Photoactivation of sulfonated polyplexes enables localized gene silencing by DsiRNA in breast cancer cells

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Abstract

Translation potential of RNA interference nanotherapeutics remains challenging due to in vivo off-target effects and poor endosomal escape. Here, we developed novel polyplexes for controlled intracellular delivery of dicer substrate siRNA, using a light activation approach. Sulfonated polyethylenimines covalently linked to pyropheophorbide-\(\alpha\) for photoactivation and bearing modified amines (sulfo-pyro-PEI) for regulated endosomal escape were investigated. Gene knock-down by the polymer-complexed DsiRNA duplexes (siRNA-NPs) was monitored in breast cancer cells. Surprisingly, sulfo-pyro-PEI/siRNA-NPs failed to downregulate the PLK1 or eGFP proteins. However, photoactivation of these cell associated-polyplexes with a 661-nm laser clearly restored knock-down of both proteins. In contrast, protein down-regulation by non-sulfonated pyro-PEI/siRNA-NPs occurred without any laser treatments, indicating cytoplasmic disposition of DsiRNA followed a common intracellular release mechanism. Therefore, sulfonated pyro-PEI holds potential as a unique trap and release light-controlled delivery platform for on-demand gene silencing bearing minimal off target effects.

Key words: RNA interference; siRNA delivery; Polymer; Photosensitizer; Endosomal escape

RNA interference (RNAi) is a biological phenomenon that modulates gene expression and significantly impacts cellular processes.\textsuperscript{1} Various small non-coding RNAs such as small interfering RNAs (siRNAs), microRNAs (miRNAs), short hairpin RNAs (shRNAs), and piwi-interacting RNAs (piRNAs) have been demonstrated to initiate the process of RNAi.\textsuperscript{2} Research is underway to develop RNA-based therapeutics that exploit RNAi for the treatment of diseases such as infections,\textsuperscript{3} cancer,\textsuperscript{4} cardiovascular diseases,\textsuperscript{5} and eye-related disorders.\textsuperscript{6,7} RNAi-based therapies can be applied either on their own merit or in combination with other drugs.\textsuperscript{8-10} Most widely studied RNAi-therapeutics utilize siRNAs as the molecules of choice with several clinical trials currently in the pipeline.\textsuperscript{11,12} siRNAs are generally constructed as a \(\sim\) 21 base balanced salt solution supplemented with Ca\textsuperscript{2+} & Mg\textsuperscript{2+} ions; BSA, Bovine serum albumin; EEA1, early endosomal antigen 1 (ThermoFisher Inc. cat # PA1-063A); PBS, Phosphate buffered saline (2.66 mM KCl, 1.47 mM KH\textsubscript{2}PO\textsubscript{4}, 138 mM NaCl, 8.06 mM Na\textsubscript{2}HPO\textsubscript{4}-7H\textsubscript{2}O) (pH 7.1)); Nucleic Acid Assembly buffer, 2 mM Mg\textsubscript{(OAc)}\textsubscript{2}, 50 mM KCl, 89 mM Tris, 89mM boric acid (pH 8.2); HEPES buffer (HBS), 10 mM HEPES, 140 mM NaCl (pH 7.2-7.5); TBE buffer, 89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8.2); ROS, Reactive oxygen species; RIPA, Radio immunoprecipitation assay

Abbreviations: FBS, Fetal bovine serum; DMEM, Dulbecco’s Modified Eagle Medium supplemented with 10% (v/v) heat-inactivated FBS, 100 i.u./mL penicillin and 100 \(\mu\)g/mL streptomycin; HBSS\textsuperscript{+}, Hanks’ balanced salt solution supplemented with Ca\textsuperscript{2+} & Mg\textsuperscript{2+} ions; BSA, Bovine serum albumin; EEA1, early endosomal antigen 1 (ThermoFisher Inc. cat # PA1-063A); PBS, Phosphate buffered saline (2.66 mM KCl, 1.47 mM KH\textsubscript{2}PO\textsubscript{4}, 138 mM NaCl, 8.06 mM Na\textsubscript{2}HPO\textsubscript{4}-7H\textsubscript{2}O) (pH 7.1)); Nucleic Acid Assembly buffer, 2 mM Mg\textsubscript{(OAc)}\textsubscript{2}, 50 mM KCl, 89 mM Tris, 89mM boric acid (pH 8.2); HEPES buffer (HBS), 10 mM HEPES, 140 mM NaCl (pH 7.2-7.5); TBE buffer, 89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8.2); ROS, Reactive oxygen species; RIPA, Radio immunoprecipitation assay

