Electronic Supplementary Material (ESI) for Polymer Chemistry

Supporting information

PolyPEGylated Nanodiamond for Intracellular Delivery of Chemotherapeutic Drug

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1. Materials and characterization

Pristine ND (denoted as ND-OH) was obtained from BeiJing Grising Co., Ltd. (BeiJing, China), 2-bromoisobutyryl bromide (Alfa Aesar, 98%), N,N,N′,N″,N″-pentamethyldiethylenetriamine (PMDETA, J&K chemica, 98%), poly(ethylene glycol) methyl ether methacrylate (PEGMA, Mn~475, J&K chemica, 98%) were used as received. CCK-8 kit and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). All other solvents and chemicals were purchased from commercial sources and used directly without further purification. Doxorubicin (DOX) was purchased from HUBEI KANGBAOTAI FINE-CHEMICALS CO., LTD (Wuhan, China).

The Fourier transform infrared (FT-IR) spectra were obtained in a transmission mode on a Perkin-Elmer Spectrum 100 spectrometer (Waltham, MA, USA). Typically, 4 scans at a resolution of 1 cm^{-1} were accumulated to obtain one spectrum. UV-Visible absorption spectra were recorded on UV/Vis/NIR Perkin-Elmer lambda750 spectrometer (Waltham, MA, USA) using quartz curettes of 1 cm path length. The fluorescence measurements were made on a PE LS-55 spectrometer equipped with quartz curettes of 1 cm path length. The X-ray photoelectron spectra (XPS) were measured using a PHI Model Quantera SXM scanning X-ray microprobe, using Al Kα as the excitation source (1486.6 eV) and binding energy calibration was based on C1s at 284.8 eV. Transmission electron microscopy (TEM) images were recorded on a Hitachi 7650B microscope operated at 80 kV; the TEM specimens were made by placing a drop of the nanoparticle suspension on a carbon-coated copper grid. Thermal gravimetric analysis (TGA) was conducted on a TA instrument Q50 with a heating rate of 20 °C/min. Samples weighing between 10 and 20 mg were heated from 25 to 500 °C in air flow (60 mL/min), N_2 as the balance gas (40 mL/min). Particle size and zeta-potential was measured by dynamic-light

scattering using a 90 Plus Particle Sizer Analyzer (Brookhaven Instruments Corp., New York, NY, USA). All measurements were carried out at room temperature. Each parameter was measured in triplicate and average values were calculated.

Abbreviations: nanodiamond (ND); atom transfer radical polymerization (ATRP); N,N,N′,N″,N″-pentamethyldiethylenetriamine (PMDETA); Poly(ethylene glycol) methyl ether methacrylate (PEGMA; molecular weight (MW); molecular weight cut-off (MWCO); as-received ND (ND-OH); polyPEGMA conjugated ND (ND-polyPEGMA); revolutions per minute (rpm); Fourier transform infrared (FT-IR); X-ray photoelectron spectra (XPS); Transmission electron microscopy (TEM); Thermal gravimetric analysis (TGA); Triethylamine (TEA).

2. Synthesis and characterization of ND-polyPEGMA

Scheme 1A shows the preparation of polyPEGMA conjugated ND by ATRP. 2-Bromoisobutyryl bromide modified ND-OH was used as initiator (ND-Br), and polyPEGMA chains were covalent linked on the ND surface through ATRP using Cu(Br)/PMDETA as the catalyst/ligand. Influence of polymerization time on the grafting ratios was studied. ND-polyPEGMA-1 and ND-polyPEGMA-2 represent polyPEGMA samples prepared with different polymerization time for 16 and 48 h, respectively. Detailed experimental procedures for synthesis and characterization of ND-polyPEGMA are described as follows.

2.1 Synthesis of ND-initiator (ND-Br)

ND-OH (1.5 g), triethylamine (1.0 mL) and anhydrous toluene (10 mL) were mixed by sonication for 30 min, then 2-bromoisobutyryl bromide (0.93 g) dissolved in 5.0 mL of anhydrous toluene was added dropwise at 0 °C for 30 min. The resulting mixture was stirred at room temperature for 24 h, the solid was then separated from the mixture by centrifugation (7000 rpm, 5 min), and washed with 60

mL of methanol for five times to remove salt and any unreacted 2-bromoisobutyryl bromide. The solid was collected and dried at 40 °C overnight under vacuum to get ND-Br (1.32 g).

