Published in final edited form as:

Adv Healthc Mater. 2016 November ; 5(22): 2882–2895. doi:10.1002/adhm.201600677.

Targeted dual pH-sensitive lipid siRNA self-assembly nanoparticles facilitate in vivo cytosolic siRNA delivery in tumor and overcome drug resistance by silencing eIF4E in breast cancer therapy

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Abstract

Down-regulation of oncogenes associated with multidrug resistance with RNAi has the potential to enhance the efficacy of cancer chemotherapy. Here, we have designed and developed targeted dual pH-sensitive lipid siRNA self-assembly nanoparticles RGD-PEG(HZ)-ECO/siRNA to enhance cytosolic siRNA delivery via systemic administration, to regulate oncogene expression, and to improve the efficacy of cancer chemotherapy. The dual pH-sensitive function of RGD-PEG(HZ)-ECO/siRNA nanoparticles facilitates effective cytosolic siRNA delivery in cancer cells both in vitro and in vivo after systemic injection. Intravenous injection of RGD-PEG(HZ)-ECO/siRNA nanoparticles (1.0 mg-siRNA/kg) results in effective gene silencing for at least a week in MDA-MB-231 tumor. Intravenous injections of RGD-PEG(HZ)-ECO/siRNA specific to eukaryotic translation initiation factor 4E (eIF4E) every 6 days for 6 weeks down-regulate the overexpression eIF4E protein in tumor and resensitize a drug-resistant MDA-MB-231 breast cancer model to paclitaxel, resulting in significant tumor regression at a low dose, with negligible side effects. RGD-PEG(HZ)-ECO/siRNA nanoparticles also result in minimal immunogenicity after repeated injections in immunocompetent mice. Silencing eIF4E with the targeted dual pH-sensitive multifunctional lipid siRNA nanoparticles has the potential to re-sensitize drug-resistant breast cancer to chemotherapy.

Graphical Abstract

Targeted dual pH-sensitive RGD-PEG(HZ)-ECO/siRNA nanoparticles facilitate effective cytosolic siRNA delivery in cancer cells and result in effective gene silencing for at least a week in MDA-MB-231 tumor after systemic injection. Intravenous injections of RGD-PEG(HZ)-ECO/siRNA nanoparticles specific to eIF4E resensitize a drug-resistant MDA-MB-231 breast cancer to paclitaxel, resulting in significant tumor regression, with negligible side effects.

Keywords

Dual pH-sensitive; ECO; cytosolic siRNA delivery; eIF4E; multidrug resistance

1. Introduction

Multidrug resistance (MDR) renders chemotherapeutics ineffective and poses a major challenge in cancer treatment. RNAi is a potent approach that can down-regulate the expression of oncogenes associated with MDR and may also circumvent the problem of acquired MDR encountered by chemotherapeutics. However, clinical application of RNAi is limited by the lack of safe and effective delivery of therapeutic siRNA.^[1] We have developed a multifunctional pH-sensitive lipid, ECO, for the cytosolic delivery of therapeutic siRNA to regulate cancer-related genes for anti-cancer therapy.^[2] ECO exhibits multifunctionalities necessary for the cytosolic delivery of siRNA, including convenient nanoparticle self-assembly with siRNA, pH-sensitive amphiphilic endosomal escape, and glutathione-mediated siRNA release in the cytoplasm.^[3] Moreover, the ECO/siRNA nanoparticles can be readily modified with maleimide-functionalized polyethylene glycol (PEG) with a target agent to improve their biocompatibility and to achieve targeted siRNA delivery.^[4]

Eukaryotic translation initiation factor 4E (eIF4E), the 5'-cap mRNA-binding protein involved in the complex and multistep process of mRNA translation, is known to specifically regulate multiple mRNA targets involved in cellular growth, proliferation, differentiation and apoptosis.^[5] Increasing evidence has shown that elevated expression of eIF4E favors the selective translation of a wide array of mRNAs implicated in tumorigenesis, including c-Myc, cyclin D1, survivin, Bcl-2, Bcl-xL, VEGF, and MMP-9,^[5d, 6] and may be associated with the development of resistance to chemotherapeutic agents.^[7] Breast cancer patients with elevated eIF4E expression have been found to exhibit worse prognosis than those with low eIF4E expression in the tumors.^[8] Therefore, eIF4E could be a potential therapeutic target to alleviate MDR for effective chemotherapy. Although the inhibition of eIF4E with chemotherapeutic agents, e.g. ribavirin, has shown promising results in treating malignant

tumors,^[9] clinical studies demonstrate that therapeutic efficacy is compromised by acquired resistance to the drug.^[10] Recent studies have revealed that significant decrease in eIF4E levels in normal organs does not affect normal mammalian development, global protein synthesis, and organ functions,^[11] suggesting that RNAi of eIF4E could be a safe therapeutic strategy for use in cancer.

Here, we report the development of peptide-targeted and dual pH-sensitive ECO/siRNA self-assembly nanoparticles for specific cytosolic siRNA delivery in cancer cells via systemic targeted delivery and explore the potential of the nanoparticles for alleviating MDR in chemotherapy by silencing eIF4E in a mouse triple negative breast cancer (TNBC) model. An acid-labile hydrazone (HZ) bond was incorporated within the peptide-targeted PEG spacer to overcome the steric effect of PEG on endosomal escape of the core multifunctional ECO/siRNA nanoparticles.^[12] The PEG layer is able to shed from the targeted nanoparticles in response to the acidic endosomal environment, following receptor-mediated endocytosis in tumor cells. Once the core nanoparticles are exposed, the intrinsic pH-sensitive amphiphilicity of ECO enables endosomal membrane destabilization, facilitating endosomal escape and cytosolic siRNA release to achieve potent gene silencing (Fig. 1). Cytoplasmic siRNA delivery and gene silencing efficiency of the targeted dual pH-sensitive ECO/siRNA nanoparticles were first assessed with a reported gene *in vitro* and in mice after intravenous injections. The capability of the targeted dual pH-sensitive ECO/siIF4E nanoparticles in resensitizing drug-resistant TNBC to chemotherapy was also investigated in a paclitaxel-resistant TNBC mouse model.

