Electronic Supplementary Material (ESI) for ChemComm.

Direct Oligonucleotide-Photosensitizer Conjugates for Photochemical Delivery of Antisense Oligonucleotides

Ahu Yuan[a,b] , Brian Laing[a] , Yiqiao Hu[b] and Xin Ming[a,*]

[a]Division of Molecular Pharmaceutics, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA.
*Tel: 919-966-4343; Fax: 919-843-3017; E-mail: xming@email.unc.edu
[b]State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, China

Supporting information

Materials and Methods:

Preparation of Oligonucleotide and Ce6 (ON-Ce6) Conjugates

The 5'-NH_2 2'-O-Me phosphorothioate ON623 [5'NH_2-GUU AUU CUU UAG AAU GGU GC-3'] and 5'-NH_2 2'-O-Me phosphorothioate Bcl-x SSO [5'NH_2-UGG UUC UUA CCC AGC CGC CG-3'] were synthesized according to a method described previously.[1] Chlorin E6 (Ce6, MedKoo Biosciences, Chapel Hill, NC) was activated to obtain Ce6-NHS by EDC and NHS (molar ratio= 1:10:10) in DMSO overnight at room temperature. Ce6-NHS was incubated with 5'-NH_2-ON623 or 5'-NH_2-Bcl-x-SSO with molar ratio of 5:1 overnight in PH7.4 PBS. ON623-Ce6 and Bcl-x-SSO-Ce6 were purified with Varian HPLC system equipped with Agilent ZORBAX SB-C18 column. Products are separated by running a linear gradient move phase. The organic solvent was acetonitrile, and the aqueous phase is 0.1M TEAA. The gradient started at 5% and ramped to 50% organic solvent in 35 minutes. ON623-Ce6 and Bcl-x-SSO-Ce6 conjugates were then desalted by Sephadex G-25 gel (GE Healthcare).

Characterization of Oligonucleotide and Ce6 Conjugates

The absorption spectrum of ON623-Ce6 conjugates was measured using Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). The fluorescence of ON623-Ce6 conjugates (10μM, in PBS) and free Ce6 (10μM, in PBS and methanol) were detected by NanoDrop 3300 fluorospectrometer (Thermo Scientific).

To evaluate the singlet oxygen generation (SOG) of ON623-Ce6 conjugates (1μM) and free Ce6 (1μM) in PBS, the singlet oxygen sensor green (SOSG, Life Technologies) was introduced at the concentration of 1μM. SOG was induced by photoirradiation with a 660nm laser at the intensity of 3.5 mW/cm^2. After photoirradiation, SOSG fluorescence was read at an excitation and emission of 488 and 525 nm, respectively, on a FLUOstar Omega microplate reader (BMG LABTECH, Cary, NC, USA). Fluorescence of solution before photoirradiation was set as negative control and subtracted from fluorescence of each group. Each point represents change of fluorescence.

Preparation of Polyplexes

To prepare polyplexes at different N/P ratios, variable amounts of jetPEI (Polyplus, Illkirch, France) were diluted with 150 mM NaCl solution and were then mixed by vortex with an equal volume of NaCl solution containing ON-Ce6 conjugates. The resultant dose solution contained concentrated 600 nM ON-Ce6 conjugates. After 20 min of incubation at room temperature, the polyplexes were then added to the cells (DMEM media with 10% FBS) to obtain 50nM ON623-Ce6 or Bcl-x-SSO-Ce6. N/P ratio was calculated based on manufacturers' instructions. The particle size and zeta potential of the PEI/ON-Ce6 polyplexes (N/P=3, 600 nM) in 150 mM NaCl solution were determined by Zetasizer Nano Z (Malvern Instruments, Westborough, MA, USA).

