Supporting information

Title: Assessment of Neurotoxicity Induced by Different-Sized Stöber Silica: Induction of Pyroptotic Cell Death in Microglia

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# *Equal contribution.*

Figure S1

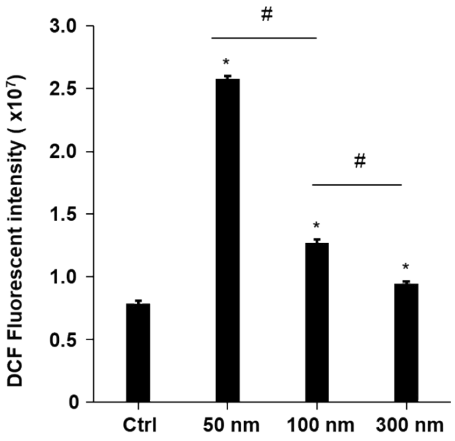


Figure S1. Abiotic ROS generation induced by Stöber silica Nanoparticles. DCF was used to determine the reactive oxygen species (ROS) generation potential of particles. $^{*}p < 0.05$ compared to negative control. $^{\#}p < 0.05$ compared to 100 nm silica.

Figure S2

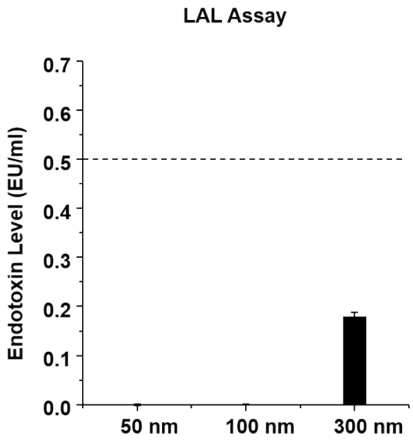


Figure S2. Endotoxin levels of Stöber silica nanoparticles.

Figure S3

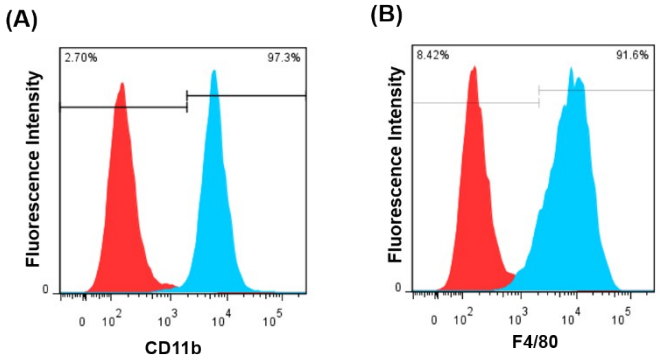


Figure S3. Flow cytometry analysis of (A) CD11b and (B) F4/80 expressions on N9 cell surfaces after treated with LPS (1 μg/mL, 4 h).

Figure S4

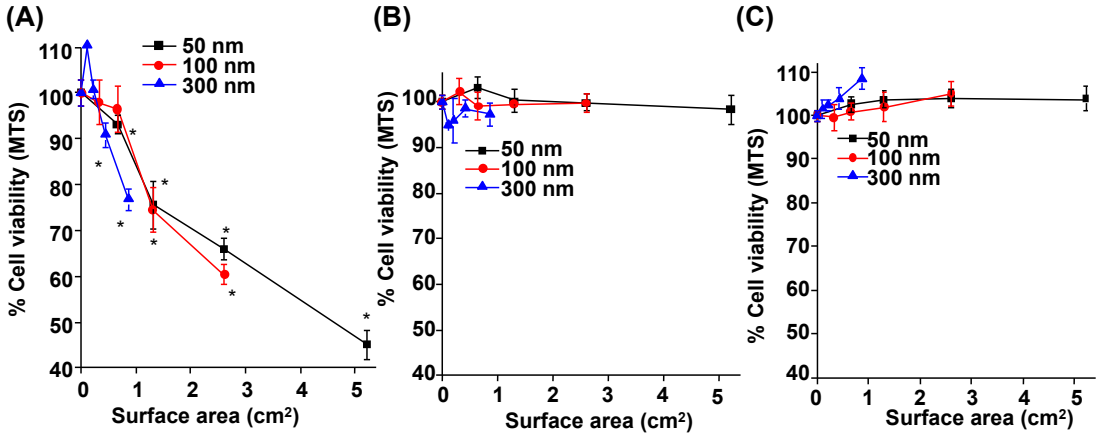


Figure S4. Cytotoxicity profiles of Stöber silica nanoparticles in N9, bEnd.3 and HT22 cells. Cell viability of (A) N9, (B) bEnd.3 and (C) HT22 cells after exposure to Stöber silica for 24 h. The results were shown in surface area dose. $^{*}p < 0.05$ compared to non-treated control cells.