2.2 PolyPEGylated ND (ND-polyPEGMA)

ND-Br (116 mg), CuBr (24 mg), PMDETA (24 mg) and PEGMA (1.43 g) were mixed with 5 mL of toluene in a 20 mL dry flask, which was then sealed with a rubber plug. The mixture was carried out freeze-pump-thaw circle for three times under N_2 atmosphere, and then kept in a 70 °C oil bath for 16 h. At the end of reaction, the polymerization was quenched by opening to the air and cooling to room temperature. The mixture was centrifuged at 7000 rpm for 30 min, and the collected precipitation was washed with deionized water and methanol for five times, respectively. The resulting product (denoted as ND-polyPEGMA-1) was obtained by dried at 40 °C overnight in vacuum. ND-polyPEGMA-2 was obtained using the similar experimental procedure but the polymerization time was extended to 48 h.

3. Cytotoxicity of ND

3.1 Cell morphology observation

A549 cells were cultured in Dubecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} of streptomycin. Cell culture was maintained at 37 °C in a humidified condition of 95% air and 5% CO_2 in culture medium. Culture medium was changed every three days for maintaining the exponential growth of the cells. The effect of ND nanoparticles on A549 cells were first examined by optical microscopy. Briefly, cells were seeded in 6-well microplates at a density of 1×10^5 cells mL^{-1} in 2 mL of respective media containing 10% FBS. After cell attachment, plates were washed with PBS and the cells were treated with complete cell culture medium, or cell culture media containing 20 or 80 μg mL^{-1} of ND nanoparticles (ND-OH, ND-polyPEGMA-1, and ND-polyPEGMA-2) for 24 h. Then all

samples were washed three times with PBS to remove the uninternalized nanoparticles. Cells were observed by using an optical microscopy (Leica, DMI3000B, Germany), and photos were taken using a charge-coupled device (CCD).

3.2 Cell viability of ND nanoparticles

Cell viability of ND nanoparticles on A549 cells was determined by WST assay according to our previous report. [1] Briefly, cells were seeded in 96-well microplates at a density of 5×10^4 cells mL^{-1} in 160 μL of respective media containing 10% FBS. After 24 h of cell attachment, the cells were incubated with 10, 20, 40, 80, 160 μg mL^{-1} ND nanoparticles（ND-OH, ND-polyPEGMA-1，ND-polyPEGMA-2）for 24 h. Then nanoparticles were removed and cells were washed with PBS for three times. 10 μL of CCK-8 dye and 100 μL of DMEM cell culture media was added to each well and incubated for 2 h at 37 °C. Plates were then analyzed with a microplate reader (VictorIII, Perkin-Elmer). Measurements of dye absorbance were carried out at 450 nm, with the reference wavelength at 620 nm. The values were proportional to the number of live cells. The percent reduction of WST was compared to controls (cells not exposed to nanoparticles), which represented 100% WST reduction. Three replicate wells were used for each control and test concentrations per microplate, and the experiment was repeated three times. Cell survival was expressed as absorbance relative to that of untreated controls. Results are presented as mean ± standard deviation (SD).

3.3 Radical oxygen species (ROS) assay

The intracellular ROS was determined using 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay according to our previous work.[2] Cells were treated with ND nanoparticles as described in cell viability assay. 200 μL of DCFH-DA at the concentration of 10 μM was added to each well. After incubation for 30 min, fluorescence data of oxidized DCFH-DA were recorded by using a microplate

reader (VictorIII, Perkin-Elmer) with the excitation and emission wavelengths set at 485 and 535 nm, respectively. The fluorescence of cells without incubation with dyes was used as the background (F_0), and cells incubated with 0.5 and 1.0 mg mL^{-1} of Rosup for 30 min were served as positive control. The values were expressed as percent of fluorescence intensity relative to control wells. All the procedures were performed without exposure to light. Three replicate wells were used for each control and test concentrations per microplate, and the experiment was repeated three times. Results are presented as mean ± SD.

