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Polyphosphoester-based cationic nanoparticles serendipitously release integral biologically-active components to serve as novel degradable inducible nitric oxide synthase inhibitors

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EXPERIMENTAL SECTION

Materials. The amphiphilic and cationic diblock copolymer consisting of poly(2-ethylbutyl phospholane) (PEBP) as a hydrophobic segment and poly(3,4-bis((2-aminoethyl)thio)butylphospholane)(PAETBP) as a hydrophilic segment was synthesized according to the literature.[1] 3,3'-(Ethane-1,2-diylbis(oxy))dipropanoic acid (commercial name: Bis-dPEG®2-acid) was used as received from Quanta BioDesign, Ltd. (Powell, Ohio). Alexa Fluor® 488 Sulfodichlorophenol Ester (Alexa Fluor® 488 5-SDP Ester) was used as received from Life Technologies Corp. (Carlsbad, CA). 2,2-Dimethoxy-2-phenylacetophenone (DMPA) and 1-[3'-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (EDCI) were used as received from Sigma-Aldrich Company (St. Louis, MO). Nanopure water (18 MΩ·cm) was acquired by means of a Milli-Q water filtration system, Millipore Corp. (St. Charles, MO). Ultrapure water (Molecular Biology Grade, Fisher BioReagents) was used as received from Thermo Fisher Scientific Inc (Pittsburgh, PA). The RAW 264.7 cell line was obtained from the American Type Culture Collection. CellTiter 96 non-radioactive cell proliferation assay and Griess assay were from Promega Co. All cell culture media, TRIzol Reagent, Lipofectamine™2000 were purchased from Invitrogen, Inc. TURBO DNA-free kit, high-capacity cDNA reverse transcription kit and Power SYBR green master mix were from Ambion. LPS (lipopolysaccharides from *Escherichia coli* 055:B5) was from Sigma and IFN-gamma (γ-IFN, mouse, recombinant, *E. coli*) was from Peprotech.

Characterization Techniques. ^{1}H NMR, ^{31}P NMR and ^{13}C NMR spectra were recorded on an Inova 300 or Mercury 300 spectrometer interfaced to a UNIX computer using VnmrJ software. Transmission electron microscopy (TEM) was conducted on a Hitachi H-7500 microscope, operating at 100 kV. Samples for TEM measurements were prepared as follows: 4 μL of the dilute solution (with a polymer concentration of 0.1 mg/mL) was deposited onto a carbon-coated copper grid, and after 2 min, the excess of the solution was quickly wicked away with a piece of filter paper. The samples were then negatively stained with 1 *wt*% phosphotungstic acid (PTA) aqueous solution. After 1 min, the excess staining solution was quickly wicked away with a piece of filter paper and the samples were left to dry under ambient conditions overnight. The average diameter of nanoparticles on the TEM grid was obtained by measuring the core domain of 200 particles at different areas of the TEM specimen and the standard deviation was calculated. Dynamic light scattering (DLS) measurements were conducted using a Delsa Nano C from Beckman Coulter, Inc. (Fullerton, CA) equipped with a laser diode operating at 658 nm. Scattered light was detected at a 165° angle and analyzed using a log correlator over 70 accumulations for a 0.5 mL of sample in a glass size cell (0.9 mL capacity). The photomultiplier aperture and the attenuator were automatically adjusted to obtain a photon counting rate of *ca.* 10 kcps. The calculations of the particle size distribution and distribution averages were performed using CONTIN particle

size distribution analysis routines using Delsa Nano 2.31 software. The peak averages of histograms from intensity, volume and number distributions out of 70 accumulations were reported as the average diameter of the particles. All determinations were repeated 10 times. The zeta potential values of the nanoparticles were determined by Delsa Nano C particle analyzer (Beckman Coulter. Fullerton, CA) equipped with a 30 mW dual laser diode (658 nm). The zeta potential of the particles in suspension was obtained by measuring the electrophoretic movement of charged particles under an applied electric field. Scattered light was detected at a 30° angle at 25 °C. The zeta potential was measured at five regions in the flow cell and a weighted mean was calculated. These five measurements were used to correct for electroosmotic flow that was induced in the cell due to the surface charge of the cell wall. All determinations were repeated 6 times.