Figure S5

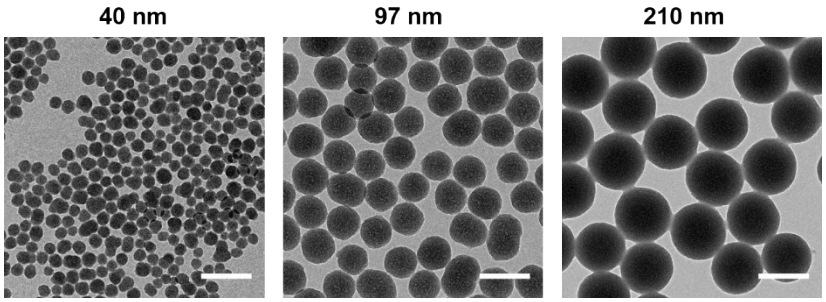


Figure S5. TEM images of in-house synthesized Stöber silica nanoparticles. Scale bar is 200 μm.

Figure S6

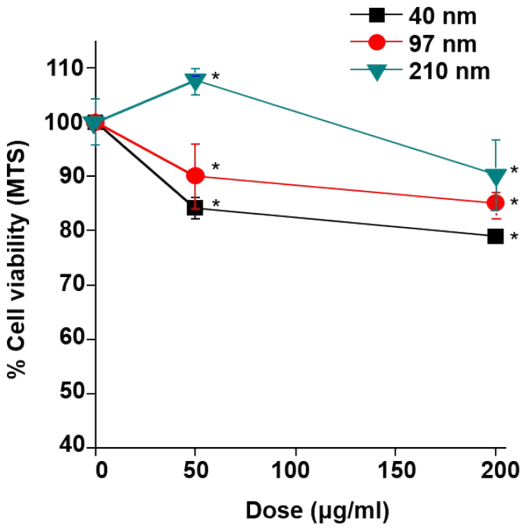


Figure S6. Cell viability of N9 cells after Stöber silica nanoparticles treatment. LPS-primed (1 µg/mL, 16 h) N9 cells were exposed to in-house synthesized Stöber silica at 50 and 200 µg/mL for 24 h, and MTS was used to determine cell viability. Cell viability of non-treated cells were regarded as 100%. $^{*}p < 0.05$ compared to nontreated control cells.

Figure S7

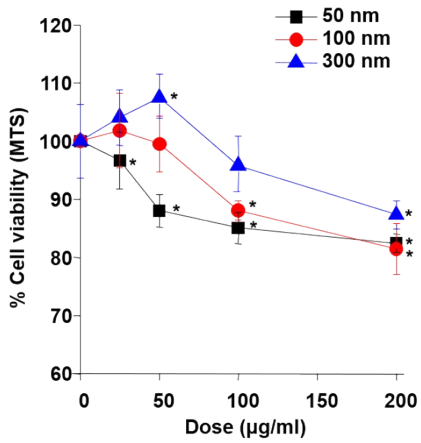


Figure S7. Cell viability of BV-2 microglial cells after exposure to Stöber silica nanoparticles. LPS-primed (1 µg/mL, 16 h) BV-2 cells were exposed to Stöber silica 24 h, and MTS was used to determine cell viability. $^{*}p < 0.05$ compared to nontreated control cells.