4. PolyPEGylated ND for drug delivery

4.1 DOX loading onto ND nanoparticles

The loading of doxorubicin hydrochloride (DOX) onto polyPEGylated ND nanoparticles was performed by simple mixing ND nanoparticles with DOX. In brief, 40 mL of polyPEG-ND nanoparticles (0.2 mg mL^{-1}) and DOX (0.1 mg mL^{-1}) dispersed in PBS were throughly mixed and stirred at room temperature. At different time interval, 2 mL of mixture was taken and centrifuged at 8000 rpm for 10 min. Then absorbance of DOX in the supernatant was determined using UV-Visible spectrometer with the wavelength at 482 nm and the concentration was calculated by a standard DOX concentration curve generated from a series of DOX solutions with various concentrations. The drug loading efficiency (DLE) was calculated from the following formula:

$$\text{DLE (w/w \%)} = (\text{weight of loaded drug/ weigh of ND nanoparticles}) \times 100\%$$

4.2 Release behavior of DOX from polyPEG-ND nanoparticles

The release behavior of DOX from the ND nanoparticles was carried out at room temperature in PBS. In brief, 8.0 mg of ND-DOX complexes (the mass of ND nanoparticles) were placed into a dialysis tube, which was dialyzed against 100 mL of PBS (release reservoir). The release reservior was

placed in a shaking bed at 37 °C with a rotation speed at 100 rpm, and 2.0 mL of release media in the reservoir was taken out at desired time interval. And the concentration of DOX was quantified using the fluorescence spectroscopy (excitation wavelength = 480 nm, emission wavelength = 561 nm).

4.3 Cellular effects of polyPEG-ND-DOX complexes

The cellular effects of polyPEG-ND-DOX were determined using the WST assay as described above. Briefly, cells were seeded in 96-well microplates at a density of 5×10^4 cells mL^{-1} in 160 μL of respective media containing 10% FBS. After 24 h of cell attachment, the cells were incubated with 10, 20, 40, 80, 160 μg mL^{-1} of ND-polyPEGMA-2-DOX (the concentration of ND nanoparticles) for 24 h. 9 μg mL^{-1} of free DOX was severed as the positive control. Then nanoparticles were removed and cells were washed with PBS for three times. 10 μL of CCK-8 dye and 100 μL of DMEM cell culture media were added into each well and incubated for 2 h at 37 °C. Plates were then analyzed with a microplate reader (VictorIII, Perkin-Elmer). Measurements of dye absorbance were carried out at 450 nm, with the reference wavelength at 620 nm. The values were proportional to the number of live cells. The percent reduction of WST was compared to controls (cells not exposed to nanoparticles), which represented 100% WST reduction. Three replicate wells were used for each control and test concentrations per microplate, and the experiment was repeated three times. Cell survival was expressed as absorbance relative to that of untreated controls. Results are presented as mean ± standard deviation (SD).

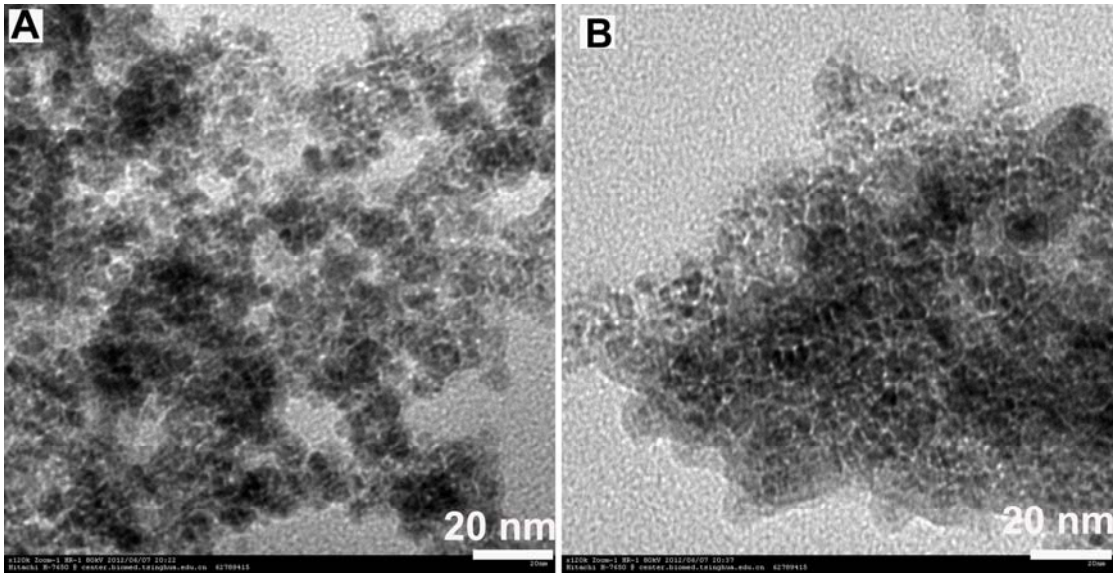
4.4 Cellular uptake of polyPEG-ND-DOX complexes

