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Supporting Information

A Two-Pronged Pulmonary Gene Delivery Strategy: A Surface-Modified Fullerene Nanoparticle and a Hypotonic Vehicle

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Supplementary Materials and Methods

Materials. N-hydroxylsuccinimide (NHS) functionalized polyethylene glycol (PEG-NHS; 5 kDa) was purchased from Creative PEG Works. Tris-buffered saline (TBS, 10×) was purchased from Corning and diluted by 10 times with ultrapure water (Invitrogen) before use. Plasmid encoding fluorescence protein ZsGreen (pZsgreen1-C1 or pZG) was obtained from Clontech Laboratories, and plasmid encoding luciferase (pd1GL3-RL or pGL3) was kindly provided by Professor Alexander M. Klibanov at the Massachusetts Institute of Technology (MIT). Luciferase assay system was purchased from Promega. Mounting medium with DAPI was purchased from Abcam. Tissue-Tek® O.C.T. Compound was from Fisher.

Synthesis of TPFE and TPFE-PEG. 1-tert-butyloxycarbonyl-protected tetra(piperazino)fullerene epoxide (Boc-TPFE) was firstly synthesized using a previously established method.[1] Briefly, 250 mg C_{60} was added to a mixture of 12.5 ml *o*-dichlorobenzene and 3.15 ml dimethyl sulfoxide (DMSO) under stirring, subjected to oxygen flow at room temperature for 5 minutes and reacted with 248 mg 1-tert-butyloxycarbonyl piperazine at room temperature for 36 hours. Saturated ammonium chloride aqueous solution was added and shaken prior to the collection of the organic phase. After washing with saturated sodium bicarbonate and ultrapure water, the organic phase was evaporated and dried at 80 ºC in vacuum overnight to remove DMSO. Boc-TPFE was purified with silica column, with toluene and acetonitrile as the mobile phase. The obtained Boc-TPFE was dissolved in $CDCl_3$ and methanol for ^{1}H-NMR and ESI-MS characterization, respectively.

Tetrapiperazino fullerene epoxide (TPFE) was obtained by deprotection of Boc-TPFE. 250 mg Boc-TPFE was dissolved in 142 ml chloroform and brought to 0 ºC while stirring and then 48 ml trifluoroacetic acid was added dropwise and reacted for 30 minutes. Subsequently, the

temperature was adjusted to room temperature and reacted for 24 hours. The organic solvent was removed by rotary evaporation and vacuum drying at 40 ºC for 4 hours. The obtained solid product was hydrated with 75 ml ultrapure water, centrifuged at 10,000 rpm for 5 minutes to remove aggregates, filtered with 0.22 μm membrane and freeze-dried for later uses. The dried TPFE was dissolved in $(CD_3)SO$ and methanol for ^{1}H-NMR and ESI-MS characterization, respectively.

TPFE was PEGylated by reacting with 5 kDa PEG-NHS. 50 mg TPFE and 1g PEG-NHS were dissolved in 5 ml DMF and stirred overnight, and then extensively dialyzed against ultrapure water for 1 week to remove DMF. The unconjugated PEG was removed by ultrafiltration (MWCO: 10 kDa; Thermo Scientific™) for three times. The TPFE-PEG product was obtained by lyophilization. A small amount of TPFE-PEG was dissolved in D_2O for ^{1}H-NMR characterization.

Formulation of plasmid DNA-loaded fullerene nanoparticles. To formulate pZG/TPFE and pGL3/TPFE, 5 mg respective plasmid DNA was dissolved in TBS, added dropwise to 4 μl TPFE in 2 mM KCl aqueous solution (10 mg/ml) to make a final total volume of 100 μl, and the mixture was incubated at room temperature for 30 minutes. Likewise, pZG/TPFE-PEG and pGL3/TPFE-PEG were prepared in the same way, except 10 μl TPFE-PEG (10 mg/ml) was mixed with TPFE prior to the dropwise addition of plasmid DNA. Hydrodynamic diameter and ζ-potential of particles were measured with a Zeta Nanosizer ZS90. In parallel, particle samples were added to copper grids and dried overnight to be imaged with a Hitachi H7600 microscope to microscopically determine morphology and geometric size.

Colloidal stability of pZG/TPFE-PEG in bronchoalveolar lavage fluid (BALF). BALF was harvested from C57BL/6 mice and the protein content was determined by a BCA protein assay

kit (Thermo Fisher Scientific). The freshly prepared pZG/TPFE-PEG and pZG/TPFE was added to the BALF, incubated at room temperature and the hydrodynamic diameters were measured over time with a Zeta Nanosizer ZS90.

Colloidal stability of pZG/TPFE-PEG in a mucin solution. An aqueous mucin solution was prepared by dissolving 5 mg porcine stomach Type II mucin (Sigma Aldrich) in 1 ml ultrapure water, followed by filtration with a 0.22 μm filter. The freshly prepared pZG/TPFE-PEG and pZG/TPFE were mixed with the mucin solution at a 1:1 volume ratio, incubated at room temperature for 5 minutes and the hydrodynamic diameters were measured over time with a Zeta Nanosizer ZS90.

***In vitro* macrophage uptake.** Mouse alveolar microphages (MH-S, ATCC) were cultured in ATCC-formulated RPIM-1640 medium supplemented with 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum. Cells were seeded to a 24 welled plate at a density of 10,000 cells per well. Cell media was first removed and pZG/TPFE and pZG/TPFE-PEG suspended in DPBS were added to the cells at a concentration of 5 μg/ml, followed by a 2-hour incubation. Subsequently, DPBS containing residual nanoparticles was collected and then cells were washed with 100 μl DPBS for three times. The nanoparticle-containing and washing DPBS were combined and used to determine the absorbance at 400 nm wavelength by Nanodrop to quantify the amounts of non-phagocytosed pZG/TPFE and pZG/TPFE-PEG. Identically processed untreated cells were served as a control.

Cell culture. A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS). Calu-3 cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and 1% PS. Cells were incubated at 37°C in a 5% CO_2 in atmosphere.

Cell viability test. A549 cells were seeded in a 96 well plate at a density of 5,000 cells per well. Twenty-four hours later, cells were treated with pGL3/TPFE or pGL3/TPFE-PEG at varying concentrations. The medium was replaced with fresh DMEM, following a 24-hour incubation, and cell viability was determined using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies).

