Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Revisiting Cellular Toxicity of Benzo[a]pyrene from the View of Nanoclusters: Size- and Nanoplastics Adsorption-Dependent Bioavailability

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Experimental Section

Quantitative Analysis for Bap Absorption on PS. PS nanoparticle solution (0.5 mg/mL, 1 mL) was incubated with 4 μL different concentration (0.025 mM, 0.25 mM, 1.25 mM, 2.5 mM) of Bap solutions (DMSO as solvent). The solutions were centrifuged at 16000 rpm for 30 min, and washed with ultrapure water three times. The supernatants were extracted by adding methylbenzene and stirring for 30 min. The fluorescent of the extracts was measured to calculate the amount of unadsorbed Bap based on a standard curve of Bap solutions in methylbenzene. Bap adsorption rate was determined as follows: Bap adsorbed (%) = (Total amount of Bap − amount of unadsorbed Bap)/Total amount of Bap×100%.

Cell culture. A549 cells were grown in RPMI 1640 medium containing 10 % (v/v) heat-inactivated FBS, 100k U/L penicillin and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere with 5 % CO_2. The medium without serum was selected for the nanoparticles exposure experiment. The cells were cultured at approximately 50 % confluence when exposed in imaging experiments, and at 70 % in MTT assay. PS was first dissolved in sterile deionized water at a concentration of 5 mg/mL and then diluted with serum-free medium to the concentration required for cell culture. S-Bap and PS@S-Bap were prepared by diluting 4 μL 0.25 mM Bap solutions (DMSO as the solvent) to 996 μL bare medium solution or 996 μL PS (50.20 μg/mL) medium solution. L-Bap and PS@L-Bap were prepared by diluting the 4 μL stock solution (50 mM) 250 times using ultrapure water, then diluted the 5 μL Bap solutions to 995 μL bare medium solution or 995 μL mL PS (50.25 μg/mL) medium solution.

Cytotoxicity Assay. Cytotoxicity was assessed by the MTT assay. A549 cells were seeded in 96-well plates. After overnight incubation, the cells were incubated with PS, S, L-Bap, PS@S, L-Bap for 24 h at 37 °C. MTT solution was added into each well and incubated for 4 h at 37 °C. Cells were lysed with 10% DMSO solution. Absorbance was determined at 570 nm using a microplate reader (Tecan, Austria).

Quantitative Analysis for Bap Internalization Amount in A549 Cells. First, A549 cells were seeded in plates. After the cell confluence reached ~70%, the cells were incubated with 1 μM S-Bap, L-Bap, PS@S-Bap (containing 50 μg/mL PS) and PS@L-Bap (containing 50 μg/mL PS) for 9 h at 37 °C. Following collection, cells were centrifuged and washed with ultrapure water three times to remove the nanoparticles adhering to the cell surface. Then, cells are counted and normalized to 4×10^6 with the same final volume. Cells were broken and the solutions were extracted by adding methylbenzene and stirring for 30 min. The amount of Bap was calculated via fluorescence intensity of Bap.

Endocytosis Determination. For internalization mechanism study, the cells were pretreated with CPZ (3 μg/mL), MβCD (5 mg/mL), EIPA (50 μM) and CyD (5 μg/mL) for 30 min, respectively. The cells were washed with PBS three times, then incubated in medium containing 1 μM S-Bap, L-Bap, PS@S-Bap (containing 50 μg/mL PS) and PS@L-Bap (containing 50 μg/mL PS). After treatment for 6 h, cells were washed and captured through CLSM.

Cellular Distribution and Cellular Trafficking Studies. To track S, L-Bap and PS@S, L-Bap

localization and intracellular delivery, cells were exposed to 1 μM S-Bap, L-Bap, PS@S-Bap (containing 50 μg/mL PS) and PS@L-Bap (containing 50 μg/mL PS). After incubation for the desired time, cells were washed with PBS three times, then labeled with different organelle trackers (Mito-Tracker, Lyso-Tracker, and ER-Tracker, λex 488 nm, λem 500−540 nm). Images were obtained using CLSM.

Image Analysis. The mean fluorescence intensity of cells was analyzed using Leica Application Suite X software equipped in Leica SP8 confocal microscope. Intensity data of the cells were collected from 30 randomly selected cells. For fluorescence colocalization investigation, colocalization ratios of two fluorescent signals were analyzed using ImageJ software (the thresholded Mander's coefficients). Data were collected from 15 randomly selected cells.

