SUPPLEMENTARY INFORMATION

Materials and methods

Materials

The carrier-free $Na^{125}I$ was purchased from the PerkinElmer CO. USA. Chloramine-T was supplied by Merck Chemicals Co., Ltd., Shanghai. Dexamethasone (DEX) was obtained from Shanghai Pharmaceutical Co., Shanghai, and other chemicals were from Sinopharm Chemical Reagent Co., LtdS., Shanghai.

Synthesis of NGO

NGO was synthesized from natural graphite by oxidation with $H_2SO_4/KMnO_4$ according to a modified Hummers method.[1] Specifically, 20 g of graphite powder was put into an 80 °C solution of concentrated H_2SO_4 (30 ml), $K_2S_2O_8$ (10 g) and P_2O_5 (10 g). After 6 hours, the resulting mixture was cooled to room temperature; then it was carefully diluted with a large volume of distilled water, filtered and washed on the filter until the pH of the rinse water was neutral. The product was dried in an air-oven at 40 °C. Approximately 20 g of the preoxidized graphite was put into 460 ml of cooled (0 °C) concentrated H_2SO_4. Approximately 60 g of $KMnO_4$ was added gradually with stirring and cooling, so that the temperature of the mixture was not allowed to reach 20 °C. The mixture was then stirred at 35 °C for 2 hours. Subsequently, distilled water (920 ml) was slowly added and the mixture was maintained at this temperature for 15 min. The reaction was terminated by the addition of 2800 ml of distilled water and 50 ml of 30% H_2O_2 solution. The solid product was separated by filtration and washed with 5 L of 1:10 HCl solution. The NGO product was suspended in distilled water and sonicated for 2 hours. The supernatant yellow-brown sol was subjected to dialysis with ultrapure water (18 MΩ.cm resistivity) from a Millipore Milli-Q water purification system for complete removal of metal ions and acids.

The resulting NGO was characterized by Fourier transform infrared spectroscopy, Raman spectroscopy, atomic force microscopy (AFM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The FTIR spectrum of the NGO was collected using a Thermo Nicolet AVATAR 370 FT-IR spectrophotometer at a resolution of 4 cm^{-1} and 32 scans in the absorbance mode. The Raman spectrum of NGO was collected using a LabRam-1B Raman spectrophotometer (J.Y., France). Colloidal suspensions of NGO were deposited on freshly cleaved mica for AFM imaging in tapping mode on a Multimode Nanoscope III from Digital Instruments (Veeco Metrology Group, NY). SEM and TEM images of the NGO were collected on a 1530VP, LEO Corporation SEM instrument and a JEOL 2010 TEM instrument with an acceleration voltage of 200 kV, respectively.

Synthesis of ^{125}I-NGO

NGO was easily labeled with ^{125}I by the Chloramine-T (N-chloro-p-toluenesulphonic acid) method. The iodine atom easily covalently binds to the position on the analogous phenyl structure of NGO. Briefly, 50 μl of NGO (ca. 50 μg), 10 μl of carrier-free $Na^{125}I$ (10 μCi ^{125}I) and 10 μl of PB (0.25 M pH=7.4) were added to the tube in sequence and mixed by a vortex mixer. To initiate the labeling, 7 μl of Chloramine-T (in a 1 mg/ml PB solution) was added to the tube. After incubation under ultrasonication for 30 min at room temperature, 7 μl of $Na_2S_2O_5$ (in a 2 mg/ml PB solution) was added to the tube to stop the labeling process. The labeling efficiency was examined by paper chromatography (PC, silica gel, eluting solvent:$V_{acetone}$:V_{water} = 9:1, $Rf_{I\text{-}NGO}^{125}$ = 0). Six different reaction times (2 min, 5 min, 10 min, 30 min, 1 hour and 2 hours) were tested to determine the best labeling condition.