***In vitro* transgene expression.** A549 cells were seeded into confocal dishes at a density of 10,000 cells per dish and incubated until reaching confluence of 70% - 80%. The medium was then removed and cells were treated with pZG/TPFE-PEG suspended in 90% (i.e., 90% media and 10% ultrapure water to create a mildly hypotonic vehicle condition) or 100% media (i.e., isotonic condition) at 0.1 mg/ml plasmid DNA concentration. The treated cells were incubated for 5 hours, replenished with fresh media and incubated for 2 days. The cells were stained with DAPI prior to imaging with a confocal laser scanning microscope for the assessment of distribution of reporter transgene expression.

To assess the overall level of transgene expression in A549 and Calu-3 cells, cells were treated as described above but with pGL3/TPFE-PEG. Cells were harvested 2 days after the treatment and subjected to cell lysis by a cell lysis reagent and three freeze-and-thaw cycles. Luciferase activity, as a measure of the overall level of transgene expression, was determined using a standard luciferase assay kit (Promega, Madison, WI) and a 20/20n luminometer (Turner Biosystems, Sunnyvale, CA). The luciferase activity was normalized by the total protein content measured by a BCA protein assay kit (Thermo Fisher Scientific).

Animal Experiments. All the animal experiments are conducted under the supervision of The Animal Care and Use Program at Johns Hopkins. The approved animal protocol number is MO19M96 and Dr. Laura Ensign is the PI and Dr. Jung Soo Suk is the co-PI.

***In vivo* transgene expression.** pZG, pZG/TPFE or pZG/TPFE-PEG (0.5 mg/ml pZG in 50 µl vehicle solution) was intratracheally instilled into the lungs of C57BL/6 or epithelial sodium channel-overexpressing transgenic (*Scnn1b*-Tg) mice (backgrounded on C57BL/6 mice) after anesthetized by ketamine and xylazine. Mice were sacrificed 2 days after the administration and lungs were subjected to vascular perfusion with 15 ml DPBS to flush out blood. The lungs were then harvested and embedded in O.C.T. before inflated with a mixture of DPBS and O.C.T at a volume ratio of 1:1. Subsequently, the lung samples were frozen overnight at -80 ºC, sectioned and stained with DAPI prior to imaging with a confocal laser scanning microscope for the assessment of distribution of reporter transgene expression.

To assess the overall level of transgene expression, animals were treated as described above but with pGL3, pGL3/TPFE or pGL3/TPFE-PEG. Lung Tissues were harvested 2 days after the administration and subjected to an extensive bead-milling, three freeze-and-thaw cycles and centrifuge at 17,000 g for 10 minutes. Luciferase activity, as a measure of the overall level of transgene expression, in the supernatant was measured using a standard luciferase assay kit (Promega, Madison, WI) and a 20/20n luminometer (Turner Biosystems, Sunnyvale, CA). The luciferase activity was normalized by the total protein content measured by a BCA protein assay kit. For the image-based quantification, 9 non-overlapping areas were randomly chosen from each slide (3 slides from each mouse) and the images were captured under 20 × objective. The percentage of positive cells was measured with QuPath. Measurement settings include: background radius: 15 px; median filter radius: 0 px; sigma: 3 px; minimum area: 10 px^2; maximum area: 1000 px^2; intensity threshold: 25, split by shape; cell expansion: 5 px, include cell nucleus; general parameters: smooth boundaries and make measurements; score

compartment: cell (green mean); single threshold: 20. number of fields used per analysis: 9; average number of cells per field: 600.

To determine the role of the osmotically-driven regulatory volume decrease (RVD) mechanism on transgene expression, pZG/TPFE-PEG and PGL3/TPFE-PEG were concomitantly administered with 100 nM suramin, an inhibitor of RVD, in vehicle solutions with varying osmolality, and the distribution and overall level of transgene expression was assessed as described above.

Blood biochemistry analysis and H&E staining. C57BL/6 mice intratracheally received pGL3/TPFE-PEG (0.5 mg/ml pGL3 in 50 µl ultrapure water) and euthanized 2 days after the administration. Whole blood was collected in BD Microtainer Lavender Tubes by removing the eyeballs of the mice and serum was collected by centrifuge at 4000 rpm for 10 minutes. The blood biochemistry analysis was conducted on an automated clinical chemistry analyzer (Alfa Wassermann, West Caldwell, NJ) to determine the liver and kidney toxicity. In parallel, major organs, including lung, liver, kidney, spleen and heart, were harvested and fixed in 10% formalin for 48 hours. The tissues were then embedded in paraffin and sectioned for H&E staining. The H&E-stained slices were imaged on an upright microscope (Nikon Eclipse NiU) for the assessment of acute inflammation and tissue damage.

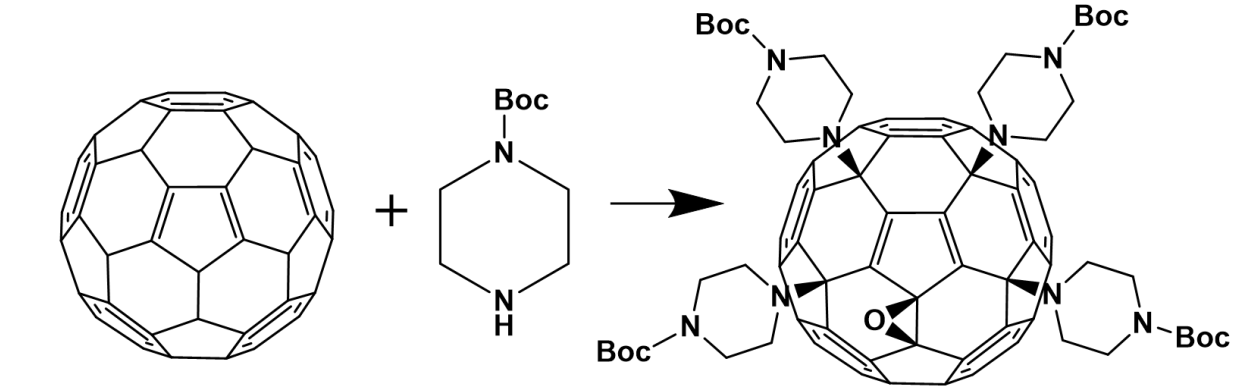
Measurement of inflammatory cells. After euthanizing C57BL/6 mice, we established a small hole was in the trachea and inserted an 18 G metal tube adaptor into the trachea through the hole. Three successive volumes of 1 ml of DPBS were instilled, gently aspirated and pooled. The obtained solution was then centrifuged at 1,500 × g for 10 minutes at 4°C and the cells were collected. The cells were then fixed in paraformaldehyde and were counted with a hemocytometer. Differential cell counting samples were prepared as follows. Cells were fixed