REVISED Manuscript Pyro-PEI mediated DsiRNA release

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pair (bp) duplex with 2 nucleotide 3’-overhangs. Cellular pathways obligatory for biological function of siRNAs have been identified and thus provide strategies for their intended therapeutic intervention. Alternatively, slightly longer RNA duplexes (25-30 bp), termed Dicer substrate interfering RNAs (DsiRNAs), can be employed to accomplish gene silencing. DsiRNAs require intracellular processing by the ribonuclease-III enzyme Dicer before loading into the RISC complex. Among these, cationic lipids are widely studied, and various lipid-based delivery systems have entered clinical trials for systemic delivery of RNAi.28 In the field of small molecule drug-loaded nanomedicine, controlled, spatial and temporal release are vital for efficient therapeutic intervention. Alternatively, slightly longer RNA nanoparticles to accomplish controlled cytoplasmic delivery of DsiRNA (release) upon photoactivation.

Intracellular delivery of siRNA in its functional form can be achieved by using chemically modified siRNAs or self-assembled RNA nanostructures with multiple functionalities.15,16 An alternative approach utilizes selected delivery agents to carry the unmodified siRNAs to their desired site(s).17,18 Among these, cationic lipids are widely studied, and various lipid-based delivery systems have entered clinical trials for systemic delivery of siRNA. The first lipid-based RNAi therapeutic (patisiran, ONPATTRO) was approved by the FDA for the treatment of Polyneuropathy of Hereditary Transthyretin-Mediated Amyloidosis (Alnylam Pharmaceuticals, Inc. Cambridge, MA). Cationic polymers are another class of widely studied molecules to deliver siRNA due to their inherent positively-charged surfaces for siRNA binding.20–23 RNAi nanotherapeutics can either enter cells by direct cytosolic delivery of the siRNA or the particles can be endocytozed. However, the RNAi activity of endocytozed nanoparticles is often hindered due to the lack of efficient and directed cytoplasmic siRNA disposition.24,25 Therefore, suitable strategies for endosomal/lysosomal escape of siRNA for controlled, spatial and temporal release are vital for efficient RNAi.26 In contrast, the endocytozed sulfo-pyro-PEI-NPs having a reduced number of available amines for pronation are predicted to mitigate ion influx and are unable to exploit osmotic swelling mechanisms for their intended cytosolic release. This restriction can be lifted by exploring a charge independent remote-strategy such as photoactivation of endosome-localized NPs enabling on-demand localized RNAi (Figure 1).

Sulfo-pyro-PEI that vary in their degree of sulfonation were examined for their ability to associate with the DsiRNA, their intracellular uptake, and the ability to enhance RNAi activity upon photoactivation. Interestingly, photoactivation of sulfo-pyro-PEI complexed-DsiRNA was found to be essential for gene silencing indicative of DsiRNA release into the cytosol in a regulated fashion. In contrast, non-sulfonated pyro-PEI complexed DsiRNA displayed gene silencing in the absence of photoactivation with no further increase in gene knock-down upon light treatments. This lack of photoactivation requirement for the non-sulfonated NPs can be interpreted as an alternate, non-specific, cytosolic DsiRNA release mechanism (potentially the proton sponge effect). Therefore, the photoactivation strategy described here presents a promising approach to facilitate regulated RNAi-induced gene silencing when using sulfo-pyro-PEI as the delivery agent. These photosensitizer-conjugated polyplexes provide a built-in engineered platform for directional activation and on demand RNAi with minimal side effects with translation potential in humans.

Methods

Materials

Nuclease and protease-free water was purchased from Quality Biological Inc. (Gaithersburg, MD). Cell titer blue reagent was obtained from Promega (Madison, WI, USA). Cell culture reagents and media were from Invitrogen (Grand Island, NY, USA). Nucleic acid sequences were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa, USA) (sequences...
provided in Supplemental section). RIPA lysis buffer and GAPDH mouse Mab (cat# sc-47724) were purchased from SANTA CRUZ Biotech., Inc. (Dallas, TX). Other antibodies for immunostaining assays were bought from ThermoFisher Scientific (Rockford, IL).