Different N/P ratios for luciferase induction

A375 human melanoma cells were stably transfected with firefly luciferase expression cassettes containing a mutated intron and were referred to as A375/Luc705 cells.[2] ON623 targets an intronic splice site, result in splicing out of the intron and allow expression of wild type luciferase mRNA and protein.[2]

A375/luc705 cells were plated on 96-well plates (8,000 cells per well) in DMEM media with 10% FBS. After culturing overnight, cells were treated with PEI/ON623-Ce6 polyplexes (50nM of ON623-Ce6) at N/P ratios of 1, 2, 3 and 6, respectively. Following the overnight treatment at 37°C, cells were washed with with fresh media and were further incubated for one hour. Then cells were photoirradiated with a 660nm laser for 30min at the light intensity of 3.5 mW/cm^2. Twenty-four hours later, cell lysates were collected for luciferase assay. Luciferase assay was performed with a FLUOstar Omega microplate reader (BMG LABTECH). Protein content was determined by the BCA protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Luciferase activity of the cells without oligonucleotide treatment was used as control. Cell viability of A375/luc705 cells under the same experimental condition was detected with AlamarBlue reagent (Life Technologies).

Time dependent luciferase expression was conducted by similar experimental procedures. Cells treated with PEI/ON623-Ce6 polyplexes (50nM, N/P=3) were photoirradiated with a 660nm laser (3.5 mW/cm^2) for 0, 5, 10, 20 and 30 minutes.

Cellular uptake

Total cellular uptake of free Ce6, ON623-Ce6 conjugates and PEI/ON623-Ce6 polyplexes (N/P=3) at the concentration of 50nM, was measured by flow cytometry using a LSR II cell analyzer (Becton-Dickenson, San Jose, CA, USA). After the treatment for 12 hours, the cells were trypsinzed and the Ce6 fluorescence was detected by flow cytometry, with a 405 nm laser coupled with a 655/20 emission filter.

Confocal Fluorescence Microscopy

Intracellular distribution of PEI/ON623-Ce6 polylexes in A375/luc705 cells was examined using an Olympus Confocal FV1200 fluorescent microscope with 60× oil immersion objectives. A375/luc705 cells were plated in 35 mm glass bottom microwell dishes (MatTek, Ashland, MA,

USA) and were then transfected with the PEI/ON623-Ce6 polyplexes (50nM of ON623-Ce6, N/P=3) for 4 hours. After that, the cells were washed and incubated with PBS containing 75nM of Lysotracker Green for 1 hour. Then cells were fixed with fresh prepared 4% paraformaldehyde and DAPI was used for nucleus staining. Cellular distribution of the PEI/ON623-Ce6 polyplexes was observed by confocal microscopy.

In addition, confocal microscopy was also performed to examine the subcellular distribution of ON623-Ce6 with or without photoirradiation. A375/luc705 cells were plated in 35 mm glass bottom dishes and were then treated with the PEI/ON623-Ce6 polyplexes (50nM of ON623-Ce6, N/P=3) for 4 hours. After that, the cells were washed and replaced with fresh medium and incubated for an additional 1 hour. After that, cells were photoirradiated by 660nm laser for 30min (3.5 mW/cm^2). Then the cells were imaged using confocal microscope.

RT-PCR and cell viability

Cells were seeded in 24-well plates at 50,000 cells/well. After culture for overnight, PEI/ON623-Ce6 polyplexes and PEI/Bcl-x-SSO-Ce6 polyplexes (50nM of ON conjugates) were added. Following the treatment for 16 hours at 37 °C, cells were washed and incubated with fresh media for an additional 1 hours. Then cells treated by the polyplexes were photoirradiated with a 660nm laser for 30min at the intensity of 3.5 mW/cm^2. After incubating overnight, total RNA from A375/luc705 cells was isolated using TRIzol® RNA Isolation Reagents (Thermo Fisher, NY) according to the manufacturer's protocol. RT-PCR for Bcl-x was performed as previously described using the specified primers.[3]

The cytotoxicity caused by photochemical delivery of Bcl-x SSO in A375/luc705 and SKOV3 cells was measured with the AlamarBlue assay. Cells were seeded in 96-well plates at 4000 cells/well. After culture for overnight, PEI/ON623-Ce6 or PEI/Bcl-x-SSO-Ce6 polyplexes were added. Following the overnight treatment at 37 °C, cells were washed and incubated with fresh media for an additional 1 hours. Then cells treated by the polyplexes were photoirradiated with a 660nm laser for 30min (3.5 mW/cm^2). Cells treated with PEI/ON623-Ce6 polyplexes in the absence of photoirradiation were set as control because no obvious dark toxicity was observed in previous experiment. After incubation for another 48 hours, the cell viability was detected with AlamarBlue reagent. The fluorescence of AlamarBlue was read in FLUOstar Omega microplate reader (BMG LABTECH, Cary, NC, USA) set at 540 nm excitation wavelength and 590 nm emission wavelength.