Radioiodinated NGO were separated from free ^{125}I ions via dialysis against Milli-Q water for several days until little radioactivity was detected in the dialysate. The final solution was measured

using a γ detector (SN-682, Shanghai Institute of Applied Physics, China). The radioactivity was represented as counts per min (cpm). To verify the effect of the purification by dialysis, the radiochemical purities of ^{125}I-NGO before and after dialysis were examined by PC; the resulting chromatographs were evaluated using a radio-TLC imaging scanner (AR-2000, Bioscan, USA).

The stability of the ^{125}I-NGO in serum was assayed as follows: 30 μl of ^{125}I-NGO was added to 180 μl of mouse serum and incubated. After a certain interval, the radiochemical purity of ^{125}I-NGO was examined by PC.

Non-radioactive iodinated NGO (I-NGO) was synthesized by the same method, in which stable iodine was substituted for ^{125}I. I-NGO was characterized by x-ray photoelectron spectroscopy (XPS, Kratos Axis Ultra DLD, UK). The chamber pressure was kept below 3×10^{-9} Torr. A binding energy of 284.8 eV for the C_{1s} level was used as an internal reference.

Animals

All animal experiments were performed in compliance with the local ethics committee. In addition, all the procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee. Male KunMing and C57BL/6 mice (18~22 g) were obtained from the Shanghai Slack Experimental Animal Center. The animals were housed in a room that was maintained at 22℃ with a daily light-dark cycle (06:00-18:00 light). Mice were fed standard mice chow and provided with water *ad libitum*.

Intratracheal instillation

The general intratracheal instillation procedure was modified from one used in previous experiments.[2, 3] Briefly, mice were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium (10 mg/ml; Sigma, St. Louis, MO) and placed in a supine position with their heads placed upward on a board tilted at 50°. A midline incision was performed in the neck and the

trachea was exposed. The solution was drawn (up to a premarked location) into a 0.45 mm intravenous infusion needle and rapidly instilled into the lung with a 1 ml plastic disposable syringe prefilled with 100 μl air. After intratracheal instillation, the mice were placed in a vertical position and rotated for 0.5-1 min to distribute the instillation evenly within the lungs. The neck incision was then sutured. The mice recovered and were active within 20 min after the intratracheal instillation.

Blood collection

Blood was collected by removing eyeballs of mice. The mouse was firmly grasped and eyeball on one side was quickly pulled out with a forcep causing it to bleed. The blood was collected in a tube. Body was also massaged to get bigger drops of blood. At the end of the procedure, the mouse was killed by cervical dislocation.

BAL fluid assay

After the mice were exsanguinated and sacrificed, the tracheas of the mice (5 mice per group) were exposed with an incision in the neck and cannulated with a 20-gauge cannula. The lungs were flushed with 0.9% saline in 0.2-ml increments. The fluid recovery rate was 87 ± 2%. The recovered fluid was centrifuged for 5 min at 300 *g*. The supernatant was assessed for biochemical index, including LDH, ALP and total protein, using commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions. The pellet from the recovered fluid was resuspended in 1 ml PBS with 1% BSA and 0.1% sodium azide. A 10-μl aliquot was used to count cells. Cell viability was determined by Trypan Blue (Sigma-Aldrich) exclusion. In addition, cytospun cells were prepared using a cytocentrifuge (Academy of Military Medical Sciences) for Wright's staining and differential cell counting.

Lung wet/dry weight ratio

Body weights were determined for each group (5 mice per group) before the mice were terminated.

After the mice were exsanguinated and sacrificed, the chests of the mice were opened by a median sternotomy, and the lungs were entirely excised. The wet weight of the exsanguinated entire lungs was measured using an electronical balance. The lungs were then wrapped loosely in aluminum foil, placed in a drying oven at 60 °C for 2 days, and weighed again for their dry weight. Finally, the lung index (lung wet weight to body weight) and wet/dry weight ratio were calculated.