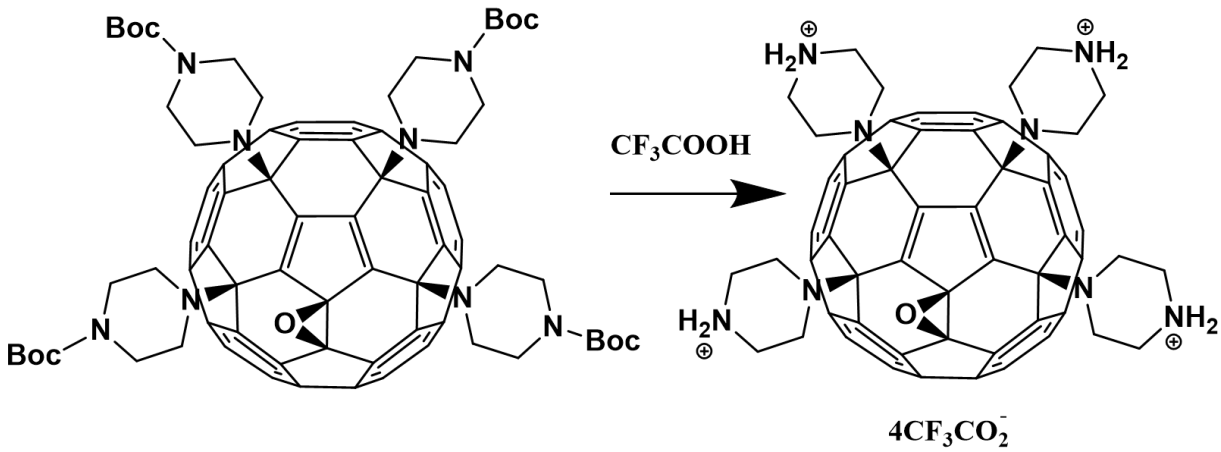
onto microscope slides and allowed to dry overnight. The cells were stained with Diff-Quik stain (Electron Microscopy Sciences). Cells were imaged with a Nikon E-600 microscope and a total of 5 - 10 images were taken per sample. A trained, blinded participant classified each cell in the images as macrophage, neutrophil, lymphocyte or other immune cells. A minimum of 300 cells were classified and counted per sample.

Quantification of cytokines. The levels of cytokines, including IL-2, IL-4, INF-γ, IL-10 in BALF form all animals were quantified by enzyme-link immunosorbent assay (ELISA) as per the manufacture's protocol. The Th1/Th2 mouse uncoated ELISA kit were purchased from Thermo Fisher Scientific.

Statistics. Statistical significance between treatment groups was determined via a two-tailed Student's t test, assuming unequal variances. Comparisons between multiple groups were determined using one-way analysis of variance (ANOVA) with Tukey's post hoc test. Calculations were performed using GraphPad Prism software. P values less than 0.05 were considered significant.

Supplementary Figures and Tables





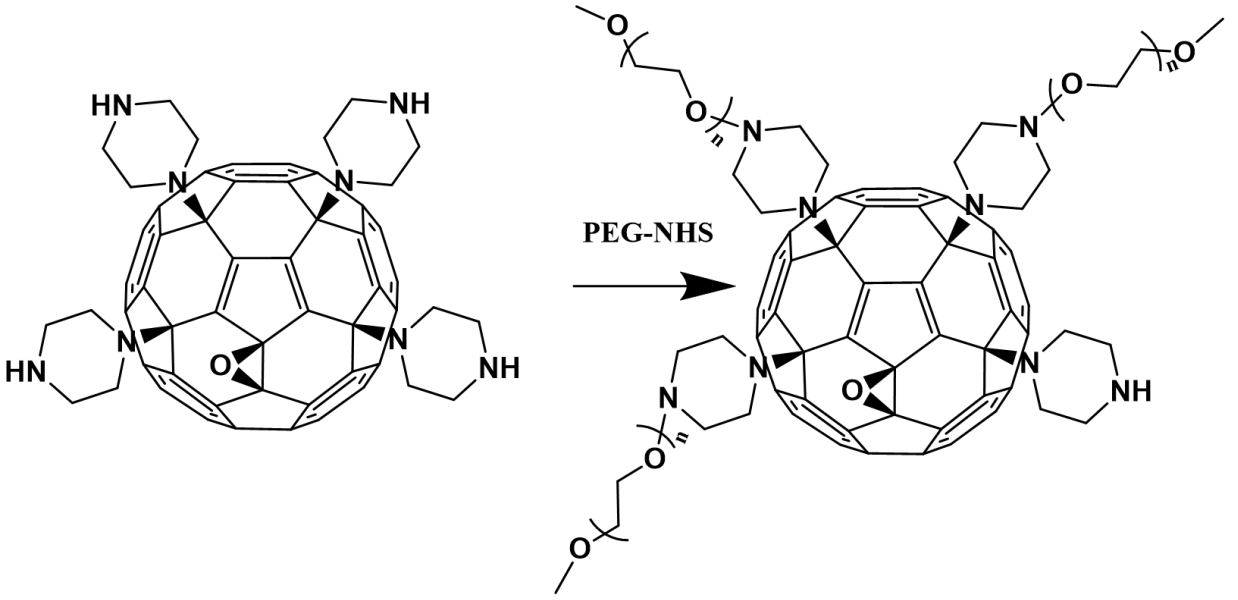


Figure S1. Synthesis of TPFE and TPFE-PEG.