Figure S8

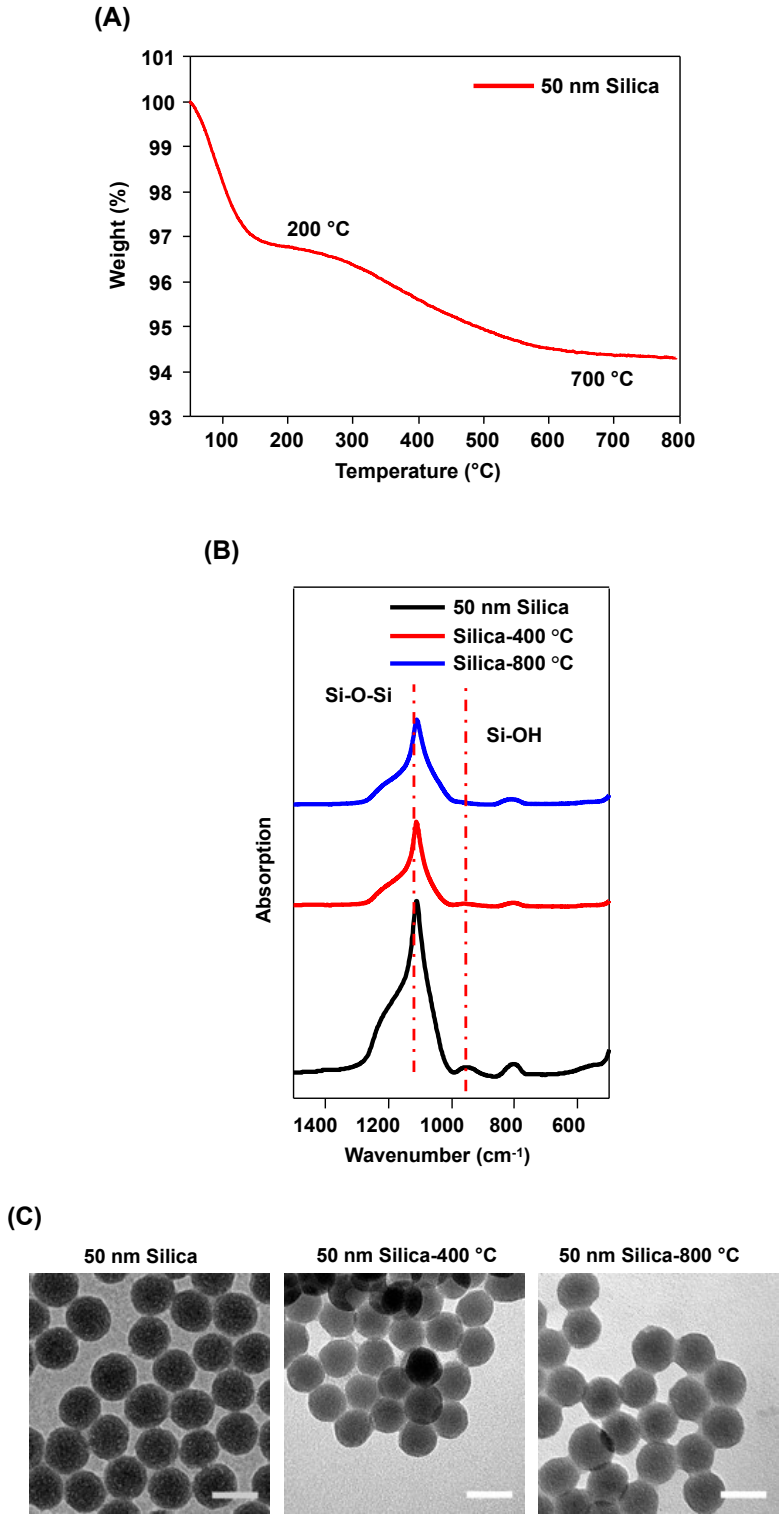


Figure S8. (A) TGA analysis of 50 nm Stöber Silica Nanoparticles. (B) FTIR analysis of 50 nm Stöber Silica Nanoparticles before and after calcination at 400 and 800 °C. (C) TEM images of 50 nm Stöber Silica Nanoparticles before and after calcination at 400 and 800 °C. The scale bar is 50 nm.

Figure S9

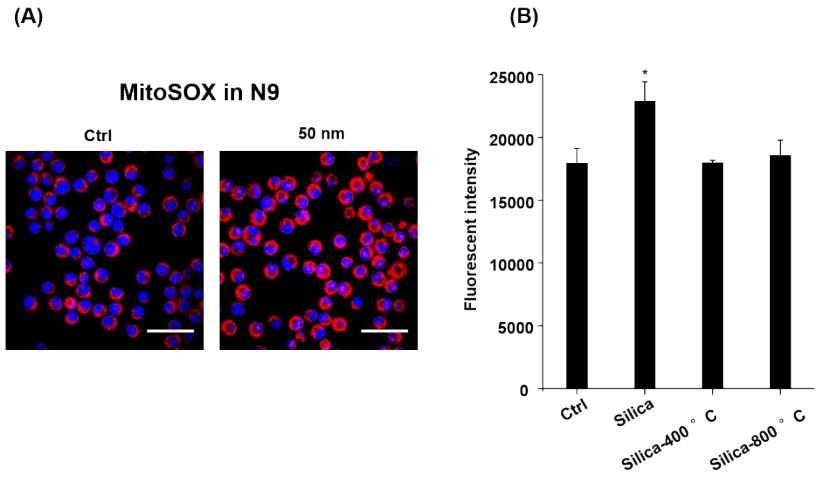


Figure S9. Production of mitochondrial ROS in N9 cells after (A) 50 nm Stöber silica or (B) calcinated 50 nm Stöber silica treatment determined by MitoSOX staining. N9 cells were treated with 50 nm silica (50 μg/mL) for 2 h, then cells were incubated with MitoSox Red (5 μM) for 20 min. Later, cells were fixed with 4% paraformaldehyde and incubated with Hoechst 33342 (10 μM) for 15 min. Images were obtained by Olympus confocal microscope (FV 1000). Scale bar is 50 μm. (B) N9 cells were exposed to calcinated 50 nm Stöber silica nanoparticles at 50 μg/mL for 2 h, then they were incubated with MitoSOX Red (5 μM) for 20 min, and fluorescence intensity was read by a SpectraMax i3x microplate reader. *$p < 0.05$ compared to nontreated control cells.

