Supporting Information

Photo-reactive oligodeoxynucleotide-embedded nanovesicles (PROsomes) with switchable stability for efficient cellular uptake and gene knockdown

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Materials and methods

Materials. α-Methoxy-ω-propylamine poly(ethylene glycol) (PEG-NH_2, M_n = 2,000) was purchased from NOF corporation (Tokyo, Japan). β-Benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) was purchased from Chuo Kaseihin Co., Inc. (Tokyo, Japan). *N,N*-Dimethylformamide (DMF), dichloromethane (DCM), *N*-methyl-2-pyrrolidone (NMP), and 1,5-diaminopentane (DAP) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and distilled before use. Hexane, ethyl acetate, acetic acid, and hydrochloric acid (HCl) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Phosphodiester oligodeoxynucleotides with or without 3-cyanovinylcarbazole modified D-threoninol (^{CNV}D)[1] (*i.e.*, PRO, PolyA, and PolyT) were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). Phosphorothioate antisense oligonulceotides (ASOs) with locked nucleic acids (LNAs) (*i.e.*, asNEAT2 and asGL3) were purchased from GeneDesign, Inc. (Osaka, Japan). Alexa Fluor 647-labeled ASO (AF647-ASO) and Alexa Fluor 546-

labeled ASO (AF546-ASO) were obtained by attaching the fluorophore to the 3′-end of asNEAT2. The detailed structures of oligodeoxynucleotides are summarized in Table S1.

Table S1. Structural information of oligodeoxynucleotides used in this study

Oligonucleotide	Structure (5′ to 3′)
PRO	AAXAAAXAAAXAAAXAAAXA
asNEAT2	**A•T•G•G•**A•G•G•T•A•T•G•A•C•A•T•A•T•A•**A•T•C**
asGL3	**T•C•G•A•**A•G•T•A•C•T•C•A•G•C•G•T•A•A•**G•T•T**
PolyA	AAAAAAAAAAAAAAAAAAAA
PolyT	TTTTTTTTTTTTTTTTTTTT

X: ^{CNV}D-installed A, •: phosphorothioate, **Bold**: LNA (**C**: LNA methylcytosine).

Synthesis of poly(ethylene glycol)-*block*-poly(β-benzyl-L-aspartate) (PEG-PBLA). PEG-PBLA was synthesized by the ring-opening polymerization of BLA-NCA initiated by the terminal primary amino group of PEG-NH_2, as previously reported.[2] Briefly, BLA-NCA (500 mg, 2 mmol) was mixed with PEG-NH_2 (100 mg, 50 μmol) as an initiator in the solution of DMF/DCM mixture (10 mL, 1:9). The solution was stirred at 35 °C for 3 days under Ar. The vanished peaks of carboxylic anhydride group of NCA indicating the completion of polymerization were confirmed by an IR Report-100 instrument (JASCO Corporation, Tokyo, Japan). The resulting solution was precipitated into an excess amount of hexane/ethyl acetate (150 mL, 3:2), followed by the vacuum drying. The molecular weight distribution (M_w/M_n) was determined to be 1.05 by size exclusion chromatography (SEC) (GPC system, Shimadzu Corporation, Kyoto, Japan) using a TSKgel SuperAW4000 column (Tosoh Corporation, Tokyo, Japan; eluent: NMP with 50 mM LiBr).

Synthesis of PEG-*block*-poly[(5-aminopentyl)-α,β-aspartamide] (PEG-P(Asp-AP)). PEG-P(Asp-AP) was synthesized by the aminolysis of benzyl groups in PBLA with DAP, as previously reported.[3] Briefly, freeze-dried PEG-PBLA (50 mg) was dissolved in distilled NMP containing 0.5 M of thiourea (2.5 mL) at 35 °C, and DAP (1.2 mL, 50 equivalent to BLA unit) was diluted with the same volume of NMP. The PEG-PBLA solution was added to the DAP solution dropwise and stirred at 5 °C for 1 h under Ar, and neutralized with HCl at a temperature below 10 °C. The resulting solution was dialyzed against 0.01 M HCl then deionized water at 4 °C, followed by the lyophilization (53 mg, 76% yield). The obtained PEG-P(Asp-AP) was characterized by SEC (HPLC system, JASCO) equipped with a Superdex 75 10/300 GL column (GE Healthcare UK Ltd., UK; eluent: 10 mM acetic acid with 500 mM NaCl) (Fig. S1). The DP of P(Asp-AP) was determined to be 39 by the ^{1}H NMR spectrum (JNM-ECS 400, JEOL Resonance Inc., Tokyo, Japan; 5 mg mL^{-1}, solvent: D_2O, temperature: 80 °C) from the peak intensity ratio of the methylene protons of PEG (δ = 3.7) to the methylene protons of P(Asp-AP) units (δ = 2.8) (Fig. S2).