Sfigures:

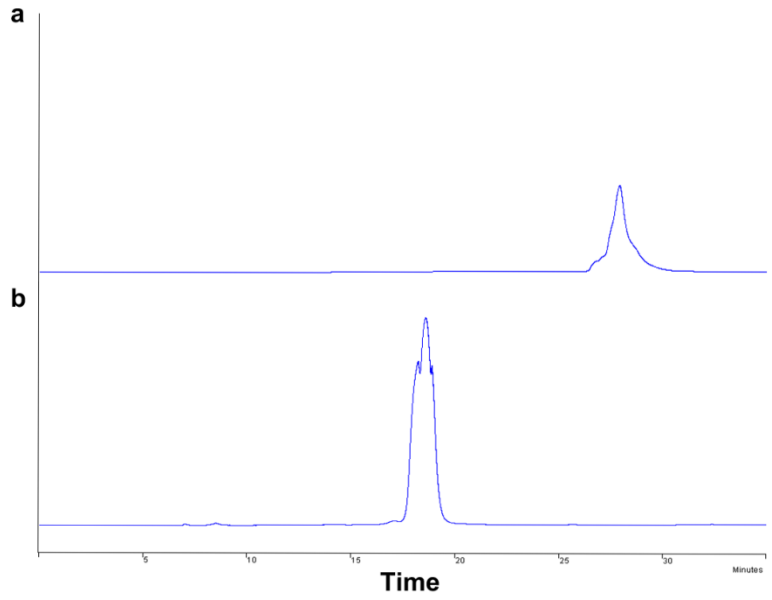

Fig. S1: HPLC chromatography of (a) ON623-Ce6 conjugates and (b) 5'-NH_2-ON623.

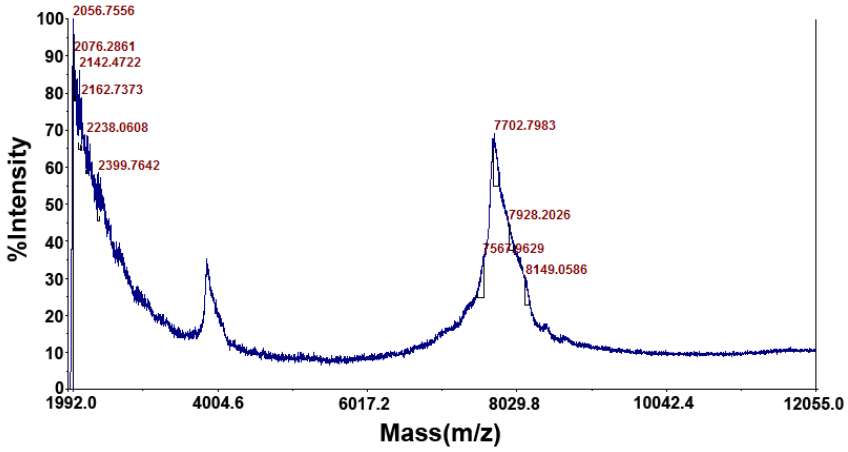

Fig. S2: The MALDI-TOF-MS of ON623-Ce6 Conjugates was carried out on an ABI 4800 MALDI-TOF with 2′,4′,6′-Trihydroxyacetophenone monohydrate (THAP) as matrix.

