Supporting Information

Ageing remarkably alters the toxicity of carbon black particles towards susceptible cells: determined by differential changes of surface oxygen groups

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Methods and materials

Ageing process of carbon black (CB) particles: Commercial carbon black particles (Printex U, abbreviated as PU) were purchased from the Degussa Inc. Corp (Germany). Ozonation of PU particles was carried out according to a well-established method, as described in previous work.[1] Briefly, PU particles (30 mg) was dispersed on a Teflon filter (ϕ 47 mm), followed by exposure to ozone mixed with ambient air with a total flow of 2.0 L/min. According to the China National Environmental Monitoring Centre (http://www.cnemc.cn/sssj/), the corresponding average concentration of O_3 is 83 ppb in June, 2018, in Beijing, China. Thus, ozone was generated by an ozone generator (UV-185, Jingxinhe, China) at a concentration of 1,992 ppb and the exposure time was 1, 4 and 7 hours, respectively, which represents the real ageing periods for 1, 4 and 7 days according to the equation S1.

$$83\ (ppb) \times 24\ (hour) \times \mathrm{d}\ (day) = \mathrm{O_3\ conc.}\ (ppb) \times \mathrm{t}\ (hour) \qquad \text{(S1)}$$

The PU particles underwent different ozonation periods were referred to as PU-0 (pristine PU), PU-1 (1 day), PU-4 (4 days) and PU-7 (7 days), respectively.

Characterization of PU particles: Physical dimensions and morphologies of the PU particles were characterized by a scanning electron microscope (SEM, SU-8020, Hitachi, Germany). Element components and energy bands were determined by X-ray photoelectron spectroscopy (XPS, ESCALAB250Xi, Thermo Fisher Scientific, USA) and analyzed by the software XPSPEAK41.[2] Hydrodynamic diameter (D_h) and zeta potential of the PU suspensions (at 10 μg/mL) were measured in deionized (DI) water at 25 ºC using a ZetaSizer Nano ZS (Malvern Nano series, Malvern, UK). The concentrations of various metals in the particles were also measured. In brief, the pristine CB samples were first digested with mixture of HNO_3/HCl (v/v = 1:3), and were then assessed using an inductively coupled plasma mass spectrometry (ICP-MS, 7500a, Agilent Technologies, USA).

Cell culture: RAW264.7 cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, USA) and 100 U/mL penicillin/streptomycin (Gibco, USA) in an incubator at 37 °C under 5% (v/v) CO_2 atmosphere.

Mouse erythroleukemia (MEL) cells were purchased from the Cell Resource Center of the Institute of Basic Medical Sciences (CAMS, China) and cultured in suspension with Roswell Park Memorial Institute 1640 medium (RPMI 1640, Gibco, USA), supplemented with 10% (v/v) FBS (Hyclone, USA) and 100 U/ml penicillin/streptomycin (Gibco, USA) in an incubator at 37 °C under 5% (v/v) CO_2 atmosphere. To induce MEL cell differentiation, cells were cultured with 1.5% (v/v) dimethyl sulfoxide (DMSO, Solarbio, China) according to a previous protocol.[3]

Hemolysis assay: Red blood cells (RBCs) were collected from male Balb/C mouse, followed by centrifugation (5 min, 2,000 rpm, 4 °C) and washing with sterile isotonic phosphate-buffered saline (PBS) for 5 times. Afterwards, 10 μL PU stock solution (1 mg/mL) were suspended in 500 μL PBS and were then mixed with 500 μL RBC suspension, giving rise to PU concentration at 10 μg/mL. The mixed samples were gently incubated for 6 h at room temperature (25 °C). DI water and PBS were used as the positive control and negative control, respectively. Afterwards, the mixtures were centrifuged at 2,000 rpm (4 °C) for 5 min, and the absorbance of supernatants was recorded at 414 nm, as described.[4] The relative hemolysis ratio was measured by normalization to the PBS group.

Cytotoxicity assay: MEL and RAW264.7 cells were seeded into 96-well plates with 2.0 × 10^4 and 1.0 × 10^4 cells/well, respectively. The cells were then exposed to different concentrations of PU suspensions for 24 h. Then, the cell viability was assessed by Cell Counting Kit-8 assay following a standard protocol provided by the manufacturer (CCK-8,

Solarbio, China).

RNA isolation and quantitative reverse-transcription polymerase chain reaction (RT-qPCR): Total RNAs were purified from cells with TRIzol reagent (Invitrogen, USA). The RNA concentrations were measured with a spectrophotometer (Nanodrop ND-2000, Thermo Fisher Scientific, USA). Total RNA (4 μg) was reversely transcribed into cDNA with Maxima™ H Minus cDNA Synthesis Master Mix (Promega, USA). Relative gene expression was determined using SYBR green master mix on a CFX96 real-time instrument (Bio-Rad, USA). eIF2α was used as an internal control for normalization. Primer sequences for the PCR reactions were listed in Table S1.

