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ELECTRONIC SUPPLEMENTARY INFORMATION

# Redox-triggered intracellular siRNA delivery

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**Abstract:** Gene silencing using small interfering RNA (siRNA) is a promising strategy for the treatment of multiple diseases. However, the low *in vivo* stability of siRNA, its poor pharmacokinetics and inability to penetrate inside cells limit its employment in the clinic. Here, we present a novel redox-sensitive micellar nanopreparation based on a triple conjugate of polyethylene glycol, polyethyleneimine and phosphatidylethanolamine, PEG-*SS*-PEI-PE (PSSPD). This non-toxic system efficiently condenses siRNA and specifically downregulates target green fluorescent protein (GFP) only under reducing conditions via intracellular siRNA release after de-shielding of PEG due to increased glutathione (GSH) levels characteristic of cancer cells.

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## Section S1. Materials and Methods

### S1.1. Materials

3,3′-Dithiodipropionic acid, N,N'-Dicyclohexylcarbodiimide (DCC) and 4-Dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich (St. Louis, MO). $mPEG_{2000}$-Amine and $mPEG_{2000}$-SVA (Methoxy PEG succinimidyl valerate) were purchased from Laysan Bio (Arab, AL). Branched PEI with a molecular weight of 1.8 kDa was purchased from Polysciences, Inc (Warrington, PA). N-glutaryl-phosphatidyl ethanolamine (NGPE) was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). The siRNA duplexes were purchased from Dharmacon (Lafayette, CO), namely, siRNA targeting green fluorescent protein (GFP-siRNA): 5'-AUGAACUUCAGGGUCAGCUdTdT-3' (sense) [1] and a non-targeting control duplex, (Negative-siRNA): 5'-AGUACUGCUUACGAUACGGdTdT-3' (sense). The CellTiter-Blue® Cell Viability Assay was purchased from Promega (Madison, WI). Nuclease-free water was purchased from Qiagen (Germantown, MD). All the other materials and solvents were in synthesis grade and purchased from Sigma-Aldrich.

### S1.2. Cell culture

C166-GFP cells encoding enhanced GFP upon stable transfection of C166 cell line with the pEGFP-N1 plasmid reporter vector was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.2 mg/mL of Geneticin (G-418, Invitrogen, CA) and cultured at 37°C with 5% $CO_2$.

### S1.3. Synthesis procedure of PEG-SS-PEI-PE and PEG-PEI-PE

The three-step synthesis of PEG-SS-PEI-DOPE is shown in the Figure S1. The first step included the conjugation of $mPEG_{2000}$-Amine to the starting material, 3,3′-dithiodipropionic acid. One of the two carboxylic acid groups of 3,3′-dithiodipropionic acid (40 µmol) was activated by using DCC (40 µmol) and DMAP (as catalyzer, 0.8 µmol) in anhydrous chloroform at room temperature for one hour. At the end of the activation period, mPEG2000-Amine (40 µmol) in dry chloroform was added drop-wise, and the reaction continued overnight with stirring at room temperature **(i)**. The reaction product, PEG-SS-COOH **(1)** was obtained after the evaporation of the solvent, freeze drying and purification by dialysis with approximate yield of 31%. The reaction product was confirmed by TLC, using chlorofom:methanol (60:40, v/v) as solvents. Dragendorff and bromocresol green reagents were used for visualization of the PEG and carboxylic acid groups, respectively.

The second step of the reaction included the conjugation of PEI to the PEG-SS-COOH. The carboxylic acid group on the PEG-SS-COOH was activated by using DCC (3 molar-fold excess) and DMAP (catalytic amount) in dry chloroform. PEI was added at a molar ratio of 1:1 **(ii)**. The reaction continued for 4 days until the PEI spot on the TLC visualized by ninhydrin reagent turned pale/disappeared. The PEG-SS-PEI **(2)** was obtained after the evaporation of the solvent, freeze drying and purification by dialysis with a yield of 51%.