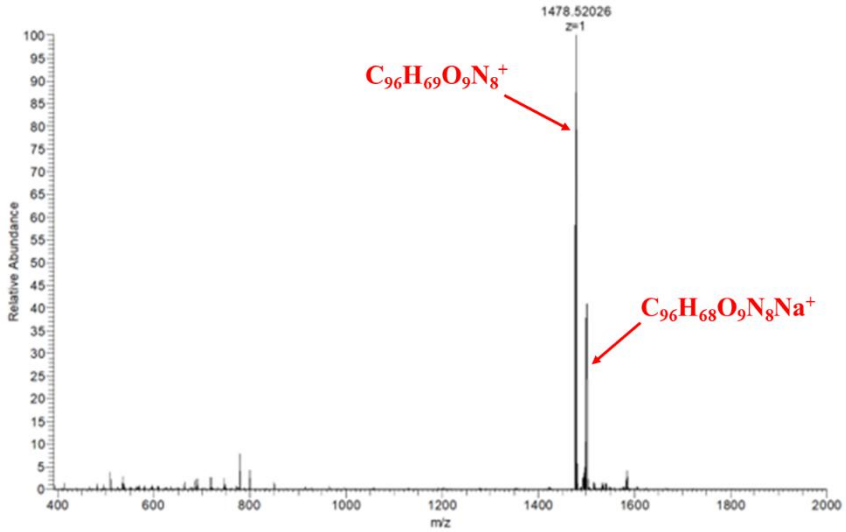


Figure S2. Mass spectrum of Boc-TPFE.

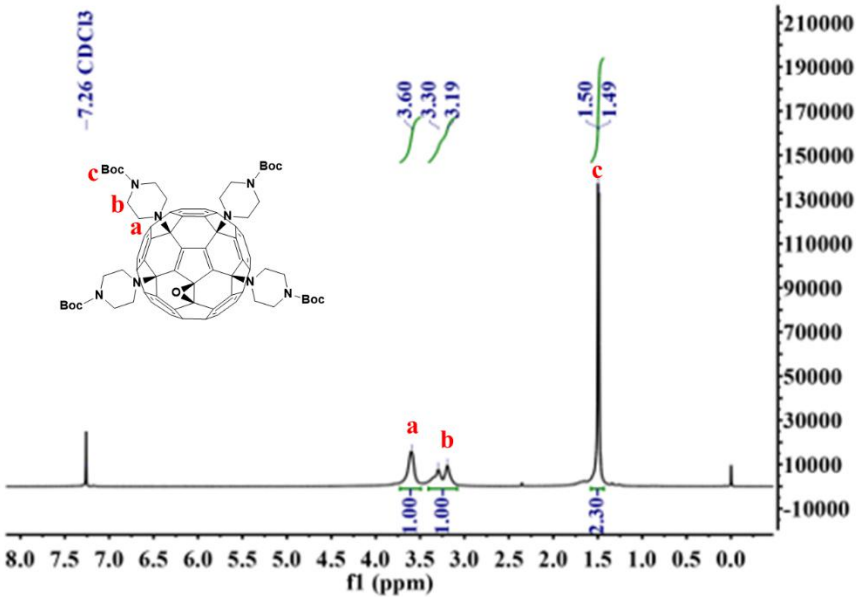


Figure S3. ^{1}H-NMR spectrum of Boc-TPFE in $CDCl_3$.

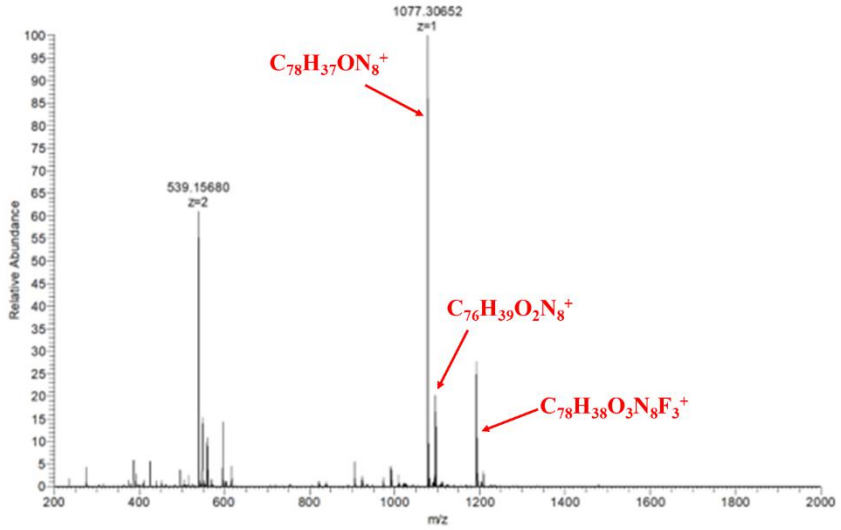


Figure S4. Mass spectrum of TPFE.

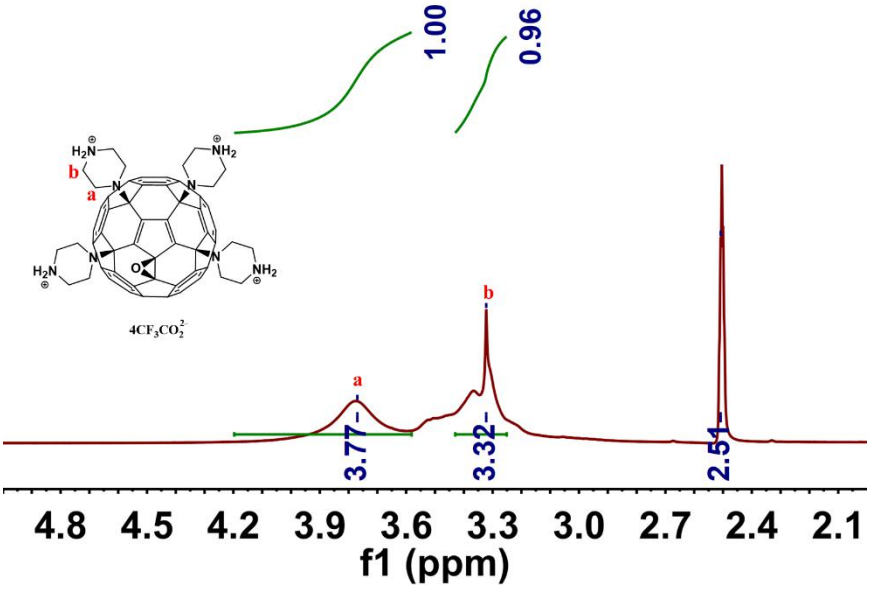


Figure S5. ^{1}H-NMR spectrum of TPFE in $(CD_3)_2SO$.