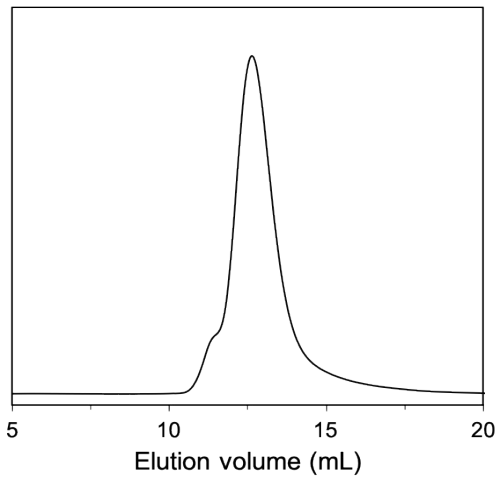

Fig. S1. SEC chart of PEG-P(Asp-AP) (column: Superdex 75 10/300, eluent: 10 mM acetic acid with 500 mM NaCl, 0.75 mL min^{-1}, and room temperature).

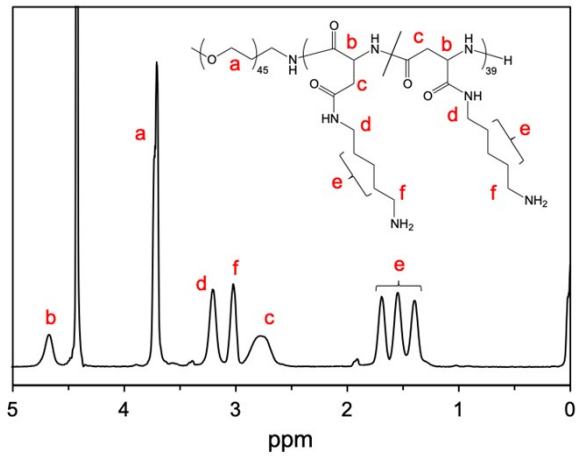

Fig. S2. ^{1}H NMR spectrum of PEG-P(Asp-AP) (concentration: 5 mg mL^{-1}, solvent: D_2O, and temperature: 80 °C).

Preparation of polyion complex (PIC) and crosslinking by UV_{365}. PICs were prepared by vortex mixing of PEG-*block*-polypeptide solution with oligodeoxynucleotide solution at a charge-stoichiometric ratio. Briefly, PEG-P(Asp-AP) was dissolved in 10 mM HEPES sterile buffer (pH 7.3) at a concentration of 1.0 mg mL^{-1} and purified by the filtration through a 220-nm membrane filter. The PEG-P(Asp-AP) solution was mixed with a solution of oligodeoxynucleotide mixture (*e.g.*, PRO/asNEAT2, 1:1) at an equivalent charge ratio of amino groups in PEG-P(Asp-AP) to phosphate (or phosphorothioate) groups in asNEAT2, followed by vortex mixing. For photo-crosslinking, the resulting PIC solution was placed on the parafilm, followed by the UV_{365} irradiation using a UV lamp

(Black-Ray B-100AP; UVP, Upland, CA, USA) (365 nm, 32 mW cm^{-2}) for 1 min, except for the stability assay under a physiological salt condition (Fig. 3a,b).

Characterization of PICs. Intensity-derived hydrodynamic diameter and polydispersity index (PDI) of PICs before and after UV_{365} irradiation were determined by dynamic light scattering (DLS) measurement using a Zetasizer Nano-ZS instrument (ZEN3600, Malvern Instruments, Worcestershire, UK) equipped with a He-Ne ion laser (λ = 633 nm) (Fig. 2a). The morphologies of UV_{365}-irradiated PICs and additionally UV_{312}-irradiated PICs were observed by transmission electron microscopy (TEM; JEM-1400, JEOL) after staining with a 50% ethanol solution containing 2 wt% uranyl acetate (Fig. 2b and Fig. S3, respectively). The additional UV_{312} irradiation to X-PROsomes was performed for 0.5 min, as described below. The obtained vesicular PICs are termed 'PROsome'. The PROsomes are further classified as '(NX-)PROsome' for the non-crosslinked PROsome before UV_{365} irradiation and as 'X-PROsome' for the crosslinked PROsome after UV_{365} irradiation.