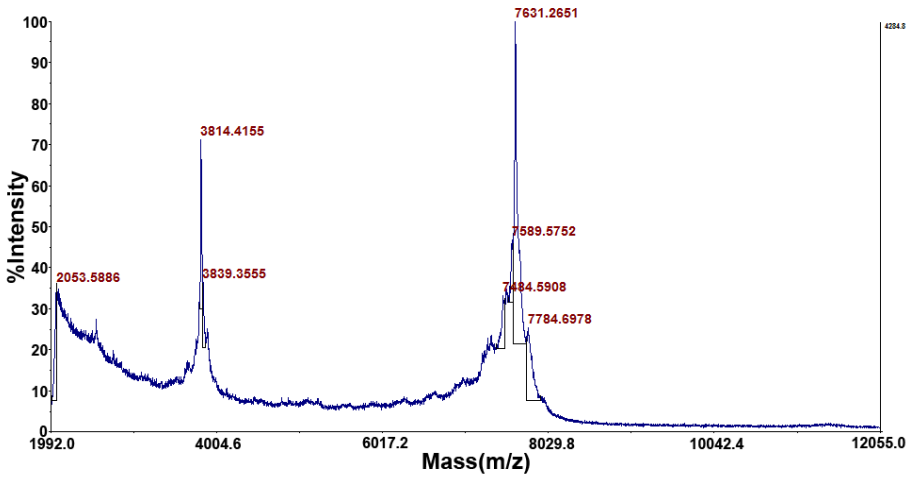

Fig. S3: The MALDI-TOF-MS of Bcl-x-SSO-Ce6 was carried out on an ABI 4800 MALDI-TOF with THAP as matrix.

Table. S1: The MALDI-TOF mass of Bclx-NH_2, Bclx-Ce6, 623- NH_2, and 623-Ce6 Conjugates.

	Calculated (m/z)	Measured (m/z)
[Bclx-NH_2+H]$^+$	7034.6	7041.5
[Bclx-Ce6+H]$^+$	7612.9	7631.2
[623- NH_2+H]$^+$	7086.6	7095.8
[623-Ce6+H]$^+$	7664.9	7702.8

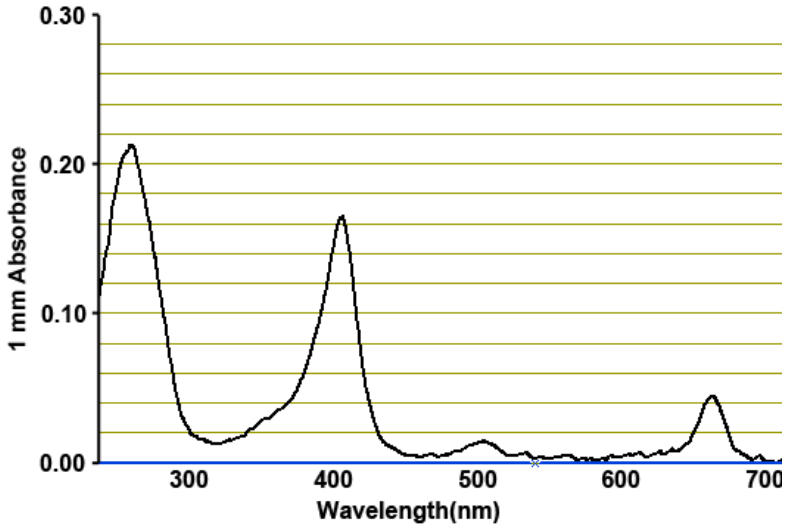

Fig. S4: Absorption spectrum of ON623-Ce6 conjugates.