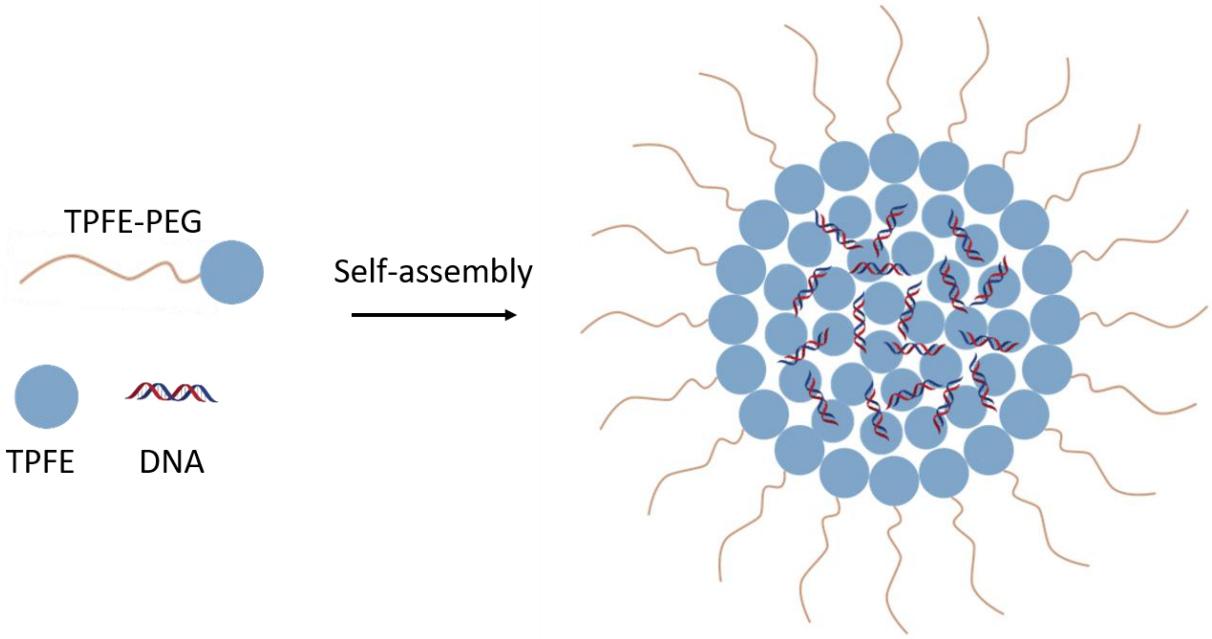


Figure S6. Schematic of DNA/TPFE-PEG.

Table S1. Physiochemical properties of plasmid DNA-loaded fullerene nanoparticles, including pZG/TPFE, pZG/TPFE-PEG, pGL3/TPFE and pGL3/TPFE-PEG. Hydrodynamic diameters and PDI were measured via dynamic light scattering (DLS) in ultrapure water and ζ-potentials were measured via laser Doppler anemometry in 10 mM NaCl at pH 7.4.

	Hydrodynamic diameter: Z-Ave (nm)	**Hydrodynamic diameter: Intensity mean (nm)**	**Hydrodynamic diameter: Number mean (nm)**	**PDI**	**ζ-potential (mV)**	**Osmolality (mOsm/kg)**
pZG/TPFE	90.4 ± 1.2	97 ± 2.6	45.1 ± 0.9	0.22 ± 0.02	32.8 ± 1.1	10 ± 3
pZG /TPFE-PEG	73.4 ± 0.7	87.6 ± 0.6	42.4 ± 3.2	0.16 ± 0.01	1.3 ± 0.2	7 ± 1
pGL3/TPFE	87.4 ± 0.2	98.8 ± 3.7	56.0 ± 4.2	0.13 ± 0.01	25.1 ± 1.3	12 ± 5
pGL3 /TPFE-PEG	83.2 ± 2.6	98.8 ± 3.1	39.1 ± 3.4	0.23 ± 0.01	3.2 ± 0.2	9 ± 2

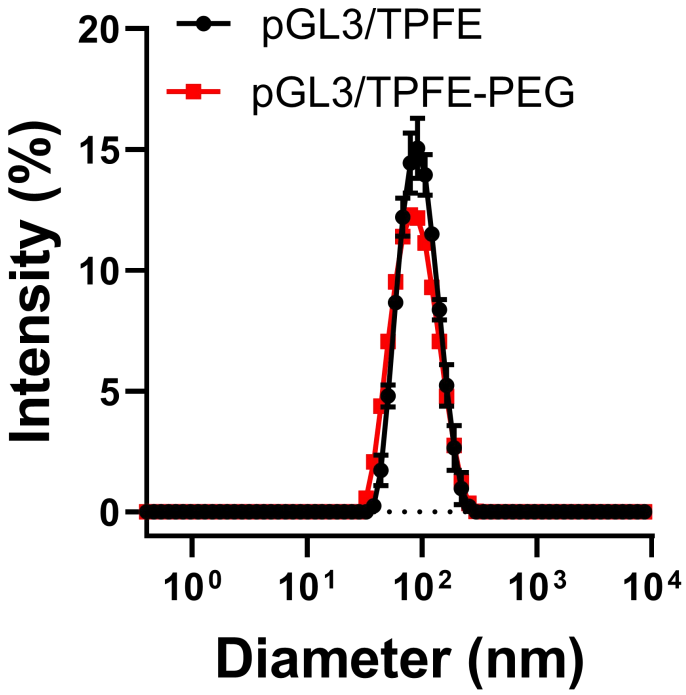


Figure S7. Hydrodynamic diameter profiles of pGL3/TPFE and pGL3/TPFE-PEG.