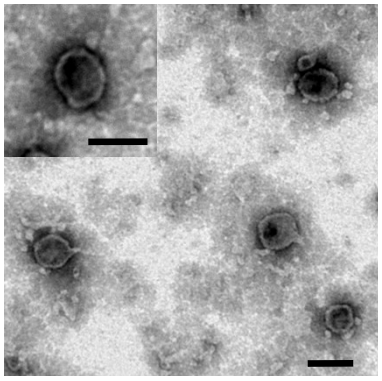

Fig. S3. TEM image of X-PROsomes after UV_{312} irradiation. Inset: high magnification. Scale bars are 100 nm.

Stability of PROsomes under a physiological salt condition. The PROsomes prepared from PEG-P(Asp-AP) and PRO/asNEAT2 in 10 mM HEPES buffer (pH 7.3) were treated with 0, 0.2, 0.5, 1, 2, or 5 min of UV_{365} irradiation and separately mixed with the same volume of 10 mM HEPES buffer (pH 7.3) containing 300 mM NaCl (final NaCl concentration: 150 mM). Time-dependent changes in relative scattered light intensity (SLI) of PROsomes were measured by static light scattering (SLS) using ZEN3600 (Fig. 3a and S4a). Time-dependent changes in hydrodynamic diameter and PDI of PROsomes were measured by DLS using ZEN3600 (Fig. 3b and S4b,c). The X-PROsomes were additionally prepared from the different combinations, *i.e.*, PEG-P(Asp-AP) and PRO/PolyA, PRO/PolyT, or PRO/asGL3. The time-dependent changes in relative SLI of X-PROsomes in 10 mM HEPES containing 150 mM NaCl were measured by SLS (Fig. 3c).

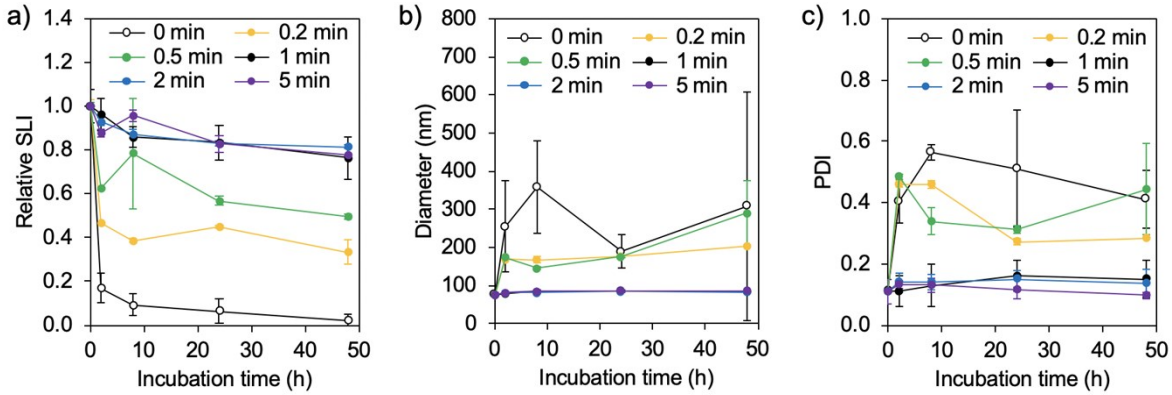

Fig. S4. Time-dependent changes in (a) relative SLI, (b) hydrodynamic diameter, and (c) PDI of PROsomes prepared from PRO/asNEAT2 with varying times of UV_{365} irradiation in 150 mM NaCl solution. All results are expressed as mean ± SD (n = 4).

De-crosslinking of X-PROsomes by UV_{312} irradiation. X-PROsomes prepared from PEG-P(Asp-AP) and PRO/asNEAT2 were immersed in 0 or 150 mM NaCl-containing buffer solution. The sample solutions were placed on a 15 W UV transilluminator (DTB-20MP, ATTO corporation, Tokyo, Japan; 312 nm) and were irradiated by UV_{312} at room temperature for 0.5, 1, 5, and 10 min to induce de-crosslinking. The relative SLI and size distribution histogram of sample solutions were determined by SLS and DLS measurements, respectively, as described above (Fig. 4a and Fig. S5, respectively).

