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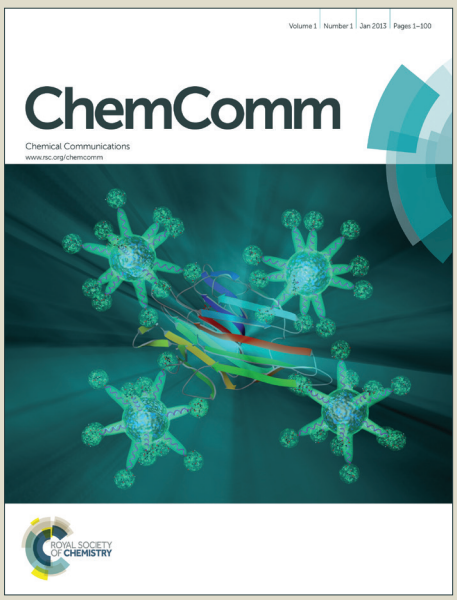

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Direct Oligonucleotide-Photosensitizer Conjugates for Photochemical Delivery of Antisense Oligonucleotides†

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Activation of photosensitizers in endosomes enables release of therapeutic macromolecules into cytosol for pharmacological actions. In this study, we demonstrate that direct conjugation of photosensitizer to oligonucleotides (ONs) allows spatial and temporal co-localization of the two modalities in the target cells, and thus leads to superior functional delivery of ONs. Further, light-activated delivery of an anticancer ON caused cancer cell killing via modulation of an oncogene and photodynamic therapy.

Oligonucleotides (ONs), including siRNA and antisense ONs, have potentials to become a new generation of medicine, due to their high specificity and low toxicity.[1] Splice switching oligonucleotides (SSOs), a new type of antisense drugs, can hybridize to pre-mRNA sequences and block access of other splicing factors to modulate alternative splicing and subsequent gene expression.[1] A SSO has been used to treat Duchenne muscular dystrophy in clinical trials;[2] while other SSOs have demonstrated anticancer activity.[3] For instance, a SSO can redirect Bcl-x splicing from anti-apoptotic Bcl-x_L to pro-apoptotic Bcl-x_S and subsequently induce apoptosis of cancer cells.[4] Unfortunately, the poor permeability of cell membrane to ONs substantially complicate the development of these macromolecules for therapeutic applications.[5] Recently, cationic lipid, polymers and nanoparticles have been widely used for ONs delivery. However, a major fraction of the internalized ONs is trapped in endo/lysosomes, which limits their full therapeutic potential.[6]

To achieve functional delivery of ONs, effective transport of ONs from endosomes into the cytosol is indispensable.[7] Recently, photochemical internalization (PCI), has been used to enhance cytoplasmic delivery of therapeutic macromolecules, such as proteins and ONs.[8] In the process of PCI, light-activation of the photosensitizer (PS) results in reactive oxygen species (ROS)-mediated disruption of the endo/lysosomal membranes followed by the subsequent release of the cargo molecules into the cytosol.[9] ONs should colocalize with PSs in endocytic vesicles to achieve maximum cytosolic delivery and avoid damage to other intracellular compartments.[8a, 10] However, in conventional PCI procedures, PSs are given separately from the ONs, and thus they may transport to different intracellular sides from ONs' and then cause side effects by damaging plasma membrane and/or mitochondria.[11]

In this study, we report that direct conjugation of PS and ONs allows spatial and temporal co-localization of the two modalities in the target cells, and thus leads to superior functional delivery of ONs. Light-activated delivery of the conjugates of Bcl-x SSO and Chlorin e6 (Ce6) caused cancer cell killing via Bcl-x splicing switching and photodynamic therapy (PDT), resulting in a novel strategy for combination therapy of solid tumors (Fig. 1).

