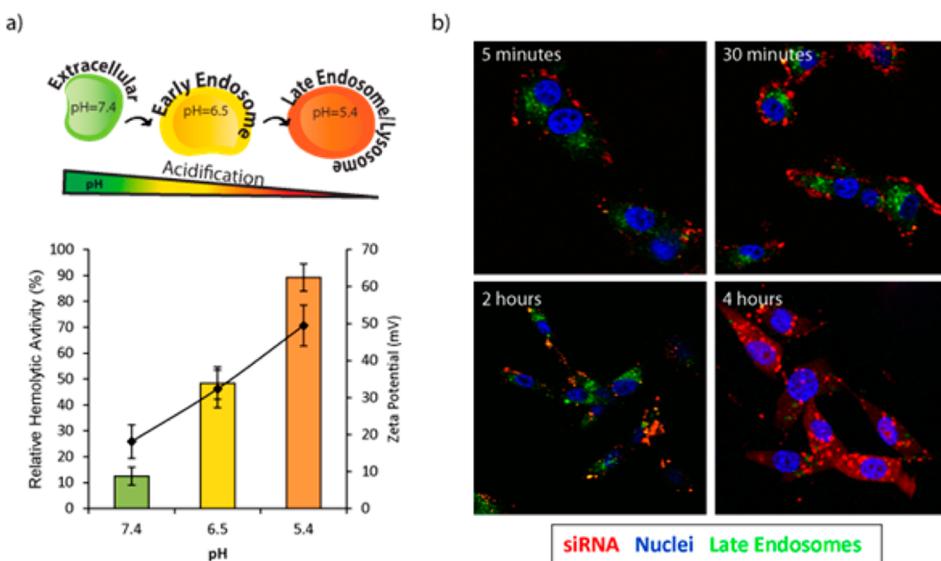


Figure 4. (a) Zeta potential measurements following incubation in PBS at various pH levels demonstrate the pH-sensitivity of the ECO/siRNA nanoparticle. The zeta potential was found to increase with increasing acidity. Hemolytic assay determined the pH-dependent membrane-disruptive ability of ECO/siRNA nanoparticles increased significantly ($p < 0.05$) with increasing acidity (pH = 7.4, 6.5, and 5.4). Relative hemolytic activity calculated with respect to the hemolytic activity of 1% Triton-X-100. (b) Immunofluorescence using a LAMP1-antibody (Alexa Fluor 488-labeled secondary antibody) to stain for late endosomes reveals colocalization of ECO/siRNA (Alexa Fluor 647-labeled siRNA) nanoparticles occurs 2 h post-transfection. At 4 h, a dispersed siRNA signal is present within the cytosol indicating that ECO/siRNA nanoparticles are able to escape from late endosomes and release the siRNA cargo.

ECO/siRNA Nanoparticles Protect siRNA and Promote Cellular Uptake in the Presence of Serum Proteins. Serum proteins may lead to dissociation of the siRNA nanoparticles and premature release and degradation of siRNA.³³ To address the question of nanoparticle stability and siRNA protection from nuclease degradation, free siRNA and ECO/siRNA nanoparticles (N/P = 10) were incubated in PBS containing 50% fetal bovine serum at 37 °C for up to 24 h. The agarose gel chromatogram of the siRNA in both formulations at various time points of the incubation revealed that free siRNA was prone to degradation within the first 30 min, and completely degraded by 6 h, while siRNA within the ECO/siRNA nanoparticles was preserved for at least 24 h (Figure 3a). The result suggests that ECO is able to sufficiently complex and pack siRNA into stable nanoparticles such to protect the siRNA from enzymatic degradation in serum. Nonspecific interaction of serum proteins with the ECO/siRNA nanoparticles may also hinder membrane adsorption, block cellular entry, and diminish the transfection efficiency, as has been demonstrated previously with lipid-based nanoparticles.^{34,35} The kinetics of cellular uptake of nanoparticles complexed with an Alexa Fluor 488-labeled siRNA was monitored over the course of 4 h in serum-free and 10% and 50% serum media. While the nanoparticle uptake is clearly higher in serum-free media than in 10% and 50% serum media ($p < 0.05$), the cellular uptake under all transfection conditions exhibits a similar biphasic trend (Figure 3b). This biphasic behavior has been speculated to originate from an initial period where nanoparticles adhere to the outer cell membrane before undergoing cellular entry and internalization.³⁶ Initial membrane adhesion is associated with slow nanoparticle internalization until a steady state is achieved with a balanced rate of nanoparticle membrane adhesion and internalization.³⁷ The lower siRNA-associated fluorescence signal observed in serum media was not due to siRNA degradation as the ECO nanoparticles were effective in protecting the cargo siRNA