2. Results

2.1. Dual pH-sensitive PEG(HZ)-ECO/siRNA nanoparticles

PEG(HZ)-ECO/siRNA nanoparticles were prepared to test for PEG shedding in response to acidic pH.^[13] The ECO/siRNA nanoparticles were modified by incorporating a hydrazone linkage mPEG (MW = 5,000Da) and maleimide to obtain cleavable mPEG(HZ)-maleimide. MALDI-TOF mass spectrometric analysis of PBS solutions of mPEG(HZ)-maleimide at pH 7.4, 6.5, and 5.4 revealed the expected shift to lower molecular weights at pH 5.4, which corresponds to the late endosomal pH, confirming their pH-sensitive cleavage (Fig. 2A and B). ECO readily formed stable nanoparticles with siRNA via self-assembly at an N/P ratio of 8, with a low zeta potential of 22.3 ± 1.73 mV at neutral pH. With a subsequent decrease in pH to 6.5 and 5.4, the ECO/siRNA nanoparticles exhibited a relatively fast and pH-sensitive increase in the zeta potential to 32.5 ± 2.7 mV and 39.8 ± 3.1 mV, respectively, due to the enhanced protonation of the amino groups of ECO (Fig. 2C). Modification of the nanoparticles with mPEG-maleimide by reacting a thiol group in 2.5 mol% of ECO decreased their zeta potential to 12.3 ± 1.39 mV (Fig. 2D). Compared to unmodified nanoparticles, the increase in zeta potential of the PEGylated ECO/siRNA nanoparticles was less pronounced at acidic pH (17.4 ± 1.1 and 18.6 ± 2.9 mV at pH 6.5 and 5.4, respectively). The PEG(HZ)-ECO/siRNA nanoparticles had a zeta potential of around 12 mV at pH 7.4 (Fig. 2F), similar to that of PEG-ECO/siRNA nanoparticles, indicating that the PEG layer is intact. At pH 6.4, the zeta potential of the nanoparticles increased, but not up to the level of the unmodified nanoparticles, indicating partial hydrazone cleavage. At pH 5.4, the zeta

potential of PEG(HZ)-ECO/siRNA nanoparticles gradually increased to 36.3 ± 1.9 in 4 hours, which is similar to the zeta potential of the ECO/siRNA nanoparticles. These results suggest that the dual pH-sensitive PEG(HZ)-ECO/siRNA nanoparticles are stable at physiological pH during systemic delivery and are able to shed the PEG in acidic pH mimicking the conditions found in endosomes.

pH-sensitive cell membrane destabilization by the PEG(HZ)-ECO/siRNA nanoparticles was determined by assessing their pH-sensitive hemolytic activity (Fig. 2F). PEG-ECO/siRNA nanoparticles showed a significantly reduced ability to induce hemolysis at pH 6.5 and 5.4 when compared to ECO/siRNA nanoparticles, indicative of PEG-mediated inhibition of nanoparticle interactions with the cell membrane. The increase in PEG surface density diminished the pH-sensitivity of the zeta potential and pH-sensitive hemolytic activity of PEG-ECO/siRNA nanoparticles (Fig. 2G,H). In contrast, PEG(HZ)-ECO/siRNA nanoparticles induced pH-sensitive hemolysis comparable to the ECO/siRNA nanoparticles, indicating that PEG(HZ)-ECO/siRNA nanoparticles are capable of pH-sensitive cell membrane destabilization in the endosomes.

2.2. Targeted PEG(HZ)-ECO/siRNA nanoparticles induce potent *in vitro* gene silencing by enhanced endosomal escape

Cyclic RGD peptide-targeted dual pH-sensitive ECO/siRNA nanoparticles (RGD-PEG(HZ)-ECO/siRNA) were prepared by conjugating an RGD-PEG-hydrazone-maleimide conjugate (RGD-PEG(HZ)-Mal) to 2.5 mol% ECO, followed by complexation with siRNA. Cytosolic siRNA delivery of RGD-PEG(HZ)-ECO/siRNA nanoparticles was determined *in vitro* in MDA-MB-231 cells using an AF647-labeled siRNA. ECO/siRNA nanoparticles exhibited robust cellular uptake, possibly due to non-specific charge interactions with the anionic cellular membrane (Fig. 3A). On the other hand, the PEGylated ECO/siRNA nanoparticles with and without hydrazone had significantly reduced cellular uptake, possibly because PEG impedes their cell interactions. The presence of RGD significantly enhanced the cellular uptake of RGD-PEG(HZ)-ECO/siRNA and RGD-PEG-ECO/siRNA nanoparticles due to receptor-mediated endocytosis via specific binding to $\alpha_v\beta_3$ integrins overexpressed on the surface of the MDA-MB-231 cells.^[14] Notably, the hydrazone had no significant effect on cellular internalization of the nanoparticles.

Gene silencing efficacy of RGD-PEG(HZ)-ECO/siRNA nanoparticles was evaluated in MDA-MB-231-Luc cells (Fig. 3B). ECO/siRNA nanoparticles mediated potent and sustained gene knockdown, achieving $92.3 \pm 2.1\%$ luciferase silencing at an N/P ratio of 8 and 100 nM siRNA within 72 hours, due to their rapid internalization, endosomal escape, and siRNA release into the cytosol.^[3] On the other hand, the silencing efficiency of PEG-ECO/siRNA nanoparticles was only $23.4 \pm 1.5\%$ after 72 hours, which improved to $32.7 \pm 2.3\%$ with the incorporation of RGD. The silencing efficiency of PEG(HZ)-ECO/siRNA nanoparticles significantly increased to $37.6 \pm 11.1\%$, while the RGD-PEG(HZ)-ECO/siRNA nanoparticles with dual pH-sensitivity exhibited the highest silencing efficiency of $83.6 \pm 4.6\%$.

After the transfection, endosomal escape of RGD-PEG(HZ)-ECO/siRNA nanoparticles was confirmed by dynamic live cell confocal fluorescence imaging (Fig. 3C). RGD-PEG(HZ)-

ECO/siRNA and RGD-PEG-ECO/siRNA nanoparticles bearing AF-647-labeled siRNA (red) showed similar interactions with MDA-MB-231 cells within 10 minutes of incubation. After 3 hours, both the nanoparticles exhibited strong co-localization (yellow) with the late endosomes (green). However, 6 hours post-transfection, RGD-PEG-ECO/siRNA nanoparticles remained within the endolysosomes. In contrast, dispersed AF-647 signal was observed within the cytoplasm with minimal co-localization of RGD-PEG(HZ)-ECO/siRNA nanoparticles with the LysoTracker signal, indicating endosomal escape and cytosolic release of siRNA from the nanoparticles, as seen with ECO/siRNA nanoparticles.^[3] The endosomal escape of the nanoparticles was further validated by the addition of chloroquine, an endosomolytic agent,^[15] which enhanced the gene silencing activity of PEG-ECO/siRNA and RGD-PEG-ECO/siRNA nanoparticles to levels exhibited by PEG(HZ)-ECO/siRNA and RGD-PEG(HZ)-ECO/siRNA nanoparticles, respectively (Fig. 3D). Chloroquine did not have a significant effect on the nanoparticles containing hydrazone. Thus, receptor-mediated endocytosis, PEG shedding in the acidic endosomes, and the enhanced endosomal escape are associated with the superior gene silencing efficiency of the RGD-PEG(HZ)-ECO/siRNA nanoparticles (Fig. 3B).