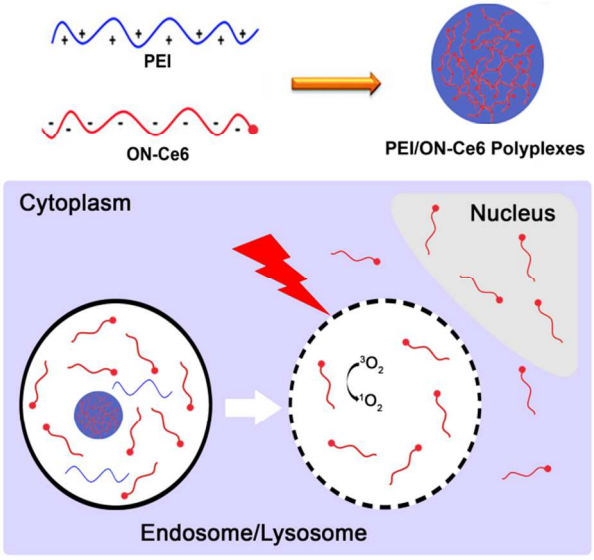

Fig. 1: Graphical illustration of PEI/ON-Ce6 polyplexes and laser irradiation mediated cytosolic and nuclear delivery of ONs.

Two ONs (ON623 and Bcl-x SSO) were synthesized according to a method described previously.[12] They were 2'-O-Me phosphorothioate ONs functionalized with a primary amine at the 5-end. To conjugate ONs to Ce6, we directly linked 5' amino-modified ONs to Ce6 via an EDC/NHS mediated amine coupling reaction. After that, the products were purified with HPLC to remove unreacted ONs and Ce6. ON-Ce6 conjugates eluted after free ONs with the retention time of 27.8 and 18.7min, respectively (Fig. S1). MALDI-TOF MS data indicated that we obtained right ON conjugates (S2 and S3 and Figs Table S1). Absorption spectra of purified ON-Ce6 conjugates showed 3 peaks at 259, 401 and 659nm, respectively (Fig. S4). The absorption peak at 259nm is attributed to ONs and the absorption peaks at 401nm and 659nm are attributed to

Ce6 in the conjugates. Free Ce6 (10μM, in PBS) exhibited only 4.1% of the fluorescence intensity of that dissolved in methanol due to the aggregation and self-quenching (Fig. S5). However, the ON-Ce6 conjugates (10μM, in PBS) showed 96.5% fluorescence (emission peak at 668nm) when compared with free Ce6 (10uM) in methanol. After the 660nm laser irradiation, dramatic increase in the SOSG fluorescence emission was observed for the ON-Ce6 conjugates (1μM, in PBS) when compared to that from free Ce6 (1μM, in PBS, Fig. S6). These results demonstrated the great solubility of ON-Ce6 conjugates in aqueous solution.

Next, ON623-Ce6 conjugates were complexed with jetPEI, a commercially available polyethylenimine (PEI), to form polyplexes for intracellular delivery. Functional delivery of ONs was measured in A375/Luc705 cells that were stably transfected by a luciferase reporter containing a mutated intron. Successful delivery of ON623 can cause splicing out of the intron and allow expression of wild type luciferase. After internalized with polyplexes and photoirradiated, dramatically higher luciferase activity was observed when compared to non-photoirradiation groups in all N/P ratios. The highest level of luciferase expression was detected at N/P ration of 3 (Fig. 2) and it was even higher than Lipofectamine 2000 lipoplexes of ON623 at the same concentration. The luciferase induction of PEI/ON623-Ce6 complexs (N/P=3) was dependent on the dose of photoirradiation (Fig. S7). By contrast, PEI/ON623 plus free Ce6 did not produce additional luciferase induction compared to single treatment of PEI/ON623 (Fig. S8). Size and zeta potential of polyplexes at N/P ration of 3 were 285.6nm and +7.1mV, respectively (Fig. S9 and S10). The positive charge of PEI/ON623-Ce6 polyplexes is crucial for efficient intracellular uptake and the N/P ration of 3 was selected for further experiments. Intracellular uptake efficiency of free Ce6, ON623-Ce6 conjugates and PEI/623-Ce6 polyplexes (N/P=3) was evaluated in A375/Luc705 cells using flow cytometry. ON623-Ce6 conjugates and PEI/ON623-Ce6 polyplexes allowed for 3.6- and 23-fold in Ce6 fluorescence compared to free Ce6, respectively (Fig. S11). In addition, positive PEI did not affect the cellular uptake of free Ce6 (Fig. S12).