Changes in Mitochondrial Membrane Potential. Mitochondrial membrane potential (MMP) was determined by a JC-1 assay kit. Cells were exposed to 1 μM S-Bap, L-Bap, PS@S-Bap (containing 50 μg/mL PS) and PS@L-Bap (containing 50 μg/mL PS) for 24 h. Following collection, cells were washed with PBS three times, stained with JC-1 solution, and incubated in the dark at 37 °C for 25 min, then detected with CLSM and Flow Cytometry (FCM, BD Biosciences, USA).

Western Blotting. Cells were exposed to 1 μM S-Bap, L-Bap, PS@S-Bap (containing 50 μg/mL PS) and PS@L-Bap (containing 50 μg/mL PS) for 24 h. Following collection, cells were washed twice with ice cold PBS on ice and lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail. Protein concentrations were determined with BCA Protein Assay Kit. Equal amounts of cell protein (20 μg) were separated by SDS-PAGE and analyzed by western blot. The primary Abs were as follows, Caspase 3, Bax, Bal-2, Cleaved Caspase 3 and anti-GAPDH Ab. The second Abs was as follows, goat anti-mouse HRP conjugated IgG and goat anti-rabbit HRP conjugated IgG. Western blotting signals were detected and scanned with a Gel Doc 2000. The intensity for autoradiogram was determined using the Image J software, and the band intensity was normalized to that of the loading control.

Cell Apoptosis. Detection by Flow Cytometry. Cells after exposure to 1 μM S-Bap, L-Bap, PS@S-Bap (containing 50 μg/mL PS) and PS@L-Bap (containing 50 μg/mL PS) for 24 h were harvested and washed three times with PBS, then stained with Annexin-V FITC and PI for 15 min in the dark, followed by FCM analysis within 1 h.