Confocal microscope images were taken to evaluate the cell uptake of polyPEG-ND-DOX. Briefly, on the day prior to treatment, cells were seeded in a glass bottom dish with a density of 1×10^5 cells per dish. On the day of treatment, the cells were incubated with polyPEG-ND-DOX at a final concentration

of 200 μg mL^{-1} (the concentration of ND) for 3 h at 37 °C. Afterward, the cells were washed three times with PBS to remove the polyPEG-ND-DOX complex and then fixed with 4% paraformaldehyde for 10 min at room temperature. The cell nuclues were stained with hoechst 43332. Cell images were taken with a Laser Scanning Confocal Microscope (LCSM) Zesis 710 3-channel (Zesis, Germany) with the excitation wavelengths of 488 nm.

Results and Discussion

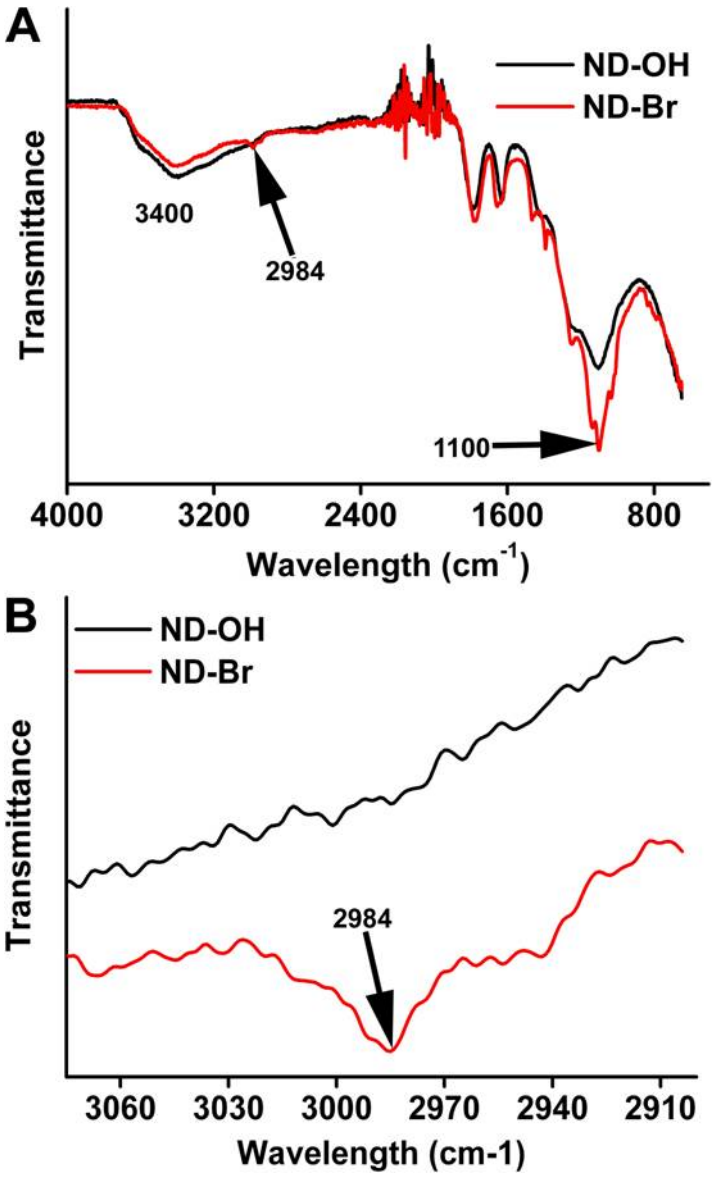
The size and morphology of ND nanoparticles were characterized by TEM. As shown in SFig. 1, the size of individual ND-OH is about several nanometers; however, they tend to aggregate into large clusters with size larger than 100 nm. After PEGylation, the pristine ND clusters appear to be drastically agglomerated. Furthermore, low contrast coating around the ND-polyPEGMA-2 was observed, providing direct evidence that polyPEGMA was covalent linked onto the surface of ND (SFig. 1B).