Finally, NGPE was successfully conjugated to PEG-SS-PEI and PEG-SS-PEI-PE **(3)** obtained with a reaction yield of approximately 90%. For the activation of NGPE, EDC and NHS were used with 50-fold molar excess ratios **(iii)**. TEA (30 µl) was also added to the flask prior to the addition of PEG-SS-PEI. The reaction continued overnight with stirring at room temperature under $N_2$. The reaction was checked by TLC both before and after the evaporation of the solvent, freeze drying and purification steps to confirm the product PEG-SS-PEI-DOPE (PSSPD).

Synthesis of the redox-insensitive polymer PEG-PEI-PE was obtained using a previously published method [2]. Briefly, $mPEG_{2000}$-SVA directly reacted with PEI (1:1 mol ratio) under $N_2$ with continuous stirring at room temperature overnight. NGPE (7.9 µmol) was activated by EDC and NHS (23.7 µmol) in dry chloroform in the presence of 30 µL TEA for 4 hours. Subsequently, the first step reaction product PEG-PEI was added drop wise to the activated NGPE under $N_2$ with continuous stirring. Following an overnight reaction, the PEG-PEI-DOPE (PPD) was purified by dialysis and stored at -80°C for further studies.

**S1.4. Determination of CMC**

The critical micelle concentration (CMC) of PSSPD was evaluated by fluorescence spectroscopy, using the standard pyrene method. Briefly, 10 µl of a 1 mg/ml pyrene solution in chloroform was added to a series of glass vials and then chloroform was evaporated under $N_2$. The polymer solutions in $dH_2O$ in the concentration range of $4.3x10^{-2}$ mM – $2.2x10^{-8}$ mM were added to the glass vials and incubated for 24 h with shaking at room temperature, protected from light. At the end of the incubation peroid, non-encapsulated pyrene was removed by filtration through 0.2 µm polycarbonate membranes. The fluorescence of filtered samples was measured at the excitation wavelength of 339 nm and emission wavelength of 390 nm using an F-2000 fluorescence spectrometer (Hitachi, Japan) [3]. Fluorescence values corresponded to each concentration of the polymer measured and the sharp increase in pyrene fluorescence in solution was observed indicating micelle formation and encapsulation of the pyrene.

**S1.5. Preparation of PSSPD/siRNA and PPD/siRNA complexes**

PSSPD/siRNA and PPD/siRNA complexes were obtained by mixing a fixed amount of siRNA with varying amounts of PSSPD or PPD. First, PSSPD/PPD was separately diluted in equal volumes with nuclease-free buffered HEPES glucose (BHG) (pH 7.4) in different tubes (nuclease-free) to vary the concentration of polymers. Then, the siRNA solution was transferred to the tubes including polymer solutions, mixed by pipetting and left for incubation at room temperature for 30 minutes. The polymer/siRNA ratio was expressed as the nitrogen/phosphate (N/P) ratio and calculated by assuming each repeating unit of PEI contained one amine per each repeating unit of siRNA containing one phosphate.

**S1.6. Gel retardation studies**

PSSPD was diluted with BHG at varying concentrations (total volume of 20 µl) corresponding to different N/P ratios and incubated with 750 ng of siRNA (20 µl, in BHG) for 30 min at room temperature for the formation of PSSPD/siRNA complexes. The same amount of siRNA as in the complexes was used as the control. Complexes were electrophoresed through a 2% agarose gel with ethidium bromide for 30 mins at 60V using the E-Gel electrophoresis system (Invitrogen, Carlsbad, CA) and evaluated under UV light.

**S1.7. Characterization of PSSPD micelles**

All size, zeta potential and polydispersity index measurements were made using Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK). The results were determined in three measurements and reported as the mean values ± standard deviation. The particle size and size distribution were determined by dynamic light scattering using a 90° fixed angle and at 25°C temperature.