Figures and Tables

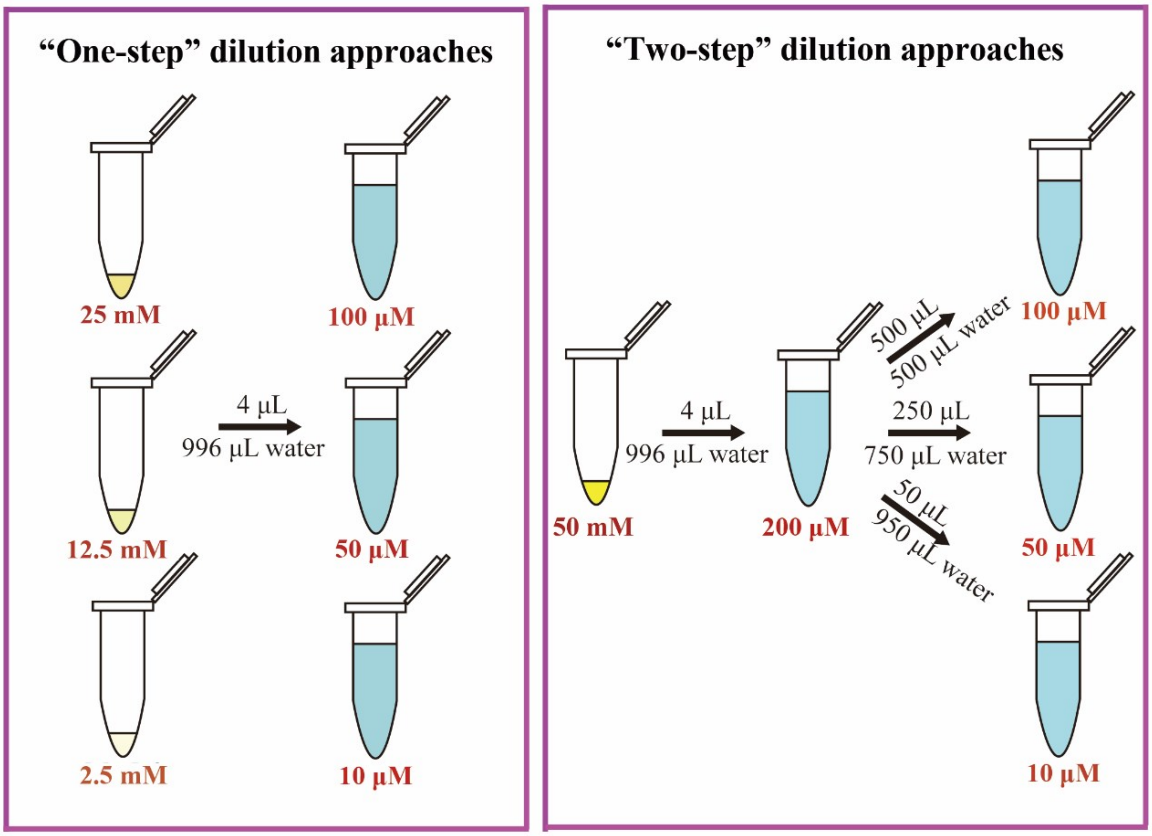

Figure S1 Schematic representation of preparation of S-Bap and L-Bap via different solution dilution approaches (one-step, two-step dilution approaches).

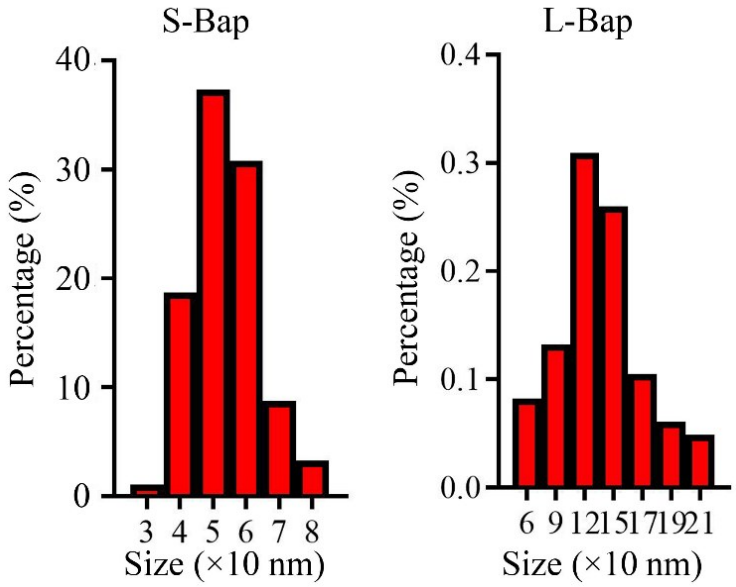

Figure S2 The SEM size of S-Bap and L-Bap.

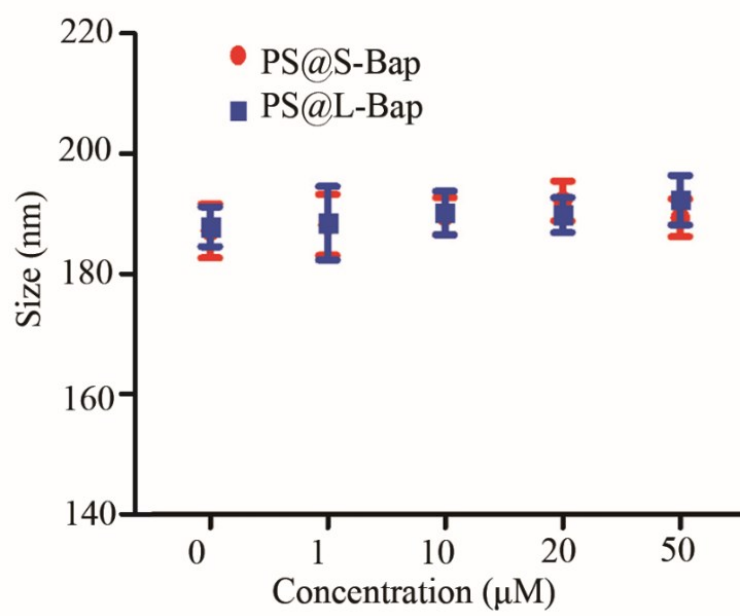

Figure S3 Size of PS@S-Bap and PS@L-Bap at different Bap concentrations (0 (PS alone), 1, 10, 20 and 50 μM), n=3.