Formation of cationic micelles. The cationic diblock copolymers (10.0 mg) were suspended into ultrapure water (2.0 mL) and sonicated for 10 min. After being stirred for 2 h, a clear solution containing the cationic micelles for the cationic SCKs was obtained.

Formation of cationic SCKs (cSCKs). The cationic diblock copolymers (10.0 mg, 33.3 μmol of amino groups) were suspended into ultrapure water (2.0 mL) and sonicated for 10 min. After being stirred for 1 h, a clear solution containing the cationic micelles was obtained. To a stirred solution of micelle in a 5-mL vial was added a solution of the diacidcrosslinker (Bis-dPEG®2-acid, 1.0 mg, 5.0 μmol) dropwise in ultrapure water (0.2 mL). The mixture solution was allowed to stir for 1 h at room temperature. To this reaction mixture was added a solution of EDCI (4.3 mg, 15 μmol) dissolved in ultrapure water (0.5 mL) dropwise. The reaction mixture was allowed to stir overnight at room temperature and was then transferred to presoaked dialysis membrane tubing (MWCO *ca*. 6-8 kDa), and dialyzed against ultrapure water for 36 h in the cold room (4-8 °C) to remove small molecules. The final concentration of cSCKs was adjusted to 2.5 mg/mL by ultrapure water. The cSCK solution was able to be lyophilized into powder and kept in the freezer (-20 °C) for long-term storage and transportation, before being tested.

Formation of dye-labeled cSCKs. The cationic diblock copolymers (10.0 mg, 33.3 μmol of amino groups) were suspended into ultrapure water (2.0 mL) and sonicated for 10 min. After being stirred for 1 h, a clear solution containing the cationic micelles was obtained. To a stirred solution of micelle in a 5-mL vial was added a solution of the diacidcrosslinker (Bis-dPEG®2-acid, 1.0 mg, 5.0 μmol) in ultrapure water (0.2 mL) dropwise, followed by a solution of Alexa Fluor® 488 5-SDP ester (0.13 mg, 0.17 μmol) in ultrapure water (0.1 mL) dropwise. The mixture solution was allowed to stir for 1 h at room temperature. To this reaction mixture was added a solution of EDCI (4.3 mg, 15 μmol) dissolved in ultrapure water (0.5 mL) dropwise. The reaction mixture was allowed to stir overnight at room temperature and was then transferred to presoaked dialysis membrane tubing (MWCO *ca*. 6-8 kDa), and dialyzed against ultrapure water for 36 h in the cold room (4-8 °C) to remove small molecules. The final concentration of dye-labeled cSCKs was adjusted to 2.5 mg/mL by the addition of ultrapure water. The dye-labeled cSCK solution was able to be lyophilized into powder and kept in the freezer (-20 °C) for the long term storage and transportation, before being tested

General procedure of degradation studies. In a typical degradation experiment, a solution of cSCKs was adjusted to pH 5.0 or pH 7.4 by 150 mM PBS buffer, 150 mM acetic buffer (for ^{31}P NMR studies) or 150 MOPS buffer (for ^{31}P NMR studies). The mixture solution was incubated in a 37 °C shaker. The size, size distribution, zeta potential and ^{31}P chemical shifts were measured by Delsa Nano C particle analyzer and Inova 300 spectrometer, respectively, during the degradation.

Preparation of degraded cSCKs. A solution of cationic SCKs (2.5 mg/mL, 8 mL) was adjusted to pH 7.4 by PBS buffer (12 mM, 2 mL, without NaCl or KCl) and incubated in the 37 ℃ shaker. After full degradation of nanoparticles (12 days), the solution was lyophilized into powder before being tested.