Lung morphological and histopathological evaluation

After the mice were exsanguinated and sacrificed, the chests of the mice (5 mice per group) were opened by a median sternotomy, and the entire lungs were excised. Subsequently, the isolated lungs were placed in a 5 ml centrifuge tube containing approximately 4 ml of the formalin (10% in neutral phosphate buffer). The lungs were fixed for at least 7 days before further processing. The formalin-fixed lungs were embedded in paraffin, thin-sectioned coronally and mounted on glass microscope slides using standard histopathological techniques. The sections were stained with hematoxylin-eosin and examined by light microscopy.

Oxidative stress assay

After the mice were exsanguinated and sacrificed, the chests of the mice (5 mice per group) were opened by a median sternotomy, and the entire lungs were excised. Subsequently, the isolated lungs were placed in the glass homogenizers containing a certain quantity of 0.9% saline, and then homogenized on ice to yield a 10% (w/v) homogenate. The homogenates were centrifuged at 2500 rpm for 10 min to obtain the supernatants. The levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) were determined using spectrophotometric diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Statistical analysis

Statistics were calculated using SPSS PASW Statistics 18 software (SPSS Inc., Chicago, IL) and all data are expressed as the means ± SEM. One-way analysis of variance (ANOVA), followed by Dunnett's post-test, was performed to determine the statistical significance ($P < 0.05$) between the indicated groups.

Results

Physicochemical characteristics of NGO

The Fourier transform infrared spectroscopy (FTIR) spectrum showed the characteristic features of NGO (Fig. S6a): O-H stretching vibrations at 1731 cm^{-1}, skeletal vibrations at 1628 cm^{-1} and C-O stretching vibrations at 1078 cm^{-1}.[4] The Raman spectrum of NGO consisted of two main features, the G mode at 1594 cm^{-1} and the D mode at 1333 cm^{-1} (Fig. S6b). The thickness of the as-made NGO sheets was ~1 nm (Fig. S6c), which led to the conclusion that the as-made NGO sheets remained separated in dispersion. Large NGO sheets of tens to several hundreds of square nanometers were individually observed using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Fig. S6d and S6e). Based on the AFM measurement, NGO was present in large sheets with a size range of 10-800 nm (Fig. S7), which was corroborated by DLS data that determined the average diameters of the NGO to be 308.4±10.4 nm.

Synthesis of ^{125}I-NGO

To obtain the best labeling conditions and highest labeling efficiency, a series of experiments using various labeling periods was performed. After removing the adsorbed ^{125}I by dialysis, the labeling efficiency was considered reasonable for the ^{125}I covalently bound to NGO. The results showed that the labeling efficiency of covalently bound NGO varied with the ultrasonication time, increasing from 51.12% (at 2 min) to 56.85% (at 30 min) then decreasing to 51.11% (at 2 hours) (Fig. S8a), indicating that the optimal ultrasonication time was 30 min.

After the dialysis against Milli-Q water for three days, the loss of the peak of Na^{125}I indicated that free ^{125}I ions had been successfully eliminated and the ^{125}I-NGO solution had been purified effectively (Fig. S8b). The radiochemical purity of ^{125}I-NGO was over 94% according to PC. The binding capacity between ^{125}I and NGO was 4 nmol/μg (0.5 μci/μg). Moreover, the stability of the

^{125}I-NGO in serum was evaluated. The results showed that ^{125}I-NGO was stable in serum. The radiochemical purity remained at more than 83% when the ^{125}I-NGO was incubated in serum for 24 hours (Fig. S8c).