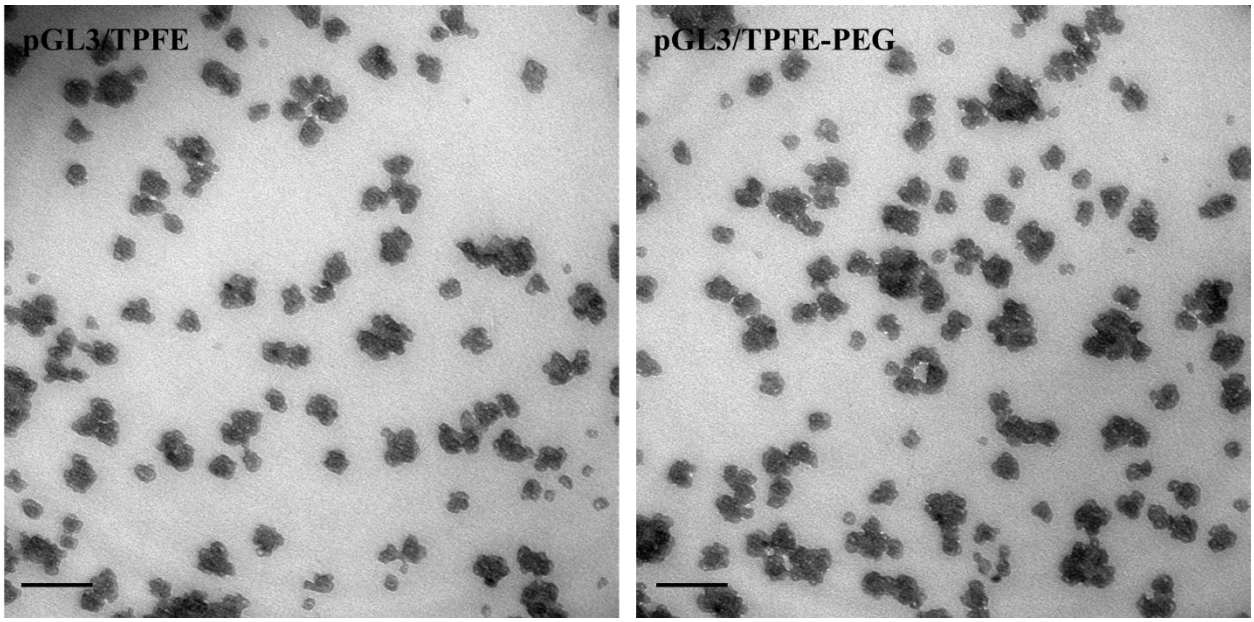


Figure S8. Representative transmission micrographs of pGL3/TPFE and pGL3/TPFE-PEG. Scale bars = 100 nm.

Table S2. Physiochemical properties of pZG/TPFE-PEG and pGL3/TPFE-PEG in vehicle solutions with varying osmolality.

NPs	Vehicle	Hydrodynamic size (nm)	PDI	ζ-potential (mV)	Osmolality (mOsm/kg)
pZG /TPFE-PEG	Isotonic	84.1 ± 3.1	0.19 ± 0.02	2.8 ± 0.4	290 ± 18
	Mildly Hypotonic	84.1 ± 1.2	0.21 ± 0.03	3.6 ± 1.0	157 ± 20
	Hypotonic	88.1 ± 2.5	0.23 ± 0.05	3.7 ± 0.6	15 ± 7
pGL3 /TPFE-PEG	Isotonic	87.6 ± 2.1	0.25 ± 0.07	3.7 ± 0.7	297 ± 18
	Mildly Hypotonic	84.7 ± 1.8	0.29 ± 0.13	4.6 ± 1.0	167 ± 16
	Hypotonic	84.1 ± 2.9	0.25 ± 0.07	3.9 ± 0.7	10 ± 5

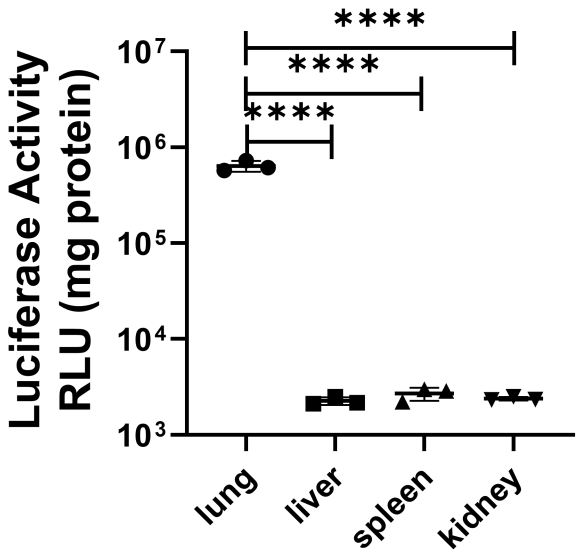


Figure S9. Luciferase activity measured in major organs harvested from C57BL/6 mice intratracheally received pGL3/TPFE-PEG (n=3).