Synthesis of 3,4-bis(2-aminoethylthio)butan-1-ol hydrochloride salt (Fragment A). A solution of 3-butyn-1-ol (0.20 g, 2.86mmol), cysteamine hydrochloride (3.24 g, 28.6 mmol), and DMPA (14.6 mg, 0.57 mmol) in 10.0 mL of methanol was sparged with nitrogen for 5 min and then irradiated under UV irradiation (365 nm) for 2 h. The reaction mixtures were precipitated from methanol into acetone four times to remove excess thiols and photoinitiator by-products to give 3,4-bis(2-aminoethylthio)butan-1-ol hydrochloride salt as a yellow liquid at a yield of 20%. ^{1}H NMR (D_2O, ppm): δ 3.74 (d, J = 5.7 Hz, HOCH_2CH$_2$), 3.34 (s, HOCH$_2$CH$_2$), 3.21 (t, J = 6.9 Hz, CH$_2$$CH_2NH_3$), 2.95 (m, SC$H_2CH_2NH_3$, SC$H_2CH$S), 2.01, 1.75 (dt, J =5.7, 6.0 Hz, HOCH$_2$$CH_2$CH). ^{13}C NMR ($CD_3OD$, ppm): δ 58.75, 42.11, 38.85, 38.42, 36.87, 35.46, 29.13, 27.15. ESI MS: calculated [M-H]$^-$ for $C_8H_{21}Cl_2N_2OS_2$: 295.0472, found: 295.0594. IR: 3600-2400, 2000, 1693, 1599, 1379, 1322, 1141, 1036, 939, 887 cm^{-1}.

Cell culture. RAW 264.7 cells (mouse monocyte-macrophage cell line) were maintained in DMEM containing 10% FBS, streptomycin (100 μg/mL), and penicillin (100 units/mL) at 37 ˚C in a humidified atmosphere with 5% CO_2.

Confocal microscopy of cSCKs cellular uptake. RAW 264.7 cells were plated in 35 mm MatTek glass bottom microwell dishes (MatTek) at a density of 2×10^4 cells per well and cultured in 100 μL DMEM containing 10% FBS. 24 hours later, AlexaFluor 488 labeled degradable cSCKs were incubated with the RAW 264.7 cells, and then cells were activated by LPS (1 μg/mL) and γ-IFN (20 ng/mL) after 6 h. At the time of confocal microscopy, 1 μL of Hoechst 33342 (10 μg/mL) was added to each dish to stain nuclei for one hour and then each dish was washed three times with PBS buffer and examined with a Nikon A1 Confocal Microscope (Nikon). In one experiment, different concentrations of AlexaFluor 488-cSCKs (0, 10, 40, 80, 150 μg/mL) were added to each dish and images were collected by confocal microscopy after 24 h incubation. In another experiment, degradable AlexaFluor 488-cSCKs (40 μg/mL) were added and images were collected at different time points (1 h, 6 h, 16 h, 24 h).

Flow cytometry of cSCKs cellular uptake. RAW 264.7 cells were plated in a 6 well plate at a density of 3×10^5 cells per well and cultured in 1 mL DMEM containing 10% FBS. After 24 h, AlexaFluor 488-cSCKs were added to RAW 264.7 cells, and cells were activated by LPS (1 μg/mL) and γ-IFN (20 ng/mL) 6 h later. At the time of flow cytometry, cells were washed 3 times with PBS, collected by trypsinization, pelleted, and resuspended in 0.5 mL PBS for flow cytometric analysis on an FACS-calibur instrument (Becton Dickinson) equipped with a 488 nm Ar laser. For each sample, 10,000 events were collected and the fluorescence from two channels was detected on a logarithmic scale, respectively. The data were processed by FlowJo software. In one experiment, different concentrations of AlexaFluor 488-cSCKs (0, 10, 20, 40, 100, 150 μg/mL) were added to each dish and data were collected after 24 h incubation. In another experiment, degradable cSCK-AlexaFluor 488 (40 μg/mL) were added and data were collected at different time points (0, 1 h, 4 h, 16 h, 21 h, 24 h).