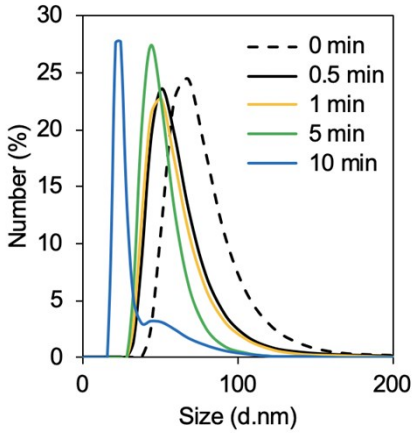

Fig. S5. Size distribution (number-weighted) histograms of X-PROsomes under a physiological salt condition at varying times of UV_{312} irradiation.

Stability of X-PROsomes in serum-containing media. The X-PROsomes prepared from PEG-P(Asp-AP) and PRO/AF647-ASO were immersed in the media containing 10% fetal bovine serum (FBS; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) and 150 mM NaCl. The sample solutions were placed on a 15 W UV transilluminator and were irradiated by UV_{312} for 0.5 min to induce de-

crosslinking. After additional incubation for 10, 30 min, 1, 3, and 6 h at 37 °C, the samples were loaded into an 8-well chamber and immediately analyzed by fluorescence correlation spectroscopy (FCS) using a confocal laser scanning microscopy equipped with a C-Apochromat 40× objective (LSM510; Carl Zeiss, Oberkochen, Germany). The fluorescence detection was carried out using a 633 nm He-Ne laser for excitation and a 650 nm long pass filter for emission. Naked AF647-ASO was used as a control. The diffusion times (D_T) were determined for the samples prepared at 10 nM AF647-ASO with a sampling time of 10 s and a repeating time of 20. The diffusion coefficients (D_C) were calculated from D_T based on a reference of Cy5 maleimide. The hydrodynamic diameter (D_H) was calculated from D_C based on the Strokes-Einstein equation: $D_H = k_B T/3\pi\eta D_C$ (k_B: Boltzmann's constant, T: absolute temperature, and η: dynamic viscosity) (Fig. 4b).

Cell culture. Human lung carcinoma cells (A549, American Type Culture Collection, Manassas, VA, USA) were cultured in a growth medium consisting of Dulbecco's modified Eagle's medium (FUJIFILM Wako Pure Chemical Corporation), 10% FBS, and 1% penicillin-streptomycin (Sigma-Aldrich Co., St. Louis, MO, USA). The ASO transfection and UV_{312} irradiation were performed when the confluence of cells reached approximately 70%.

Cellular uptake. A549 cells were seeded to 12 well plates at 2×10^5 cells per well and incubated for 24 h in the growth medium. PROsomes were prepared from PEG-P(Asp-AP) and PRO/AF647-ASO. Naked AF647-ASO, NX-PROsomes, and X-PROsomes were separately applied to cultured A549 cells at 100 nM AF647-ASO in the growth medium. After 6 h incubation, the cells were rinsed and suspended in PBS. The fluorescence intensity of cells (1×10^4 cells) was measured by flow cytometer (BD™ LSR II, BD Biosciences, San Jose, CA, USA) equipped with FACSDiva software (BD Biosciences) (Fig. 5a). In uptake inhibition assays, A549 cells were seeded to 48 well plates at 2×10^4 cells per well and incubated for 24 h in the growth medium. Then, the cells were incubated at 4 or 37 °C for 4 h in the growth medium with X-PROsomes prepared from PRO/AF647-ASO at 25, 50, 100, and 200 nM AF647-ASO concentrations. Alternatively, the cells were treated with a macropinocytosis inhibitor, cytochalasin D (FUJIFILM Wako Pure Chemical Corporation),[4] at varying concentrations for 1 h, and then, incubated with X-PROsomes at 100 nM AF647-ASO concentration for 24 h. After incubation, the cells were washed twice with PBS and their fluorescence intensities were measured by a microplate reader (Spark®, Tecan Group Ltd., Männedorf, Switzerland) (Fig. S6).