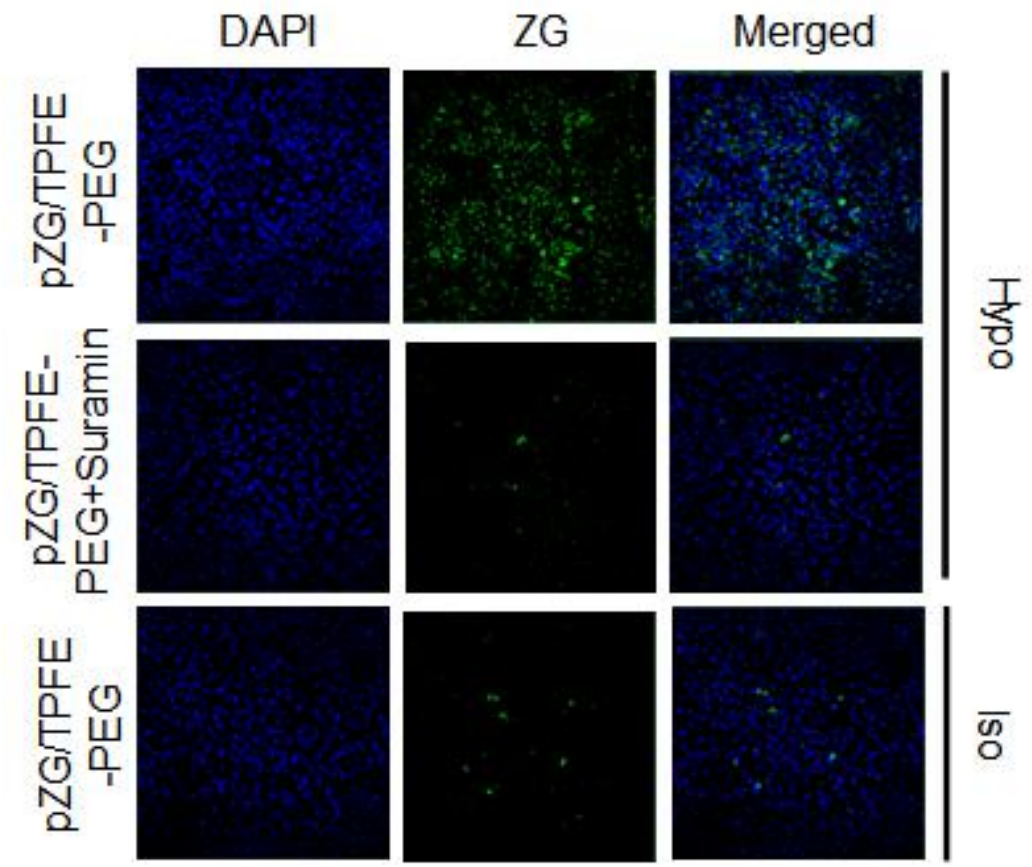


Figure S10. Representative confocal images demonstrating reporter transgene expression in A549 cells mediated by pZG/TPFE-PEG in an isotonic vehicle or in a mildly hypotonic vehicle (± 100 nM suramin).

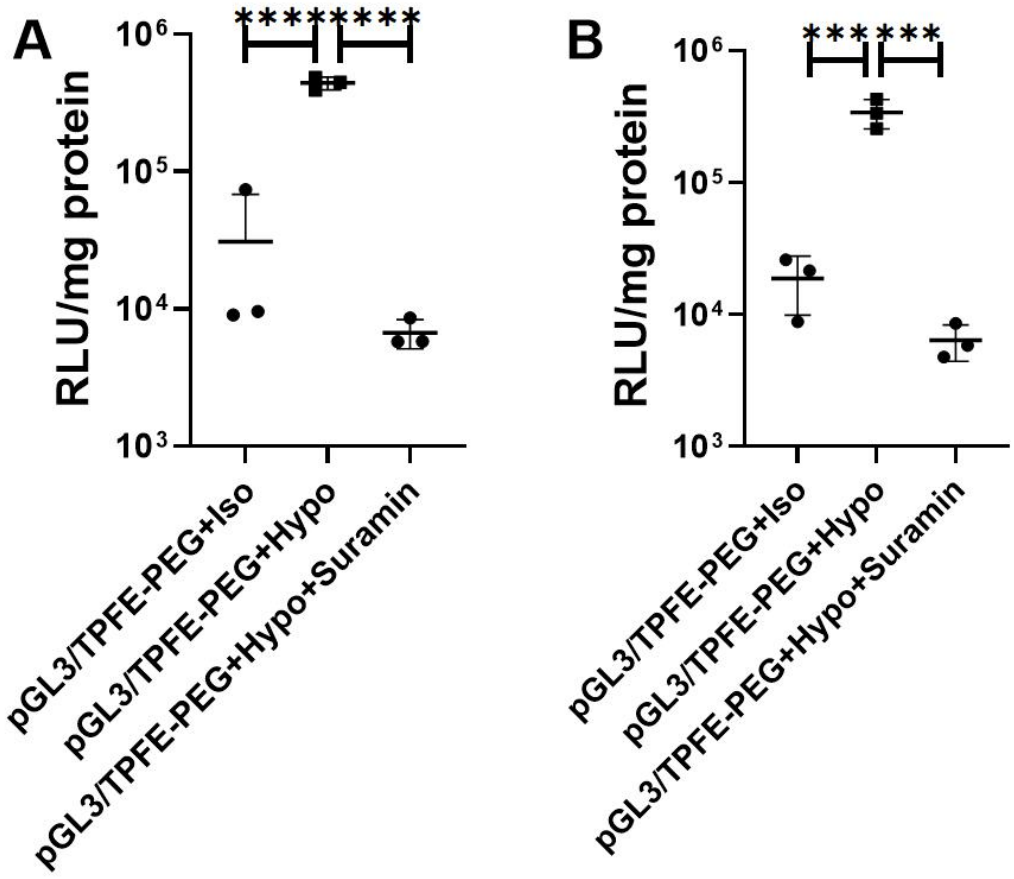


Figure S11. Overall level of reporter transgene expression mediated by pGL3/TPFE-PEG in an isotonic vehicle or in a mildly hypotonic vehicle (± 100 nM suramin) in **(A)** A549 and **(B)** Calu-3 cells (n=3).

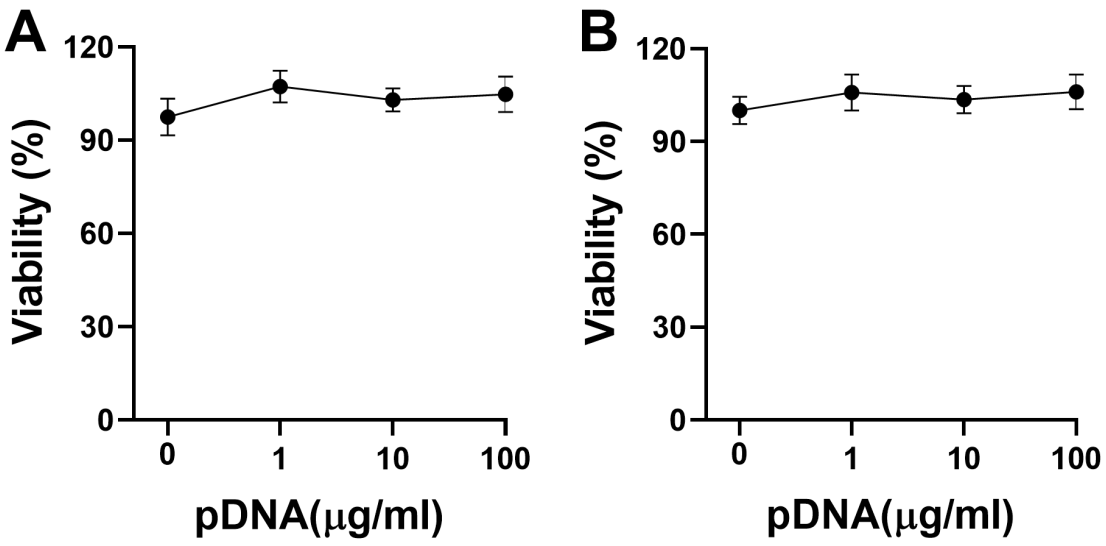


Figure S12. A549 cell viability after incubation with **(A)** pGL3/TPFE and **(B)** pGL3/TPFE-PEG at varying concentrations.