**Preparation of pyro-PEI/nucleic acid complexes**

Pyro-PEI and sulfo-pyro-PEI were synthesized and characterized as described42 (see supplemental material, Scheme S1). A dose response study was performed to optimize pyro-PEI/nucleic acid binding ratios. ATTO488-DNA duplexes were diluted in the assembly buffer at a concentration of 1 nmol/mL, and Pyro-PEI molecules were diluted in RNase/DNase free water as desired. A known concentration of DNA (3 pmol) was mixed with various amounts of the diluted pyro-PEI (containing 0-5 μg), and samples were analyzed on a 2% agarose gel. DNA mobility was detected by image analysis using the Typhoon Trio variable mode imager (GE Healthcare) with a filter set of Ex/Em 488/532 nm (for ATTO488) and Ex/Em 488/670 nm (for pyro fluorescence).

For routine binding/uptake studies, pyro-PEI or sulfo-pyro-PEI were placed in 0.5 mL tubes and mixed with ATTO488-DNA duplexes to a final DNA/pyro-PEI ratio of 1:165. These ATTO488-DNA-NPs were incubated for 30 min at room temperature, diluted 10-fold with phosphate buffered saline (PBS without Ca²⁺/Mg²⁺), and then used immediately for various experiments.

For gene silencing studies, anti-eGFP-DsiRNA-NPs or anti-PLK1-DsiRNA-NPs were prepared at a ratio of 10 nmol pyro-PEI or sulfo-pyro-PEI and 10 pmol DsiRNA in a volume of 50 μL of the corresponding DsiRNA duplexes. Following incubations for 30 min at room temperature, the samples were diluted in 1 mL DMEM and were used immediately for gene silencing studies.

**Sizing and zeta potential analysis of DsiRNA-NP complexes**

DsiRNA-NPs were prepared using the anti-eGFP DsiRNA as described above and analyzed for their hydrodynamic size, particle concentration and zeta potential. For sizing, DsiRNA-NPs (10 μL) were diluted in Hepes-buffered saline (HBS, pH 7.4) to a final volume of 400 μL in 10 mM NaCl in a microcuvette. Dynamic light scattering measurements (12-24 acquisitions each) were acquired in triplicate using a Zeta Sizer Nano ZS (Malvern Instruments, MA). For zeta potential

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**Figure 1. Proposed hypothesis of selective siRNA delivery via photoactivation.** Pyro-PEI complexed siRNA-NPs are delivered into the target cell and accumulate in the endosomes. (A) Non-sulfonated pyro-PEI-NPs escape endosomes by way of the osmotic swelling mechanism. (B) The sulfo-pyro-PEI NPs on the other hand, remain trapped in the endosomes absent osmotic swelling. Photoactivation results in disruption of endosomal membrane, releasing NPs into the cytoplasm and initiating gene silencing (B, bottom panel).
measurements, DsiRNA-NPs or pyro-PEI samples without bound nucleic acids were diluted in 10 mM NaCl, in order to compare the effect of nucleic acid binding on reduction of overall charge.

**Cell lines**

Human breast cancer cells, MDA-MB-231 and green fluorescent protein expressing MDA-MB-231/GFP cells were procured from ATCC (Manassas, VA, USA) and Cell Biolabs Inc. (San Diego, CA, USA), respectively, and were maintained in DMEM supplemented with 10% FBS, 100 i.u./mL penicillin and 100 μg/mL streptomycin in 5% CO₂ at 37 °C.

**Cell binding/uptake studies**

ATTO488-DNA-NPs were incubated with MDA-MB-231 cells at a density of 10⁶/mL at desired temperatures for 2 h. Details of pyro-PEI/DNA ratios and exact incubation conditions are provided in the corresponding figure legends. Following incubations, cells were pelleted, washed, and analyzed for cell-associated ATTO488 fluorescence by flow cytometry. Analyses were performed using the Amersham Typhoon 5 imager (GE Healthcare) with excitation/emission at 665 nm/720 nm.