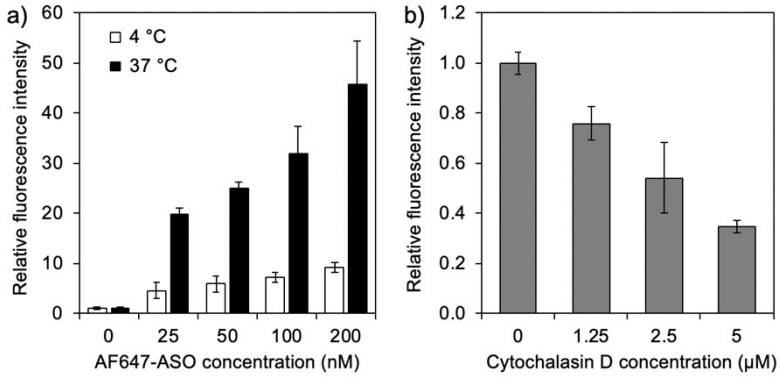

Fig. S6. Uptake inhibition assays of X-PROsomes prepared from PRO/AF647-ASO. Relative fluorescence intensities of cultured A549 cells treated with (a) varying concentrations of X-PROsomes at 4 or 37 °C for 4 h and (b) X-PROsomes at 100 nM AF647-ASO for 24 h after 1 h pretreatment with varying concentrations of cytochalasin D at 37 °C. Relative fluorescence intensities were calculated by normalizing the obtained values to those from nontreated cells at 37 °C. All results are expressed as mean ± SD (n = 4).

Cytotoxicity associated with UV_{312} irradiation. A549 cells were seeded to 96 well plates at 1×10^4 cells per well and incubated for 24 h in the growth medium. After replacing the growth medium with PBS, the cells were irradiated by UV_{312} on a 15 W UV transilluminator at different exposure times. After irradiation, PBS was replaced with the growth medium and the cells were incubated in a CO_2 incubator for 2 h. The cell viability of A549 cells was evaluated using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instruction. The absorbance was measured at 450 nm using a microplate reader (Spark®) (Fig. S7).

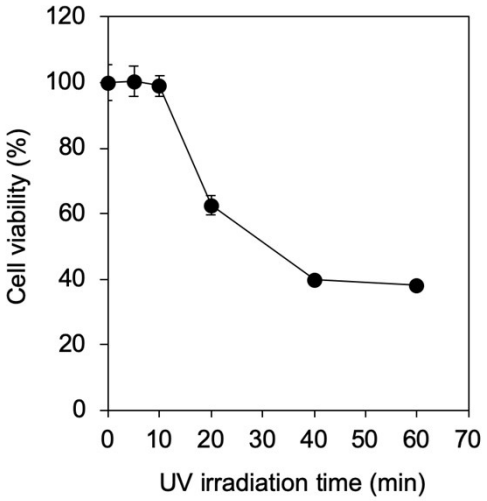

Fig. S7. Viability of cultured A549 cells after UV_{312} irradiation at varying times (mean ± SD, n = 4).

RNA amount in cells after UV_{312} irradiation. A549 cells were seeded to 24 well plates at 5×10^4 cells per well and incubated for 24 h in the growth medium. After replacing the growth medium with PBS, A549 cells were irradiated by UV_{312} on a 15 W UV transilluminator at different exposure times. Then, PBS was replaced with the growth medium and the cells were incubated in a CO_2 incubator for 24 h. After incubation, RNA was extracted from the cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instruction. The total amount of extracted RNA was determined from the absorbance at 260 nm (A_{260}) using NanoDrop One (Thermo Fischer Scientific, Wilmington, DE, USA) (Fig. S8).

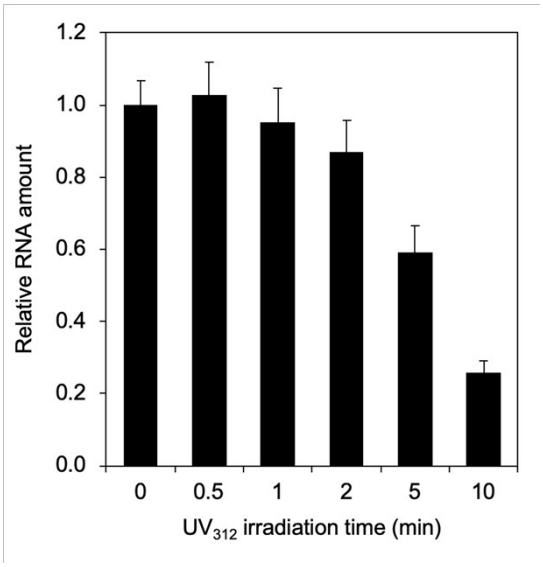

Fig. S8. Relative RNA amount extracted from A549 cells treated with UV_{312} irradiation at varying times (mean ± SD, $n = 4$).