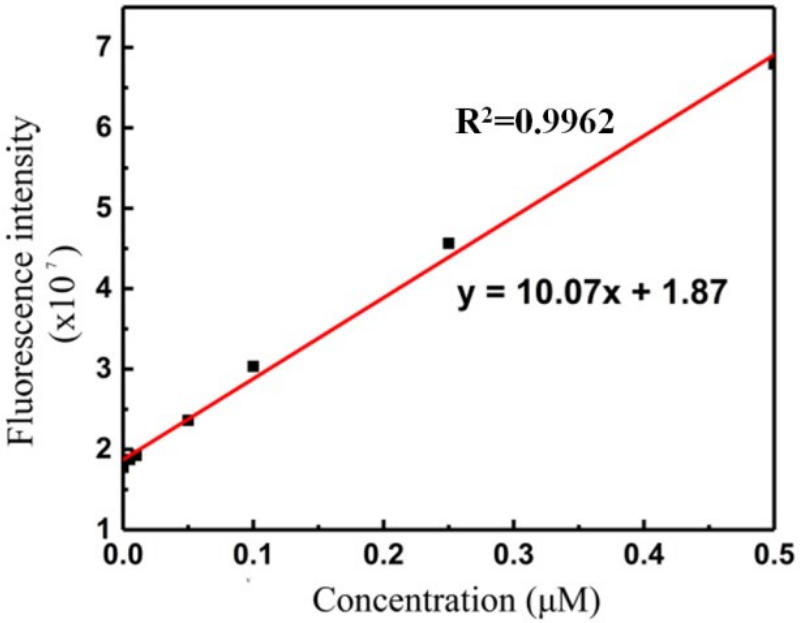

Figure S4 The standard curve revealed the linear relationship between Bap fluorescence intensity and Bap concentrations (0.005–0.5 μM) (R^2=0.9962).

Table S1 The adsorption amount of Bap on PS. PS (0.5 mg/mL) was incubated with Bap (100 nM-10 μM), followed by the separation of PS@S, L-Bap complex and dissolution of the adsorbed Bap with methylbenzene. The adsorption rate of Bap was calculated via fluorescence intensity of Bap.

Bap Concentration	100 nM	1 μM	5 μM	10 μM
Adsorption Rate (%)	92.43 %	96.42 %	93.17 %	90.08 %

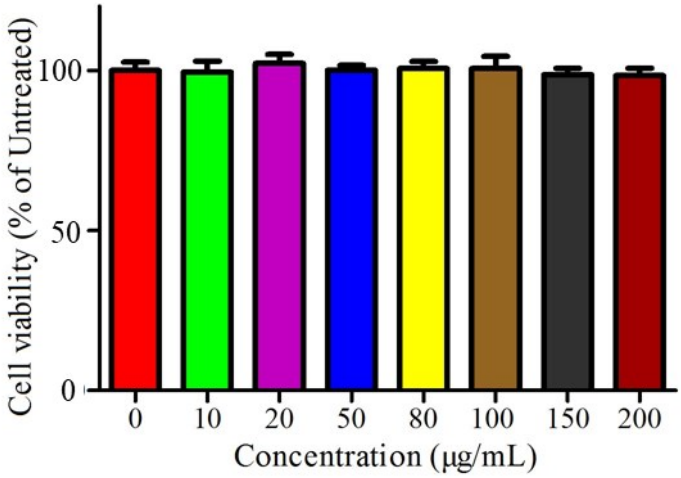

Figure S5 A549 cells cytotoxicity assessment after exposure to indicated concentrations of PS for 24 h.

