

Development of a reduction-sensitive diselenide-conjugated oligoethylenimine nanoparticulate system as a gene carrier

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Background: The reduction-sensitive cationic polymer is a promising nonviral carrier for gene delivery. Until now, disulfide bonds have been the only golden standard for its design. The aim of this research was to develop a novel reduction-responsive cationic polymer as a gene carrier.

Methods: Polycationic carriers were synthesized by addition of branched oligoethylenimine 800 Da (OEI₈₀₀) via an active ester containing diselenide bonds. Disulfide bonds cross-linked with OEI₈₀₀-SS_x and monoselenide bonds linked with OEI₈₀₀-Se_x were synthesized and compared. Their molecular weights and degradation properties were determined using gel permeation chromatography. Changes in particle size, morphology, and DNA binding were investigated by dynamic light scattering, transmission electron microscopy, and electrophoresis assay in a reduction environment. Cytotoxicity and transfection in vitro were evaluated in a murine melanoma cell line (B16F10) and a human cervical epithelial carcinoma cell line (HeLa), while intracellular degradation and dissociation with DNA were studied by confocal laser scanning microscopy with FITC-labeled OEI₈₀₀ derivatives and Cy5-labeled DNA.

Results: Diselenide-conjugated OEI₈₀₀ (OEI₈₀₀-SeSe_x) polymer carriers of high molecular weight were successfully synthesized. After compacting with DNA, the OEI₈₀₀-SeSe_x polymers formed nanoparticles with an average size of 140 nm at an adequate C/P ratio. OEI₈₀₀-SeSe_x showed reduction-responsive degradation properties similar to those of the OEI₈₀₀-SS_x via gel permeation chromatography, dynamic light scattering, and transmission electron microscopy. OEI₈₀₀-SeSe_x showed much lower cytotoxicity than PEI_{25k}, and significantly higher transfection efficiency than OEI₈₀₀ in both B16F10 and HeLa cells. Transfection of luciferase in the OEI₈₀₀-SeSe_x group was comparable with that of standard PEI_{25k} and traditional reduction-sensitive polymer OEI₈₀₀-SS_x groups. Furthermore, intracellular degradation of OEI₈₀₀-SeSe_x and dissociation with DNA were also confirmed by confocal laser scanning microscopy.

Conclusion: The OEI₈₀₀-SeSe_x obtained was able to bind plasmid DNA efficiently to yield nanosized particles and had reduction sensitivity which is as efficient as that for OEI₈₀₀-SS_x. In vitro experiments confirmed its low cytotoxicity and high transfection ability. Diselenide bonds can be used as effective and novel reduction-sensitive linkages for gene delivery.

Keywords: diselenide, oligoethylenimine, reduction-sensitive, gene carriers

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Introduction

Cationic polymer-mediated nonviral gene delivery has emerged as a viable alternative to viral vectors because of its ease of manufacturing, no limitation on DNA size, lesser inflammatory response, and lack of immunogenicity.^{1,2} However, cationic polymers are problematic in terms of the delivery process. Between the time when the complexes are assembled and when they are taken up by endosomes, the nucleic acids

need to be well compacted within the cationic polymers to form complexes, thus requiring strong binding interactions to prevent gene degradation as a result of mechanical stress and enzymatic activity.³ On the other hand, after entry into the cell or endosomal escape, the complexes should dissociate to enable efficient gene release, which involves movement of DNA into the nucleus for transcription or targeting siRNA to mRNA for RNA interference.^{1,4,5} Moreover, high transfection efficiency is often associated with high toxicity of the vector. A typical example is polyethylenimine (PEI), which is one of the most effective nonviral gene delivery carriers due to its DNA-condensing capability, proton sponge effect for osmotic swelling, and physical rupture of the endosome.^{6–10} PEI with its high molecular weight is necessary for effective gene transfection, but its presence also results in high cytotoxicity. In contrast, low molecular weight PEI has low toxicity but cannot condense DNA effectively and has poor transfection ability.¹¹ Therefore, one approach is to design biodegradable polycations which allow efficient gene transfer and stimulus-responsive degradation into low molecular weight products with minimal toxicity.

Intracellular and extracellular environments differ in their pH, enzymatic activity, and redox potential. For example, some intracellular compartments, such as late endosomes and lysosomes, are more acidic than normal physiological pH.^{12,13} High matrix metalloproteinase expression is observed in tumor cells,¹⁴ and glutathione levels are higher intracellularly than extracellularly,^{15–17} being approximately 1000-fold higher than the concentration in plasma.¹⁸ Furthermore, other intracellular reductive reagents, such as nicotinamide adenosine dinucleotide phosphate (NADPH), can change oxidative glutathione to reduced glutathione.¹⁹ These factors can be used as triggers for disassociation of polycation and gene payload. Various chemical linkers responsive to different triggers have been designed, including acetals,^{20–22} hydrazone bonds,^{23–26} esters,⁶ disulfides,^{27–33} and matrix metalloproteinase substrate peptide-containing linkers.¹⁴ Among them, disulfide bonds have been widely used as the only golden standard for reductive design in gene and drug delivery.^{27,29,34–37} After it was found that low molecular weight oligoethylenimine (OEI) cross-linked by dithiobis (succinimidylpropionate) or dimethyl-3,3'-dithiobispropionimidate·2HCl achieved highly efficient transfection in vitro and had lower cytotoxicity,³⁴ much research attention has been focused on modification of different disulfide bonds containing linkers^{27–29,35} and effect of the molecular weight of cross-linked OEI.³⁵

Inspired by the success of disulfide bonds, the selenium (Se) element listed in the same family as sulfur (S) in the

periodic table of elements has attracted attention. Diselenide bonds have also been considered as potential candidates for stimuli-sensitive design. As reported recently, diselenide bonds possess reductive biodegradable ability similar to that of disulfide bonds.^{19,40} Micelles formed by polymers containing diselenide bonds were quite stable under physiological conditions, but were sensitive to reductive stimuli.³⁶ In addition, disulfide bonds were too fragile for the reductive environment, but can be cleaved by a reductive reagent in low concentration.³⁵ This property is not beneficial for transfection in vivo, because the disulfide may be destroyed in the circulation, so the loaded genes cannot be delivered to their desired location.

In this study, diselenide bonds were introduced into a non-viral gene delivery system for the first time. The diselenide bonds were designed to be cleavable due to reduction, resulting in release of DNA inside the cell. Specifically, branched OEI 800 Da (OEI₈₀₀) was conjugated with diselenide bonds and with disulfide bonds, and their potential for reduction sensitivity was tested and compared.

Materials and methods

Materials

Selenium powder and sodium borohydride (NaBH₄) were obtained from Kelong Chemical Company (Chengdu, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Life Technologies Corporation (Gibco, Grand Island, NY), and 3-chloropropanoic acid, 3, 3'-disulfanediyldipropanoic acid (DSDPA), reduced glutathione, antibiotics (penicillin and streptomycin), branched polyethylenimine 25 kDa (PEI_{25k}), oligoethylenimine (OEI₈₀₀), and fluorescein isothiocyanate (FITC) were obtained from Sigma-Aldrich (Shanghai, China). N-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC) were purchased from AstaTech Pharmaceutical (Chengdu, China). 1,4-dithiothreitol (DTT) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were commercially available from Amresco (Solon, OH). Cell lysate and the luciferase reporter gene assay kit were purchased from Promega (Madison, WI). A bicinchoninic acid protein assay kit was purchased from Pierce (Rockford, IL). The DNA labeling kit, Label IT[®]Cy5, was from Mirus Bio Corporation (Madison, WI), plasmid pGL3 (encoding *Photinus pyralis* luciferase under control of the SV40 enhancer/promoter) and enhanced green fluorescent protein encoding plasmid (pEGFP) was purified using the EndoFree plasmid kit from Qiagen (Hilden, Germany). All buffers were prepared in

MilliQ ultrapure water and filtered (0.22 μm) prior to use. All other chemicals were purchased from Sigma-Aldrich and used as received.

Synthesis of DSeDPA and SeDPA

3, 3'-Diselanediyldipropanoic acid (DSeDPA) was synthesized in the same manner as in previous studies, with some modifications.^{37,38} Selenium powder (2.37 g, 30 mmol) in 10 mL of water was added to a three-necked flask under a nitrogen atmosphere. NaBH_4 (2.27 g, 60 mmol) dissolved in 25 mL of cold H_2O was syringed dropwise into the selenium suspension. The reaction mixture was stirred at 0°C and reacted until colorless for complete dissolution of the selenium powder. Another quantity of selenium powder (2.37 g, 30 mmol) was added, and the mixture was heated to 105°C for 20 minutes until reddish brown. 3-Chloropropanoic acid (6.5 g, 60 mmol) was dissolved in 15 mL of water, and its pH was adjusted to 8.0 with sodium carbonate (Na_2CO_3). This was added to the reddish brown solution, with stirring overnight at room temperature under nitrogen. After another 4 hours of stirring and exposure to the atmosphere, the reaction mixture was filtered. The yellow supernatant was adjusted to pH 3–4 using 1 mol/L HCl solution and extracted twice with ethyl acetate. The combined organic layers were washed with water, dried with anhydrous magnesium sulfate, filtered, and recrystallized from ethyl acetate to give a product of 5.29 g (a 58% yield). $^1\text{H-NMR}$ (400 MHz, d_6 -DMSO): δ 2.71 (t, 2H), 3.05 (t, 2H). $^{13}\text{C-NMR}$ (d_6 -DMSO): δ 23.93, 35.41, 173.05. $^{77}\text{Se-NMR}$ (400 MHz, d_6 -DMSO): δ 318.98. MS *m/z*: 302/306 ($^{78}\text{Se}/^{80}\text{Se}$) (shown in Supplementary Figure 1).