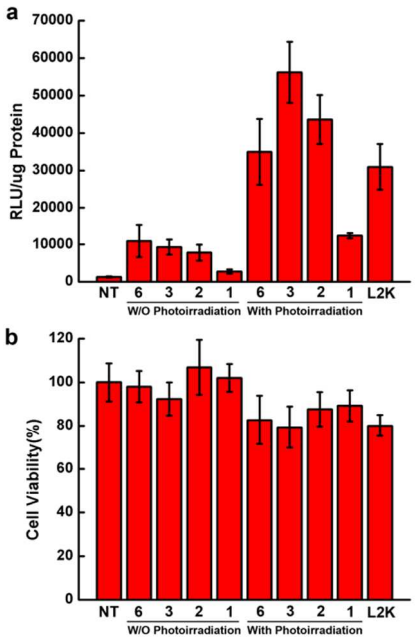

Fig. 2: Comparison of luciferase induction (a) and cytotoxicity (b) of PEI/ON623-Ce6 polyplexes (50nM of ON623-Ce6) with N/P ratios of 1, 2, 3 and 6, respectively. L2K represents the luciferase induction by Lipofectamine 2000 complexes of ON623 (50nM).

Next, confocal laser scanning microscopy was used to observe subcellular distribution of PEI/ON623-Ce6 polyplexes after 4 hours incubation with A375/luc705 cells. Most of the ON623-Ce6 polyplexes were observed as red dots in the cytoplasm around the nucleus and significant colocalization between the polyplexes and lysotracker green was also observed (Fig. S13), indicating that most ON623-Ce6 molecules transported to late endosome/lysosome after cellular entry. To assess endosomal release, PEI/623-Ce6 polyplexes were incubated with cells for 4 hours to allow cellular uptake. Once internalized, ON623-Ce6 remained largely trapped within endosome/lysosomes with little escape to cytosol, as these molecules were initially localized in a punctate distribution. Yet, most of the cells displayed a cytosolic distribution of ON623-Ce6 conjugates after photoirradiation (Fig. 3). In addition, cells with cytosolic distribution were brighter than cells with a punctate distribution, which was likely due to the self-quenching of Ce6 when they were trapped within endosome/lysosomes.

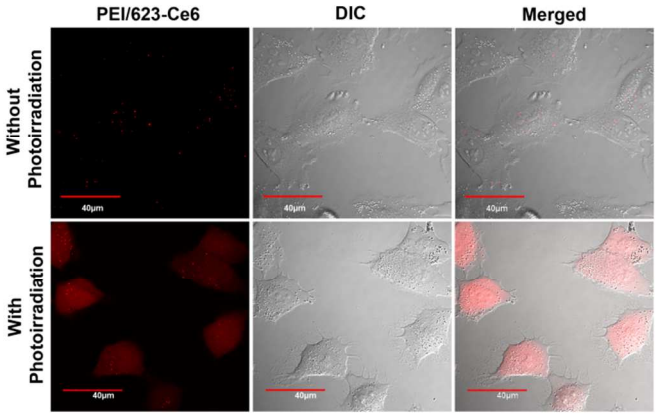

Fig. 3: Photoirradiation mediated cytosolic delivery of ONs. Scale bars, 40μm.

Photochemical delivery of direct ON-PS conjugates was further examined using a therapeutic SSO Bcl-x SSO. The Bcl-x pre-mRNA can be alternatively spliced to produce two isoforms: anti-apoptotic Bcl-x_L and pro-apoptotic Bcl-x_S. Bcl-x SSO that is complementary to the Bcl-x_L isoform splice site can shift splicing of Bcl-x_L to the Bcl-x_S isoform, and was adequate to induce apoptosis in cancer cells. PEI/Bcl-x-SSO-Ce6 polyplexes (N/P=3) showed similar size and zeta potential with PEI/ON623-Ce6 (Figs. S7 and S8). RT-PCR analysis indicated that treatment of PEI/Bcl-x-SSO-Ce6 polyplexes followed by photoirradiation induced a dramatic shift in splicing of Bcl-x pre-mRNA from anti-apoptotic Bcl-x_L to pro-apoptotic Bcl-x_S, while control treatments, including PEI/ON623-Ce6 with or without photoirradiation, and PEI/Bcl-x-SSO-Ce6 without photoirradiation, had no effect on Bcl-x splicing (Fig. 4a).