2.3. RGD-PEG(HZ)-ECO/siRNA nanoparticles mediate potent and sustained tumor gene silencing *in vivo*

Intravenous injections of RGD-PEG(HZ)-ECO/siRNA nanoparticles bearing siLuc resulted in robust and prolonged gene silencing in MDA-MB-231-Luc breast tumors in mice at an siRNA dose of 1.0 mg/kg (Fig. 4A and B). All the targeted and non-targeted PEGylated ECO/siLuc nanoparticles resulted in substantial gene silencing by day 1 post-treatment, with 77% silencing for RGD-PEG(HZ)-ECO/siLuc, higher than that of the non-targeted nanoparticles PEG(HZ)-ECO/siLuc (45%), PEG-ECO/siLuc (34%), and the targeted nanoparticles RGD-PEG-ECO/siLuc (54%) when compared to the control. The targeted RGD-PEG(HZ)-ECO/siLuc and RGD-PEG-ECO/siLuc nanoparticles mediated significantly prolonged luciferase silencing than the non-targeted ones, with 52% silencing for RGD-PEG-ECO/siLuc and 85% for RGD-PEG(HZ)-ECO/siLuc, as compared to the control on day 7. RGD-PEG(HZ)-ECO/siLuc not only demonstrated the highest tumor gene silencing efficiency but also maintained the reduced luciferase expression for at least 7 days.

The efficient tumor gene silencing of the targeted nanoparticles was then validated by high tumor delivery efficiency of an AF-647-labeled siRNA in mice after intravenous injection (Fig. 4C and D). Although all 4 nanoparticles showed a similar pattern of initial tumor localization in the first 4 hours, RGD-PEG(HZ)-ECO/siRNA and RGD-PEG-ECO/siRNA nanoparticles resulted in prolonged tumor siRNA retention than their non-targeted counterparts for at least 24 hours (Fig. 4D). The tumors were excised at 48 hours post-injection, disaggregated into single cell suspensions, and stained for epithelial cellular adhesion molecule (EpCAM) using HEA-FITC to distinguish between the human MDA-MB-231 cancer cells and murine stromal cells.^[16] Flow cytometry analysis of the cell suspensions in the FITC channel revealed two distinct cellular populations: EpCAM(+) human cancer cells and EpCAM(−) murine stromal cells (Fig. 4E). Gating for EpCAM(−) cells in the AF-647 channel revealed a minimal shift in fluorescence between both the targeted and non-targeted nanoparticles compared to the PBS negative control (Fig. 4F). A

distinct shift was observed in EpCAM(+) cells for targeted nanoparticles compared to both the non-targeted and PBS control groups, suggesting that the RGD-targeted nanoparticles are internalized preferentially by the human cancer cells (Fig. 4G). Contour plots shown in Fig. 4H highlight the two distinct EpCAM(+) and EpCAM(−) cellular populations. While the siRNA signal from the non-targeted nanoparticles was evenly distributed throughout both populations, the siRNA signal in EpCAM(+) cells was 4.6-fold greater for the RGD-targeted nanoparticles. No difference was observed between the nanoparticles with and without hydrazone.

Following flow cytometry, the cell suspensions were investigated under a confocal microscope, revealing that both RGD-PEG(HZ)-ECO/siRNA and RGD-PEG-ECO/siRNA nanoparticles were internalized by the EpCAM(+) cells, whereas, in comparison, the non-targeted formulations showed minimal uptake (Fig. 4I). Thus, the presence of RGD enables cell-specific recognition and internalization of the targeted nanoparticles for prolonged tumor retention (Fig. 4D) and sustained gene silencing of the targeted nanoparticles (Fig. 4A and B). Although both RGD-PEG(HZ)-ECO/siRNA and RGD-PEG-ECO/siRNA had similar efficiencies of tumor intracellular siRNA delivery, the dual pH-sensitive PEG shedding and endosomal escape of RGD-PEG(HZ)-ECO/siRNA nanoparticles allowed for more efficient and prolonged tumor gene silencing. Taken together, these results demonstrate that the targeted dual pH-sensitive RGD-PEG(HZ)-ECO/siRNA nanoparticles are highly efficient for targeted, systemic, and cytosolic delivery of siRNA into tumors.

2.4. Silencing eIF4E by RGD-PEG(HZ)ECO/siRNA nanoparticles sensitizes drug-resistant TNBC cells and tumors to paclitaxel

As shown in Fig. 5A and B, eIF4E is overexpressed at both mRNA and protein levels in paclitaxel (PTX)-resistant MDA-MB-231 cells (MDA-MB-231.DR). RGD-PEG(HZ)-ECO/siEIF4E nanoparticles mediated significant downregulation of eIF4E in both the wild-type and drug-resistant cells, while the nanoparticles bearing non-specific siRNA (siNS) did not affect eIF4E expression. The MDA-MB-231.DR cells were less responsive to PTX than MDA-MB-231 cells. The treatment with RGD-PEG(HZ)-ECO/siEIF4E nanoparticles enhanced the sensitivity of both MDA-MB-231 and MDA-MB-231.DR breast cancer cells to PTX to achieve similar cytotoxicity (Fig. 5C).

Treatment of nude mice injected with orthotopic drug-resistant MDA-MB-231.DR tumors with PTX and RGD-PEG(HZ)ECO/siNS nanoparticles enhanced growth of the PTX-resistant tumors, when compared to the no treatment control (Fig. 6A, B, and C). On the other hand, intravenous injections of RGD-PEG(HZ)ECO/siEIF4E (1.5 mg/kg siRNA, every 6 days) significantly inhibited tumor growth. Alternating treatments with RGD-PEG(HZ)ECO/siEIF4E (1.5 mg/kg siRNA) and PTX (5 mg/kg) every 6 days also resulted in significant tumor regression. After 42 days of treatment, the average tumor volume and weight in the group treated with RGD-PEG(HZ)ECO/siEIF4E and PTX were $50.2 \pm 35.7 \text{ mm}^3$ and $103.2 \pm 67.4 \text{ mg}$, and were significantly lower than those in the control ($430.1 \pm 37.8 \text{ mm}^3$ and $404.9 \pm 43.6 \text{ mg}$), siNS + PTX ($560.3 \pm 46.2 \text{ mm}^3$ and $715.3 \pm 101.5 \text{ mg}$), and RGD-PEG(HZ)ECO/siEIF4E ($240.4 \pm 35.7 \text{ mm}^3$ and $256.8 \pm 33.5 \text{ mg}$) treatment groups, respectively (Fig. 6D and E).

RT-PCR analysis revealed that tumors in both the groups treated with RGD-PEG(HZ)-ECO/siIF4E showed significant down-regulation of eIF4E (Fig. 6F). In comparison, treatment with PTX and non-specific siRNA nanoparticles enhanced the expression of eIF4E compared to the control. This observation was confirmed by immunofluorescent staining of coexpression of eIF4E with other cancer-related genes, including survivin, cyclin D1, and VEGF in the primary tumor sections (Fig. 6G). Thus, elevated expression of eIF4E is associated with a significant increase in the expression of other genes associated with cancer cell survival and proliferation. Our results show that down-regulation of eIF4E with RGD-PEG(HZ)-ECO/siIF4E nanoparticles re-sensitized the MDA-MB-231.DR cells to the cytotoxic effects of PTX and enhanced its therapeutic efficacy.