Synthesis of 3, 3'-selenodipropanoic acid (SeDPA) was similar to the process used for DSeDPA, but with a different ratio of selenium powder to NaBH_4 . Selenium powder (2.37 g, 30 mmol) was mixed with NaBH_4 (2.27 g, 60 mmol) to obtain a colorless solution without adding another quantity of selenium powder, and then reacted with 3-chloropropanoic acid, with the same procedure followed after treatment. A final product of 3.85 g was obtained (a 57% yield). $^1\text{H-NMR}$ (400 MHz, d_6 -DMSO): δ 2.62 (t, 2H), 2.69 (t, 2H). $^{13}\text{C-NMR}$ (d_6 -DMSO): δ 17.70, 35.40, 173.26 (as shown in Supplementary Figure 2).

Synthesis of disulfide, diselenide and monoselenide bonds for conjugation with OEI

DSeDPA (0.365 g, 1.2 mmol), DSDPA (0.252 g, 1.2 mmol), or SeDPA (0.270 g, 1.2 mmol) and NHS

(0.331 g, 2.88 mmol) dissolved in 5 mL of anhydrous tetrahydrofuran were added to a three-necked flask under nitrogen with magnetic stirring. EDC (0.445 g, 2.88 mmol) dissolved in 5 mL of anhydrous tetrahydrofuran was added dropwise into the mixture at 0°C . The reaction mixture was stirred overnight at room temperature. After filtration, evaporation, and redissolution in 0.5 mL of anhydrous dimethyl sulfoxide, the active ester solution (DSeDPA-NHS, DSDPA-NHS or SeDPA-NHS) obtained was ready for further use.

Prior to cross-linking, 0.8 g (1 mmol) of oligoethylenimine (OEI_{800}) was dissolved in water with pH adjusted to 7.4 by HCl solution, and lyophilized. It was redissolved in 2 mL of dimethyl sulfoxide, and mixed with various active ester solutions (DSeDPA-NHS, DSDPA-NHS, or SeDPA-NHS) under nitrogen. The solution was stirred continuously for 2 days at 35°C , and dialyzed against water using cellulose dialysis bags (molecular weight cutoff 7 kDa). The final products of $\text{OEI}_{800}\text{-SeSe}_x$, $\text{OEI}_{800}\text{-SS}_x$, and $\text{OEI}_{800}\text{-Se}_x$ were obtained after lyophilization.

NMR characterization

NMR spectra for ^1H , ^{13}C , and ^{77}Se were recorded on a Bruker Avance II NMR spectrometer at 400 MHz using $\text{DMSO-}d_6$ as solvents, with 0.5% tetramethylsilane as the internal standard.

Molecular weight characterization

The molecular weight of the products was determined by gel permeation chromatography (Waters, Milford, MA). A Waters 2690D high-pressure liquid chromatography system was equipped with Ultrahydrogel 1000 and 120 columns, as well as a refractive index detector. NaCl solution 0.1 M with pH adjusted to 2.8 by HCOOH was used as the eluent at a flow rate of 1.0 mL per minute. The external and column temperatures were kept at 35°C . Pullulan of a different molecular weight (1 mg/mL) was used as the standard for determination of the calibration curve.

Measurement of degradation

To measure degradation, 5 mg of $\text{OEI}_{800}\text{-SeSe}_x$ or $\text{OEI}_{800}\text{-SS}_x$ polymer dissolved in 0.9 mL of water was treated with 0.1 mL of glutathione (1.0 mol/L), DTT (1.0 mol/L), or NaBH_4 (1.0 mol/L) at 37°C for 2 hours, respectively. $\text{OEI}_{800}\text{-Se}_x$, PEI_{25k}, and OEI_{800} were used as controls.²⁷ Degradation of OEI_{800} and other analogs cross-linked with disulfide and diselenide in reductive conditions was evaluated by gel permeation chromatography.

Complex formation, particle size, zeta potential, and morphology

A cationic polymer solution in HEPES buffered glucose (20 mM HEPES and 5% [w/v] glucose, pH 7.4) was gently mixed with plasmid DNA HEPES buffered glucose (100 µg/mL) for preparation of complexes with different C/P ratios (ie, weight ratios of cationic polymer to plasmid DNA). The nanoparticle suspension was incubated at room temperature for 20 minutes before use.

Particle sizes and zeta potentials of the complexes were characterized using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The incubated complexes (C/P 1.2, 3, or 6) were diluted to 1 mL with MilliQ water to a final plasmid DNA concentration of 3 µg/mL. For measurement in a reductive environment, DTT or NaBH₄ 25 mM was added to the complexes and incubated at 37°C for 30 minutes prior to dilution. The morphology of the complexes was characterized by transmission electron microscopy. A drop of complex (C/P 6) suspension incubated with or without a reductive reagent was deposited on amorphous carbon-coated copper grids, dried, dyed using 2% (w/w) phosphomolybdic acid, and detected using a JEOL JEM-100CX electron microscope (Tokyo, Japan).

DNA binding determined by electrophoresis assay

Suspensions of various complexes (OEI₈₀₀-SeSe_x, OEI₈₀₀-SS_x, OEI₈₀₀-Se_x, PEI_{25k}, or OEI₈₀₀) with different C/P ratios (0.02–1.4) were loaded onto 1% agarose gel. Electrophoresis was performed in Tris-acetate-EDTA buffer (pH 8) running at 85 V for 40 minutes. The gel was stained with ethidium bromide and analyzed on an ultraviolet illuminator (BioRad, Hercules, CA) to visualize the location of the DNA bands. Compaction of the complexes under reduction conditions was evaluated after incubation with DTT at a final concentration of 25 mM at 37°C for 30 minutes.

Cell culture and cytotoxicity assay

Murine melanoma cells (B16F10) and human cervical epithelial carcinoma cells (HeLa) obtained from the Shanghai Institute for Biological Science (Shanghai, China) were used for the cytotoxicity study. The B16F10 and HeLa cells were cultured in DMEM containing penicillin 100 U/mL, streptomycin 100 µg/mL, and fetal bovine serum 10% at 37°C in the presence of 5% CO₂.

Relative cell viability when incubated with the cationic polymer solution was determined by MTT assay. The B16F10 and HeLa cells were seeded into a 96-well tissue

culture plate at a density of 8×10^3 cells per well in 100 µL of DMEM. A designated amount of cationic polymer solution (OEI₈₀₀-SeSe_x, OEI₈₀₀-SS_x, OEI₈₀₀-Se_x, PEI_{25k}, and OEI₈₀₀ at a final concentration of 5–50 µg/mL) were added once cell confluence had reached 70%–80%. After 24 hours of incubation, the cytotoxicity measurement was performed according to the usual laboratory protocol, and the ultraviolet value of the dissolved formazan crystals was recorded at 570 nm using a BioRad 550 microplate reader.

Gene transfection of pEGFP and pGL3

pEGFP and pGL3 was used to determine the transfection efficiency of the OEI₈₀₀ derivatives. B16F10 and HeLa cells were seeded into 96-well plates at a density of 8×10^3 cells per well in 100 µL of DMEM, and the cells were incubated overnight for attachment. Directly before transfection, the medium in each well was replaced with 100 µL of fresh serum-free DMEM containing various amount of complexes (200 ng of plasmid DNA per well, C/P ratios 4–14). After 4 hours of incubation, the medium was replaced with 100 µL of fresh serum-containing medium. Qualitative evaluation of pEGFP transfection was observed under a fluorescence microscope (Leica, Germany) after further incubation for 48 hours. Subsequently, the cells were washed with ice-cold phosphate-buffered solution, harvested, and placed in 1 mL of phosphate-buffered solution containing 10% fetal bovine serum. The percentage of transfected cells was determined by flow cytometry (BD FACSAria™ II, Franklin Lakes, NJ), and was obtained by determining the statistics of cells fluorescing above the control level, where nontransfected cells were used as the control. Quantitative measurement using the luciferase assay was carried out after 24 hours of incubation according to the manufacturer's protocol. Relative light units were measured using a BioRad 550 microplate reader while total protein was measured using a bicinchoninic acid protein assay kit. Luciferase activity was expressed as relative fluorescence intensity per mg protein.

Intracellular fate of nanoparticles by confocal laser scanning microscopy

B16F10 cells were seeded at a density of 2×10^4 cells per well in a 35 × 12 mm glass-bottomed chamber (NEST, Wuxi, China) the day before use. Cy5-labeled pEGFP DNA was prepared according to the DNA labeling kit (Label IT®Cy5), while FITC-labeled cationic polymer was obtained via conjugation of FITC to the cationic polymer.³⁹ Complexes containing 40% Cy5-labeled pEGFP and FITC-labeled cationic polymer with a C/P ratio of 6 were added to serum-free fresh medium at

300 ng of plasmid per chamber. At the desired incubation time points (0.5, 1, 2, 4 and 8 hours), the cells were washed twice with phosphate-buffered solution and observed with a confocal laser scanning microscope (Leica TCS SP5, Germany). The Cy5 fluorophore was excited at 543 nm and emission was detected using a 565–615 nm band pass filter, while FITC was excited at 495 nm and emission was detected using a 515–545 nm band pass filter. Data were analyzed using LAS AF Lite 2.3 software (Leica, Germany).⁴⁰

Data and statistical analysis

Group data were reported as the mean \pm standard deviation. Differences between groups were analyzed by two-tailed heteroscedastic *t*-tests using Microsoft Excel 2010, and statistical significance was accepted at $P < 0.05$.