Confocal laser scanning microscopic (CLSM) observation. A549 cells were seeded to 8 well Lab-Tek chambers at 1×10^4 cells per well and incubated for 24 h in the growth medium. X-PROsomes were prepared from PRO/AF647-ASO/AF546-ASO (2:1:1) to a final concentration of 10 μM PRO and 5 μM AF647-ASO and AF546-ASO, and were applied to the cells at 200 nM AF647-(and AF546-)ASO. At 24 h post-transfection, cells were treated with 0.5 min UV_{312} irradiation. After 24 or 48 h incubation, the cell nuclei were stained with Hoechst 33342 (Dojindo, Kumamoto, Japan), and then, the cells were washed with PBS twice and supplemented with fresh medium. The CLSM images were obtained by LSM 880 equipped with a C-Apochromat 40× objective (ZEISS). Excitation wavelengths were set at 633 nm (He-Ne laser), 488 nm (Ar laser), and 405 nm (Diode laser) for excitation of AF647-ASO, AF546-ASO, and Hoechst 33342, respectively (Fig. S9a). Colocalization of AF647-ASO and AF546-ASO in the cells was statistically analyzed by calculating Pearson's correlation coefficients using ImageJ. Results are represented as the mean ± SD from six cells (Fig. S9b).

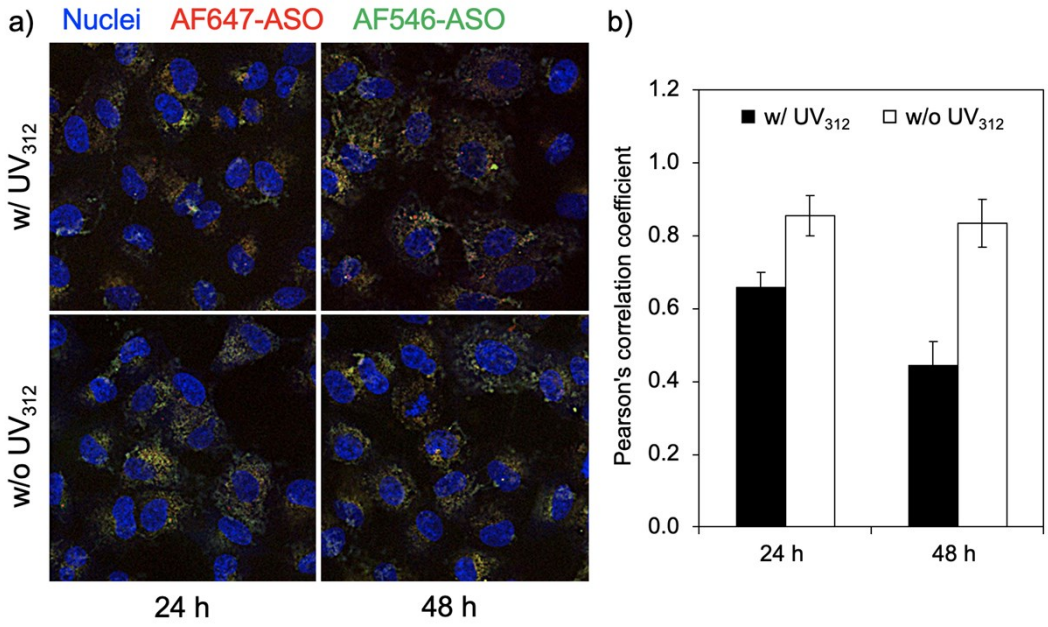

Fig. S9. (a) CLSM images of cultured A549 cells treated with X-PROsomes at 200 nM ASO for 24 and 48 h after UV_{312} irradiation for 0.5 min. The X-PROsomes were prepared by AF647-ASOs (red) and AF546-ASOs (green) at a molar ratio of 1:1. (b) Pearson's correlation coefficients between AF647-ASOs and AF546-ASOs in cultured A549 cells, which were determined using ImageJ. Results are expressed as mean ± SD (n = 6).