(Figure 3a). The difference in cellular uptake may then be in part due to the nonspecific interaction of serum proteins with nanoparticles and the consequent reduction of zeta potential, diminishing the ability of the nanoparticles to adhere to the outer cellular membrane and to undergo cellular internalization. This was confirmed (Figure 3c) by the observation that ECO/siRNA nanoparticles had a reduced zeta potential in 10% and 50% serum media compared to serum-free media ($p < 0.05$). Cellular uptake and zeta potential was not found to be significantly different between 10% and 50% serum transfection conditions suggesting that serum protein binding to the ECO nanoparticles may reach a point of saturation. To determine this, the binding of bovine serum albumin (BSA), the major protein constituent of fetal bovine serum, to ECO/siRNA nanoparticles was quantified following incubation over a range of BSA concentrations (Figure 3d). Indeed a saturation point of BSA adsorption was observed for BSA concentrations ≥ 0.25 mg/mL.

The cellular uptake of ECO/siRNA nanoparticles in the presence of serum was further investigated with confocal microscopy using an Alexa Fluor 488-labeled siRNA (Figure 3e). Intracellular internalization and dispersed cytosolic siRNA distribution was observed as early as 4 h post-transfection. In accordance with the observed sustained luciferase silencing (Figure 2b), the dispersed signal intensity increased over time and persisted at least 72 h post-transfection. From these images, it is clear that even with a reduced zeta potential ECO/siRNA nanoparticles were effectively taken up by the cells in serum and that siRNA was released into the cytosol.

ECO/siRNA Nanoparticles Are pH-Sensitive and Promote Endosomal Escape. Following successful internalization, one of the most crucial events for effective intracellular siRNA delivery is the escape from the endosomal–lysosomal pathway.³⁸ It is imperative for the carrier to promote the escape from such pathways for the purpose that siRNA must be available within the cytosol to initiate RNAi. If the siRNA

nanoparticles remain in these transport vesicles, they will be at risk to lysosomal degradation.³⁹ It has been proposed that the multifunctional nanoparticles are able to escape endosomal–lysosomal pathways with their ability to disrupt the membrane of the acidic endosomes and lysosomes in a pH-sensitive manner.^{19,40} To validate this hypothesis, the zeta potential and membrane disruption ability of ECO/siRNA nanoparticles at pH levels of the extracellular (7.4) and endosomal and lysosomal environments (6.5 and 5.4) was studied. As the pH level decreased and became more acidic, amine groups within the cationic headgroup of ECO become protonated, and consequently, the zeta potential increased from 18.1 mV at pH = 7.4 to 32.4 mV at pH = 6.5 and 49.5 mV at pH = 5.4 (Figure 4a). The relative hemolytic activity of ECO/siRNA nanoparticles in rat blood cells (RBCs), normalized to the hemolytic activity of 1% Triton-X-100, a known hemolytic agent, was found to increase with acidity in a similar manner through which maturing endocytic vesicles are acidified (Figure 4a). Hemolysis of $48.5 \pm 6.2\%$ at pH of 6.5 and $89.2 \pm 5.4\%$ at pH of 5.4 demonstrated the ability of these nanoparticles to interact with the membrane of late endosomes and/or lysosomes in response to the pH changes. The low hemolytic activity of ECO/siRNA nanoparticles at pH of 7.4 ($12.5 \pm 3.5\%$) is consistent with the observation that ECO/siRNA nanoparticles elicit a low cytotoxic effect on cells, as minimal membrane disruption was observed.