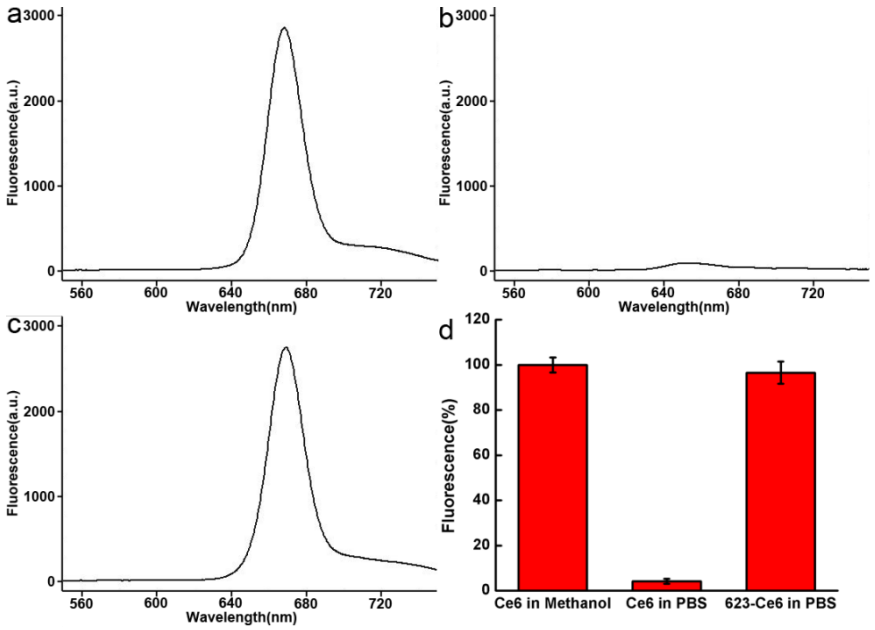

Fig. S5: Fluorescence of 10μM of (a) free Ce6 in methanol, (b) free Ce6 in PBS, (c) ON623-Ce6 conjugates in PBS and (d) quantitative determination. The data was depicted as mean ±SD, n=3.

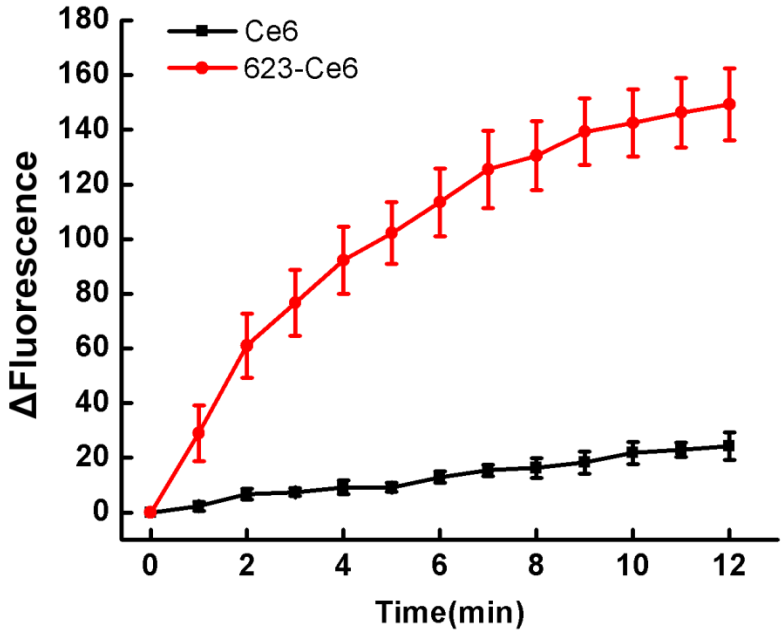

Fig. S6: Change in SOSG fluorescence due to the generation of singlet oxygen by 623-Ce6 conjugates and free Ce6 (1μM) in PBS.

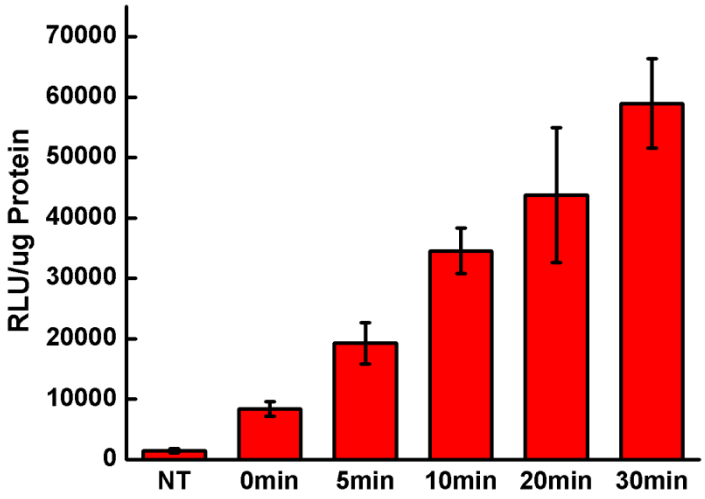

Fig. S7: Luciferase induction of PEI/ON623-Ce6 polyplexes (50nM of ON623-Ce6, N/P=3) after 0, 5, 10, 20, 30 min photoirradiation.