Cytotoxicity assay of cSCKs in RAW 264.7 cells. The cytotoxicties of cSCKs were examined by CellTiter 96 non-radioactive cell proliferation assay (Promega). RAW 264.7 cells were seeded in a 96 well plate at a density of 2×10^4 cells per well and cultured in 100 μL

DMEM containing 10% FBS. After 24 h, the medium was replaced with 100 μL fresh culture medium containing cSCKs (fresh), cSCKs (degraded), fragment A, fragment B, fragment C and fragment D with different concentrations. 6 h later, cells were activated with LPS (1 μg/mL) and γ-IFN (20 ng/mL) to induce iNOS expression. After another 24 h, 15 μL of dye solution was added to each well and incubated at 37 °C in a humidified CO_2 incubator. 4 h later, 100 μL of stop solution was added to each well and incubated overnight. The absorbance of colored formazan product was recorded at 570 nm using a microplate reader (Molecular Devices) and normalized to the control group with no treatment. An average of three determinations was made.

Inhibition of iNOS expression by cSCKs determined by Griess assay. RAW 264.7 cells were seeded in a 96 well plate at a density of 2×10^4 cells per well and cultured in 100 μL DMEM containing 10% FBS. After 24 h, the medium was replaced with 100 μL fresh culture medium containing cSCKs (fresh), cSCKs (degraded), fragment A, fragment B, fragment C and fragment D with different concentrations. 6 h later, cells were activated with LPS (1 μg/mL) and γ-IFN (20 ng/mL). After another 24 h, nitrite level was measured by Griess assay (Promega) at 540 nm using a microplate reader (Molecular Devices) and normalized to the control group with no treatment. An average of three determinations was made.

Inhibition of iNOS mRNA by cSCKs determined by qRT-PCR. RAW 264.7 cells were seeded in 6 well plate at a density of 3×10^5 cells per well and cultured in 1000 μL DMEM containing 10% FBS. 24 h later, cells were transfected with cSCKs (fresh), cSCKs (degraded), fragment A, fragment B, fragment C and fragment D with different concentrations. Cells were then activated with LPS (1 μg/mL) and γ-IFN (20 ng/mL) after 6 h. After another 24 h, cells were washed twice with PBS and total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction and quantified spectrophotometrically by Nanophotometer (Implen). Approximately 500 ng of total RNA was treated with TURBO DNA-free kit (Ambion) and reverse transcribed with high-capacity cDNA reverse transcription kit (Ambion) according to standard protocols supplied by the manufacturer. cDNA synthesis was performed for 10 min at 25 °C and 2 h at 37 °C, followed by heat inactivation for 5 min at 85 °C. The real-time PCR assay was performed using the Applied Biosystems StepOne plus Real-Time PCR System (Applied Biosystems) with 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. PCR reactions were carried out in a 25 μL of reaction mixture consisting of 12.5 μL Power SYBR Green Master Mix (2*) (Ambion), 200 nM primers, cDNA and water. The primers used to amplify iNOS were d(TGGTGGTGACAAGCACATTT) and d(AAGGCCAAACACAGCATACC), and to amplify GAPDH were d(TGGAGAAACCTGCCAAGTATG) and d(GTTGAAGTCGCAGGAGACAAC). The threshold cycle (Ct) was calculated by the instrument's software (StepOne). The expression of each mRNA was calculated by the comparative Ct method (ΔΔCt). The average of 3 determinations was calculated.