**Results**

**Description of variant pyro-PEI polymers**

To develop photoactivatable RNAi therapeutics for localized delivery of DsiRNA, we explored a branched 10 kDa polyethylenimine polymer (PEI) covalently linked to the photosensitizing molecule pyro. Pyro was chosen for its desired spectral properties for future in vivo applications. Sulfonated pyro-PEI molecules (sulfo-pyro-PEI) differ in their overall positive charges due to the chemical modification of amines through introduction of sulfonate groups (Scheme 1, Figure S1). Previous studies show that selective sulfonation of pyro-PEI results in lowered cytotoxicity in CHO cells in vitro. In this study, we examined three pyro-PEIs: (i) non-sulfonated pyro-PEI (pyro-PEI), (ii) sulfo-pyro-PEI containing 6% amine modifications (pyro-s₆-PEI), and (iii) sulfo-pyro-PEI containing 34% amine modifications (pyro-s₃₄-PEI). The ability to generate reactive oxygen species after photoactivation (Figure S1) and the degree of photoactivation in the presence and absence of DsiRNA (Figure S2) was examined for pyro-PEIs differing in their extent of sulfonation. Ultimately, those pyro-PEIs with differing degrees of amine modification were investigated for their ability to promote conditional gene silencing by way of photo-triggered released of DsiRNA.

**Sulfonation of pyro-PEI modulates binding with nucleic acid duplexes**

A fluorescently labeled DNA duplex (ATTO488-DNA) was used to determine the effect of sulfonation on pyro-PEI binding affinity for nucleic acids. ATTO488-DNA duplexes incubated with various doses of the different pyro-PEI molecules were analyzed by agarose gel electrophoresis. A clear difference in binding affinity was observed for pyro-PEI and sulfo-pyro-PEI and correlated with the overall positive charge on the pyro-PEI, as expected (Figure 2, A). However, the differences in the dose response curves for the pyro-s₆-PEI and pyro-s₃₄-PEI was less significant. Based on binding data, DNA/pyro-PEI ratios of 1:165 were used for further cellular uptake studies.
Photocatalytic treatments had no effects on the stability of ATTO488-DNA-NPs (supplemental section, Figure S3).

Next, the hydrodynamic size of anti-eGFP DsiRNA-NPs was determined by dynamic light scattering (DLS) measurements. Figure 2, B shows that the average diameter of pyro-PEI-DsiRNA-NPs decreased as the degree of sulfonation increased. Pyro-PEI NPs exhibited an average diameter of 310 nm (Pdl 0.224), whereas pyro-s6-PEI and pyro-s34-PEI NPs had an average diameter of 269 nm (Pdl 0.229) and 219 nm (Pdl 0.183), respectively. Zeta potential values for pyro-PEI in absence of DsiRNA were +7.5 mV ± 1.6, whereas anti-eGFP DsiRNA-NPs showed a reduction in zeta potential (+4.3 mV ± 0.8) (Figure 2, C). Similar effects were observed for pyro-s34-PEI before (+ 5.7 mV ± 2.5) and after complexation with anti-eGFP DsiRNA duplex (+4.1 mV ± 1.3). In contrast, pyro-s6-PEI ((+6.3 mV ± 1.1) after the DsiRNA binding showed a steep reduction in the zeta potential (+0.2 mV ± 0.2). This large drop in zeta potential for the pyro-s6-PEI NPs is a phenomenon we do not have a clear explanation for at this time.

Cellular uptake of pyro-PEI-complexed ATTO488-DNA

To monitor the effects of sulfonation of pyro-PEI on subsequent interactions with cells, we examined cellular uptake (representing the surface-bound and/or internalized NPs) of ATTO488-DNA-NPs with MDA-MB-231 cells under various conditions. Various concentrations of ATTO488-DNA duplexes (2.5, 5 or 10 pmol/10⁶ cells) complexed with pyro-PEIs were incubated with MDA-MB-231 cells at 37 °C, and cell-associated fluorescence was determined by flow cytometry (Figure S4, A). The extent of ATTO488-DNA-NP binding varied linearly with concentration across the range of doses examined. Pyro-PEI complexed DNA displayed 1.5-2-fold greater binding to cells as compared to sulfo-pyro-PEI complexed DNA. However, differences in binding observed between pyro-s6 and pyro-s34-complexed DNA were relatively minor.