**S1.8. Disassembly of PSSPD micelles triggered by GSH**

The disassemble behaviors of redox-sensitive PSSPD micelles under reductive condition were monitored in GSH contained in PBS (pH 7.4) mimicking the reduction conditions in the human body. PSSPD micelles in PBS without the addition of GSH were used as controls. Briefly, various concentrations of GSH (0.2 µM, 1 mM, 10 mM,) were added to PSSPD micelles to control different reduction conditions. These solutions were incubated in a thermostatic shaking bath at 37 °C. The size distribution and zeta potential changes of PSSPD micelles were observed by DLS over 24 h at different time intervals (0, 2 h, 4 h, 6 h and 24 h) [4].
Particle size and zeta potential characterization of the redox-insensitive PPD nanopreparation was performed following 4h incubation with 10 mM GSH concentration as the PSSPD samples.

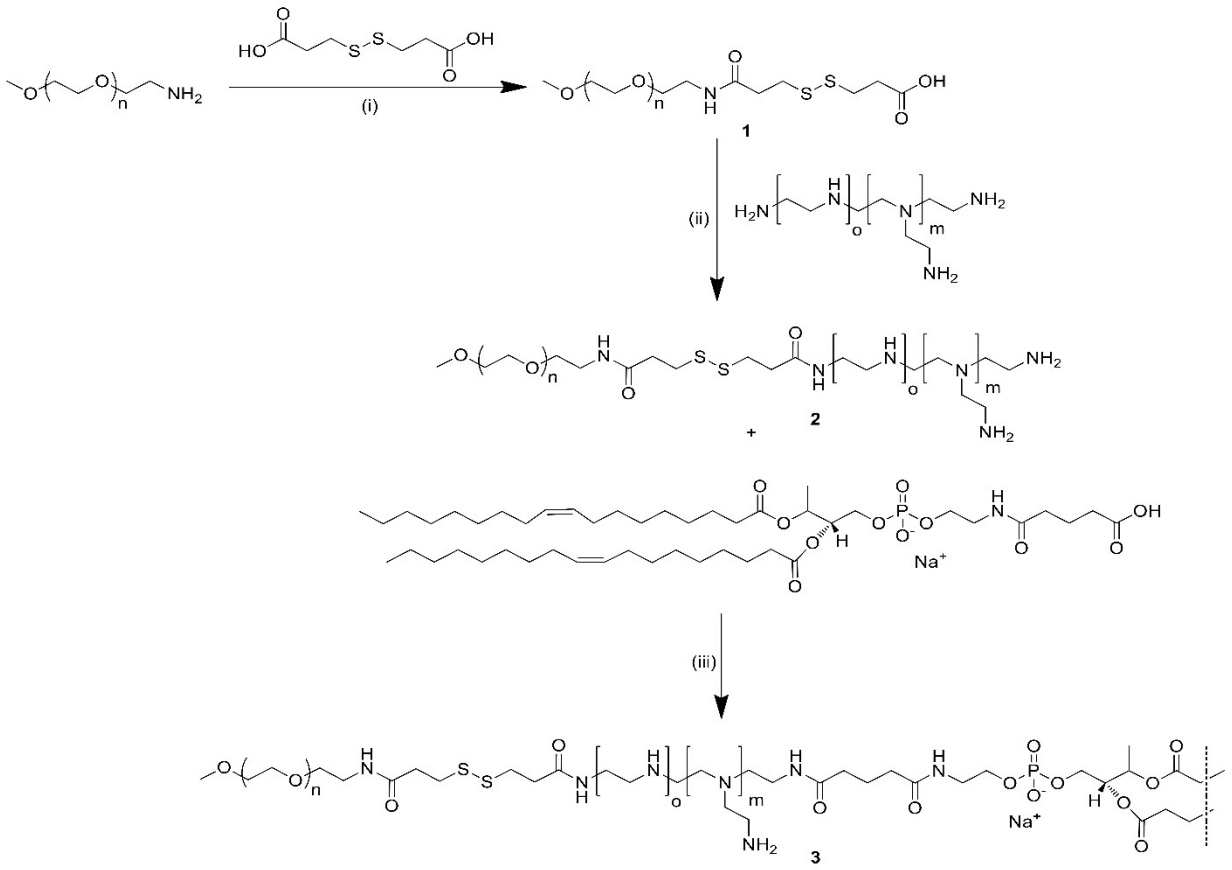
**S1.9. *In vitro* cytotoxicity studies**