Intracellular trafficking of ECO/siRNA nanoparticles was further determined using fluorescence confocal microscopy based on the localization of an Alexa Fluor 647-labeled siRNA with respect to a specific marker for late endosomes and lysosomes (anti-LAMP1). As shown, the ECO/siRNA nanoparticles began interacting with the cell membrane with no visible colocalization with LAMP1-stained vesicles within the first 5 min of transfection (Figure 4b). At 30 min, areas of colocalization of nanoparticles and late endosomes arose, and colocalization increased at 2 h, where the majority of the siRNA-based fluorescent signal is coalescent with the vesicles that are characteristic for late endosomes. By 4 h, a dispersed siRNA signal distribution appeared and the colocalization of the siRNA with LAMP1 was diminished. This data suggests that the ECO/siRNA nanoparticles were trafficked through the endosomal–lysosomal pathway to the late endosomes, whereupon they were able to escape from the vesicles to release their cargo in the cytosol. Although the exact pathways responsible for internalization and trafficking of the nanoparticles have yet to be explored and defined, it has recently been suggested that most nanosized particles are trafficked to the lysosomes regardless of their method of endocytosis.⁴¹ The result here suggests that, irrespective of the endocytic pathway, the multifunctional carrier ECO can promote effective early escape in the endosomal–lysosomal pathway, a key feature responsible for its success in inducing gene silencing.

Cytosolic Reduction of ECO/siRNA Nanoparticles Is Crucial for siRNA Release and RNAi Activity. Once escaped from the endosomal–lysosomal pathway, the final step of the multistage process of intracellular siRNA delivery is to ensure the cytosolic release of the siRNA cargo whereupon it will be available to bind to the RNA induced silencing complex (RISC) and initiate RNAi. During nanoparticle formation, the ECO/siRNA nanoparticles are stabilized by disulfide bonds. The cleavage of these linkages within the reductive cytosolic environment, via disulfide–thiol exchange initiated by endogenous glutathione, can facilitate the release of the

complexed siRNA.^{42,43} This bioreducible functionality of ECO was demonstrated by incubating nanoparticles at the physiological concentration of glutathione (5 mM) for 1 h at 37 °C. Agarose gel electrophoresis was used to evaluate whether the siRNA cargo could be released in the presence of the reducing agent. In the absence of glutathione, ECO successfully complexed siRNA into nanoparticles, while in the presence of glutathione the siRNA cargo disassociated from the nanoparticles, indicating that disulfide reduction by glutathione was sufficient to unpack the ECO/siRNA nanoparticles (Figure 5a).