To confirm the formation of a covalent bond (C–I) between iodine and NGO, non-radioactive I-NGO was synthesized. The iodine 3d XPS spectrum of I-NGO is shown in Fig. S8d. The peak split corresponds to the orbital split of iodine 3d into $3d_{3/2}$ and $3d_{5/2}$ levels. The binding energies of 620.3 and 631.3 eV were in quite good agreement with those of the reference compound, *p*-I-C6H4NO2 (620.4 and 631.9 eV).[5] No ionic iodine (3d5/2, 618.6 eV and 3d3/2, 630.1 eV) was observed in the sample.[6] Thus, we conclude that the iodine atoms are covalently bound to NGO at the *meta* position of the carboxyl groups on NGO. It is well known that the covalent bond is remarkably stronger than the other weak binding forms *in vivo.*

Body weight, lung wet weight and index

Body weight, lung wet weight and lung index were used to access the effects of the intratracheally instilled NGO. NGO induced a dosage-dependent decrease in body weight and an increase in lung wet weight and index (Fig. S9, a,c,e). The lowest value of body weight and the peaks of lung wet weight and index were observed at 48 hours (Fig. S9, b,d,f). These results indicate the possibility of NGO pulmonary toxicity (most severe at 48 hours).

Lung morphological evaluation

Morphological evaluation was used to observe the distribution of NGO and gross lesions in the lungs. As illustrated on Fig. S10, a dorsal view shows the distribution of NGO (black region) in the lungs. It was obvious that NGO presented a dosage-dependent distribution according to the range and degree of the black color (Fig. S10, a-e). In addition, the different dosages of NGO induced different degrees of lung edema in accordance with the increased lung volume. Furthermore, the

lung edema induced by a constant quantity of NGO was closely related to the time following exposure. After intratracheal instillation (0 hour), NGO began to induce lung edema, which appeared to be most severe at 48 hours and then was gradually relieved up to 1 week (Fig. S10, f-j).