Results

Polymer synthesis and structural analysis

The synthetic route of various cross-linked OEI₈₀₀ (reduction-responsive and stable derivatives) is shown in Figure 1. Diselenide bonds cross-linked with OEI₈₀₀ (OEI₈₀₀-SeSe_x) was started by preparation of diselenide bonds containing the linker DSeDPA. The carboxyl group at the terminal end of DSeDPA was activated by NHS, using EDC as the dehydrolyzing agent in tetrahydrofuran. The active ester reacted with the primary amine group of the OEI₈₀₀, resulting in diselenide bonds containing OEI₈₀₀-SeSe_x. For controls, the

corresponding stable analog OEI₈₀₀-Se_x, and the standard reduction-responsive polymer OEI₈₀₀-SS_x containing disulfide bonds, were prepared (Figure 1). All the critical steps were confirmed by ¹H NMR. The chemical environment of the hydrogen close to selenium was different between DSeDP and SeDPA. The peak at 3.05 ppm reflects the signal of CH₂ in DSeDPA, while the peak of 2.62 ppm is assigned to the protons of CH₂ in SeDPA.⁴¹ Accordingly, the peak of CH₂ close to selenium in DSeDPA detected by ¹³C-NMR is 23.93 ppm, while that in SeDPA is 17.70 ppm. (Supplementary Figures 1 and 2).

Molecular weight and degradation of diselenide, monoselenide, and disulfide bonds conjugated with OEI

The molecular weights of the reduction-sensitive OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x polymers and the stable OEI₈₀₀-Se_x polymer were detected by gel permeation chromatography in the absence or presence of the reduction substance (glutathione, DTT, or NaBH₄, Table 1). Standard PEI_{25k} and OEI₈₀₀ showed average molecular weights of 16 kDa and 0.76 kDa, while the molecular weights of the cross-linked OEI₈₀₀-SS_x and OEI₈₀₀-SeSe_x polymers were about 12 kDa and 18 kDa, respectively. OEI₈₀₀-Se_x was also cross-linked as a high molecular weight polymer of about 15 kDa. After incubation with the reductive reagents, the peak shift of the different polymers in gel permeation chromatography

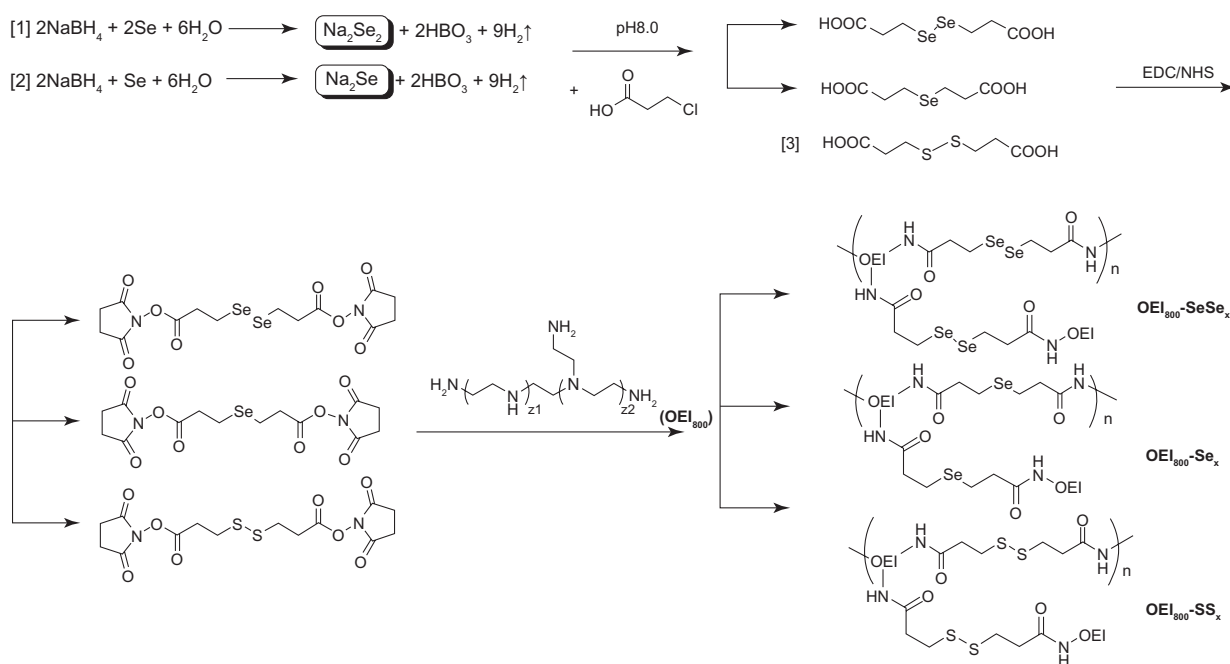


Figure 1 Synthetic schema for OEI₈₀₀-SeSe_x (1), OEI₈₀₀-Se_x (2), and OEI₈₀₀-SS_x (3).

Abbreviations: OEI, oligoethylenimine; NHS, N-hydroxysuccinimide; EDC, 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide.

Table 1 Molecular weights of various polymers detected by gel permeation chromatography in the absence or presence of reduction reagents

Polymers	$M_w/10^3$ Da	$M_n/10^3$ Da	PDI
OEI ₈₀₀ -SS _x	12	3.9	3.1
OEI ₈₀₀ -SS _x + GSH	1.6	0.78	2.1
OEI ₈₀₀ -SS _x + DTT	0.99	0.75	1.3
OEI ₈₀₀ -SeSe _x	18	5.3	3.4
OEI ₈₀₀ -SeSe _x + GSH	8.1	2.5	3.2
OEI ₈₀₀ -SeSe _x + DTT	3.9	2.6	1.5
OEI ₈₀₀ -SeSe _x + NaBH ₄	1.4	0.79	1.8
OEI ₈₀₀ -Se _x	15	5.7	2.8
OEI ₈₀₀ -Se _x + NaBH ₄	15	5.8	2.7
PEI _{25k}	16	4.5	3.6
OEI ₈₀₀	0.76	0.52	1.5

Abbreviations: DTT, 1,4-dithiothreitol; M_w , weight-average molecular weight; M_n , number-average molecular weight; PDI, polydispersity index; OEI, oligoethylenimine; PEI, polyethylenimine; GSH, glutathione.

became diversified (Supplementary Figure 3). The peaks of OEI₈₀₀-SS_x and OEI₈₀₀-SeSe_x obviously shifted to a longer time point, indicating a decrease in molecular weight due to degradation, while the molecular weight of OEI₈₀₀-Se_x remained constant. It was interesting that the molecular

weight of OEI₈₀₀-SS_x decreased to 1.6 kDa after treatment with glutathione, but to 0.99 kDa after DTT treatment, which is very close to the molecular weight of OEI₈₀₀ (0.76 kDa). The molecular weight of OEI₈₀₀-SeSe_x reduced to 8.1, 3.9, and 1.4 kDa after being incubated with glutathione, DTT and NaBH₄, respectively.

Characterization of particle size, zeta potential, and morphology

Dynamic light scattering measurements were used to examine the hydrodynamic particle size of the complexes (Figure 2A). The polymers of PEI_{25k}, OEI₈₀₀-SeSe_x, OEI₈₀₀-SS_x, and OEI₈₀₀-Se_x were able to condense plasmid DNA in the form of complexes (C/P 1.2 for PEI_{25k}, and C/P 1.2–6 for OEI₈₀₀ derivatives), with an approximate diameter of 140–250 nm and a narrow distribution. The size of the complexes with OEI₈₀₀ derivatives depended on the C/P ratio. For instance, the sizes of the OEI₈₀₀-SeSe_x complexes decreased from 250 nm, to 190 nm, to about 140 nm, as the C/P increased from 1.2, to 3, to 6. In contrast, the complexes formed by OEI₈₀₀ and plasmid DNA tended to aggregate or disperse