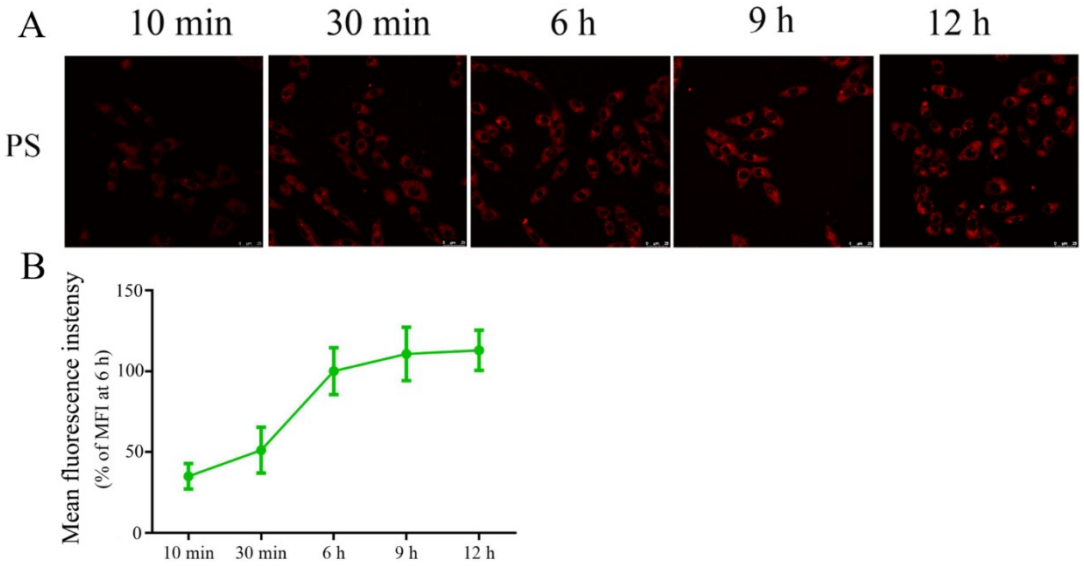

Figure S6 Dynamics of accumulation of PS in A 549 cells. Confocal images of A549 cells incubated with PS (50 μg/mL) for indicated time periods (10 min, 0.5, 6, 9 and 12 h, A). Mean fluorescence intensity of internalized 50 μg/mL PS for indicated time periods (10 min, 0.5, 6, 9 and 12 h) was obtained from confocal images by analyzing with Leica Application Suite X software equipped in Leica SP8 confocal microscope (B).

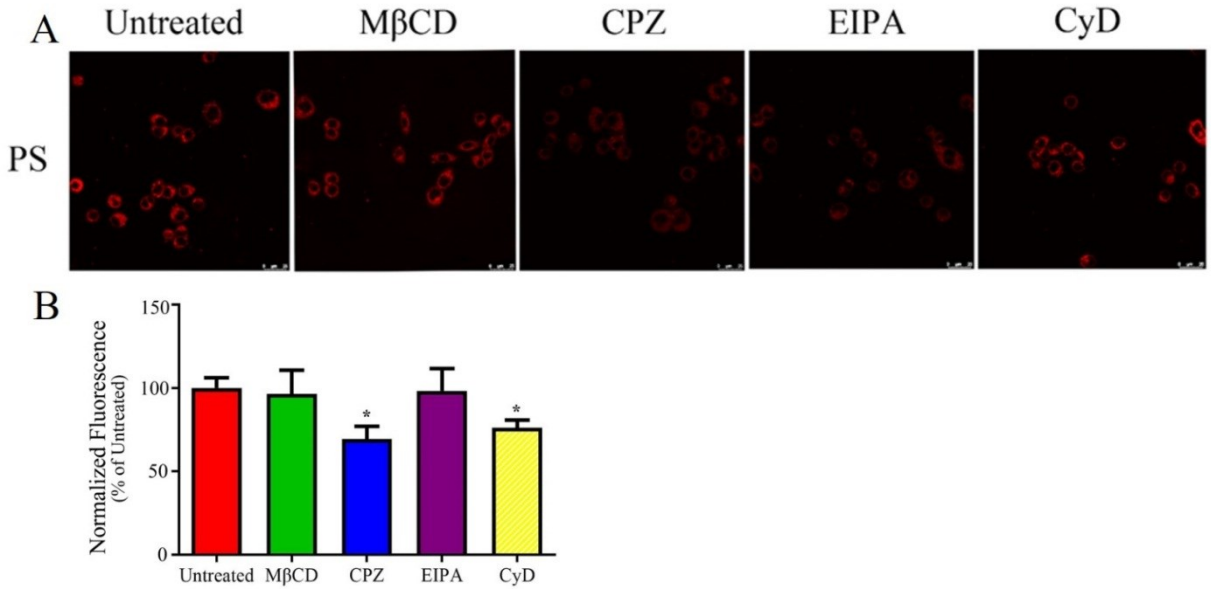

Figure S7 Effects of different pharmacological inhibitors on the uptake of PS (50 μg/mL) by A549 cells after 6 h of incubation (A). Histogram of intracellular fluorescence of PS in cells pre-incubated with different pharmacological inhibitors, * $P < 0.05$, versus Untreated (B).