SFig. 1 Representative TEM images of ND particles before and after PEGylation for 48 h, (A) ND-OH, (B) ND-polyPEGMA-2, scale bar = 20 nm. Low contrast coating around the ND-polyPEGMA-2 was

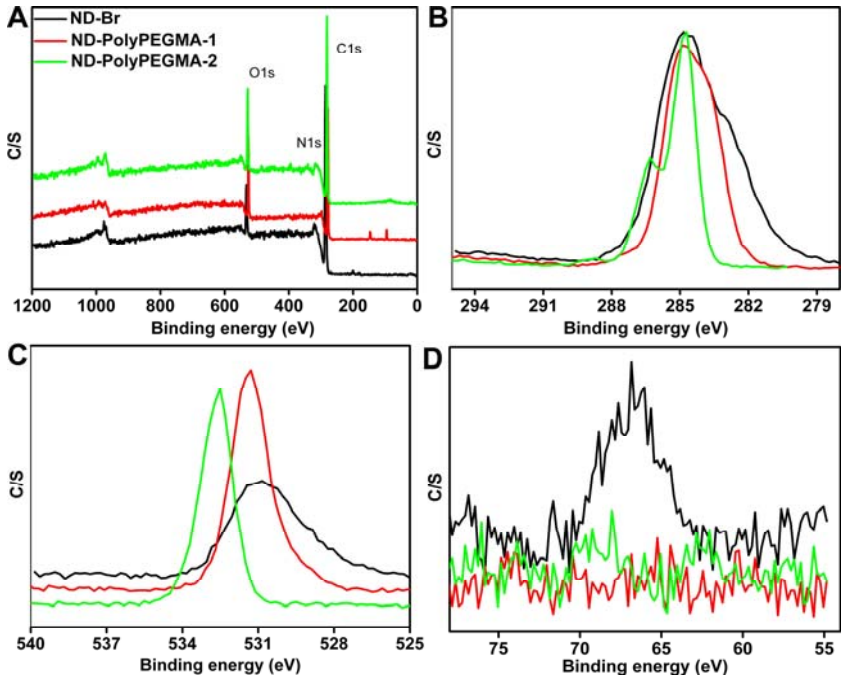
observed.

FT-IR spectra were further used to evidence the successful surface modification of ND with polyPEGMA. As shown in SFig. 2, the absorption due to the -OH stretches shows at 3400 cm^{-1} is weakened in these spectra, while bands appear at 1100 and 2984 cm^{-1}, characteristic of the C-O and C-H stretches were strengthen, revealing the immobilization of 2-bromoisobutyryl bromide on the surface of ND.



SFig. 2 FT-IR spectra of ND-OH and ND-Br (A) and the enlarged of spectra (B).

A rough survey with scan range from 0-1200 eV was carried out to identify the elements present in the different ND samples. XPS scan studies of these materials revealed the existence of C, N and O atoms (SFig. 3A). Compared to the survey spectra obtained from ND-Br, , the C1s, O1s peaks still appeared after polyPEGylation, but the relative intensity of the C1s peak significantly decreased while that of O1s peak increased, indicating the efficient modification of polyPEGMA on ND surface (SFig. 3. B and C). Furthermore, the peaks of O1s were shifted to the high binding energy (532.2–533.4 eV) corresponding to C-OH and/or C-O-C groups, further confirming that polyPEGMA was conjugated onto the surface of ND (SFig. 3C).



SFig. 3 XPS spectra of ND-Br, ND-polyPEGMA-1, and ND-polyPEGMA-2. (A) Survey scans the spectral region from 0 to 1200 eV. (B) The carbon 1s region, (C) the oxygen 1s region, and (D) the bromine 3d region.