Figure S10

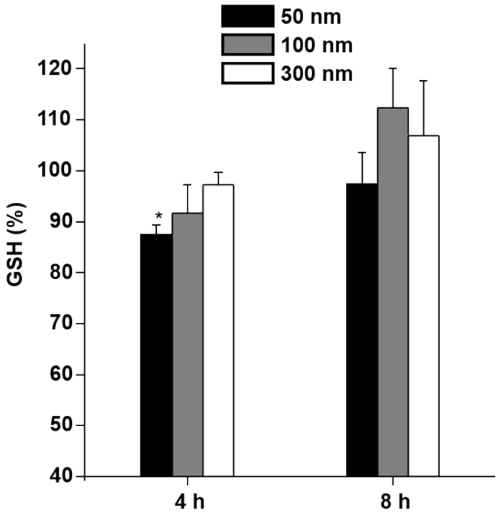


Figure S10. Content of glutathione (GSH) inside N9 cells after Stöber silica nanoparticles treatment. Cells were exposed to Stöber silica (50 μg/mL) for 4 h and 8 h, and then GSH-Glo^{TM} Glutathione assay kit was used to determine GSH content.

Figure S11

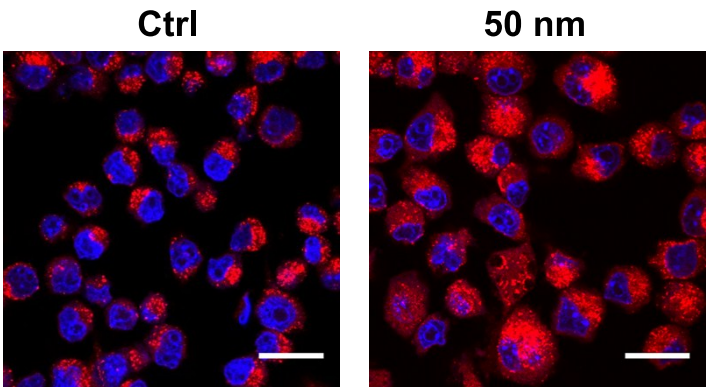
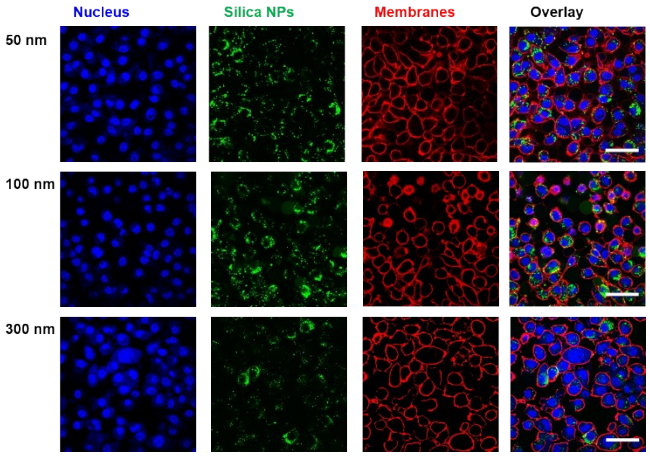


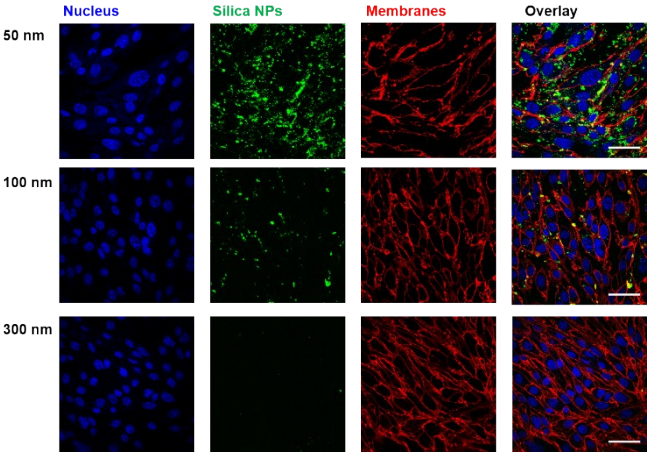
Figure S11. Lysosomal damage and cathepsin B release was determined by confocal microscopy, using a Magic Red-labeled cathepsin B substrate. N9 cells were seeded into a 8-well chamber slide and exposed to Stöber silica nanoparticles for 11 h. Cells were stained with Magic Red-labeled cathepsin B substrate for 1 h. The scale bar is 25 µm.