References

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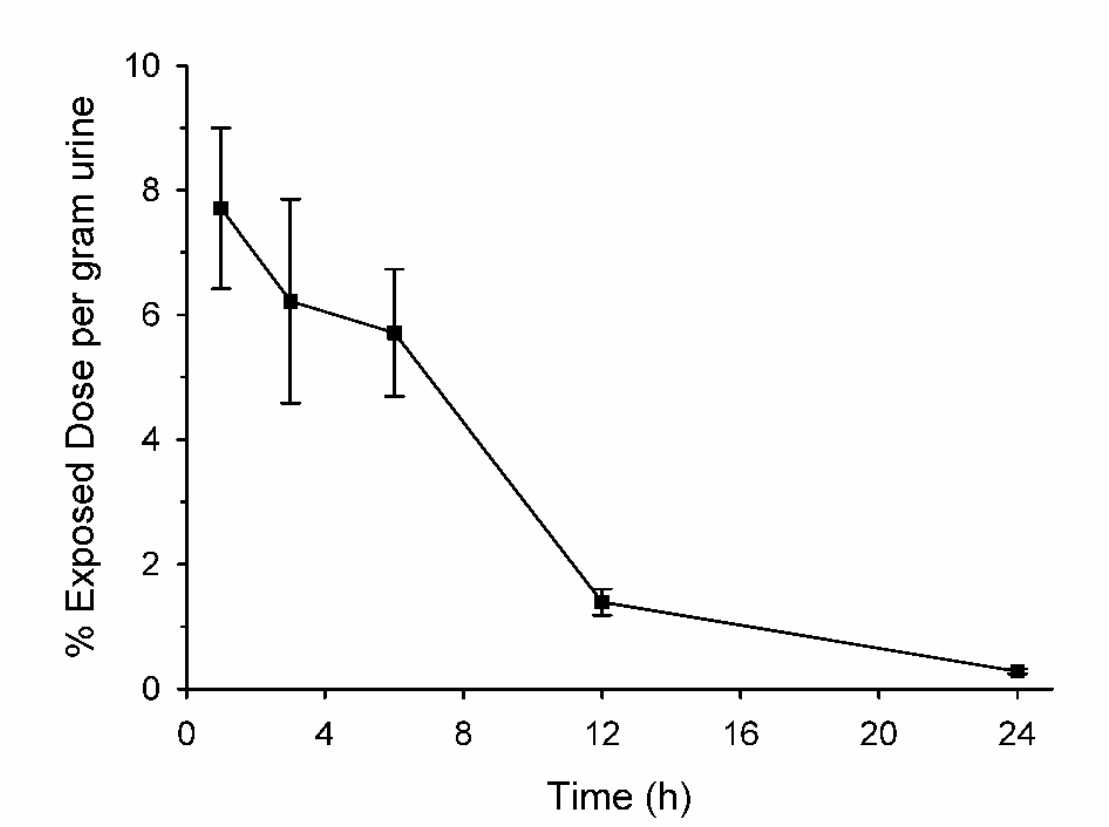