Table S1 lists the element concentrations of ND-Br, ND-polyPEGMA-1 and ND-polyPEGMA-2. It shows that the concentration of C atom is decreased from 88.35% to 81.52 % and 81.53%. While the

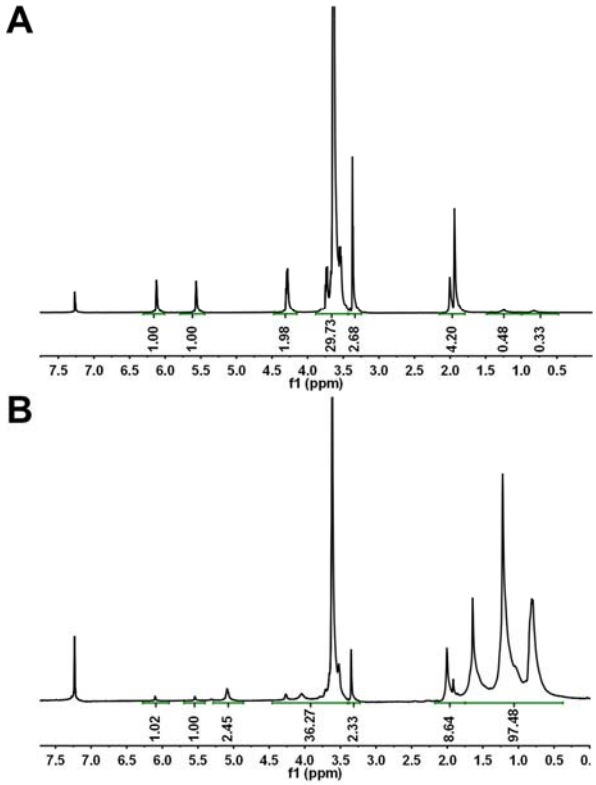
concentration of O atom increased from 9.89% to 17.31% and 18.31% after PEGylation. The grafting polymer weight ratios of grafting from calculated from the XPS results was about 29% and 33.4%. Of note, decrease of intensity of peaks for Br3d after PEGylation further revealed that PEGMA was immobilized on ND successfully (SFig. 3D).

Table S1 Element contents of ND-Br, ND-polyPEGMA-1, and ND-polyPEGMA-2 based on the XPS analysis.

	C1s	N1s	O1s	Br3d
ND-Br	88.35	1.62	9.89	0.13
ND-polyPEGMA-1	81.52	1.12	17.31	0.05
ND-polyPEGMA-2	81.53	0.14	18.31	0.02

Table S2 Zeta-potential and size distribution of ND-OH, ND-polyPEGMA-1, and ND-polyPEGMA-2 dispersed in H_2O and PBS.

	Zeta-potential (mV)		Size distribution (nm)	
	H_2O	PBS	H_2O	PBS
ND-OH	19.2±4.4	-15.9±3.9	344.5±19.5	2834.9±356.3
ND-polyPEGMA-1	-22.5±7.5	-20. 8±4.9	743.5±52.2	1275.9±199.1
ND-polyPEGMA-2	-28.2±2.1	-23.8±6.5	570.6±21.6	968.8±32.8



SFig. 4. Typical ^{1}H NMR spectra of PEGMA (A) and ND-polyPEGMA (B).