2.5. RGD-PEG(HZ)-ECO/siIF4E nanoparticles elicit minimal tissue damage and immunogenicity

Histological analyses of the liver and kidney from mice receiving the long-term PTX and nanoparticle treatments revealed no overt tissue damage to the organs, suggesting that systemic delivery of siIF4E harbors minimal toxicity (Fig. 7A). Immune response to RGD-PEG(HZ)-ECO/siRNA nanoparticles was also tested in immunocompetent BALB/c mice by measuring the levels of TNF- α , IL-6, IL-12, and IFN- γ in the blood collected at 2 h and 24 h after the first, third, and fifth injections, where mice were intravenously administered with RGD-PEG(HZ)-ECO/siIF4E every 5 days (Fig. 7B). Intravenous injections of ECO/siRNA nanoparticles induced significant activation of all the cytokines at 2 and 24 hours post-injection, whereas RGD-PEG(HZ)-ECO/siRNA nanoparticles significantly attenuated the immune response for all the tested cytokines. Compared to the baseline, the TNF- α levels remained unchanged, while IL-6, IL-12, and IFN- γ levels increased slightly at 2 h, before falling to the baseline levels at 24 h, indicative of a transient response. More importantly, serum levels of the examined cytokines did not meaningfully differ over the course of 5 repeated injections, further suggesting only a transient response upon intravenous administration of the nanoparticles. Taken together, these results are indicative of the safety of long-term administration of RGD-PEG(HZ)-ECO/siIF4E nanoparticles.

3. Discussion

Cytosolic delivery of therapeutic siRNA is essential for the clinical application of RNAi therapeutics. Our targeted dual pH-sensitive multifunctional lipid/siRNA nanoparticles are rationally designed to overcome the barriers to systemic delivery of therapeutic siRNA into cancer cells.^[3] The multifunctional pH-sensitive lipid ECO effectively facilitates the endosomal escape of siRNA nanoparticles via pH-sensitive amphiphilic membrane destabilization in acidic endosomes, resulting in cytosolic siRNA delivery and high gene silencing efficiency.^[2b] Modification of the ECO/siRNA nanoparticles with a targeting peptide via PEG spacer further improves their biocompatibility and tumor specificity following systemic administration.^[4] However, PEGylation of the nanoparticles diminishes their endosomal escape ability. Incorporation of acid-labile hydrazone into the PEG spacer greatly improves both *in vitro* and *in vivo* gene silencing efficiency in tumor cells by pH-sensitive PEG shedding in acidic endosomes, subsequent pH-sensitive endosomal escape of the core ECO/siRNA nanoparticles, and reductive dissociation of the nanoparticles to release

siRNA in cytosol.^[17] As a result, the dual pH-sensitive RGD-PEG(HZ)-ECO/siRNA nanoparticles mediate efficient cytosolic siRNA delivery and silencing of luciferase in tumor for at least one week after intravenous injection at a low siRNA dose (1.0 mg siRNA/kg).

The dual pH-sensitive ECO/siRNA nanoparticle-mediated RNAi of eIF4E is a promising therapeutic strategy for enhancing the efficacy of paclitaxel for the treatment of PTX-resistant TNBC. Our data demonstrate that treatment of drug-resistant MDA-MB-231.DR tumors with PTX further elevated eIF4E expression and other oncogenes, which in turn facilitated more aggressive cancer proliferation. Although previous studies have shown that down-regulation of eIF4E blocks tumorigenesis in animal models,^[11a] data from clinical trials reveal that treatment with eIF4E-specific antisense oligonucleotides only leads to tumor cytostasis without regression.^[18] RGD-PEG(HZ)-ECO/siIF4E nanoparticles mediate highly efficient down-regulation of eIF4E in MDA-MB-231.DR cells after intravenous injections at a low siRNA dose (1.5 mg siRNA/kg), which is much lower than that of antisense oligonucleotides (25 mg/kg).^[11c] Robust silencing of eIF4E with RGD-PEG(HZ)-ECO/siRNA nanoparticles also resulted in cytostasis of the drug-resistant tumors. Silencing eIF4E is known to down-regulate other cancer-related genes, including survivin, which also contribute to PTX resistance.^[19] Survivin downregulation can also resensitize the cancer cells to PTX-induced apoptosis, resulting in tumor regression by the combination therapy. As expected, the down-regulation of eIF4E with RGD-PEG(HZ)-ECO/siRNA re-sensitized MDA-MB-231.DR cells to low doses of PTX, resulting in significant tumor regression in combination with PTX. These results suggest that targeting eIF4E alone may not be effective for cancer regression although it is involved in multiple facets of cancer signaling. The combination of eIF4E down-regulation with existing cancer therapies may be an effective approach to treating human cancer, especially drug-resistant cancers. Silencing of eIF4E with RGD-PEG(HZ)-ECO/siRNA may also circumvent acquired drug resistance and compromised therapeutic efficacy of other therapeutics, as observed in clinical studies.^[10]

The prolonged treatment with RGD-PEG(HZ)-ECO/siIF4E appears safe and does not cause any overt adverse effects on major organs in mice. Off-target gene silencing is often a safety concern for systemic administration of siRNA nanoparticles. A recent report has shown that maintaining eIF4E at 50% of the normal level does not affect normal mammalian development and global protein synthesis control.^[11b] Also, 80% knockdown of eIF4E in essential organs using antisense oligonucleotides does not cause any systemic toxicity.^[11a, 11c] A reduction in eIF4E levels would therefore have a minimal effect on normal biological functions. RGD-PEG(HZ)-ECO/siRNA nanoparticles also exhibited low immune stimulation in immunocompetent mice after prolonged exposure, which is critical for the clinical development of this therapy. Going forward, our future work will focus on optimizing the targeted dual pH-sensitive ECO/siRNA nanoparticles to improve therapeutic efficacy at further reduced doses and dosing frequency and to minimize potential side effects, particularly immunogenicity. The ability of silencing eIF4E with RGD-PEG(HZ)-ECO/siRNA nanoparticles to resensitize MDR tumors to other chemotherapies will also be explored to broaden the potential of the molecular target and therapy.

In summary, our targeted dual pH-sensitive siRNA delivery system, RGD-PEG(HZ)-ECO/siRNA nanoparticles, is capable of systemic targeted delivery of therapeutic siRNA and

facilitates endosomal escape, cytosolic siRNA release, and efficient silencing of target genes in cancer cells. Silencing eIF4E with the targeted nanoparticles inhibits tumor proliferation and re-sensitizes drug-resistant TNBC to paclitaxel. The combination of RGD-PEG(HZ)-ECO/siEIF4E with chemotherapeutics has the potential to effectively treat multidrug resistant tumors, including TNBC. The targeted dual pH-sensitive multifunctional ECO/siRNA nanoparticles constitute a facile and versatile platform and can be used to deliver other therapeutic siRNA or oligonucleotides for treating multiple human diseases.

4. Materials and Methods

Cell Culture

Human triple-negative breast cancer MDA-MB-231 cells expressing a luciferase reporter enzyme (MDA-MB-231-Luc) were obtained from ATCC (American Type Culture Collection) and cultured in Dulbecco's modified Eagle's media (Invitrogen), supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 units/mL penicillin, at 37°C in 5% CO₂. A paclitaxel-resistant subline (MDA-MB-231.DR) was induced by chronic exposure of MDA-MB-231-Luc cells to 5 nM paclitaxel (PTX, Sigma Aldrich) with increasing concentration at each passage over 8 weeks to reach a final concentration of 20 nM. The IC₅₀ of MDA-MB-231 cells was calculated to be 0.32 ng/mL while the IC₅₀ of MDA-MB-231.DR was 5.0 ng/mL. Once resistance to PTX was confirmed via the shift in IC₅₀, the MDA-MB-231.DR cells were maintained at 5 nM PTX.