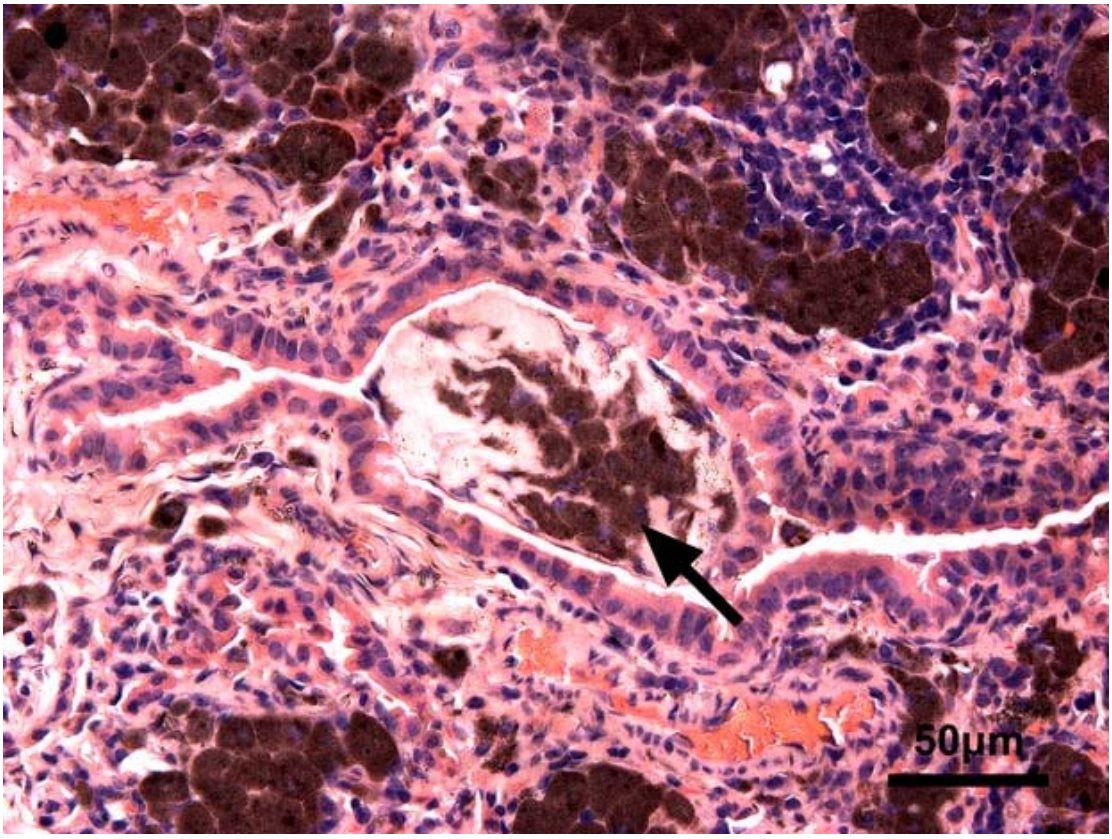
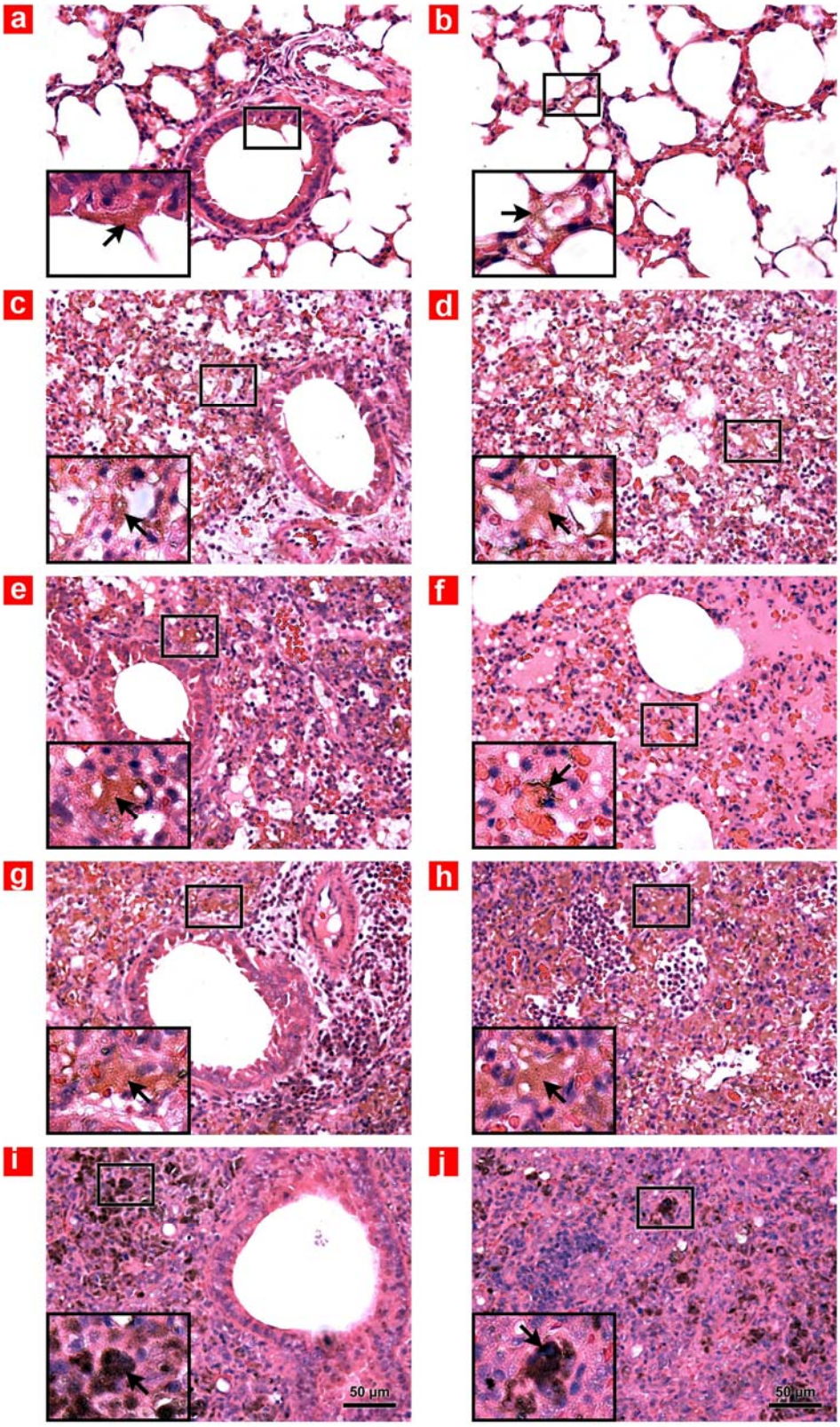
SUPPORTING FIGURES