Finally, cell viability assay was performed after photochemical delivery of Bcl-x SSO in A375/luc705 (Fig. 4b) and SKOV3 cells (Fig. S14) in order to assess combinational effects of photodynamic therapy and pro-apoptotic splicing switching. Cells treated with PEI/ON623-Ce6 polyplexes in the absence of photoirradiation served as control, in which no obvious dark toxicity was observed. Cells treated with PEI/Bcl-x-SSO-Ce6 polyplexes in the absence of photoirradiation showed slightly decreased viability, but the difference was not significant. When there was not light-triggered endosomal release, the PEI/Bcl-x-SSO-Ce6 polyplexes only showed very weak gene therapeutic efficiency. Upon 660nm laser irradiation, cells treated with PEI/ON623-Ce6 polyplexes exhibited decreased viability, indicating the photodynamic toxicity of direct conjugates of ON and PS. Importantly, the combination of photodynamic and gene therapies of the cells by the PEI/Bcl-x-SSO-

Ce6 polyplexes treatment and photoirradiation showed much lower cell viability compared to other three treatments. These results revealed that light-induced endosomal escape significantly enhanced the Bcl-x SSO-mediated toxicity, allowing for concerted photodynamic and gene therapies against cancer cells.

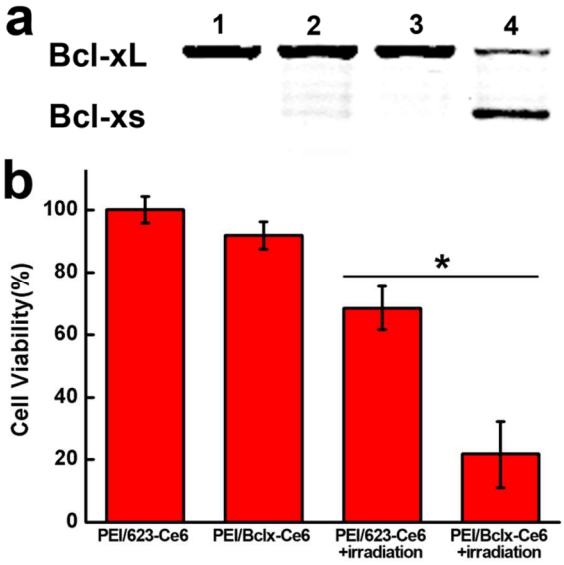

Fig. 4: In vitro photoirradiation induced photodynamic and gene therapies in A375/luc705 cells. (a) The pre-mRNA of Bcl-xL and Bcl-xs determined by RT-PCR **(**Reverse transcription polymerase chain reaction**);** 1, PEI/ON623-Ce6; 2, PEI/Bcl-x-SSO-Ce6; 3, PEI/ON623-Ce6+photoirradiation; 4, PEI/Bcl-x-SSO-Ce6+photoirradiation. (b) In vitro cytotoxicity effect in A375/luc705 cells induced by PDT, gene therapy, and combined photodynamic and gene therapy. The combined therapy allowed for a significantly higher tumor cell therapeutic effect compared to PDT or gene therapy alone. *p<0.05.

Conclusions

In this study, we have successfully prepared direct conjugate of antisense ON and PS for light-activated delivery of antisense ONs that leads to combination therapy of cancer. PSs have been conjugated to aptamers, single-stranded ONs with tertiary structures that bind to specific target molecules, for the purpose of targeted PDTs.[13] However, this method has not been used for intracellular delivery of therapeutic ONs including siRNA, microRNA, and antisense ONs. The direct conjugates of ONs and PSs may provide advantages including enhanced water solubility of PSs and consistent spatial distribution of the two modalities in the endosome/lysosomes of target cells. After cellular entry using polyplexes, photoirradiation controlled the endosomal escape of therapeutic ONs in the cancer cells. Ultimately, the polyplexes of the conjugates achieved the significant growth inhibition of cancer cells via concerted photodynamic and gene therapies. This study may thus provide a novel platform technology for cytosolic delivery of therapeutic ONs including siRNA, antisense, SSOs, and miRNA antagomirs.

Notes and references

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