Synthesis of PEG(HZ)-MAL and RGD-PEG(HZ)-MAL

NHS-PEG-SH (MW 3400) and mPEG-hydrazide (MW 5000) were obtained from Nanocs (New York, NY) and Laysan Bio (Arab, AL), respectively. cRGDfk was obtained from Peptides International (Louisville, KY). RGD-PEG-MAL was prepared as previously described.^[20]

To synthesize the non-targeted, pH-cleavable PEG spacer, mPEG5000-hydrazide was reacted with N-4-acetylphenyl maleimide. First, 87.8 mg (MW=5000, 1 equivalent, 17.5 µmol) of mPEG5000-hydrazide was dissolved in 10 mL DCM/MeOH (50/50) and 100 mg Na₂SO₄ was added. Next, 11.7 mg (MW = 215.2, 3.1 equivalent, 54.44 µmol) of N-4-acetylphenyl maleimide was dissolved in 1 mL DCM/MeOH (50/50) and added drop-wise into the mPEG-hydrazide solution. After the addition, acetic acid (1.77 µL of 34% v/v solution in DCM, 0.6 equivalent, 10.54 µmol) was added. The reaction was stirred for 24 hours at room temperature under nitrogen. After 24 hours, the solution was precipitated into cold diethyl ether (3X) to obtain a product. The product mPEG5000(HZ)-maleimide (PEG(HZ)-MAL) was characterized by ¹H-NMR spectroscopy (CDCl₃, ppm): 8.43 (s, 1H, -NH-), 8.06 (d, 2H, in phenyl), 7.54 (d, 2H, in phenyl), 6.91 (2, 2H, two olefinic protons of maleimide), 3.48–3.58 (m, 438H, PEG).

A three-step reaction was used to synthesize the cRGD-targeted pH-cleavable PEG-hydrazone moiety (RGD-PEG(HZ)-MAL).^[13, 21] NHS-PEG3400-SH (70.4 mg, 20.7 µmol) was dissolved in 1 mL of DMF and added drop-wise into the c(RGD)fk (25 mg, 2 equivalent, 41.4 µmol) in 5 mL DMF. After addition, 100 µL DIPEA was added to the

solution. The solution was stirred at room temperature for 4 hours. The solution was precipitated into an excess of diethyl ether (3X) to remove excess c(RGD)fk and obtain cRGD-PEG3400-SH. To ensure free thiol availability, 100 mg dithiothreitol (DTT) was added to the solution and stirred overnight to reduce any disulfide bonds present in the synthesized cRGD-PEG-SH. Free DTT was removed using a desalting spin column (1.8K MWCO). The product was lyophilized, re-suspended in chloroform and stored at -80°C. The product was confirmed by MALDI-TOF mass spectrometry and an increase of molecular weight of ~600 was observed. To create a hydrazide-activated cRGD-PEG, cRGD-PEG-SH (25.2 mg, 6.3 μ mol) was dissolved in 5 mL chloroform and reacted with N- ϵ -maleimidocaproic acid hydrazide (4.25 mg, 3 equivalents, 18.9 μ mol) and 4.39 μ L trimethylamine was added to the reaction. The reaction was carried out for 4 hours at room temperature with stirring. The reaction solution was then purified using a spin column (1.8K MWCO). The product cRGD-PEG-Hydrazide was lyophilized, re-suspended in chloroform, and stored at -80°C. Finally, cRGD-PEG-Hydrazide (12 mg, 2.84 μ mol) was reacted with N-4-acetylphenyl maleimide (1.2 mg, 2 equivalent, 5.68 μ mol) overnight at room temperature with stirring. The reaction mixture was purified on a silica gel column using chloroform:methanol mobile phase (9:1 v/v). The final product was concentrated and lyophilized. cRGD-PEG3400(HZ)-maleimide was characterized using ^1H -NMR spectroscopy (DMSO- d_6 , ppm): 8.48 (s, 1H, -NH-), 8.0 (d, 2H, in phenyl), 7.6–7.8 (m, cRGD), 7.45 (d, 2H, in phenyl), 7.2 (2, 2H, two olefinic protons of maleimide), 3.1–3.5 (m, 304H, PEG).

Preparation of PEG-modified ECO/siRNA nanoparticles

The multifunctional lipid ECO was synthesized as described previously.^[3] ECO (MW=1023) was dissolved in ethanol to give a stock solution with a concentration of 2.5 mM for *in vitro* experiments and 50 mM for *in vivo* experiments. The siRNA was reconstituted in RNase-free water to a concentration of 18.8 μ M for *in vitro* experiments and 25 μ M for *in vivo* experiments. For *in vitro* experiments, siRNA transfection concentration of 100 nM was used. ECO/siRNA nanoparticles were prepared at an N/P ratio of 8 by mixing predetermined volumes of ECO and siRNA for a period of 30 minutes in RNase-free water (pH 5.5) at room temperature under gentle agitation to enable complexation between ECO and siRNA. The total volume of water was determined such that the volume ratio of ethanol:water remained fixed at 1:20. For RGD-PEG(HZ)-ECO/siRNA nanoparticles, RGD-PEG(HZ)-MAL was first reacted with ECO in RNase-free water at 2.5 mol% for 30 minutes under gentle agitation and subsequently mixed with siRNA in RNase-free water for an additional 30 min. A stock solution of RGD-PEG(HZ)-MAL was prepared at a concentration of 0.32 mM in RNase-free water. The total volume of water was determined such that the volume ratio of ethanol:water remained fixed at 1:20.

Nanoparticle Characterization

The zeta potential of unmodified, mPEG, and mPEG(HZ) modified ECO/siRNA nanoparticles was determined at different pH values in PBS with a Brookhaven ZetaPALS Particle Size and Zeta Potential Analyzer (Brookhaven Instruments, Holtsville, NY). The nanoparticles were diluted in PBS solutions at pH 7.4, 6.5, or 5.4. The zeta potential

measurement was taken at predetermined time points up to 4 hours. Data represents the mean of three independently conducted experiments.

pH-Dependent Membrane Disruption Hemolysis Measurement

Hemolytic activity was measured to determine the membrane-disruptive ability of unmodified, mPEG and mPEG(HZ) modified ECO/siRNA at pH levels corresponding to various stages of intracellular trafficking. Rat red blood cells (RBCs) (Innovative Research Inc., Novi, MI) were diluted 1:50 in PBS solutions at pH 7.4, 6.5, and 5.4. The nanoparticles were prepared at a volume of 150 μ L at a final amine concentration of 150 μ M and incubated with an equal volume of the various RBC solutions in a 96-well plate at 37°C for 2 hours. Following incubation, the 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 non-ionic surfactant. Each experiment was conducted in triplicate and the data presented represents the mean and standard deviation.