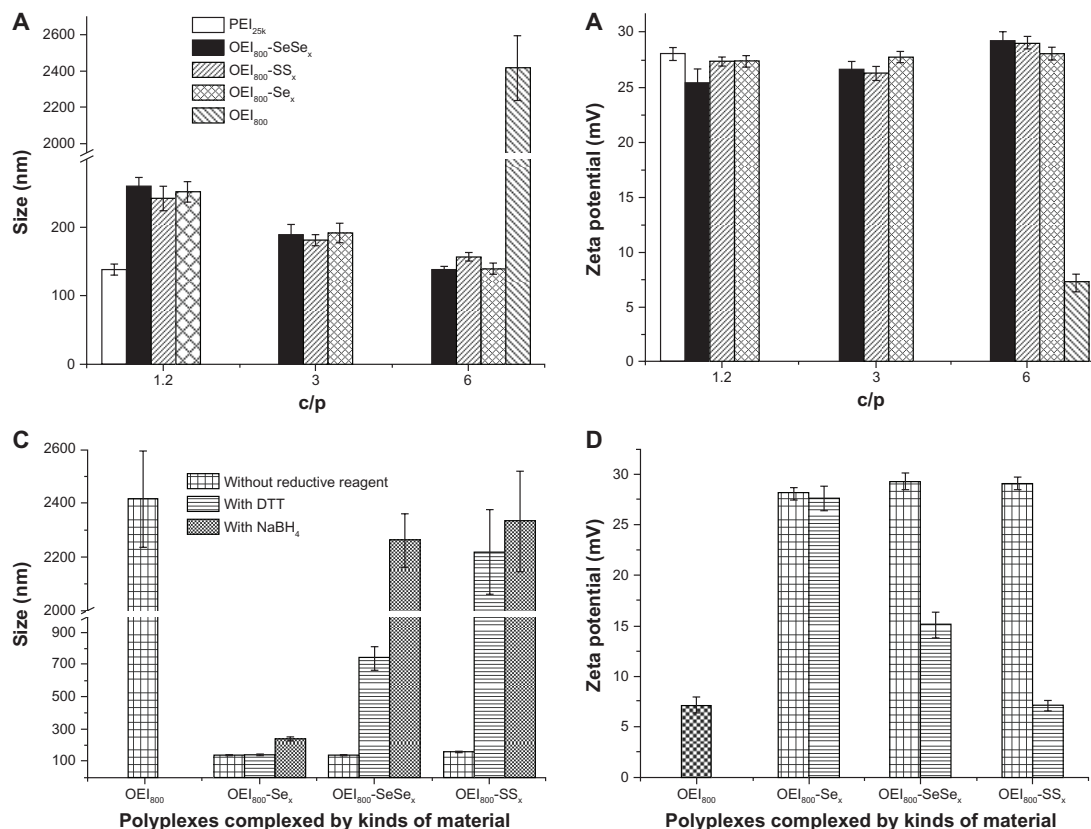


Figure 2 Sizes and zeta potentials of various complexes determined by Zetasizer Nano ZS. (A) Sizes of complexes composed of PEI_{25k}, OEI₈₀₀, or OEI₈₀₀ derivatives and genes at different C/P ratios, (B) zeta potentials of complexes composed of PEI_{25k}, OEI₈₀₀ or OEI₈₀₀ derivatives and genes at different C/P ratios, (C) changes in size with or without the reductive reagent (C/P 6), and (D) zeta potential changes with or without the reductive reagent (C/P 6, n = 3).

Abbreviations: DTT, 1,4-dithiothreitol; OEI, oligoethylenimine; PEI, polyethylenimine.

at a diameter over 2000 nm, even at a high C/P ratio of 6. Accordingly, the well compacted nanoparticles composed of high molecular weight polycations (PEI_{25k} and OEI₈₀₀ derivatives) and DNA were all positively charged, with about +27 mV zeta potential, while that of low molecular weight OEI₈₀₀ complexes was only about +7 mV (Figure 2B). After being treated with different reductive reagents, the size of the OEI₈₀₀-SeSe_x, OEI₈₀₀-SS_x, and OEI-Se_x complexes was detected at an appropriate C/P of 6 (Figure 2C). The average diameter of OEI₈₀₀-SeSe_x/DNA complexes with NaBH₄ and OEI₈₀₀-SS_x/DNA complexes in DTT and NaBH₄ was obviously increased to over 2200 nm, and the size of OEI₈₀₀-SeSe_x/DNA (C/P 6) treated with DTT increased to about 740 nm. As controls, the stable analog OEI₈₀₀-Se_x/DNA (C/P 6) complexes showed no obvious changes after being treated with reductive reagents. Changes in the zeta potential were also observed after treatment with DTT (Figure 2D). The mean surface charge of well compacted nanoparticles containing disulfide bonds and diselenide bonds reduced to approximately 15 mV and 7 mV, respectively, while no change was detectable in the OEI₈₀₀-Se_x group ($P > 0.05$).

In addition, the morphology of the complexes investigated by transmission electron microscopy was in agreement with the size measurement by dynamic light scattering (Figure 3). PEI_{25k}, OEI₈₀₀-SeSe_x, and OEI₈₀₀-SS_x could compact plasmid DNA to form nanoparticles at an appropriate C/P ratio. All diameters of the PEI_{25k}/DNA (C/P 1.2), OEI₈₀₀-SeSe_x/DNA (C/P 6), and OEI₈₀₀-SS_x/DNA (C/P 6) complexes were less than 200 nm, whereas samples of DNA condensed by OEI₈₀₀ was partly aggregated and dispersed.

After treatment with NaBH₄, the diameter of OEI₈₀₀-SeSe_x/DNA increased to more than 400 nm, which was similar to what phenomena happened to OEI₈₀₀-SS_x/DNA upon treatment with DTT. It was obvious that the OEI₈₀₀-SeSe_x/DNA and OEI₈₀₀-SS_x/DNA complexes became looser after incubation with the reductive substance, indicating that both OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x had reduction-sensitive ability and would degrade to low molecular weight OEI in the reductive environment.

DNA binding ability in presence and absence of reduction reagents

The gel retardation assay verified that all the cationic polymers could retard DNA at an appropriate C/P ratio, and as the polymer concentration increased, their capacity to integrate with plasmid DNA was enhanced (Figure 4). Polycations containing high molecular weight polymers, including OEI₈₀₀-SS_x (Figure 4A), OEI₈₀₀-SeSe_x (Figure 4B), OEI₈₀₀-Se_x (Figure 4C), and PEI_{25k} (Figure 4E), could completely retard DNA migration at a C/P ratio of 0.4, while OEI₈₀₀ formed stable complexes with DNA only at C/P ratios higher than 0.6. This indicated that cross-linked OEI₈₀₀ can condense plasmid DNA at C/P ratios lower than that of the original monomer. After incubation with DTT, the reduction-responsive polymers of OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x released part of their DNA at C/P ratios of 0.4 and 0.5, respectively, returning to a condensation ability similar to that of OEI₈₀₀. On the other hand, the corresponding stable analog (OEI₈₀₀-Se_x) showed no detectable changes after treatment with DTT.

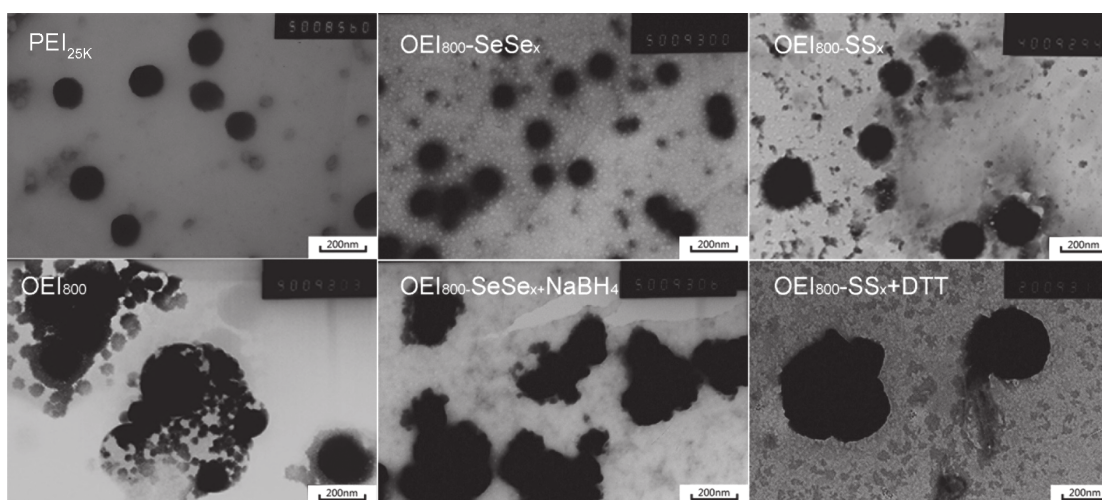


Figure 3 Transmission electron microscopy results for PEI_{25k}, OEI₈₀₀, and OEI₈₀₀-derived complexes dropped onto amorphous carbon-coated copper grids, dried, and dyed using phosphomolybdic acid.

Note: Bar = 200 nm.

Abbreviations: DTT, 1,4-dithiothreitol; OEI, oligoethylenimine; PEI, polyethylenimine.

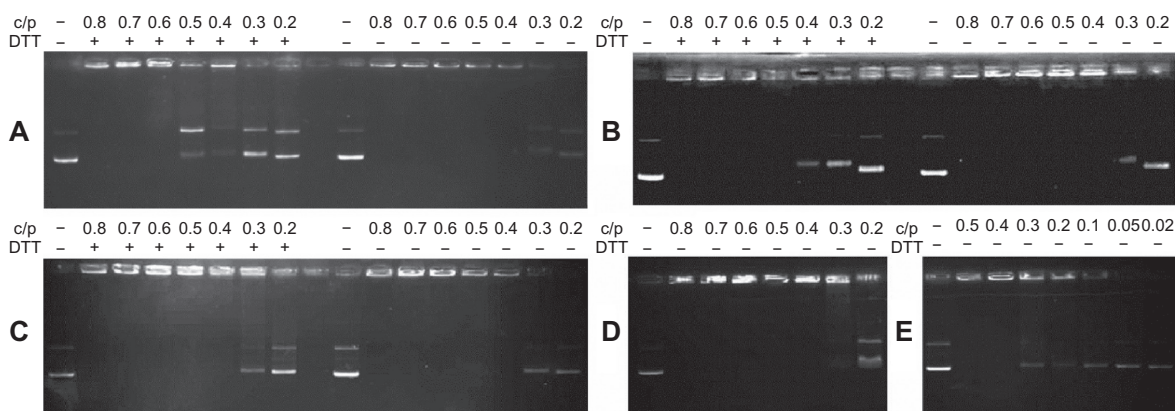


Figure 4 Gel retardation assay of various cationic polymers complexed with plasmid DNA. (A) OEI₈₀₀-SS_x, (B) OEI₈₀₀-SeSe_x, (C) OEI₈₀₀-Se_x, (D) OEI₈₀₀ and (E) PEI_{25k}.
Notes: Polymers and plasmid DNA were mixed at various mass ratios and incubated at 37°C for 30 minutes with or without DTT. The mixed suspension was subsequently applied to the agarose gel and electrophoresed at 85 V for 40 minutes.
Abbreviations: DTT, 1,4-dithiothreitol; OEI, oligoethylenimine; PEI, polyethylenimine.