To further understand the nature of interactions between ATTO488-DNA-NPs and cells, we examined the effect of incubation temperature on binding, as the active endocytic mechanisms are known to be retarded at lower temperatures. Binding assays performed at a decreased temperature (4 °C) were compared with binding at 37 °C and clearly show that pyro-PEI-DNA complexes exhibit relatively higher cellular uptake compared to that for pyro-s34-PEI at both temperatures studied (Figure 3, A). Interestingly, these differences were more prominent at 4 °C, indicating that uptake of sulfo-pyro-PEI is more reliant on energy-dependent cellular processes (Figure 3, B). To further substantiate the observed temperature-dependent effects, we tested binding of ATTO488-DNA-NPs to cells across a range of temperatures (Figure 3, C). Enhanced binding of pyro-PEI-DNA complexes was observed at all the temperatures tested, while pyro-s6-PEI and pyro-s34-PEI exhibited similar binding at each temperature. Taken together, cellular entry pathways for pyro- and sulfo-pyro-PEI complexed DNA duplexes are related to energy-dependent processes. Future detailed studies are warranted to map the exact intracellular uptake mechanism of these particles.

To gain further insight into the cellular uptake mechanisms of various pyro-PEI complexes, we examined the effects of trypsin treatment on the cell-associated ATTO488 fluorescence under various conditions (Figure S4, B). We observed that the trypsin sensitivity was similar for pyro-PEI and sulfo-pyro-PEIs. Most of the bound pyro-PEIs were cleaved by trypsin when binding was done at 4 °C, suggesting that at a lower temperature, the complexes remain surface bound. On the other hand, trypsin treatment resulted in only a partial decrease of ATTO488-DNA-NP fluorescence at 37 °C. These data suggest that the pyro-PEI and sulfo-pyro-PEI complexed nucleic acids are taken up via a similar mechanism. Again, detailed and thorough experiments are needed to further dissect out the exact cellular uptake patterns of these nanoparticles.
interactions with negatively charged nucleic acids, as well as subsequent uptake by cells. We speculated that sulfonated DNA-NPs at various temperatures.

A dose-dependent study was initially conducted to assess non-specific cellular toxicity of Pyro-PEI complexed DsiRNAs (Figure S5) prior to pursuing eGFP silencing studies. Anti-eGFP DsiRNA duplexes were complexed with various pyro-PEIs and gene silencing activity was measured in MDA-MB-231/eGFP+ cells. Clearly, only non-sulfonated pyro-PEI complexes mediated eGFP gene silencing in absence of laser treatment (Figure 4, A). However, this gene silencing was observed only at higher doses of the pyro-PEI (Figure 4, A, left panel, red curve as indicated by the arrow), but not at a lower dose (Figure 4, A, i, green curve). In contrast, sulfo-pyro-PEI complexed DsiRNA under identical conditions failed to show measurable eGFP silencing even when higher doses of the complexes were used (Figure 4, A, ii and iii, red curves). This lack of eGFP silencing was observed irrespective of the degree of sulfonation; neither pyro-s6-PEI (Figure 4, A, ii) nor pyro-s34-PEI (Figure 4, A, iii) complexed DsiRNA was able to downregulate eGFP under the experimental conditions examined.

Photoactivation rescues sulfo-pyro-PEI complexed DsiRNA mediated eGFP gene silencing in MDA-MB-231/eGFP+ cells

Various pyro-PEI-DsiRNA-NPs were bound to MDA-MB231/eGFP+ cells at two different doses for 4 h, after which cells were treated with a 661 nm laser for 0-15 min. Cells were then incubated at 37 °C for an additional 72 h after which eGFP expression levels were examined. When pyro-PEI was used for DsiRNA delivery, we did not observe any eGFP silencing at lower doses compared to control cells (Figure 4, B, i). At higher doses, a clear downregulation in eGFP levels was observed in samples that were not treated with the laser, and photoactivation resulted only in a slight enhancement of eGFP downregulation at this higher dose (Figure 4, B, iv). These data show that pyro-PEI complexed DsiRNA gene silencing occurs via an intracellular pathway that does not rely on the photoactivation of pyro-PEI.