Flow Cytometry for Nanoparticle Cellular Uptake Measurements

Cellular uptake and intracellular delivery of mPEG, mPEG(HZ), RGD-PEG, and RGD-PEG(HZ)-modified ECO/siRNA nanoparticles were evaluated with flow cytometry. The nanoparticles were prepared with AlexaFluor647-labeled siRNA (Qiagen, Valencia, CA). Approximately 2.5×10^4 MDA-MB-231 cells were seeded onto 12-well plates and grown for an additional 24 hours. The cells were transfected with each ECO/siRNA nanoparticle formulation (25 nM siRNA) in 10% serum media. After 4 hours, 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, Waltham, MA) 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 AlexaFluor 647 fluorescence for a total of 10,000 cells per each sample using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). Each formulation was conducted in triplicate and the data were presented as the mean fluorescence intensity and standard deviation.

Confocal Microscopic Imaging of Nanoparticle Uptake and Intracellular Release of siRNA

Live cell fluorescence confocal microscopy was used to assess the cellular uptake, endosomal escape, and intracellular release of siRNA. Approximately 1×10^5 MDA-MB-231 cells were seeded onto glass-bottom micro-well dishes. After 24 hours, the cells were stained for 30 minutes each with 5 μ g/mL Hoechst 33342 (Invitrogen) and with 50 nM LysoTracker Green DND-26 (Molecular Probes, Eugene, OR). RGD-PEG and RGD-PEG(HZ)-modified ECO/siRNA nanoparticles were formed at N/P = 8 and 25 nM siRNA concentration with an AlexaFluor 647-labeled siRNA. Images were taken using an Olympus FV1000 confocal microscope while the cells were housed in a humidified weather station under 5% CO₂.

In Vitro Luciferase Silencing Efficiency

MDA-MB-231-Luc cells were seeded in 24-well plates at a density of 2×10^4 cells and allowed to grow for 24 hours. Transfections were carried out in 10% serum media with the nanoparticles at 100 nM for siLuc (Dharmacon, Lafayette, CO: sense sequence: 5'-CUUACGCUGAGUACUUCGAdTdT-3', anti-sense sequence: 5'-UCGAAGUACUCAGCGUAAGdTdT-3'). Following a 4 hour transfection period, the media was replaced with fresh serum-containing media and the cells continued to grow for up to 72 hours. For experiments using chloroquine (Sigma Aldrich, St. Louis, MO), transfections were conducted in a similar manner either with or without 100 μ M chloroquine. As above, following a 4 hour transfection period, the media was replaced with fresh serum-containing media and the cells continued to grow for up to 48 hours. At each time point for luciferase silencing experiments, the cells were rinsed twice with PBS and lysed using the reporter lysis buffer provided in the Promega Luciferase Assay kit (Madison, WI). Following lysis, the samples were centrifuged at 10,000 g for 5 minutes 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, Sunnyvale, CA). Luciferase activity was normalized to the total protein content measured from the cell lysate of each well using the BCA assay (Thermo Scientific, Waltham, MA). Data was presented relative to the control, which received no siRNA treatment.

In Vivo Luciferase Silencing Efficiency

All animal experiments were performed in accordance with the guidelines and an approved animal protocol by the Institutional Animal Care and Use Committee of Case Western Reserve University. MDA-MB-231-Luc cells were engrafted into the mammary fat pad of female nude mice (2×10^6 cells/mouse). Once the tumors reached an average of 250 mm³, the mice were randomly sorted into 5 groups (n=3): 1) PBS control, 2) PEG-ECO/siLuc, 3) PEG(HZ)-ECO/siLuc, 4) RGD-PEG-ECO/siLuc, and 5) RGD-PEG(HZ)-ECO/siLuc. All the siRNA nanoparticle variations were formulated at 1.0 mg/kg siRNA in a total injection volume of 150 μ L. All mice received a single intravenous tail vein injection of the formulations following bioluminescent imaging on day 0. Expression of luciferase was quantified using bioluminescence imaging on day 0, 1, 3, 5, and 7. The bioluminescence signal intensity was quantified from a region of interest (ROI) placed over the tumor area. Data was normalized to the average signal intensity at day 0.

Fluorescence Molecular Tomography

Fluorescence imaging of the accumulation of the siRNA nanoparticles within primary MDA-MB-231 tumors in mammary fat pad was performed using the FMT 2500 quantitative fluorescence tomography system (Perkin-Elmer, Waltham, MA). Mice were treated with an intravenous tail vein administration of various ECO nanoparticles with AlexaFluor 647-conjugated siRNA (1.0 mg/kg) in a total injection volume of 150 μ L. The mice were imaged before and after intravenous injection of the nanoparticles at 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h.

Ex vivo flow cytometry and confocal microscopy

Mice treated with ECO nanoparticles bearing AF 647-siRNA were sacrificed at 48 hours post-injection, whereupon the primary tumor was resected and disaggregated into single cell suspensions using mechanical force and disaggregation solution [16]. The cell suspension was stained with FITC-conjugated mouse MAb against human epithelial antigen (EpCAM) (HEA125; Miltenyi Biotec, Auburn, CA) in the dark and on ice for 10 minutes. After staining, the cells were washed and centrifuged, fixed with paraformaldehyde, and finally passed through a 35 μ m cell strainer (BD Biosciences). Flow cytometry was conducted using the fluorescein channel for HEA-FITC and Cy5 channel for AF647-conjugated siRNA ECO nanoparticles for a total of 10,000 cells per sample using a BD FACSCalibur flow cytometer. Gating within the fluorescein channel was used to identify EpCAM(+) and EpCAM(−) populations. Each formulation was conducted in triplicate and the data presented as the mean fluorescence intensity and standard deviation. Following flow cytometry, the cell suspensions were examined under an Olympus FV1000 confocal microscope (Center Valley, PA).

Semi-quantitative real-time PCR analyses

MDA-MB-231 or MDA-MB-231.DR cells (100,000 cells/well) were seeded overnight onto 6-well plates. The cells were then treated with ECO nanoparticles with a non-specific siRNA or eIF4E-specific siRNA (eIF4E: AAGCAAACCUGCGGCUGAUCU (Dharmacon)).^[22] At each indicated time point, total RNA was isolated using the RNeasy Plus Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis System (Bio-Rad, Hercules, CA). Semi-quantitative real-time PCR was conducted using iQ-SYBR Green (Bio-Rad) according to manufacturer's recommendations. In all cases, differences in RNA expression for each individual gene were normalized to their corresponding GAPDH RNA signals. Primer sequence for eIF4E is 5'-CTACTAAGAGCGGCTCCACCAC-3' (sense) and 5'-TCGATTGCTTGACGCAGTCTCC-3' (antisense), GAPDH 5'-ACGGATTTGGTTCGTATTGGGCG-3' (sense) and 5'-CTCCTGGAAGATGGTGATGG-3' (antisense).