Cytotoxicity of DNA-free copolymers

The cytotoxicity of the OEI₈₀₀ derivatives in B16F10 and HeLa cells was tested by MTT assay, with OEI₈₀₀ and PEI_{25k} as controls (Figure 5). PEI_{25k} was the most toxic polymer, while cells treated with OEI₈₀₀ retained more than 90% of their metabolic activity. Both reduction-sensitive bonds of cross-linked copolymers (OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x) showed lower cytotoxicity than PEI_{25k} and stable bonds of cross-linked polymers (OEI₈₀₀-Se_x) in B16F10 cells (Figure 5A), with the same trend observed in HeLa (Figure 5B). For instance, at the same concentration of 50 µg/mL, only 40%–50% viability was found after treatment with PEI_{25k} and OEI₈₀₀-Se_x in B16F10 cells, while that for OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x remained at 76% and 52%, respectively.

EGFP and luciferase reporter gene expression in carcinoma cells

The transfection activity of OEI₈₀₀-derived nanoparticles, including OEI₈₀₀-SeSe_x, OEI₈₀₀-SS_x, and OEI₈₀₀-Se_x, was evaluated in B16F10 (Figure 6A) and HeLa (Figure 6B) cells using pEGFP (Figure 7) and pGL3 (Figure 6). All of the OEI₈₀₀-derived nanoparticles had significantly higher transfection ability than that of OEI₈₀₀ alone. Incubation of cells with OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x nanoparticles resulted in slightly higher luciferase expression than that for PEI_{25k} in B16F10. For example, pGL3 transfection of the reduction-sensitive nanoparticles (OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x) was 3.3×10^9 and 5.8×10^9 relative light units/mg protein at the optimum C/P ratio in B16F10 cells, respectively, which was four orders of magnitude higher than that for the OEI₈₀₀

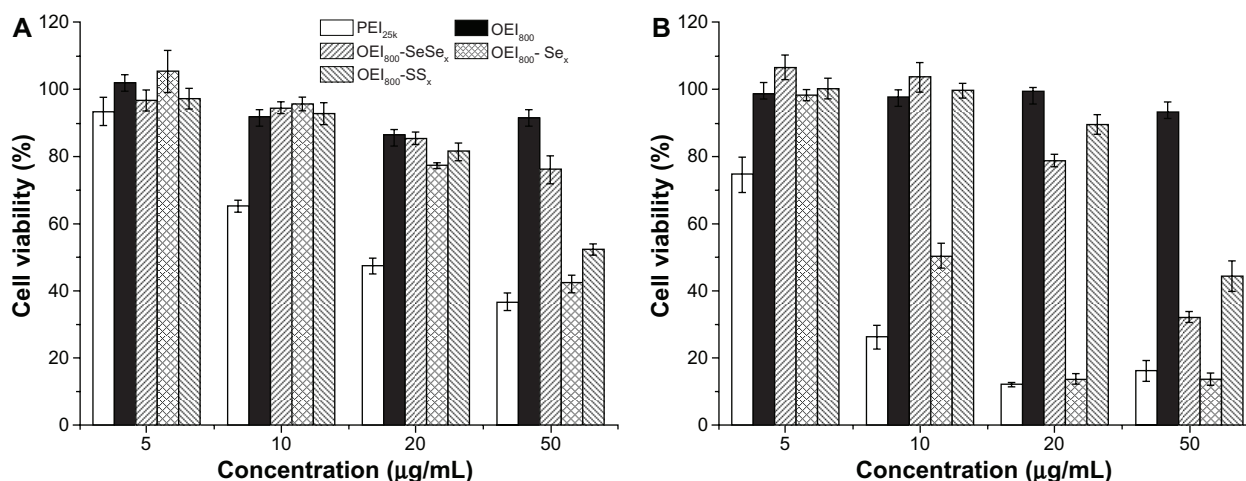


Figure 5 Cytotoxicity assays of the OEI₈₀₀ derivatives, OEI₈₀₀, and PEI_{25k} in (A) B16F10 and (B) HeLa cells.
Note: The polymers were incubated with the cells for 24 hours (n = 4).
Abbreviations: OEI, oligoethylenimine; PEI, polyethylenimine.

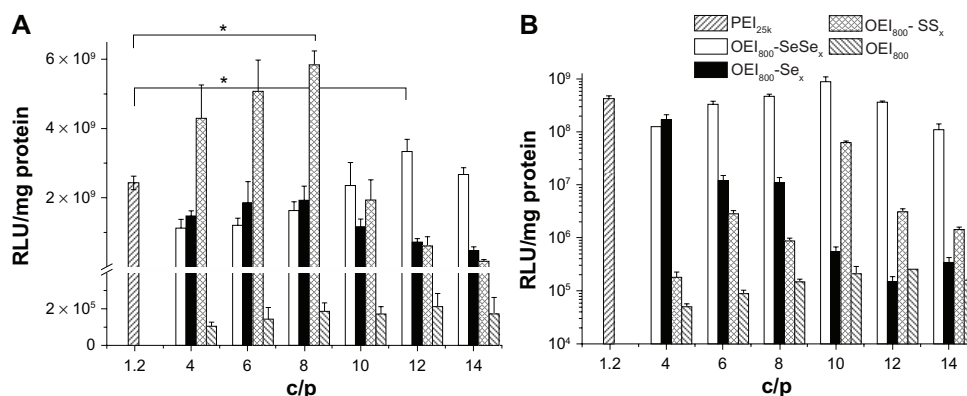


Figure 6 Luciferase transfection of the polycation-pGL3 complexes in (A) B16F10 cells and (B) HeLa cells.

Notes: All the complexes were prepared in HEPES buffered glucose, and 200 ng of pGL3 per well was used for the 96-well tissue culture plate. The data show the average for experiments performed at least three times ($n = 3$, $*P < 0.05$).

Abbreviations: OEI, oligoethylenimine; PEI, polyethylenimine; RLU, relative light units.

nanoparticles (only about 1.0×10^5 relative light units/mg protein). In addition, transfection of $\text{OEI}_{800}\text{-SeSe}_x$ and $\text{OEI}_{800}\text{-SS}_x$ nanoparticles was up to about 1.5-fold and 2.0-fold higher compared with that of PEI_{25k} (C/P 1.2, 2.4×10^9 relative light units/mg protein, $P < 0.05$). Interestingly, transfection of $\text{OEI}_{800}\text{-SeSe}_x$ was higher than that of $\text{OEI}_{800}\text{-SS}_x$ at $C/P \geq 10$, and was lower than $\text{OEI}_{800}\text{-SS}_x$ at $C/P \leq 8$ in B16F10 cells. For example, luciferase expression in the $\text{OEI}_{800}\text{-SeSe}_x$ group was up to approximately 5-fold higher compared with that in the $\text{OEI}_{800}\text{-SS}_x$ group at C/P 12 (Figure 6A). On the other hand, transfection of $\text{OEI}_{800}\text{-SeSe}_x$ was higher than that of $\text{OEI}_{800}\text{-SS}_x$ in HeLa cells at all C/P ratios in the experiment. In particular, it was three orders of magnitude higher than that of $\text{OEI}_{800}\text{-SS}_x$ at C/P 4 (Figure 6B).

The results of qualitative studies on transfection of plasmid DNA encoding EGFP with various OEI_{800} -derived nanoparticles were consistent with those for luciferase expression, which was visualized with a fluorescent microscope in B16F10 cells. After 48 hours of transfection, cells incubated with OEI_{800} derivatives ($\text{OEI}_{800}\text{-SeSe}_x$, $\text{OEI}_{800}\text{-SS}_x$, $\text{OEI}_{800}\text{-Se}_x$) and PEI_{25k} nanoparticles showed more bright

green fluorescent spots, especially at a C/P ratio of 8, for $\text{OEI}_{800}\text{-SeSe}_x$ or $\text{OEI}_{800}\text{-SS}_x$, and a C/P ratio of 4 for $\text{OEI}_{800}\text{-Se}_x$ (Figure 7), indicating more expression of green fluorescent protein. In addition, there were almost no fluorescence spots in the $\text{OEI}_{800}\text{-SS}_x$ group at C/P 14, while a large number of fluorescent spots was found in the $\text{OEI}_{800}\text{-SeSe}_x$ group. The percentage of transfected cells detected by flow cytometry showed the same trend as the results for fluorescent microscopy (Figure 8 and Supplementary Figure 4).