Protein concentration determination and Western blot analysis: MEL cells were induced by 1.5% DMSO for differentiation, and cells were simultaneously treated with PU particles at a sublethal concentration of 1 μg/mL for 72 h. Meanwhile, RAW 264.7 cells were exposed to PU particles at 50 μg/mL for 24 h. Thereafter, cells were collected, followed by washing with ice-cold PBS and lysis in RIPA lysis buffer (Thermo Fisher Scientific, USA) containing a protease inhibitor cocktail (Roche, Switzerland) for 30 min. An equal amount of each lysate (50 μg/sample) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, as described previously.[5] All antibodies used for experiments were purchased from the Proteintech (USA), including the primary antibodies against α-globin (1:1,000 (v/v)), β-globin (1:1,000 (v/v)), GATA-1 (1:1,000 (v/v)), γ-H2AX (1:1,000 (v/v)), EIF2α (1:1,000 (v/v)) and β-actin (1:1,000 (v/v)). EIF2α and β-actin were used as loading controls. The secondary antibodies were goat anti-mouse HRP-conjugated IgG (1:2,000 (v/v)) and goat anti-rabbit HRP-conjugated IgG (1:2,000 (v/v)), respectively.

Cell death determination by flow cytometry: RAW264.7 cells were first seeded in 12-well plates at 1.0×10^5 cells/well, and cell death was evaluated after treatment with different

concentrations of PU for 24 h. H_2O_2 was used as the positive control. Then, cells were collected and washed with PBS, and then stained using 50 μg/mL propidium iodide (PI, BD Pharmingen, USA) in PBS. PI-positive cells were determined through flow cytometry analysis on a flow cytometer (ACEA BIO, China), as described previously.[6]

Cellular reactive oxygen species (ROS) measurement: To measure intracellular ROS production, cells were first seeded in 96-well plates overnight, and then pre-incubated with 10 mM dichloro-fluorescein diacetate (DCF-DA, Sigma Aldrich, China) probes for 30 min following the manufacturer's instructions. H_2O_2 was used as the positive control. Cells were thereafter rinsed with PBS 3 times prior to PU exposures at different concentrations. Finally, DCF fluorescence was monitored over the time course at 525 nm with an excitation wavelength of 488 nm on a microplate reader (Varioskan Flash, Thermo, USA).

Statistical analysis: Independent *t-test* and one-way ANOVA test using the SPSS Statistics 17.0 software were applied for the statistical analysis. All data are shown as mean ± standard deviation (SD). Statistical significance is defined as *: $P < 0.05$ and #: $P < 0.001$.

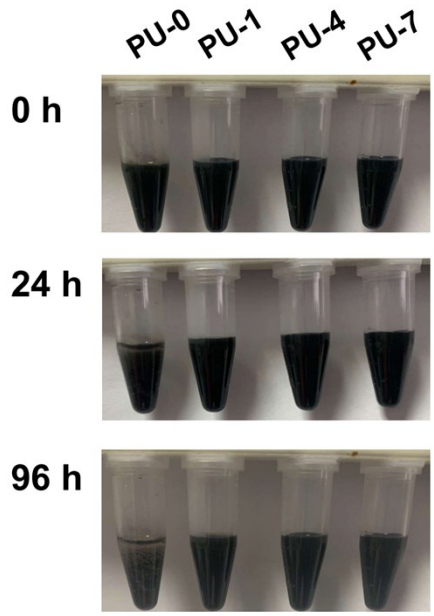

Figure S1. Colloidal stability assessment of 4 PU particles in DI water for 24 h and 96 h.

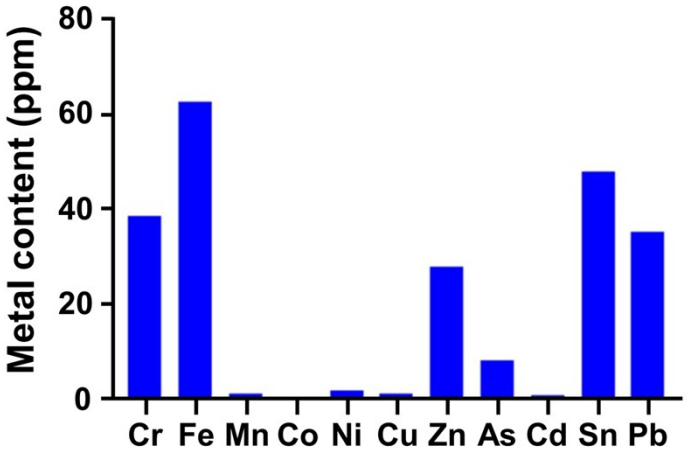

Figure S2. Concentrations of metals in the CB samples, as determined by ICP-MS.