Western blot analyses

MDA-MB-231 and MDA-MB-231.DR cells were seeded into 6-well plates (1.5×10^5 cells/well) and allowed to adhere overnight. The cells were then treated with RGD-PEG(HZ)-ECO/siRNA complexes (N/P=8, siRNA concentration of 100 nM) in complete growth medium. After 5 days, detergent-solubilized whole cell extracts were prepared by lysing the cells in Buffer H (50 mM β -glycerophosphate, 1.5 mM EGTA, 1 mM DTT, 0.2 mM sodium orthovanadate, 1 mM benzamidine, 10 mg/mL leupeptin, and 10 mg/mL aprotinin, pH 7.3). The clarified extracts (20 mg/lane) were separated through 10% SDS-PAGE, transferred electrophoretically to nitrocellulose membranes, and immunoblotted with the primary antibodies, anti-eIF4E (1:1000; Abcam, Cambridge, MA) and anti- β -actin (1:1000; Santa Cruz Biotechnology, Dallas, Texas).

Cytotoxicity Assay

Cytotoxicity assays were performed in a 96-well plate by seeding 2,000 MDA-MB-231 or MDA-MB-231.DR cells/well. First, RGD-PEG(HZ)-modified ECO/siRNA nanoparticles were used to transfect MDA-MB-231 or MDA-MB-231.DR with either non-specific (siRNA) siNS or siEIF4E (100 nM) for 48 hours. Next, the cells were washed twice with PBS and incubated with various concentrations of PTX in fresh media. After 2 additional days, the MTT reagent (Invitrogen) was added to the cells for 4 hours followed by the addition of SDS-HCl and further incubation for 4 hours. The absorbance in 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 PTX concentration and was normalized to the no treatment control.

In vivo tumor growth inhibition study

For *in vivo* anti-tumor efficacy studies, MDA-MB-231.DR cells (2×10^6 cells/mouse) were inoculated in the mammary fat pads of female nu/nu mice. When the tumors reached an average size of 150 mm^3 , the mice were randomly sorted into 4 groups (n=5): 1) PBS control, 2) RGD-PEG(HZ)-ECO/siNS (1.5 mg/kg siRNA) + PTX (5 mg/kg), 3) RGD-PEG(HZ)-ECO/siEIF4E (1.5 mg/kg siRNA) + PTX (5 mg/kg), 4) RGD-PEG(HZ)-ECO/siEIF4E (1.5 mg/kg siRNA). RGD-PEG(HZ)-ECO/siRNA nanoparticles were administered by intravenous injection via the lateral tail vein while PTX was administered in 10% DMSO/PBS with intraperitoneal injection (i.p.). Tumor growth was monitored by bioluminescence imaging and tumor size was monitored using caliper measurements. Three days after the final treatment, the mice were sacrificed to harvest tumor tissues.

Bioluminescence imaging

Bioluminescence imaging of the mice was performed using the Xenogen IVIS 100 imaging system (Alameda, CA). D-Luciferin (Xenogen) was dissolved in PBS (15 mg/mL), and 200 μL of the luciferin stock solution (15 mg/mL) was injected i.p., 5 minutes before measuring the light emission. Mice were anesthetized and maintained under 2.5% isoflurane. Bioluminescence signal was quantified using Living Image software (Xenogen) by drawing an ROI over the tumor area.

Immunofluorescence and immunohistochemical staining

For immunohistochemistry, primary tumor samples were embedded in optimum cutting temperature (O.C.T.) compound (Tissue-Tek; Torrence, CA) in preparation for cryostat sectioning and immediately frozen. The samples were then sectioned, fixed in paraffin, and maintained at -80°C . The samples were stained with H&E to evaluate the presence of tumor tissue. Briefly, the samples were fixed in 10% formalin, rehydrated in 70% ethanol and rinsed in deionized water prior to hematoxylin staining. Samples were then rinsed in tap water, decolorized in acid alcohol, immersed in lithium carbonate and rinsed again in tap water. Next, the eosin counterstain was applied and the slides were dehydrated in 100% ethanol, rinsed in xylene and finally mounted on a coverslip with Biomount.

For immunofluorescence detection of eIF4E (Abcam: ab1126), survivin (Abcam: ab76424), Cyclin D1 (Abcam: ab16663), and VEGF (Abcam: ab46154), the paraffin-embedded slides

were first deparaffinized using a series of washes in xylene and decreasing concentrations of ethanol. Heat-induced antigen retrieval was performed using a pressure cooker in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) for 20 minutes. Following heat-induced antigen retrieval, the samples were blocked in TBST solution containing donkey serum and washed three times with TBST. The primary antibody was applied at a dilution of 1:100 in blocking solution for 1 h followed by three rinses with TBST. The Alexa Fluor 647 secondary antibody (Abcam: ab150079) was applied at a dilution of 1:1000 in blocking solution for 1 h followed by three washes with TBST and counterstained with DAPI at a dilution of 1:2500 in blocking solution. After washing with TBST and mounting in an anti-fade mounting solution (Molecular Probes), the samples were imaged using a confocal microscope.

Immune response

Female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used to study the immune response induced by the systemic treatment with ECO/siRNA and RGD-PEG(HZ)-ECO/siRNA nanoparticles. The mice were intravenously injected with the nanoparticles every five days with a total of 5 injections (n=5 for each injection). Blood samples were collected at 2 h and 24 h after the 1st, 3rd, and 5th. Plasma was isolated from blood samples using Microtainer tubes (BD Biosciences). To measure plasma cytokine levels, TNF α , IL-6, IL1-2, and INF γ were quantified by ELISA, according to the manufacturer's instructions (Invitrogen).

Statistical analyses

Statistical values were defined using unpaired Student's *t*-test, with $p < 0.05$ considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research support was provided, in part, by grants from the National Institutes of Health to Z-R.L. (EB00489 and CA194518), and by a National Science Foundation Graduate Research Fellowship to M.D.G. (DGE-0951783).

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Figure 1.

Design and working of the dual pH-sensitive PEG(HZ)-ECO/siRNA nanoparticles. **(A)** Formation of targeted dual pH-sensitive ECO/siRNA nanoparticles by 1) reaction of peptide-PEG-hydrazone-maleimide (RGD-PEG(HZ)-MAL) with one of the thiol groups of small portion of multifunctional lipid ECO and 2) self-assembly with siRNA electrostatic complexation, hydrophobic condensation, and disulfide cross-linking and PEG shedding in acidic endosomes. **(B)** RGD-PEG(HZ)-ECO/siRNA nanoparticles facilitate receptor-mediated endocytosis, resulting in trafficking of the nanoparticles into endosomes. Within late endosomes, the increasingly acidic environment cleaves the hydrazone linkage to facilitate shedding of the PEG layer and to expose the core ECO/siRNA nanoparticles. Next, pH-sensitive amphiphilicity of ECO promotes endosomal escape by enhanced amphiphilic interaction of the nanoparticles with the lipid bilayer of the endosomes. Once release into the cytosol, endogenous glutathione (GSH) mediates reduction of disulfide bonds within ECO/siRNA nanoparticles to facilitate dissociation of the nanoparticles and siRNA release. Upon release, free siRNA is able to initiate RNAi-induced gene silencing.