However, when sulfo-pyro-PEIs were used, the results were quite different and interesting. We did not observe any eGFP gene downregulation for these samples in the absence of laser treatments, at either low or high doses. This lack of eGFP silencing in the absence of photoactivation was observed for both pyro-s6-PEI (Figure 4, B, ii & v) and pyro-s34-PEI (Figure 4, B iii & vi) incubated cells. This observation contrasts with the pyro-PEI, where eGFP silencing was observed for high doses in absence of photoactivation (Figure 4, B, iv). Therefore, we conclude that the inability of sulfo-pyro-PEIs to promote eGFP silencing is not due to the limited DsiRNA in the cells.

Photoactivation of cells treated with sulfo-pyro-PEI complexes resulted in a clear induction of eGFP silencing, and this effect increased with longer duration of laser treatments. This effect of photoactivation-dependent silencing was observed for both sulfo-pyro-PEIs tested. eGFP downregulation occurred at both doses of the pyro-s6-PEIDsiRNA complexes (Figure 4, B, ii & v), but the effect of photoactivation was more pronounced at higher doses of the pyro-s6-PEI. Cells incubated with pyro-s34-PEI/DsiRNA complexes clearly showed eGFP downregulation at both doses and silencing increased as a function of increased laser exposure. We observed that the efficiency of eGFP knockdown was similar between the low and high doses of the pyro-s34-PEI (Figure 4, B, iii & vi), and low dose pyro-s34-PEI.

Sulfo-pyro-PEI-complexed DsiRNA promote eGFP silencing in MDA-MB-231/eGFP+ cells only upon photoactivation

Surface modification of nanoparticles is known to modulate interactions with negatively charged nucleic acids, as well as subsequent uptake by cells. We speculated that sulfonated polymers may present an advantage for intracellular trafficking and sequestration into endosomes due to their inefficient protonation and impaired release (Figure 1). As a result of the study was...
showed markedly better silencing than low dose pyro-s6-PEI following photoactivation (Figure 4, B, ii & iii).

The possibility of any deleterious effects of laser treatment on eGFP expression was tested by treatment of the cells (i) in the absence of the pyro-PEI/DsiRNA NPs, (ii) incubations with scrambled DsiRNA/pyro-s34-PEI complexes (at high dose concentrations), and (iii) using lipofectamine as a transfection agent (Figure 4, C). Importantly, laser treatments of eGFP expressing cells alone, or pre-incubated with a non-targeting sulfo-pyro-DsiRNA-NP, did not show any effects on eGFP expression. Additionally, laser treatments of cells transfected with anti-eGFP DsiRNA complexed with a non-photoreactive carrier had no impact on the extent of eGFP downregulation. These controls clearly show that the laser treatment conditions used in our experiments had no non-specific effects on eGFP expression or RNAi-based gene silencing. Taken together, sulfonated PEIs present a system for selective and on-demand DsiRNA delivery with minimal off-target effects.
Laser pretreatment downregulates PLK1 expression when anti-PLK1 DsiRNAs are delivered by sulfo-pyro-PEI NPs

Data presented above show that eGFP gene silencing can be selectively enhanced upon light treatment using the sulfo-pyro-PEI complexed DsiRNAs. Moreover, laser mediated restoration of eGFP silencing was clearly observed at lower doses of the NPs (1 nmol NPs/10^5 cells) where no gene silencing was observed for non-sulfonated NPs. Therefore, in our next set of experiments, we examined the effects of photoactivation on cellular expression of a protooncogene, PLK1, overexpressed in MDA-MB-231 cells, at low doses (1 nmol NPs/10^5 cells) of the NPs. PLK1 targeted RNAi was previously studied in clinical trials using a lipid-based formulation (TKM-080301) for the treatment of adrenocortical cancer and also in other related in vitro and in vivo studies.50,52