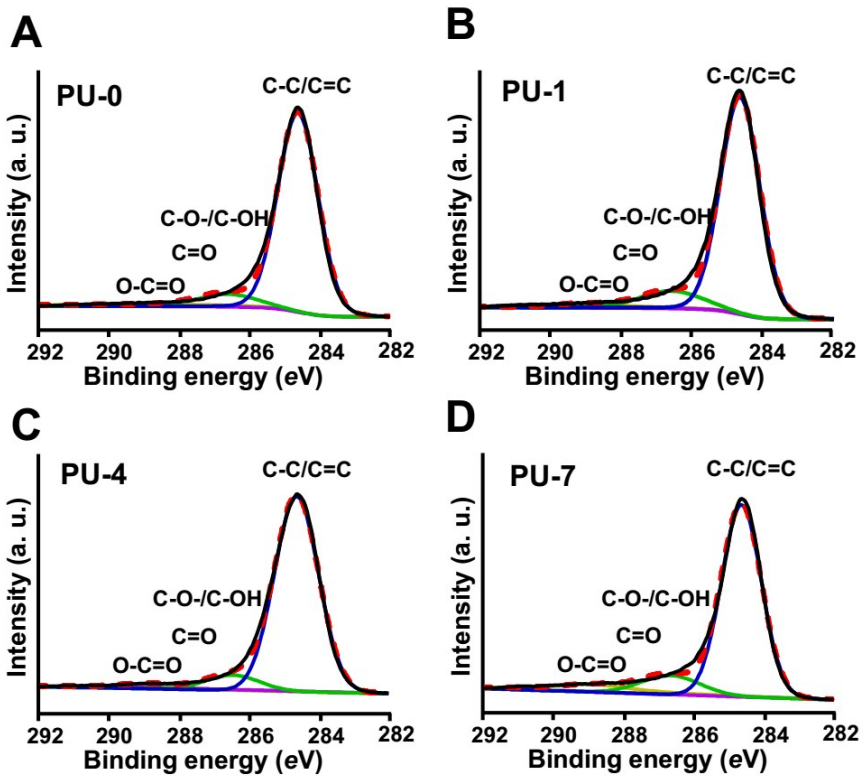

Figure S3. XPS spectra with C1 peaks deconvoluted for PU-0 (A), PU-1 (B), PU-4 (C) and PU-7 (D). The peaks with the binding energy of 284.6, 286.6, 287.8 eV and 288.8 eV were assigned to the carbon atoms in aromatic rings (C-C/C=C), epoxy/hydroxyl (C-O-C/C-OH), carbonyl (C=O) and carboxyl (O-C=O), respectively.

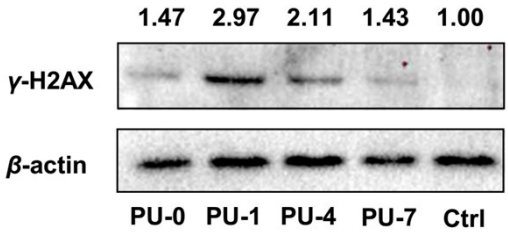

Figure S4. Determination of γ-H2AX protein mass in RAW264.7 cells upon CB treatment (at 50 μg/mL) for 24 h.

Table S1. The primers for the PCR reactions in the current study.

Genes	Forward (5′-3′)	Reverse (5′-3′)
α-globin	CACCACCAAGACCTACTTTCC	CAGTGGCTCAGGAGCTTGA
β-globin	GGAAAGGTGAACGCCGATGAA	GGGTCCAAGGGTAGACAACC
GATA-1 [a]	TGGGGACCTCAGAACCCTTG	GGCTGCATTTGGGGAAGTG
FOG-1 [b]	AGGAAACAGAGCAATCCCCG	CAGGTGGGCTCACATCTTCT
NF-E2 [c]	TGAGCCAAGCTATAAGCCATGA	AATGGTTCTTGTGCCTGTGAA
eIF2α [d]	GGAAGCAATCAAATGTGAGGACA	GCACCGTATCCAGGTCTCTTG

[a] GATA-1: GATA binding protein 1.

[b] FOG-1, also known as ZFPM1: zinc finger protein, multitype 1.

[c] NF-E2: nuclear factor, erythroid derived 2.

[d] eIF2α: eukaryotic translation initiation factor 2, subunit 1 alpha.

References

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