Figure S12

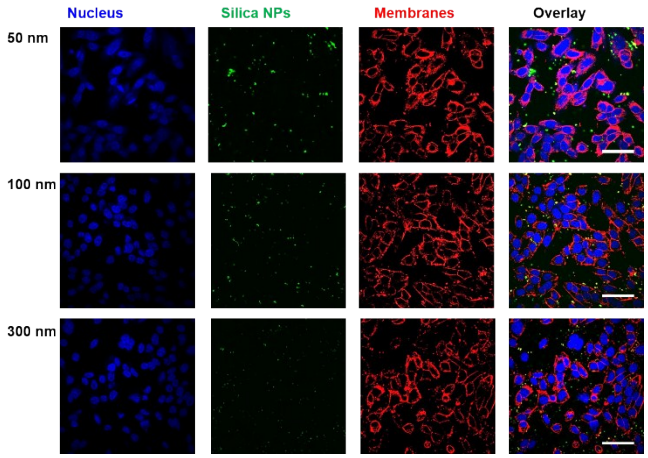
(A)



(B)



(C)



(D)

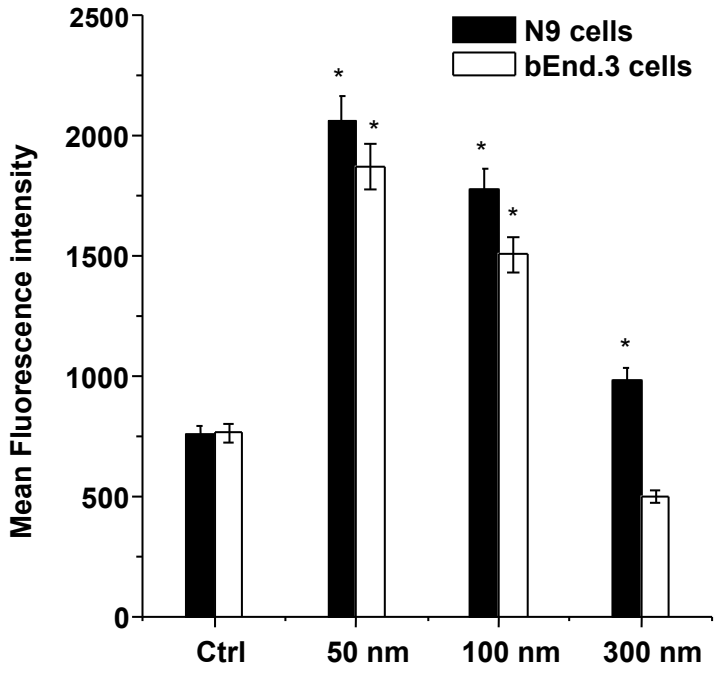


Figure S12. Confocal microscopy images showing cellular uptake of Stöber silica nanoparticles in (A) N9, (B) bEnd.3 and (C) HT22 cells. FITC-labeled NPs (12.5 μg/mL) were incubated with N9, bEnd.3 and HT22 cells for 8 h. Cells were fixed with 4% paraformaldehyde and stained with WGA Alexa Fluor® 594 conjugate and Hoechst 33342 for labeling of the cell membranes and nucleus. Scale bar is 50 μm. (D) Flow cytometry analysis showing cellular uptake of Stöber silica nanoparticles in N9 and bEnd.3 cells. FITC-labeled NPs (50 μg/mL) were incubated with N9 and bEnd.3 cells for 10 h. *$p < 0.05$ compared to nontreated control cells.

Figure S13

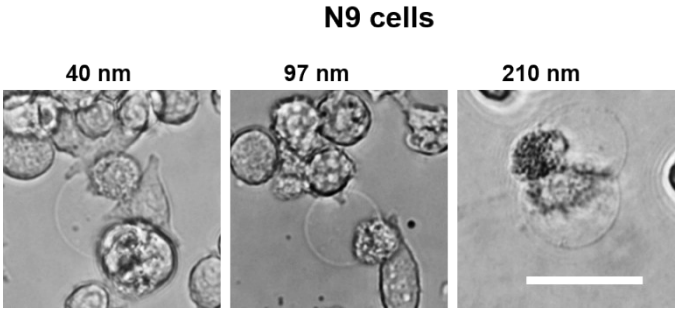


Figure S13. Optical microscopy images of N9 cells after they were exposed to in-house synthesized Stöber silica at 200 μg/mL for 24 h. Scale bar=25 μm.