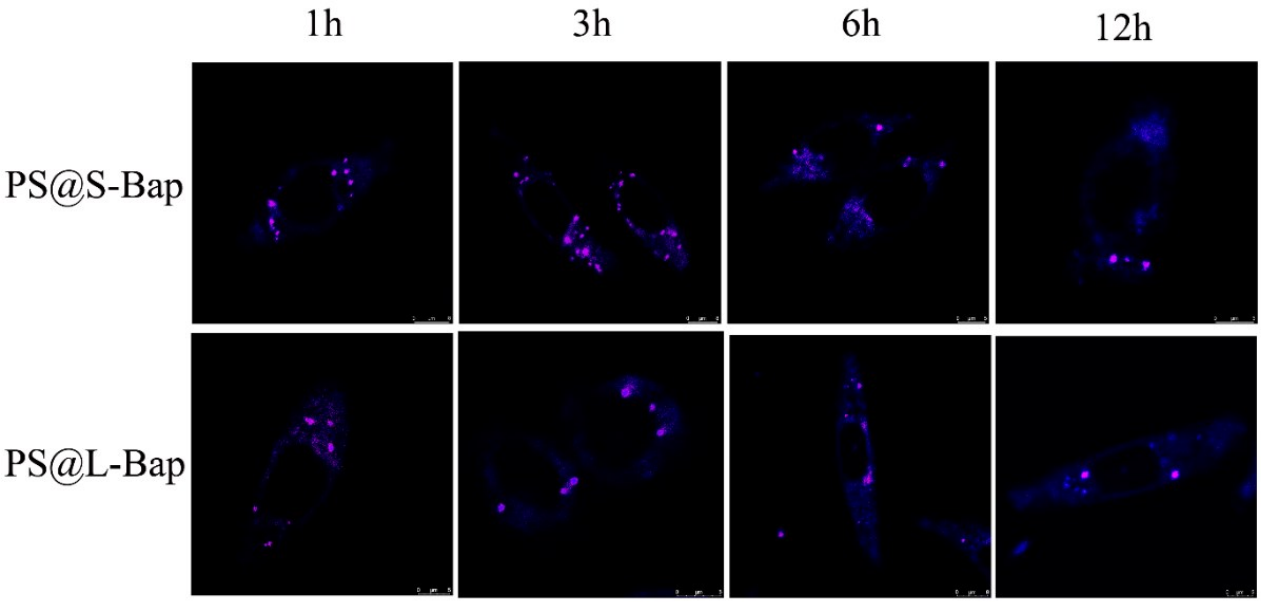

Figure S8 Confocal images of A549 cells incubated with PS@S-Bap and PS@L-Bap for different time. The red fluorescence originated from Nile red-labeled PS nanoparticles and blue fluorescence originated from surface adsorbed Bap nanoclusters co-located at 1–3h, and was isolated after 6 h incubation, indicating that Bap adsorbed on PS at the early stages of the endocytosis and subsequently desorbed from PS with prolonged exposure time.