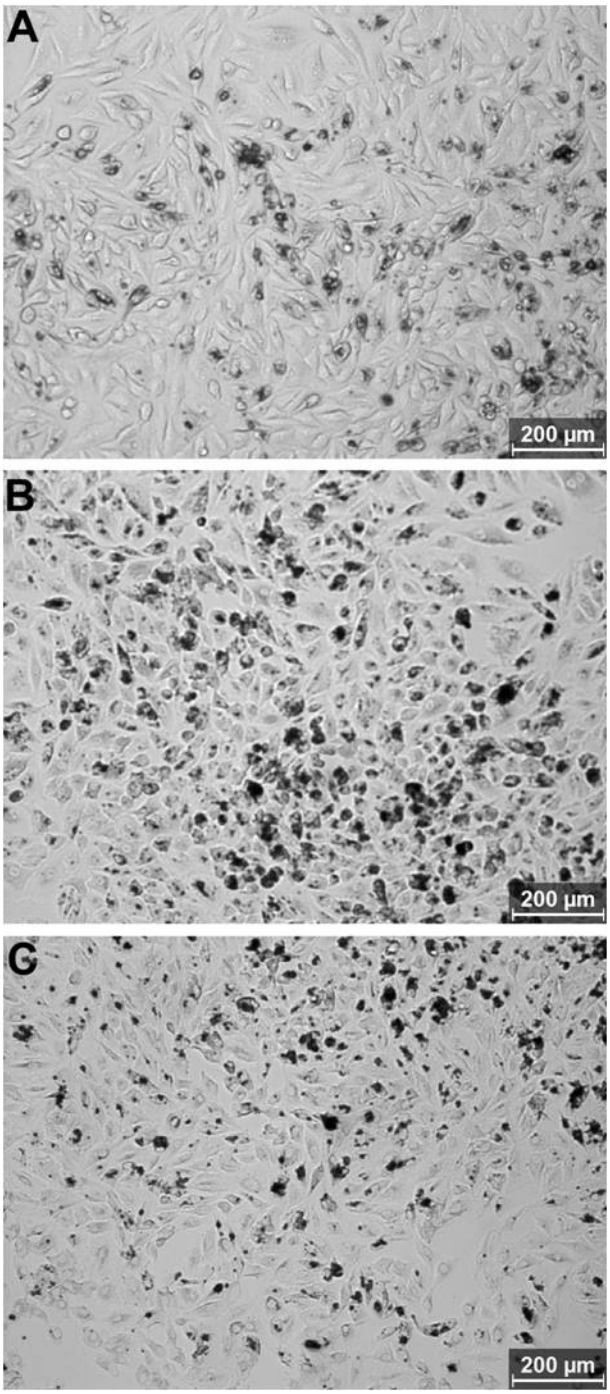


Fig. S5 Optical images of cells incubated with 80 μg mL^{-1} of ND particles for 24 h. (A) ND-OH, (B) ND-polyPEGMA-1, (C) ND-polyPEGMA-2. Scale bar = 200 μm.

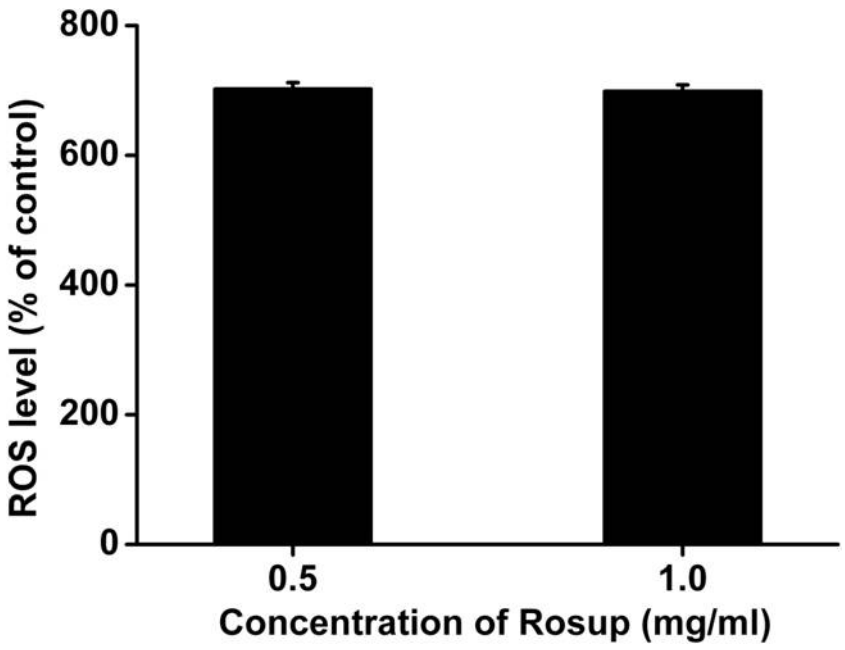


Fig. S6 Generation of reactive oxygen species (ROS) from A549 cells determined by the hydrolysis of DCHF-DA after incubation with 0.5 and 1 mg mL^{-1} of Rosup for 30 min.

1. Y. Zhu, W. Li, Q. Li, Y. Li, X. Zhang and Q. Huang, *Carbon*, 2009, **47**, 1351-1358.
2. X. Zhang, Y. Zhu, J. Li, Z. Zhu, W. Li and Q. Huang, *J Nanopart Res*, 2011, **13**, 6941-6952.