Figure S14

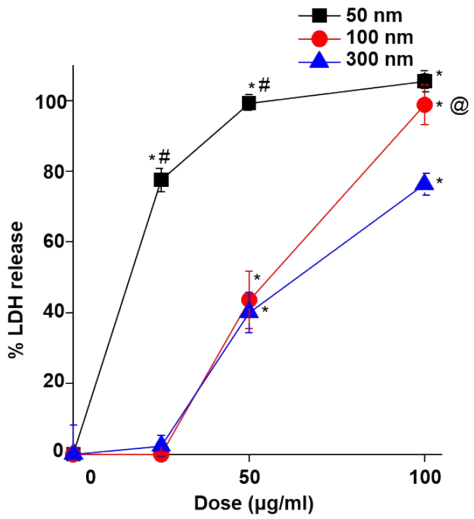


Figure S14. LDH release after N9 cells were exposed to Stöber silica nanoparticles. LPS-primed (1 µg/mL, 16 h) N9 cells were treated with Stöber silica nanoparticles for 24 h and the LDH release was determined using a CytoTox 96 Non-Radioactive Cytotoxicity (LDH) assay. *$p < 0.05$ compared to non-treated control cells; $^{\#}p < 0.05$ compared to cells treated with 100 nm silica; $^{@}p < 0.05$ compared to cells treated with 300 nm silica.

Figure S15

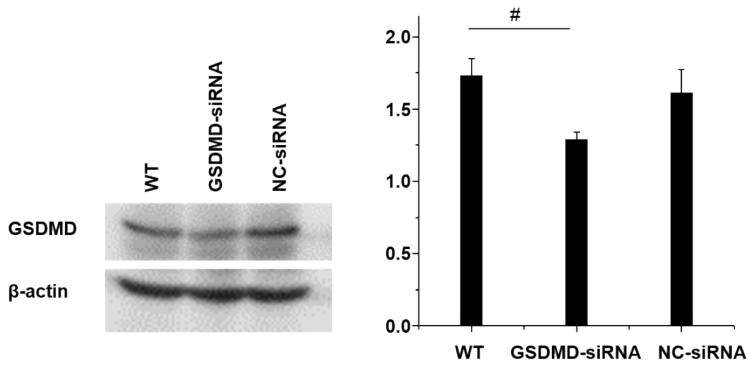


Figure S15. Confirmation of GSDMD gene silencing siRNA electrotransfection in N9 cells. The levels of GSDMD were detected using immune blotting. Expression of GSDMD in N9 cells decreased after GSDMD siRNA was transfected.

Figure S16

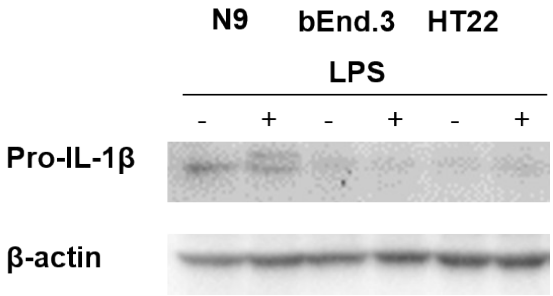


Figure S16. The levels of pro-IL-1β in N9, bEnd.3, and HT22 cells with or without LPS (1 μg/mL, 12 h) treatment.