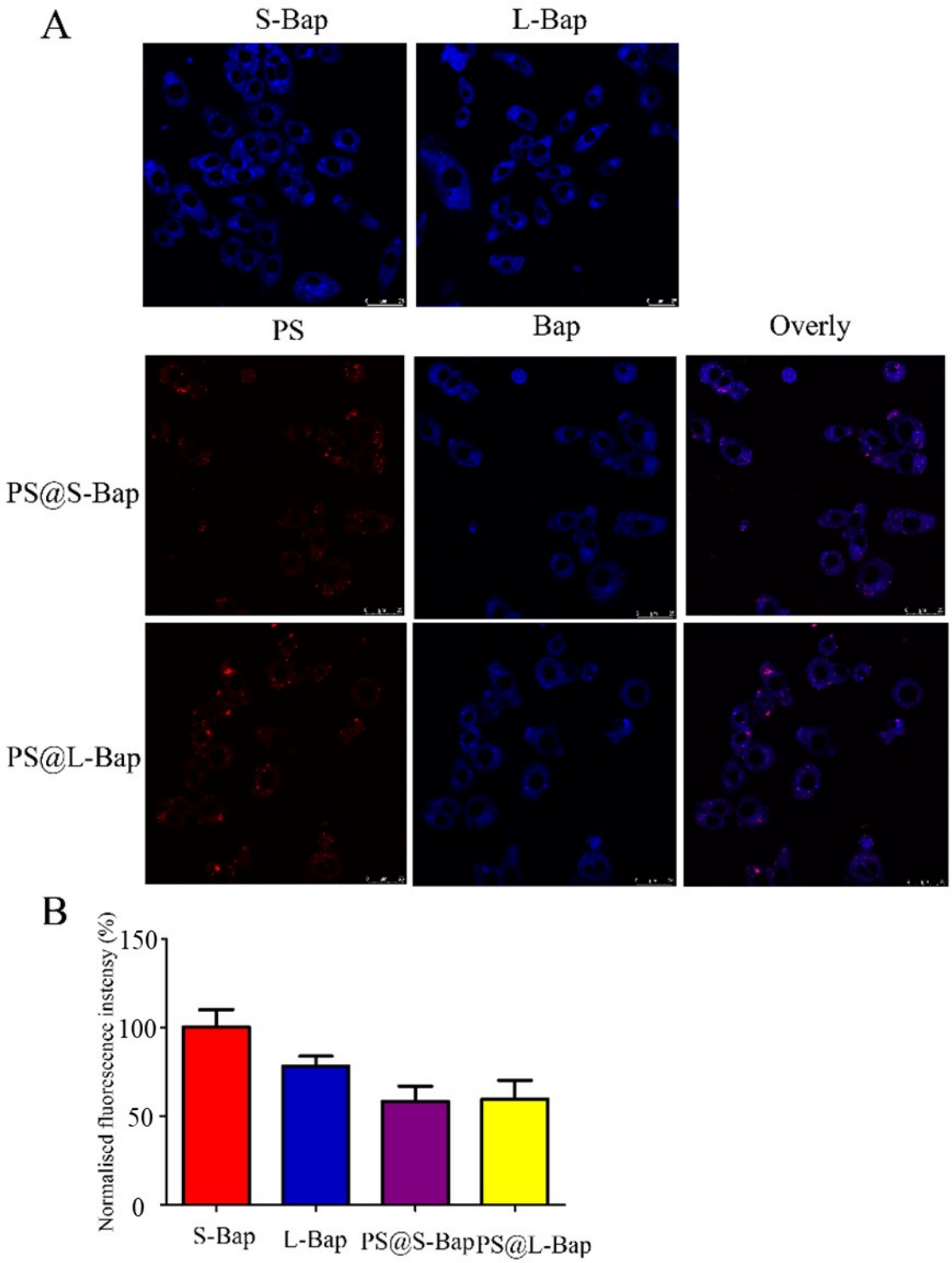

Figure S9 Confocal images of A549 cells incubated with S-Bap, L-Bap, PS@S-Bap and PS@L-Bap for 24 h (A). Histogram of intracellular fluorescence of Bap (B).

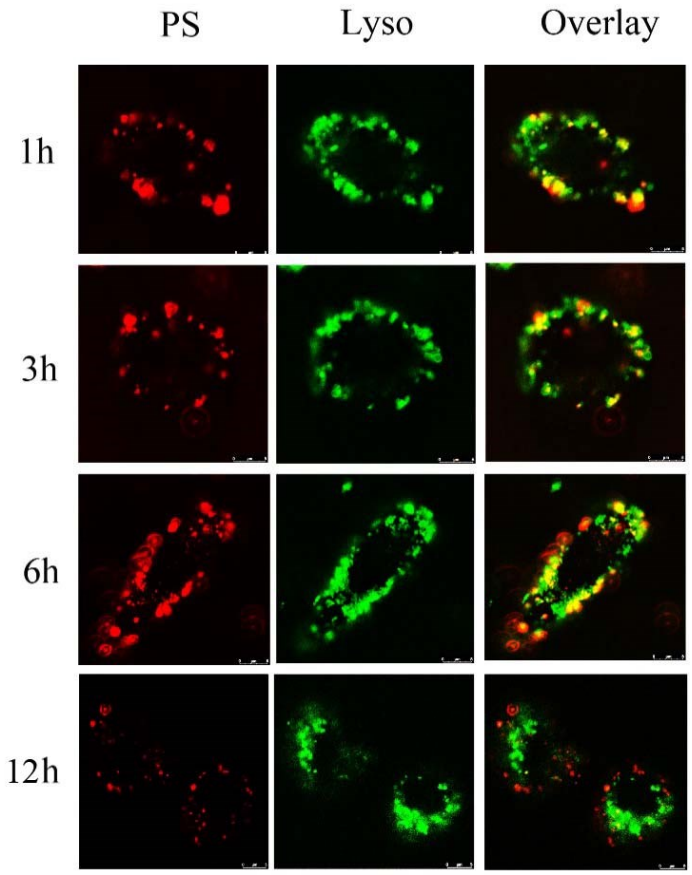

Figure S10 Kinetics of colocalization between lysosome and PS. A549 cells expressing lysosome (Lyso, green) were incubated with PS (red) and imaged by confocal microscopy at indicated time points (1, 3, 6 and 12 h). The strong colocalization of LysoTracker and PS fluorescence signals clearly indicated that the endocytic PS particles fused with the endosome/lysosome.