Gene knockdown assay. A549 cells were seeded to 24 well plates at 5 × 10^4 cells per well and incubated for 24 h in the growth medium. The A549 cells were transfected in the growth medium with PROsomes prepared from PRO/asNEAT2 (or PRO/asGL3 as a control) with/without UV_{365} irradiation at 100 nM asNEAT2 (or asGL3) concentration. After 24 h incubation, the growth medium was replaced with PBS, followed by UV_{312} irradiation for 0.5 min. PBS was replaced with the growth medium and the cells were incubated in a CO_2 incubator for another 24 or 48 h (Fig. S10). At total 48 or 72 h incubation, RNA was extracted using RNeasy Mini Kit, according to the manufacturer's instruction. The total amount of extracted RNA was determined from the absorbance at 260 nm (A_{260}) using NanoDrop One. Genomic DNA elimination and cDNA synthesis were performed using a QuantiTect reverse transcription kit (Qiagen). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using FastStart Universal SYBR-Green Master (Roche, Basel, Switzerland). The reaction was carried out on the ABI 7500 Fast Real-time RT-PCR System (Applied Biosystems, Foster City, CA, USA). A house-keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used as an internal standard to determine a relative expression level of NEAT2 lncRNA. Primer sequences were for GAPDH: forward 5′-CCA CCC ATG GCA AAT TCC-3′, reverse 5′-CAG GAG GCA TTG CTG ATG AT-3′[5] and for NEAT2: forward 5′-GAC GGA GGT TGA GAT GAA GC-3′, reverse 5′-ATT CGG GGC TCT GTA GTC CT-3′ (Fig. 5b).[6]

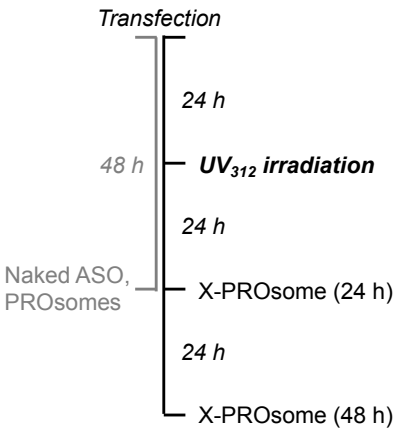

Fig. S10. Transfection and irradiation schedules for the gene knockdown study.

Cytotoxicity of X-PROsomes. A549 cells were seeded to 96 well plates at 1×10^4 cells per well and incubated for 24 h in the growth medium. The A549 cells were transfected in the growth medium with naked asGL3 or X-PROsomes prepared from PRO/asGL3 at 100–800 nM asGL3 concentrations. After 24 h incubation, the growth medium was replaced with PBS, followed by UV_{312} exposure for 10 min, as described above. After irradiation, PBS was replaced with the growth medium and the cells were incubated in a CO_2 incubator for another 24 h. At total 48 h incubation, the cell viability was evaluated using Cell Counting Kit-8 (Fig. S11).

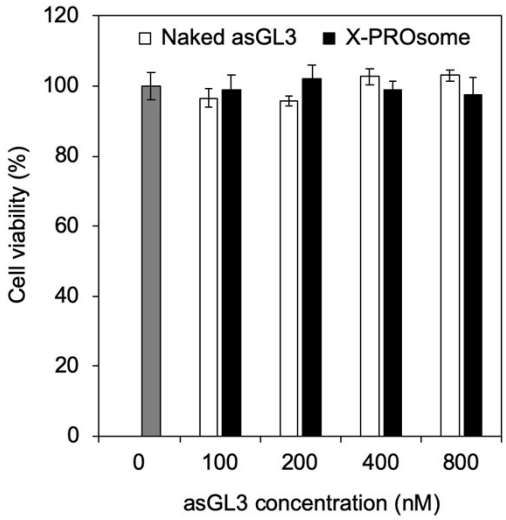

Fig. S11. Viability of cultured A549 cells treated with naked asGL3 or X-PROsomes at varying asGL3 concentrations and UV_{312} irradiation for 10 min (mean ± SD, $n = 4$).

Statistical analysis. The comparison among groups was performed by a one-way ANOVA with post-hoc test. Differences were considered statistically significant when $p < 0.05$.

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