Figure S17

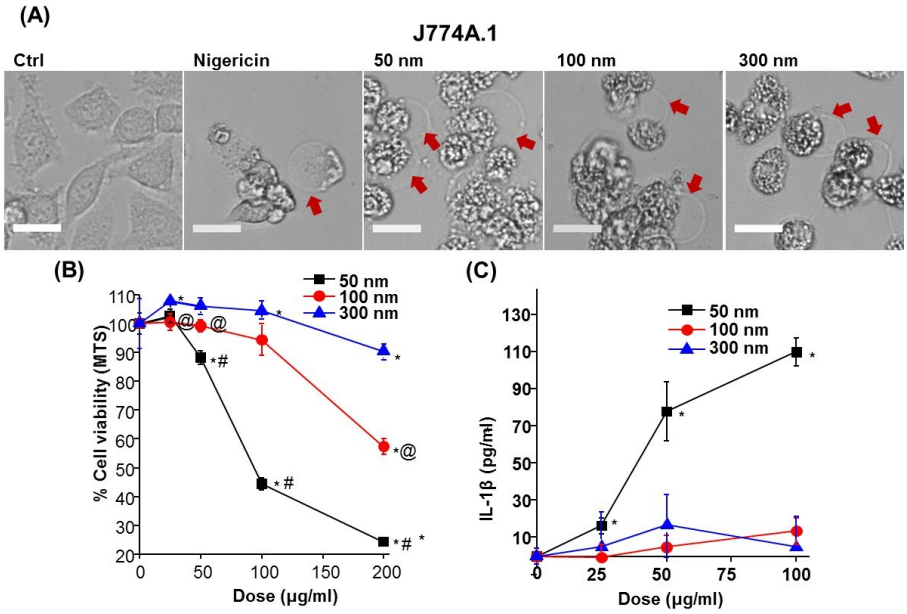


Figure S17. Toxicological profiles of Stöber silica NPs in J774A.1 cells. (A) Optical microscopy images of J774A.1 showed cell swelling and membrane blebbing after cells were treated with Stöber silica (200 µg/mL) for 24 h. (B) Cell viability of J774A.1 after exposure to silica (25-200 µg/mL) for 24 h. (C) IL-1β levels of J774A.1 was quantified using ELISA assay after 24 h exposure to silica. $^{*}p < 0.05$ compared to non-treated N9 control cells.

Table S1

Sample	Primary Size (nm)	Hydrodynamic size (nm)		Zeta potential (mv)
		Water	PDI	Water
Silica-40 nm	40±6	78±2	0.10±0.02	-35±2
Silica-90 nm	97±7	134±4	0.05±0.02	-38±2
Silica-210 nm	210±8	455±9	0.03±0.03	-36±3

Table S1. The primary sizes, hydrodynamic sizes, and zeta potentials of in-house synthesized Stöber silica nanoparticles. Hydrodynamic sizes, and zeta potentials were determined in deionized water.

Table S2

Silica	Si-OH (A)	Si-O-Si (B)	A/B	Si-OH/m^2
50 nm	4.31	216.90	0.019871	3.05E+12
100 nm	1.31	69.60	0.018822	7.11E+11
300 nm	1.86	120.58	0.015425	5.25E+10

Table S2. Silanol concentration per area of Stöber silica nanoparticles based on FTIR analysis.

Supplemental Experimental Section

Preparation and Characterization of Stöber silica NPs. 40/97/210 nm Stöber silica NPs were synthesized using traditional Stöber method. 2 mL ethanol (200 proof), 50 µL H_2O and 375/500/1000 µL NH_4OH was added in a 20 mL glass vial and mixed completely using a stir bar. Then a mixture of 300 µL TEOs and 8 mL ethanol was slowly added at room temperature and stirred overnight at 600 rpm. Silica NPs were washed with ethanol 3 times by centrifugation. TEM (JEM-2100, JEOL, Japan) was used to assay the morphologies and primary sizes of silica NPs. A ZetaPALS instrument (90Plus Zeta, Brookhaven, USA) was used to determine the hydrodynamic sizes and zeta potentials of particles. Thermal Gravimetric Analyzer (TGA 4000, PerkinElmer, USA) was utilized to measure the amount of silanol in silica nanoparticles under nitrogen atmosphere at a heating of 10 °C/min from 40 to 800 °C. Fourier transform infrared spectroscopy (FTIR, 6700, Thermo Fisher, USA) was adopted to analyze the surface silanol of the silica nanoparticles.

Fluorescence labeling of Stöber silica NPs. 10 mg/mL of Stöber silica nanoparticles dispersed in methanol (pH=3-4) were acidified with HNO_3 and then shocked at 37 °C for 2 h. 10 µL of APTEs were added and reacted with silica for 4 h. Then the solution was centrifuged and the supernatant was removed. The precipitation was dispersed in 0.5 mL of DMF mixed with 0.5 mg of FITC, and the solution was shocked for 30 min before centrifuged again. The precipitation was washed with water for 3 times and finally dispersed in 0.5 mL of water.

Dispersion of Stöber silica NPs. Silica nanoparticles were diluted in complete cell culture medium at the desired concentrations. Then the particle suspensions were sonicated for 15 min in a water bath sonicator before exposure to the cells.

Determination of abiotic ROS in Stöber silica NPs. CM-H_2DCFDA working buffer was prepared divided into pipes of 1.5 mL. Then Stöber silica NPs were added into CM-H_2DCFDA working buffer to get a final concentration of 50 μg/mL and incubated in the dark place for 3 h at room temperature. The pipes were centrifuged for 15 min at 7500 rpm, and the supernatant was transferred to wells of a black 96-well plate with 100 μL per well. Fluorescence was read by a SpectraMax i3x microplate reader (Molecular Devices, Sunnyvale, CA) at Ex 492/Em 527.