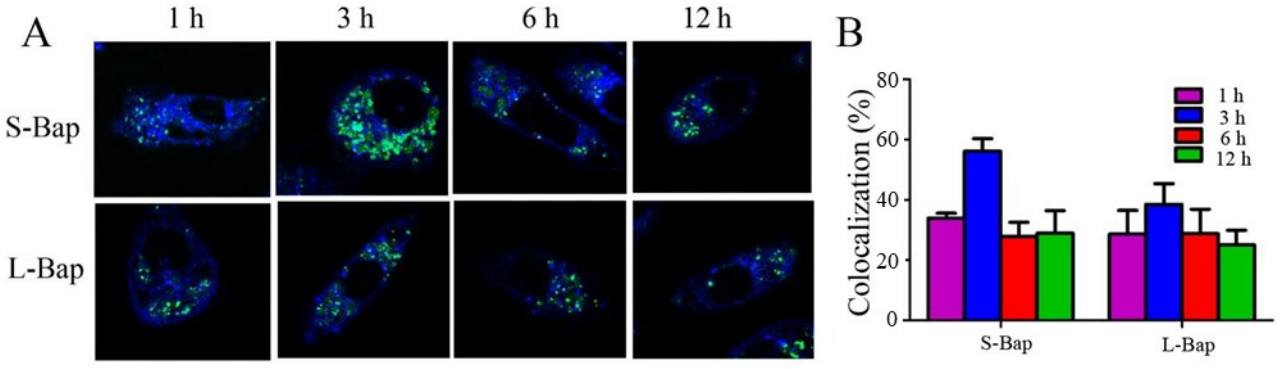

Figure S11 Kinetics of colocalization between lysosome and S, L-Bap. A549 cells expressing lysosome (green) were incubated with S, L-Bap (blue) and imaged by confocal microscopy at indicated time points (1, 3, 6 and 12 h) (A). The percentages of Bap colocalized withendosome/lysosome at dicated time points were quantified using Image J software (B).

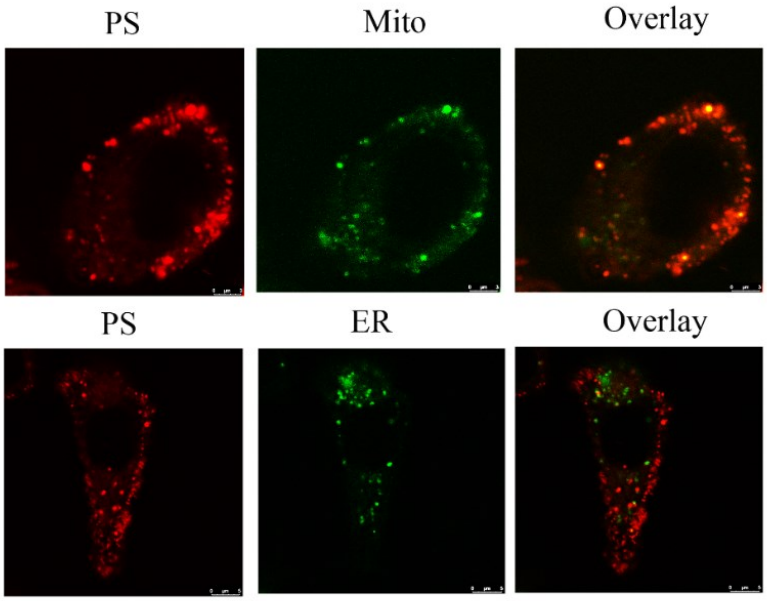

Figure S12 The colocalization between mitochondria, endoplasmic reticulum and PS. A549 cells expressing endoplasmic reticulum (ER, green), mitochondria (Mito, green) were incubated with PS (red) and imaged by confocal microscopy. PS were more inclined to enter into the mitochondria, while only a negligible accumulation of PS existed in the endoplasmic reticulum.