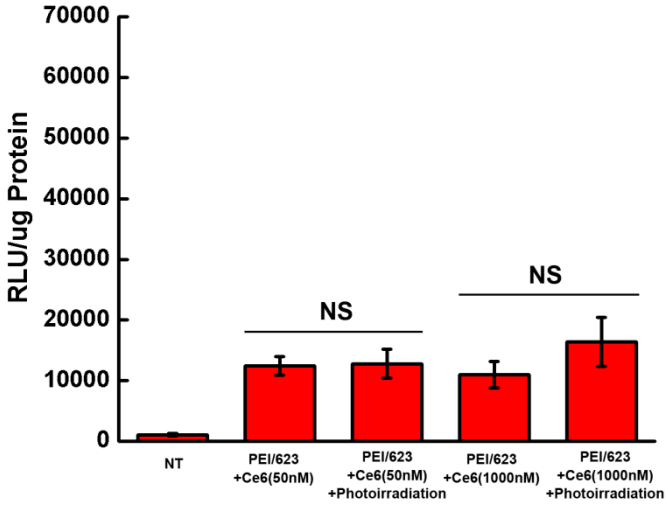

Fig. S8: Luciferase induction of PEI/ON623 (50nM of ON623, N/P=3) with 50 and 1000nM free Ce6 in A375/luc705 cells.

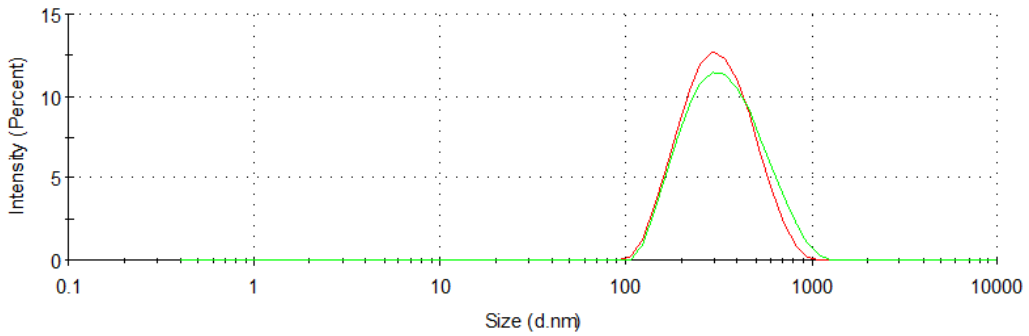

Fig. S9: Size distribution of PEI/ON623-Ce6 (red) and PEI/Bcl-x-SSO-Ce6 (green).

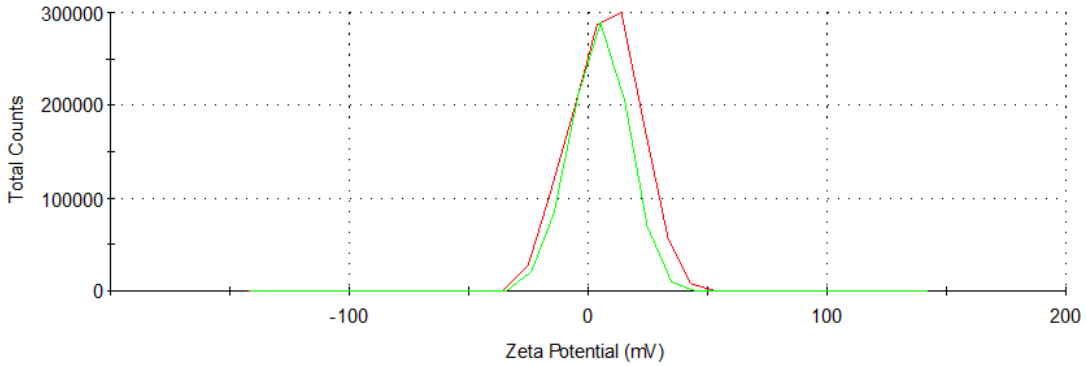

Fig. S10: Zeta potential of PEI/ON623-Ce6 (red) and PEI/Bcl-x-SSO-Ce6 (green).