Determination of endotoxin levels in Stöber silica NPs. Limulus Amebocyte Lysate assay kit was used to determine the endotoxin levels in Stöber silica NPs. Firstly, 5 μg of silica NPs in 50 μL water for bacterial endotoxin test were mixed with 50 μL of Limulus Amebocyte Lysate (LAL) reagent in the wells of a 96-well plate and were incubated at 37 °C for 14 min. Then 100 μL of chromogenic substrate solution was added to each well, and incubated at 37 °C for 6 min. Finally, 50 μL of stop solution was added to each well and the absorbance was read at 405 nm by a SpectraMax i3x microplate reader (Molecular Devices, Sunnyvale, CA). The endotoxin levels in silica NPs were calculate using a standard curve with known concentrations of endotoxin.

Flow cytometry assay of N9 and primary microglia surface markers. Determining the expression of F4/80 and CD11b to confirm the phenotypes of

N9 cells and primary microglia. Cells were cultured in 6-well plate overnight (8.5 x10^5 cells/well). LPS-primed (1 μg/mL, 4 h) N9 cells were collected and washed twice with PBS. Blocking antibody was added and incubated with cells at 4 °C for 10 min. Subsequently, cells were divided into 3 tubes, and incubated with Rat anti-mouse CD11b antibody, and Rat anti-mouse F4/80 antibody at 4 °C for 30min. Supernatant was removed by centrifugation and 300 μL of PBS (containing 1% FBS) was added for flow cytometry assay (BD, FACSCanto). For primary microglia, there was no LPS stimulation.

Flow cytometry assay of particle uptake in N9 and bEnd.3 cells. N9 and bEnd.3 cells were cultured in 6-well plate overnight (8.5 x10^5 cells/well). LPS-primed (1 μg/mL, 12 h) cells treated with FITC-labelled particles for 10h. Then the cells were collected and washed twice with PBS before flow cytometry analysis (BD, FACSCanto). The Trypan Blue was used to quench surface-associated particles before flow cytometry analysis.

Determination of LDH release. CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, USA) was used to determine the release of LDH. After treatment with silica NPs, 50 μL of cell supernatant was mixed with 50 μL of CytoTox 96® Reagent, and then the mixture was incubated for 30 min at room temperature. Subsequently, 50 μL of stop solution was added, and the absorbance was read with a SpectraMax i3x microplate reader (Molecular Devices, Sunnyvale, CA).

Determination of particle cellular uptake by confocal microscopy. N9, bEnd.3 and HT22 cells were seeded

into three 8-well Lab-Tek chamber slides at 1.5 x 10^5, 1.2 x 10^5 and 1.3 x 10^5 cells/well respectively overnight. LPS-primed (1 μg/mL, 16 h) cells were treated with FITC-labeled silica NPs at a concentration of 12.5 μg/mL for 8 hours. Then the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 15 min. A mixture of WGA Alexa Fluor® 594 conjugate (1 μg/mL) and Hoechst 33342 (10 μM) was added to the cells, and incubated with cells for 20 min at room temperature. The cells were washed with PBS twice and the fluorescent images were acquired with Olympus confocal microscope (FV 1000). The images were analyzed by ImageJ software.

Determination of intracellular GSH levels. GSH-Glo™ Glutathione Assay (Madison, WI) was performed to determine the intracellular GSH levels. N9 cells were treated with silica nanoparticles at 50 μg/mL for 4 h and 8 h in a 96-well plate. Then the media was removed and GSH-Glo reagent was added into the plate immediately. After 30-minute incubation, reconstituted luciferin detection reagent was added and incubated for 15 min. A SpectraMax i3x microplate reader (Molecular Devices, Sunnyvale, CA) was used to measure the luminescence.

Determination of mitochondrial ROS. N9 cells were cultured in 8-well Lab-Tek chamber slide overnight. LPS-primed (1 μg/mL, 16 h) N9 cells were exposed to silica NPs at 50 μg/mL for 2 hours. Subsequently, cells were washed three times with PBS, and incubated with 5 μM of MitoSOX in HBSS for 20 min. Then the cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. Following three times wash with PBS, cells were stained with 10 μM Hoechst 33342 at room

temperature for 20 min. Finally, the cells were washed twice with PBS and observed with Olympus confocal microscope (FV 1000). Image J was used to deal with the fluorescent images.

Determination of lysosomal damage. LPS-treated (12 h, 1 μg/mL) N9 cells were exposed to particles for 11 h in an 8-well chamber slides at 1×10^5 cells/400 μL media at 37 °C and 5% CO_2. After incubation, the cells were washed two times with PBS and stained with Magic Red (Immunochemistry Technologies, Bloomington, MN) in 420 μL of Magic Red working solution for 1 h at 37 °C and 5% CO_2. Further, cells were washed two more times with PBS, and fixed with 4% of PFA at room temperature for 15 min. Then cells were stained with 10 μM of Hoechst 33342 (Invitrogen, Carlsbad, CA) at room temperature for another 20 min. Finally, cells were washed with PBS for two more times and examined using a Olympus confocal microscope (FV 1000).