C166-GFP cells were seeded in 96-well plates ($5x10^3$ cells/well) and incubated at 37 °C in a humidified atmosphere with 5% $CO_2$. After 24 h, the medium was replaced with 100 µl/well of serial dilutions of PSSPD and PPD in serum-free cell culture media. At the end of the 4h incubation period, the cells were washed with PBS and replenished with serum-complete media (100 µl). After 48 h incubation, 20 µl of CellTiter-Blue® was added to each well and the plates were incubated for an additional 1 h. The fluorescence was measured at excitation and emission wavelengths of 560 and 590 nm, respectively. Cell viability (%) was calculated using the following equation;

$$Cell\ viability\ (\%) = \frac{FL\ (test)}{FL\ (control)} \times 100$$

**S1.10. Green fluorescence protein downregulation**

C166-GFP cells were seeded in 12-well plates at a density of 5 x $10^4$ cells/well the day before transfection. The PSSPD-siRNA complexes were prepared with GFP siRNA at N/P ratios of 40, 60 and 100, at a final siRNA concentration of 150 nM. The complexes were added to cells in serum-free media and incubated for 4 h. At the end of the incubation period, cells were washed and incubated for an additional 44 h in serum complete medium. GFP down-regulation of PSSPD was evaluated by flow cytometry using blue laser and FL1 channel (530/30 bandpass filter). 10,000 events were collected per repetition from the gated live cells.
To confirm the specificity of the GFP siRNA and the formulations, PPD and PSSPD nanopreparations were loaded with scrambled (mock) siRNA (siSCR) at N/P ratio of 100. C166-GFP cells were treated at a final siSCR concentration of 150 nM under the same conditions as the GFP siRNA samples.




## Section S2. Supporting Figures

**Figure S1.** Synthesis procedure of PEG-SS-PEI-DOPE. Reagents and conditions: **(i)** 3,3´-dithiodipropionic acid, DCC, DMAP, rt; **(ii)** PEI, rt; **(iii)** NGPE, EDC, NHS, TEA, $N_2$, rt.

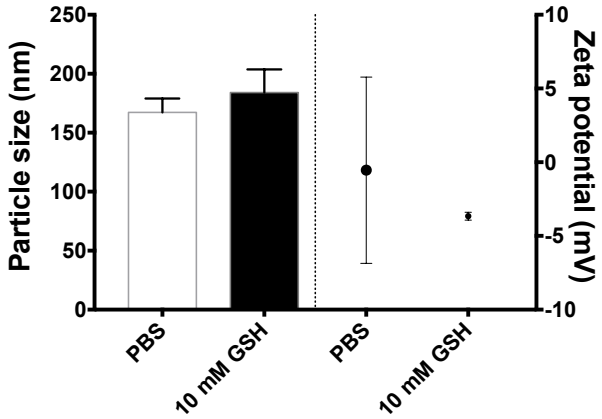



**Figure S2.** Particle size (bars, left y-axis) and zeta potential (mean spots, right y-axis) of PPD nanopreparation following exposure to 10 mM GSH for 4h at 37 ℃. Error bars represent n=3 independent measurements, ±SD.

## Author Contributions

N.B. Mutlu-Agardan performed the synthesis of compounds, characterization of the nanoformulations, wrote the manuscript. C. Sarisozen designed and performed the protein downregulation and cytotoxicity experiments, wrote the manuscript. V. P. Torchilin conceived the idea, coordinated the research and wrote the manuscript. N.B. Mutlu-Agardan and C. Sarisozen contributed equally to this work and should be considered as first authors.

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