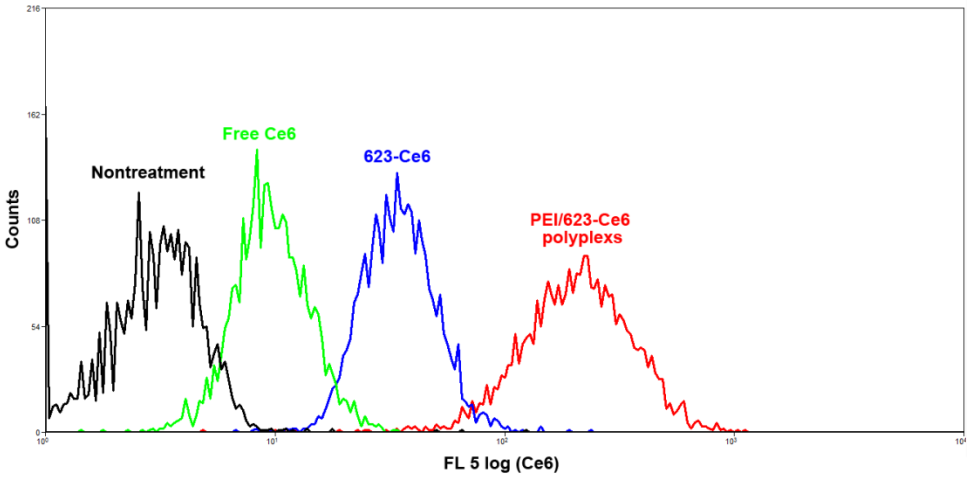

Fig. S11: Cellular uptake of free Ce6, ON623-Ce6 conjugates and PEI/ON623-Ce6 polyplexes (N/P=3,) at the concentration of 50nM in A375/Luc705 cells. After treatments for 12 hours, the fluorescence of Ce6 was measured by flow cytometry.

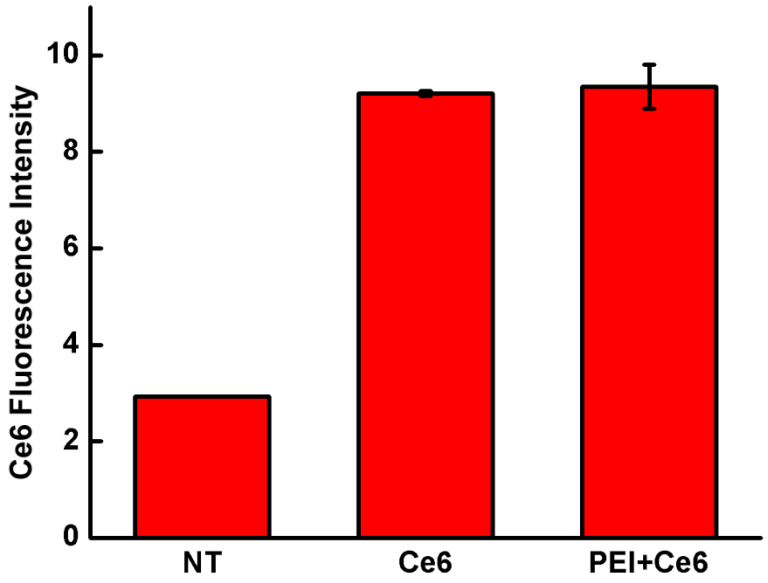

Fig. S12: Cellular uptake of free Ce6 (50nM) with or without PEI. After 12-hour treatment, the fluorescence of Ce6 was measured by flow cytometry. The Ce6 fluorescence intensity was depicted as Mean ±SD, n=3.