Intracellular investigation of labeled DNA and cationic polymers

Dual-labeled nanoparticles were prepared using Cy5-labeled DNA and FITC-labeled cationic polymers. Interaction between the polymers, DNA, and cells was monitored by confocal laser scanning microscopy. For ease of graphical representation, images of B16F10 cells incubated with different types of nanoparticles (including PEI_{25k} , $\text{OEI}_{800}\text{-SeSe}_x$ and $\text{OEI}_{800}\text{-SS}_x$) for 4 hours and 8 hours are shown (Figure 9). At 4 hours, approximately 80% of the nanoparticles assembled around the cell membrane, while more fluorescent spots

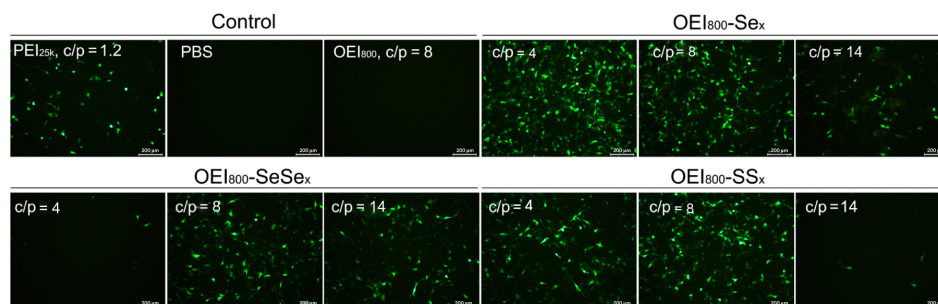


Figure 7 In vitro transfection efficiency of the complexes of pEGFP with $\text{OEI}_{800}\text{-SeSe}_x$, $\text{OEI}_{800}\text{-Se}_x$, $\text{OEI}_{800}\text{-SS}_x$, PEI_{25k} , and OEI_{800} in B16F10 cell lines at 48 hours.

Note: Bar = 200 μm .

Abbreviations: OEI, oligoethylenimine; PEI, polyethylenimine; pEGFP, enhanced green fluorescent protein encoding plasmid.

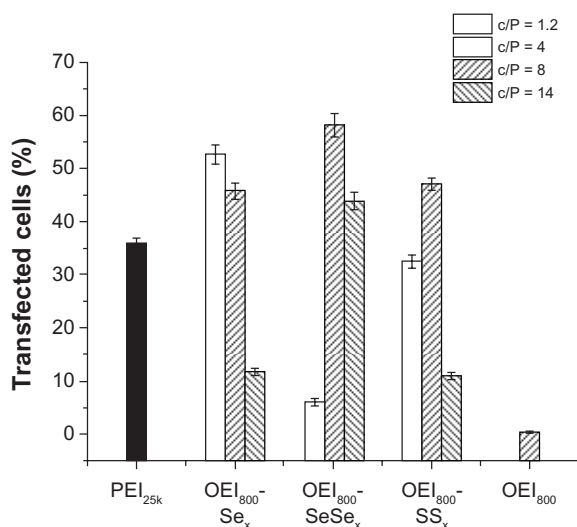


Figure 8 Percentages of transfected cells for the complexes of pEGFP with OEI₈₀₀-SeSe_x, OEI₈₀₀-Se_x, OEI₈₀₀-SS_x, PEI_{25k}, and OEI₈₀₀ quantified by flow cytometry analysis in B16F10 cells at 48 hours

Note: n = 3.

Abbreviations: OEI, oligoethylenimine; PEI, polyethylenimine; pEGFP, enhanced green fluorescent protein encoding plasmid.

located inside the cells after 8 hours of incubation. This shows that nanoparticles tend to enter the cytoplasm from the surface of cell along with the time. Before 4 hours, good compaction of the polycations and negatively charged DNA is indicated by the orange color, caused by overlay of colors from the green FITC and red Cy5. Importantly, at the 8-hour time point, the intracellular fluorescent dots in the PEI_{25k} group remained orange, but more dots in the OEI₈₀₀-SS_x and

OEI₈₀₀-SeSe_x groups were red. These changes were not seen on the membrane of cells in the OEI₈₀₀-derived group.

Discussion

For decades, disulfide bonds have been intensively explored in drug and gene delivery. No other reduction-sensitive chemical linkage has been developed that could work as a substitution for it until now. Recently, diselenide bonds have been reported for the dual redox-sensitive design,^{36,42} but their utility has not been extended to gene carrier systems. In the current study, diselenide bonds for cross-linked OEI₈₀₀ were synthesized in order to construct reduction-sensitive gene carriers with high transfection ability and low cytotoxicity. According to the design (Figure 10), cross-linked OEI₈₀₀-SeSe_x with a high molecular weight should efficiently compact with DNA to form cationic complexes. After entering an environment of higher redox potential, such as the cytoplasm, the diselenide bonds would be expected to be cleaved, breaking into subunits of OEI₈₀₀ and leading to dissociation of the complexes. This would help to move DNA into the nucleus for transcription, and keep a balance between high transfection ability and low cytotoxicity on delivery.

In order to achieve cross-linking of diselenide bonds with OEI₈₀₀, DSeDPA linkage was undertaken. The corresponding reduction-stable linker (SeDPA) was prepared by a similar method and was used as a negative control (Figure 1). DSeDPA and SeDPA were synthesized by reacting 3-chloropropanoic acid with Na₂Se₂ and Na₂Se, respectively.

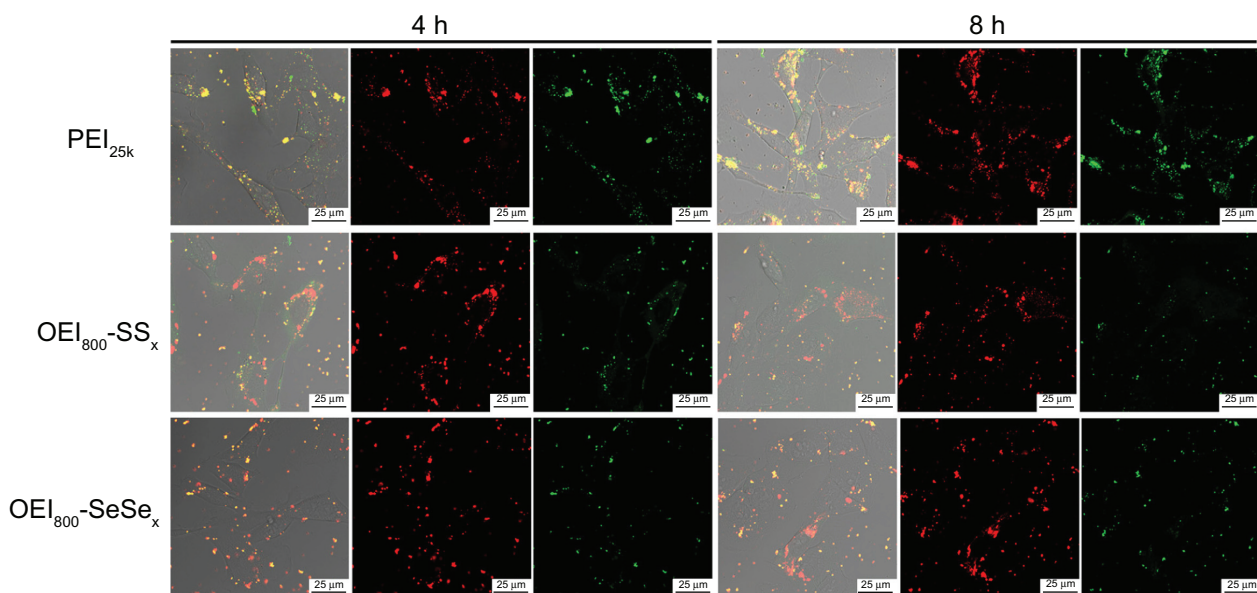


Figure 9 Labeled DNA and cationic polymer for intracellular investigation in B16F10 cells. Red dots: Cy5-labeled DNA; green dots: FITC-labeled PEI or OEI₈₀₀ derivatives.

Note: Bar = 25 μm.

Abbreviations: OEI, oligoethylenimine; PEI, polyethylenimine.

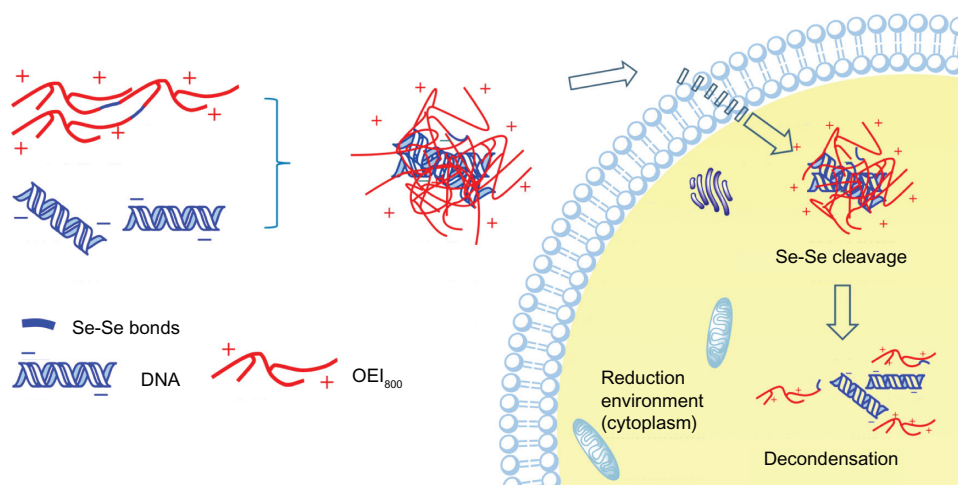


Figure 10 Schematic illustration of decondensation of reduction-sensitive complexes in the intracellular environment.