Figure 2. pH-sensitive shedding of PEG from PEG(HZ)-ECO/siRNA nanoparticles. MALDI-TOF mass spectra of mPEG (A) and mPEG(HZ) (B) at pH 7.4, 6.5, and 5.4, the pH at different stages of intracellular trafficking. Dynamic changes of zeta potential of ECO/siRNA (C), PEG-ECO/siRNA (D), and PEG(HZ)-ECO/siRNA (E) nanoparticles incubated in PBS solutions at pH 7.4, 6.5, 5.4. Comparison of hemolytic activity of ECO/siRNA, PEG-ECO/siRNA, and PEG(HZ)-ECO/siRNA nanoparticles at pH 7.4, 6.5, 5.4 (F). The pH sensitivity (G) and hemolytic activity (H) of PEGylated ECO/siRNA nanoparticles at a PEGylation degree of 0, 1, 2.5, 5, 10 mol-% measured by changes in zeta potential at pH 7.4, 6.5, and 5.4. Relative hemolytic activity was calculated with respect to the hemolytic activity of 1% Triton-X-100.

Figure 3.

Targeted PEG(HZ)-ECO/siRNA nanoparticles induce potent *in vitro* gene silencing by enhanced endosomal escape. (A) Cellular uptake of unmodified, PEG, PEG(HZ), RGD-PEG, and RGD-PEG(HZ) modified ECO/siRNA nanoparticles quantified with flow cytometry using an AF647-labeled siRNA. (B) Luciferase silencing of unmodified, PEG, PEG(HZ), RGD-PEG, and RGD-PEG(HZ) modified ECO/siRNA nanoparticles in MDA-MB-231-luc TNBC cells compared to no treatment control group. (C) Fluorescence confocal microscopy images of live MDA-MB-231 cells incubated with RGD-PEG, and RGD-PEG(HZ) modified ECO/siRNA nanoparticles at 10 min, 3 hr, and 6 hr. DAPI, cell nucleus (blue); Lysotracker DND-26, late endosomes and lysosomes (green); siRNA, AF-647 (red). (D) Luciferase silencing efficiency of different ECO/siRNA nanoparticles after 48 hours in MDA-MB-231-luc cells transfected with or without the endosomolytic agent chloroquine (100 μ M).

Figure 4.

RGD-PEG(HZ)-ECO/siRNA nanoparticles mediate potent and sustained tumor gene silencing *in vivo*. Luciferase silencing efficiency in orthotopic MDA-MB-231-Luc tumors of mice following a single intravenous injection of various surface-modified ECO/siRNA nanoparticles (1.0 mg/kg siRNA dose) compared to PBS-treated control group. (A) Bioluminescence intensity from the tumor at different time points after the treatment (siLuc). (B) Representative bioluminescence images of the different treatment groups (siLuc). (C) Representative FMT images showing tumor accumulation and retention of the surface-modified ECO/siRNA nanoparticles of an AF647-tagged siRNA within 24 hours after intravenous administration. (D) Fluorescence intensity of AF647-tagged siRNA quantifying tumor accumulation and retention of the modified nanoparticles in tumor. Cell suspensions obtained from primary MDA-MB-231 mammary fat pad tumors following intravenous administration of the modified ECO/siRNA nanoparticles were analyzed using flow cytometry and confocal microscopy analysis. (E) FACS analysis following staining for EpCAM expression using a FITC labeled anti-EpCAM antibody in tumor cell suspensions in the FITC channel revealed two populations of EpCAM(+) and EpCAM(-) cells. (F) Gating for the EpCAM(-) cell population and examining in the AF647 channel revealed minimal uptake of both non-targeted and RGD-targeted nanoparticles when compared to PBS control (data shown for the systems with hydrazone). (G) Gating for the EpCAM (+)

cell population in the AF647 channel revealed a significant shift for RGD-targeted nanoparticles compared to the non-targeted and PBS control (data shown for the systems with hydrazone). **(H)** Data represented as a two dimensional contour plot highlights the EpCAM (–) and EpCAM (+) populations along the FITC channel axis. For targeted nanoparticles, fluorescent signal from siRNA in AF647 channel axis is distinctly greater in the EpCAM (+) population. For non-targeted nanoparticles, the AF647 signal is evenly distributed between EpCAM (–) and EpCAM (+) populations. **(I)** Cell suspensions from the tumors were examined under a confocal microscope. Targeted nanoparticles display a greater amount of siRNA signal (red) in the EpCAM (+) (green) cells.

Figure 5. Silencing eIF4E by RGD-PEG(HZ)ECO/siRNA nanoparticles sensitizes drug-resistant TNBC cells to paclitaxel. Expression of eIF4E at mRNA and protein levels determined by qRT-PCR (A) and western blotting analysis (B) in MDA-MB-231 and MDA-MB-231.DR cells at 5 days following treatment with RGD-PEG(HZ)-ECO/siRNA nanoparticles (N/P=8) delivering siEIF4E or siNS (100 nM) compared to no treatment control group. (C) Cell viability of MDA-MB-231 and drug resistant MDA-MB-231.DR cells treated with varying concentrations of PTX following prior treatment with RGD-PEG(HZ)-ECO/siRNA nanoparticles delivering siEIF4E or siNS. The cells were first treated with RGD-PEG(HZ)-ECO/siRNA nanoparticles for 48 hours followed by treatment with varying concentrations of PTX for an additional 48 hours.

Figure 6.

The efficacy of combination therapy of PTX and RGD-PEG(HZ)-ECO/siIF4E nanoparticles in treating orthotopic luciferase labeled MDA-MB-231.DR TNBC tumors in mice compared to PBS-treated control group. Alternating treatment of the siIF4E nanoparticles and PTX every 6 days began at 4 weeks once the primary tumors reach an average of 150 mm³. (A) Bioluminescence intensity over the course of the experiment (data represents mean $\pm$ SE, n=5, *p 0.05, **p 0.01 comparing to the no treatment control) and (B) bioluminescence images at week 10. (C) Tumor growth was monitored using digital caliper measurements (data represents mean $\pm$ SE, n=5, *p 0.05, **p 0.01). (D) Primary tumors resected at week 10 and (E) final tumor weights (data represents mean $\pm$ SE, n=5, *p 0.05, **p 0.01). (F) Relative eIF4E mRNA expression in the resected primary tumors determined by qRT-PCR (data represents mean $\pm$ SE, n=5, *p 0.05, **p 0.01). (G) Immunofluorescence staining of eIF4E, VEGF, cyclin D1, and survivin from primary tumors.

Figure 7. (A) Histological evaluation of liver and kidney (10X) from different treatment groups. (B) Immunogenicity of ECO/siRNA and RGD-PEG(HZ)-ECO/siRNA nanoparticles in immunocompetent mice following intravenous administrations. At 2 h and 24 h following the first, third, and fifth injection, blood samples were collected and the plasma was isolated to be used for cytokine ELISA measurements of TNF- α , IL-12, IFN- γ , and IL-6 (data represents mean $\pm$ SE, n=5, *p 0.05, **p 0.01 comparing to the baseline). Solid and dotted lines indicate the mean $\pm$ SE pertaining to baseline levels of each cytokine.