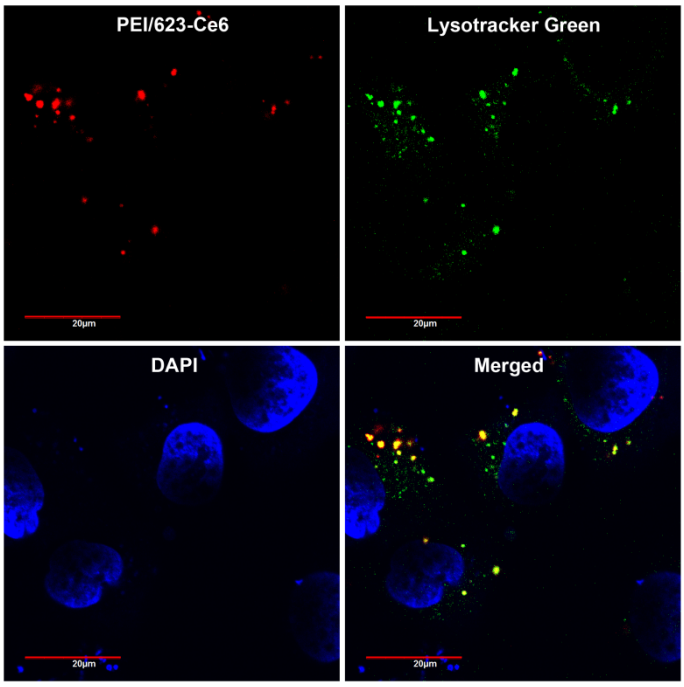

Fig. S13: Subcellular localization of PEI/ON623-Ce6 polyplexes (50nM) in A375/Luc705 cells. Red image, Ce6 fluorescence; Green image, Lysotrakcer Green for endo/lysosomes; Blue image, DAPI for nucleus. Scale bar, 20μm.

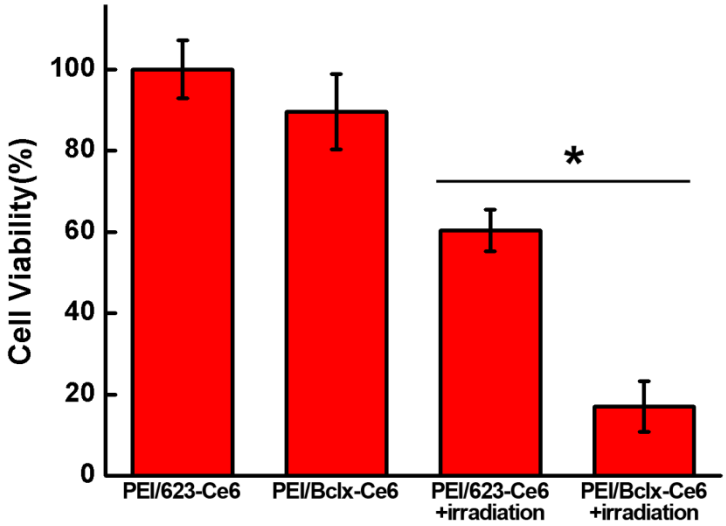

Fig. S14: In vitro cytotoxicity effect in SKOV3 cells induced by PDT, gene therapy, and combined photodynamic and gene therapy. The combined therapy allowed for a significantly higher tumor cell therapeutic effect compared to PDT or gene therapy alone. *p<0.05.

References:

1 X. Ming, M. R. Alam, M. Fisher, Y. Yan, X. Chen and R. L. Juliano, *Nucleic Acids Res*, 2010, **38**, 6567.

2 M. R. Alam, V. Dixit, H. Kang, Z. B. Li, X. Chen, J. Trejo, M. Fisher and R. L. Juliano, *Nucleic Acids Res*, 2008, **36**, 2764.

3 X. Ming, K. Carver, M. Fisher, R. Noel, J. C. Cintrat, D. Gillet, J. Barbier, C. Cao, J. Bauman and R. L. Juliano, *Nucleic acids research*, 2013, **41**, 3673.