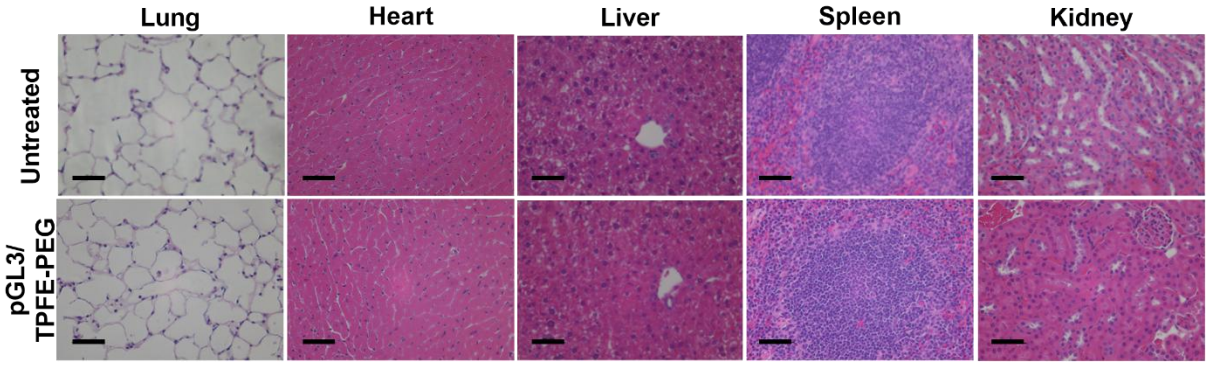


Figure S13. Representative histological images demonstrating *in vivo* safety of pGL3/TPFE-PEG. H&E staining of various tissues, including lung, heart, liver, spleen and kidney, from untreated C57BL/6 mice (Upper) and C57BL/6 mice received a single intratracheal dose of pGL3/TPFE-PEG in ultrapure water (Lower).

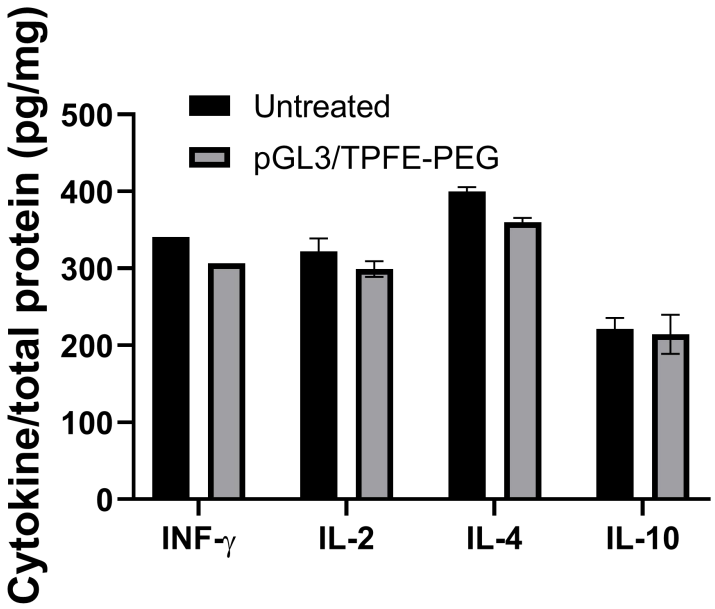


Figure S14. Quantification of Th1/Th2 cytokines in BALF from untreated mice and mice received a single intratracheal dose of pGL3/TPFE-PEG in ultrapure water by ELISA (n = 5).

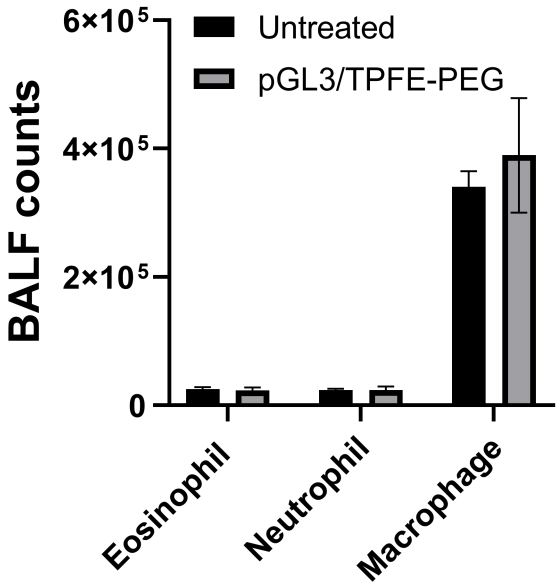


Figure S15. Quantification of cellularity in the BALF from untreated mice and mice received a single intratracheal dose of pGL3/TPFE-PEG in ultrapure water (n = 5).

Table S3. Blood biochemistry analysis of C57BL/6 mice and C57BL/6 mice intratracheally received a single dose of pGL3/TPFE-PEG in ultrapure water. Blood samples were collected 48 hours after the administration.

	Untreated	**pGL3/TPFE-PEG**	**Normal range**
ALT (U/L)	37.8 ± 2.6	40.8 ± 1.9	18 - 82
AST (U/L)	112.6 ± 8.5	117 ± 6.1	178 - 526
BUN (mg/dL)	25.6 ± 2.2	25.6 ± 2.2	18 - 36
B/C ratio	110.4 ± 14.3	118.4 ± 11.6	--
CREA (mg/dL)	0.24 ± 0.03	0.22 ± 0.02	0.2 - 0.5

References

[1] H. Isobe, T. Tanaka, W. Nakanishi, L. Lemiègre, E. Nakamura, *J. Org. Chem.* **2005**, *70*, 4826-4832.