To examine the effect of photoactivation on PLK1 downregulation, MDA-MB-231 cells incubated with anti-PLK1 DsiRNA-NPs were treated with the laser and PLK1 expression was determined post 48-h incubations in the cell lysates. Results are presented in Figure 5. A statistically significant decrease in PLK1 expression was observed upon photoactivation in the cells incubated with only sulfo-pyro-PEI complexed anti-PLK1 DsiRNA (Figure 5, B). No obvious changes in PLK1 expression were observed in either control cells or in the cells incubated with non-sulfonated pyro-PEI complexed PLK1 siRNA, regardless of exposure to photoactivation. These results further confirm that the PLK1 downregulation occurs only upon photoactivation in samples incubated with sulfo-pyro-PEI-NPs. These results are in accordance with our observations on eGFP down-modulation (Figure 4, B). We noticed a slight increase in PLK1 expression upon laser treatments in control cells as well as in the cells incubated with non-sulfonated pyro-PEI NPs. However, these differences were found to be not statistically significant, confirming that laser treatment does not have any non-specific deleterious effects on gene expression. Taken together, these results indicate the potential of selective photoactivation strategy for enhanced RNAi to treat cancer.

Sulfo-pyro-PEI bound ATTO488-DNA preferentially localizes in the endosomal compartments

Based on our data obtained from temperature dependence uptake and trypsin treatments, we concluded that pyro-PEI and sulfo-pyro-PEI molecules carry the nucleic acid to the cells presumably via similar pathways. The extent of cellular uptake by pyro-PEI NPs was increased presumably due to the overall higher positive charged residues on the surface of these NPs. We examined intracellular distribution of these NPs relative to the endosomes by fluorescence microscopy. Here, we used EEA1, an endosomal marker to mark intracellular location in the cells (identified by rhodamine-labeled secondary antibody) and ATTO488 fluorescence to monitor the NPs. Results presented in Figure 6 show representative images for a given slice from confocal microscope images. These data suggest that sulfo-pyro-PEI NPs tend to preferentially localize in the vicinity of the endosomes whereas non-sulfonated particles appear randomly distributed. It is possible that the non-sulfonated PEI complexes are capable of escaping the endosomes without laser treatment and therefore do not show enhanced eGFP silencing upon photoactivation. A detailed study, subject to future investigations will be needed to map exact intracellular location of these NPs.

Discussion

Nanomedicine-based RNAi therapeutics are currently being explored and several formulations have paved their way to clinical trials. Site-specific delivery of the RNAi therapeutics as well as spatial and temporal release of the siRNA for its actions remains a challenge. In addition, unregulated siRNA release causes off-target effects.
The inability of endocytosed siRNA-NPs to escape into the cytoplasm, one of the major hurdles is an area of intense research. Several studies include pH, redox or light activatable systems; photochemical internalization (PCI) being reported as a promising system with translational potential. The PCI approach relies on the delivery of the siRNA and the photosensitizer as separate entities with an assumption that both components will travel to the same intracellular compartment for their action. Our initial efforts to enhance cytosolic delivery of siRNA included a PDT molecule (HPPH) non-covalently partitioned into the positively charged DOTAP:DOPE liposomes. Although we observed an RNAi enhancement upon photoactivation, gene silencing prior to photoactivation was also clearly observed. Hence, this system failed to decrease off-target effects (unpublished data).

Polymer-based siRNA therapeutics have been developed; however, their utility as light-sensitive NPs has not yet been explored. Our PEI-based siRNA NPs contain a covalently linked tunable photosensitizing molecule (pyro) and hence present an advantage for intracellular site-specific co-delivery of the siRNA and the photoactivation molecule. Moreover, pyro can be activated by using wavelengths amenable for future applications in vivo (similar PDT molecules are currently used in the clinical settings). Sulfonated-Pyro PEIs also bear an advantage of reduced cellular toxicity of the carrier itself for their potential future clinical applications. Chemical synthesis of the pyro-conjugated PEIs involves simple steps with high yields providing the possibility of scale-up production of these molecules. Sulfo-pyro-PEIs only function upon light-activation (no off-target effects) and show gene silencing at significantly lower doses of siRNA as compared to their non-sulfonated counterparts. Therefore, the photoactivation strategy described here presents a promising approach to facilitate regulated RNAi-induced gene silencing when using sulfo-pyro-PEI as the delivery agent. These photosensitizer-conjugated polyplexes provide a built-in engineered platform for directional activation and on demand RNAi with minimal side effects with translation potential in humans. Targeted RNAi therapies mediated through this technology are likely to improve cancer treatment due to the selective action of RNAi at the tumor site and are likely to have positive impact in the RNAi nanotherapeutics field.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2020.102176.

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