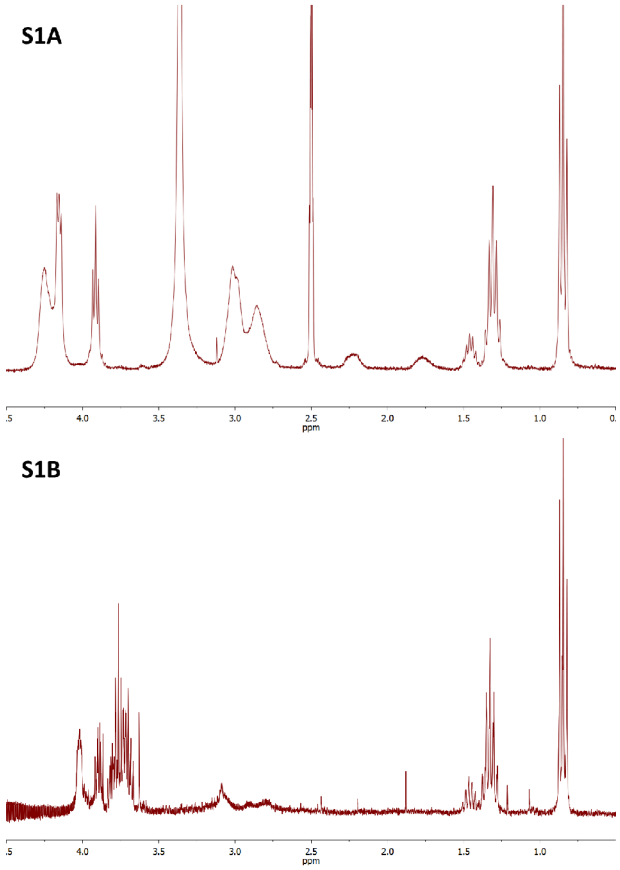

Figure S1. A) ^{1}H NMR (in d_6-DMSO) of cationic amphiphilic diblock PPE. B) ^{1}H NMR (in D_2O) of collected precipitated polymer/nanoparticle (around isoelectric point) after 1-2 days degradation. The disapperance of signals between 1.7 ppm and 3.2 ppm (on the Fragment A) verfied that the loss of cationic feature was due to the release of Fragment A after the cleavage of phosphoester linkages.

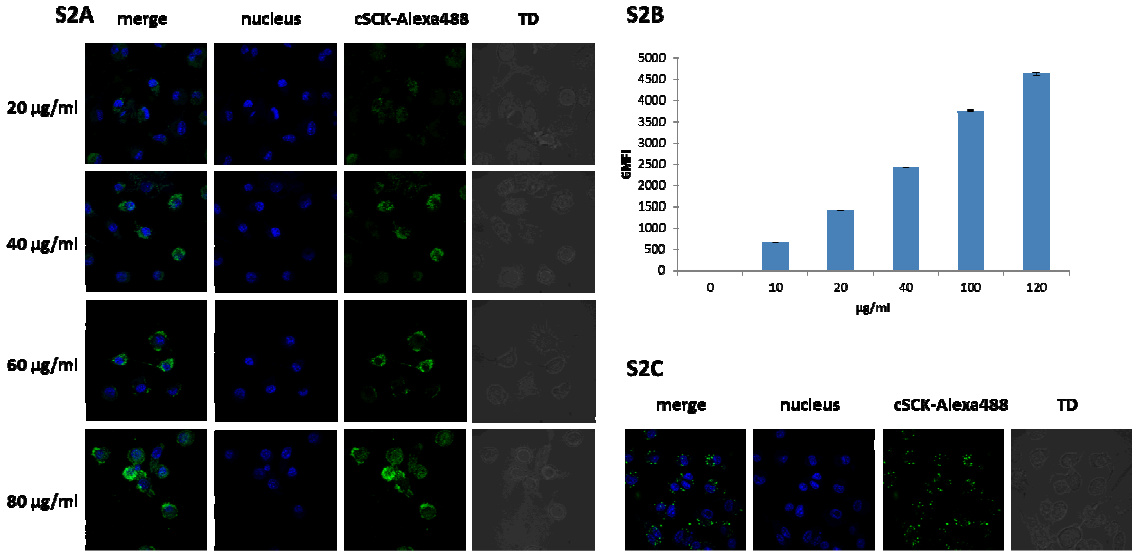

Figure S2. A) Cellular uptake of degraded cSCKs. Images were obtained under confocal microscopy. B) Degraded cSCKs with different concentrations at 24 h incubation. GMFI was quantitatively measured by Flow Cytometry. C) Cellular uptake of non-degradable cSCKs[2] (10 µg/mL) at 24 h incubation.

REFERENCES:

[1] Zhang, S.;Zou, J.; Zhang, F.; Elsabahy M.; Felder, S.; Zhu, J.; Pochan, D. J.; Wooley, K. L. *J. Am. Chem. Soc.* **2012**, *134*, 18467.
[2] Shen, Y.; Shrestha, R.; Ibricevic, A.; Gunsten, S. P.; Welch, M. J.; Wooley, K. L.; Brody, S. L.; Taylor, J.-S. A.; Liu, Y. *Interface Focus* **2013**, *3,* 3.