The key to obtaining distinct Na_2Se_2 and Na_2Se depended on different ratios of NaBH_4 to selenium powder. When the molecular ratio of NaBH_4 to selenium was 2, colorless Na_2Se was given as the product, and reddish brown Na_2Se_2 was obtained at a ratio of 1.³⁸ To avoid H_2Se_2 gas production as a side reaction, the pH of 3-chloropropanoic acid should be adjusted to 8.0. After reacting with 3-chloropropanoic acid overnight, Na_2Se_2 or Na_2Se residues should be oxidized to selenium by exposure to the atmosphere for easy purification via filtration. Unreacted DSeDPA and SeDPA was removed by adjusting the pH of the supernatant for formation of a precipitate at pH 3–4.³⁸ In view of the instability of diselenide bonds in an alkaline environment (pH > 10),⁴³ the alkaline OEI₈₀₀ solution should be neutralized to pH 7.4 prior to cross-linking. At the cross-linking step, the concentration of OEI₈₀₀ must be controlled, and is significantly related to the molecular weight of the final product.³⁵ The higher molecular weight of the cross-linked OEI₈₀₀ would be obtained, because the intermolecular reaction occurs easily at high OEI₈₀₀ concentrations. In our synthesis reaction, the OEI₈₀₀ concentration was 25% (w/w).

According to molecular weight measurement by gel permeation chromatography, OEI₈₀₀-SeSe_x (about 18 kDa), OEI₈₀₀-SS_x (about 12 kDa), and OEI₈₀₀-Se_x (about 16 kDa) of high molecular weight were synthesized successfully. It was seen that only the OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x degraded after being treated with reductive reagents (Table 1 and Supplementary Figure 3). Interestingly, OEI₈₀₀-SS_x could degrade to OEI₈₀₀ after treatment with DTT or glutathione. However, under the same conditions, the OEI₈₀₀-SeSe_x did not degrade completely, and would degrade to smaller OEI₈₀₀ pieces only when treated with a much more efficient

reductive reagent, ie, NaBH_4 . After complexing with DNA, dynamic light scattering measurements of the complexes showed concordant results (Figure 2). The size of particles formed by OEI₈₀₀-SS_x increased to over 2000 nm after incubation with DTT, while that of the OEI₈₀₀-SeSe_x group increased to approximately 740 nm. The increased size in the OEI₈₀₀-SS_x and OEI₈₀₀-SeSe_x groups reached over 2000 nm only after treatment with NaBH_4 . In addition, the reduction-sensitive ability of the diselenide bonds was demonstrated by characterization of morphology (Figure 3 and Table 1). OEI₈₀₀-SeSe_x could complex with plasmid DNA efficiently to form nanosized particles, and the results of transmission electron microscopy indicated the mean diameter of the particles to be less than 200 nm. OEI₈₀₀-SeSe_x nanoparticles in NaBH_4 showed similar swelling with OEI₈₀₀-SS_x in DTT. After treatment with reduction reagents, changes detected by transmission electron microscopy were not as obvious as the changes determined by dynamic light scattering. This difference was due to the fact that the dynamic light scattering method gives the hydration diameter rather than the actual (dry) diameter detected by transmission electron microscopy.⁴⁴ The changes in molecular weight, particle size, and morphology all confirmed that the disulfide and diselenide bonds in OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x were cleavable in a reductive environment, and that cleavage of the diselenide bonds needs a more efficient reducible reagent. The likely reason for this is that diselenide bonds are more stable than disulfide bonds, with a lower redox potential.¹⁹ This property is beneficial for transfection in vivo because disulfide bonds may be cleaved by glutathione at the concentrations present in blood, while diselenide bonds remain stable.^{18,35}

In the gel retardation assay, the ability of OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x to complex with the DNA decreased after treatment with DTT, which implies a reduction-sensitive ability for diselenide bonds and that the reductive reaction between DTT and OEI₈₀₀-SeSe_x was already efficient enough for release of DNA (Figure 4). Accordingly, the MTT assay of OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x showed similarly lower toxicity in comparison with stable OEI₈₀₀-Se_x and PEI_{25k} complexes (Figure 5). Although the diselenide bonds were much more stable than disulfide bonds, in the cytotoxicity assay, there was not only glutathione present as a reductive reagent in the cytoplasm but also high amounts of NADPH. As reported elsewhere, selenogluthathione can oxidize the common biological cofactor NADPH, and reacts strongly with glutathione reductase, which is an NADPH-dependent enzyme.¹⁹ Therefore, diselenide bonds can be efficiently cleaved in a cell reduction environment and have the same reduction-sensitive ability as disulfide bonds.

In the *in vitro* transfection assay, OEI₈₀₀-SeSe_x had much higher transfection ability than others based on OEI₈₀₀, and even similar or higher transfection ability than PEI_{25k} (Figure 6). Compared with OEI₈₀₀, OEI₈₀₀-SeSe_x with its high molecular weight possessed a better ability to condense plasmid DNA, and could form tight nanoparticles and endocytose easily. It was hypothesized that the reduction-sensitive disulfide and diselenide bonds could be cleaved in the intracellular environment, triggered by glutathione or NADPH,¹⁹ and the OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x degraded to pieces of OEI₈₀₀, which led to release of plasmid DNA to get high transfection ability and lower cytotoxicity. These results are in agreement with those of other studies of OEI₈₀₀ cross-linked by degradable bonds.^{6,28,29} In addition, the optimum C/P of cross-linked OEI₈₀₀ for transfection was found to be higher than for PEI_{25k}, which may be because the OEI content was lower in cross-linked OEI₈₀₀ or because of the increased hydrophobicity.⁶ At a high C/P ratio (C/P ≥ 10 in quantitative transfection and C/P 14 in qualitative transfection), transfection of OEI₈₀₀-SeSe_x was higher than that of OEI₈₀₀-SS_x in B16F10 cells (Figures 6A and 7), which might have been because of the cytotoxicity of OEI₈₀₀-SS_x is higher than that of OEI₈₀₀-SeSe_x at high concentrations (Figure 5A). The difference in effect between OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x in B16F10 and HeLa cells indicates the cell-dependent reduction sensitivity of diselenide. Based on these results, the diselenide bond-conjugated OEI is more suitable as a reduction-sensitive gene carrier than disulfide bond-conjugated OEI. Moreover, they had small differences

in stability,¹⁹ which might affect transfection ability. Further studies will be carried out on the sensitivity of diselenide and disulfide under physiological conditions and the time-dependence of response to a reducing agent. Like disulfide bonds, diselenide bonds can be used widely in smart drugs and for gene delivery.

In order to explore the intracellular fate of the nanoparticles, three kinds of cationic polymer-plasmid DNA complexes were investigated. It was obvious that they could be endocytosed easily, and many red or orange dots were observed in the cytoplasm (Figure 9). More red dots were observed in the groups containing reduction bonds and more orange dots were seen in the groups containing stable bonds. The number of red fluorescent dots increased with the passage of time in the OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x groups, while no significant change was observed in the PEI_{25k} group during the experiment. The PEI_{25k} group contained stable bonds which could condense DNA tightly and showed orange fluorescent dots which were the overlay of the red from Cy5 and the green from FITC. For the OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x groups, which contained reduction-sensitive bonds, cleavage of the disulfide bonds or diselenide bonds would cause parts of the FITC to be released from the nanoparticles, and more red fluorescent dots were observed with the passage of time. This phenomenon implies that diselenide and disulfide bonds are reduction-sensitive.

Conclusion

This study describes the design and synthesis of diselenide bonds cross-linked to OEI₈₀₀ for use as reduction-sensitive gene carriers. The results show that diselenide-containing OEI₈₀₀-SeSe_x was able to bind plasmid DNA efficiently to yield nanosized particles and had the same reduction sensitivity as OEI₈₀₀-SS_x. *In vitro* experiments demonstrated that the reducible OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x had much lower cytotoxicity and higher transfection activity compared with the nondegradable PEI_{25k} control. The transfection ability of OEI₈₀₀-SeSe_x was higher than that of OEI₈₀₀-SS_x in B16F10 cells at a C/P ratio ≥ 10, while the transfection ability of OEI₈₀₀-SeSe_x was higher than that of OEI₈₀₀-SS_x in HeLa cells at all C/P ratios used in this experiment. The reduction sensitivity of diselenide was cell-dependent. The results of our confocal laser scanning microscopy experiments demonstrated directly that both OEI₈₀₀-SeSe_x and OEI₈₀₀-SS_x could be cleavable in a cell-reductive environment. Our results imply that OEI conjugated with diselenide bonds is suitable for use as a reduction-sensitive gene carrier.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary figures