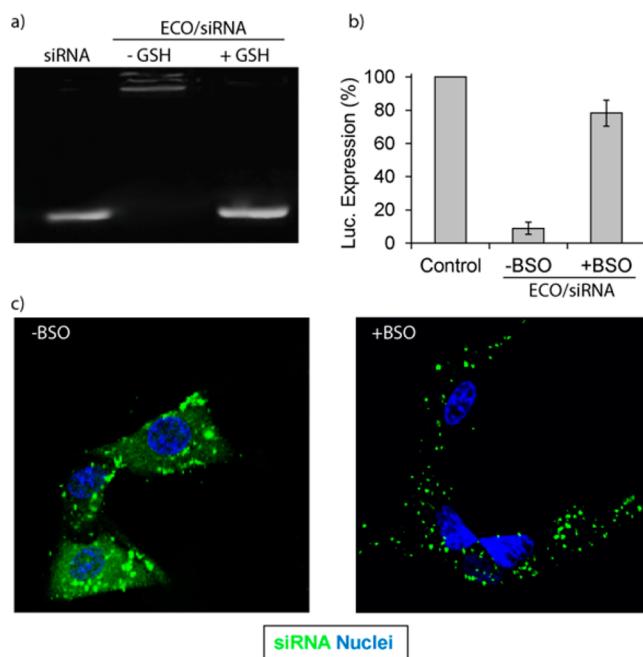


Figure 5. (a) Sensitivity of ECO/siRNA nanoparticles to reduction by endogenous levels of glutathione (GSH). Nanoparticles were incubated in the presence of 5 mM GSH for 1 h at 37 °C. Release of complexed siRNA was evaluated with an agarose gel electrophoresis assay. (b) The ability of ECO/siRNA nanoparticles to induce luciferase silencing is inhibited by the pretreatment of U87 cells with BSO for 24 h prior to transfection ($p < 0.05$). (c) Confocal imaging of cytosolic distribution of Alexa Fluor 488-labeled siRNA in U87 cells 4 h post-transfection. Compared to no treatment (left), pretreatment with BSO for 24 h (right) reduced cytosolic distribution of siRNA through inhibition of glutathione-mediated nanoparticle reduction.

To further demonstrate the significance of glutathione-dependent reduction of the nanoparticles for cytosolic release of siRNA and RNAi activity, U87-Luc cells were treated with buthionine sulfoximine (BSO) prior to transfection with ECO/siRNA nanoparticles. BSO was implemented to deplete intracellular glutathione by inhibiting γ -glutamylcysteine synthetase, the enzyme required to initiate glutathione synthesis.^{44,45} The ability of ECO/siRNA nanoparticles to silence luciferase expression was significantly inhibited by the BSO treatment (Figure 5b). Confocal microscopy further revealed that pretreatment of U87-Luc cells with BSO prevented the dispersed cytosolic distribution of siRNA 4 h post-transfection (Figure 5c). Unlike the dispersed siRNA-associated fluorescence observed in the cytosol of untreated cells (left), the fluorescence signal of the labeled siRNA in BSO-treated cells remained punctate, indicative of intact nanoparticles (right). The result demonstrates that the intracellular reduction of the

nanoparticle, the final step in the arduous intracellular delivery process of siRNA, plays a vital role and is a requisite for achieving effective intracellular siRNA delivery and high gene silencing efficiency of ECO/siRNA nanoparticles. The inclusion of the cysteine residues within the structure of ECO is a key feature to stabilize the siRNA nanoparticles and for cytosol-specific controlled siRNA release.

CONCLUSIONS

We have developed a cationic lipid-based ECO/siRNA nanoparticle delivery system that demonstrates the multifunctionality critical for efficient intracellular siRNA delivery and RNAi activity. The N/P ratio had a significant impact on the size, zeta potential, and siRNA complexation of the ECO/siRNA nanoparticles. Higher N/P ratio produced more compacted nanoparticles with improved siRNA entrapment and increased zeta potential within the N/P ratio range of 6–20. In serum-free media, ECO/siRNA nanoparticles mediated high luciferase silencing in U87-Luc, while the silencing efficiency decreased at N/P < 10 under serum media transfection conditions. For the U87 cell line, an N/P ratio of 10 was the optimal formulation for balancing robust gene silencing with minimal adverse cytotoxic effects. ECO/siRNA nanoparticles were effective to protect siRNA from degradation in serum, able to escape from the late endosomes via their pH-sensitive ability to induce membrane disruption at endosomal pH levels, and released the siRNA payload in the reductive cytosolic environment through cleavage of disulfide bonds within the nanoparticle. These functionalities of the ECO/siRNA nanoparticles are critical for their capability to mediate efficient intracellular siRNA delivery and effective gene silencing. The multifunctional ECO/siRNA nanoparticles provide a promising platform delivery system for efficient delivery of therapeutic siRNA in vitro and certainly warrant further development and evaluation of their potential for in vivo delivery.

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Notes

The authors declare no competing financial interest.

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