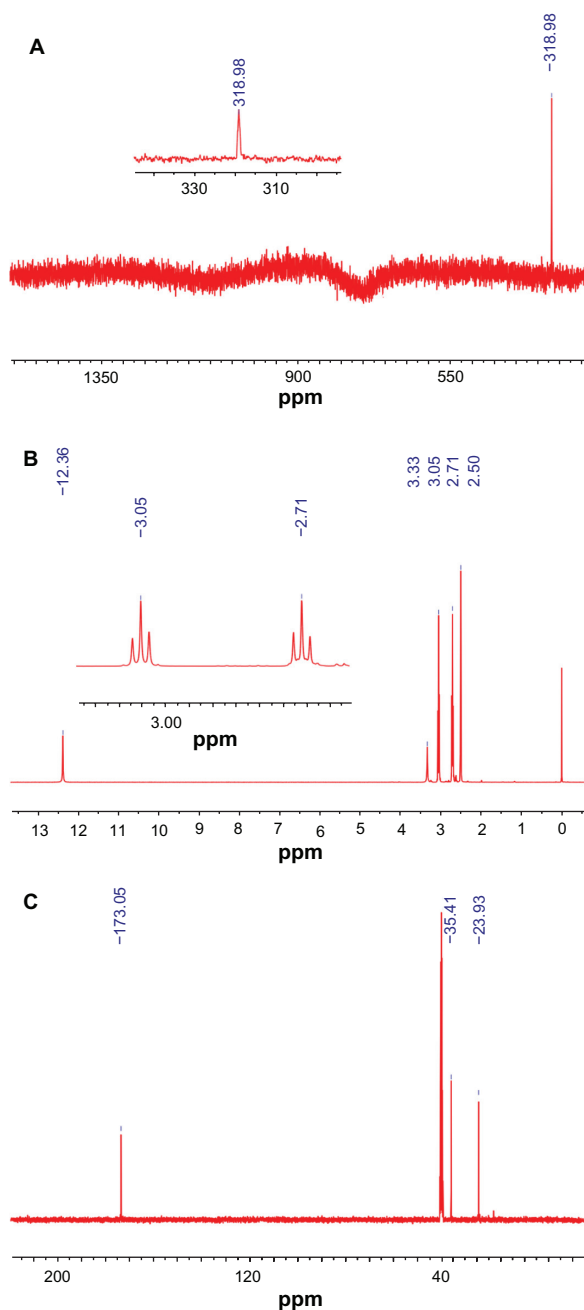


Figure S1 NMR spectrum of DSeDPA. (A) ^{77}Se NMR spectrum; (B) ^1H NMR spectrum; (C) ^{13}C NMR spectrum.

Abbreviation: DSeDPA, 3, 3'-Diselenediylpropanoic acid; NMR, nuclear magnetic resonance.

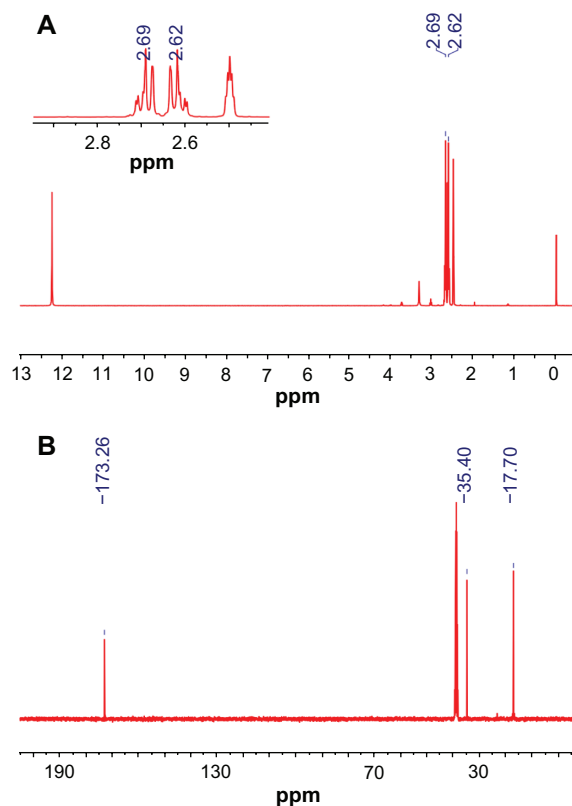


Figure S2 NMR spectrum of SeDPA. (A) ^1H NMR spectrum; (B) ^{13}C NMR spectrum. **Abbreviation:** SeDPA, 3, 3'-selenodipropanoic acid; NMR, nuclear magnetic resonance.

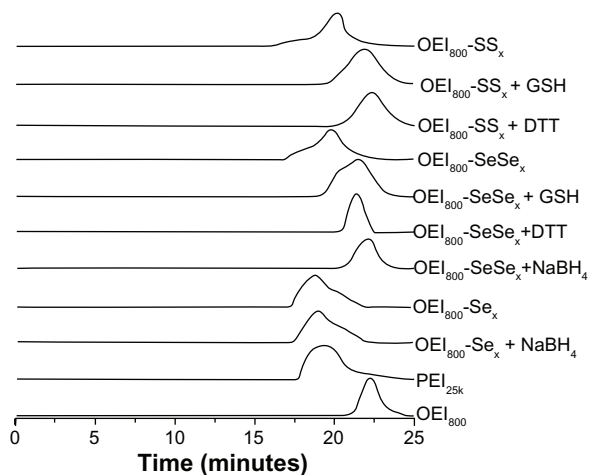


Figure S3 GPC traces of OEI₈₀₀-SS_x, OEI₈₀₀-SeSe_x, OEI₈₀₀-Se_x and the OEI₈₀₀ derivatives in the absence or presence of reduction treatment.

Abbreviations: GPC, gel permeation chromatography; OEI, oligoethylenimine; DTT, 1,4-dithiothreitol; GSH, glutathione.

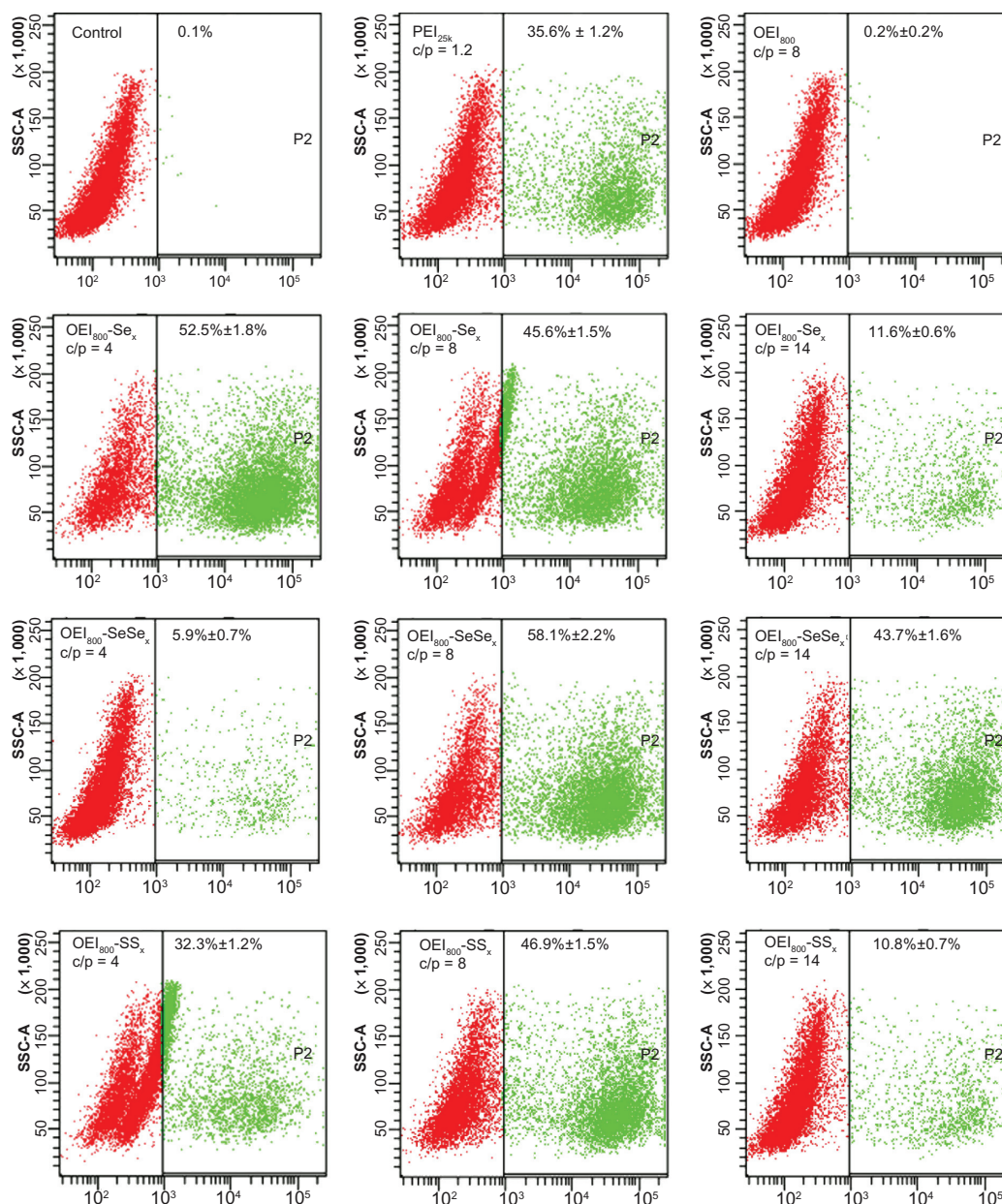


Figure S4 The percentage of transfected cell for the complexes of pEGFP with OEI₈₀₀-SeSe_x, OEI₈₀₀-Se_x, OEI₈₀₀-SS_x, PEI_{25k} and OEI₈₀₀ was quantified by flow cytometry analysis in B16F10 cell lines at 48 hours.

Note: n = 3.

Abbreviations: pEGFP, enhanced green fluorescent protein encoding plasmid; PEI, polyethylenimine; OEI, oligoethylenimine.

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