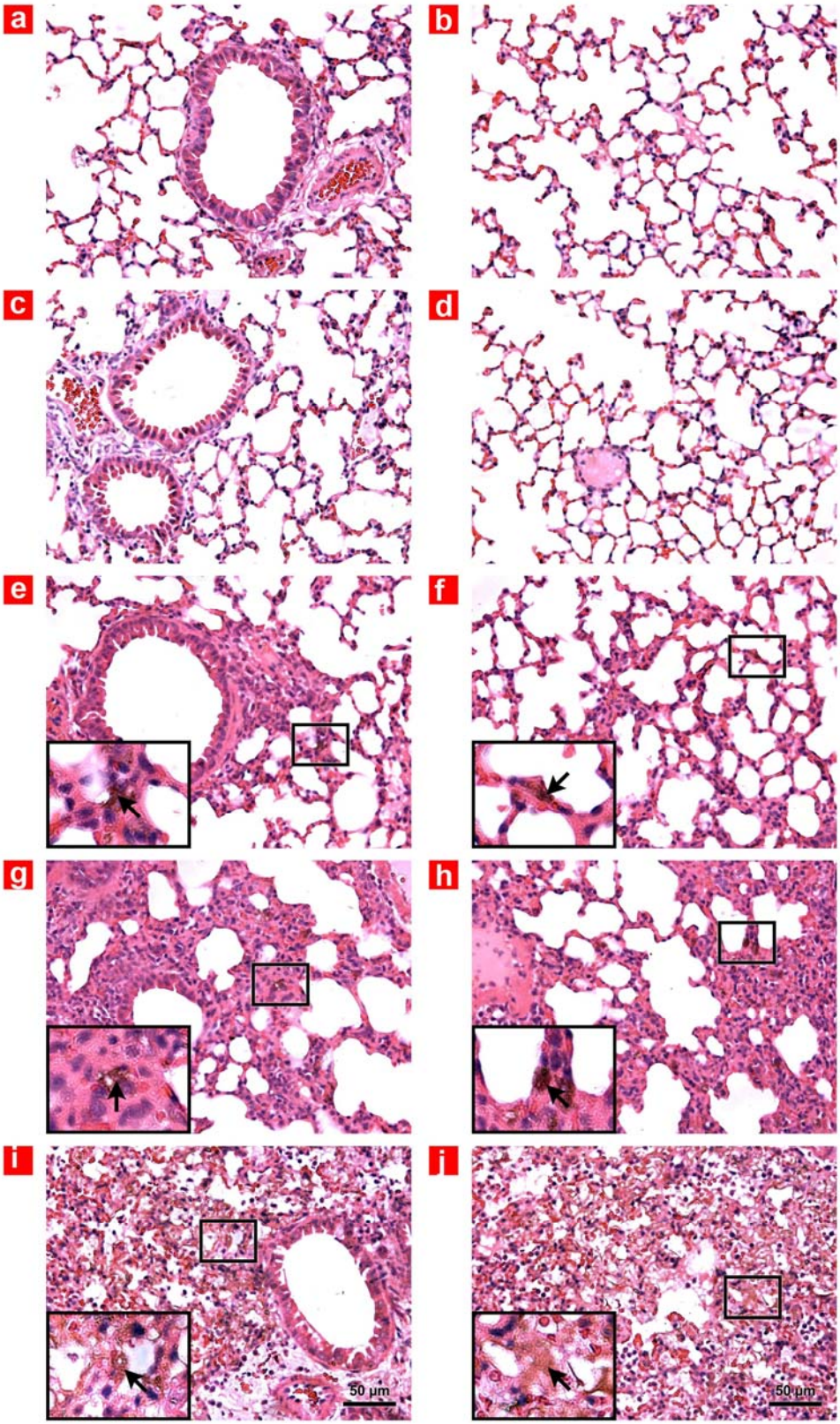
Supplementary Figure 1: **Clearance of NGO in the urine of mice.** N=5 in each group. Values are presented as the mean ± SEM.



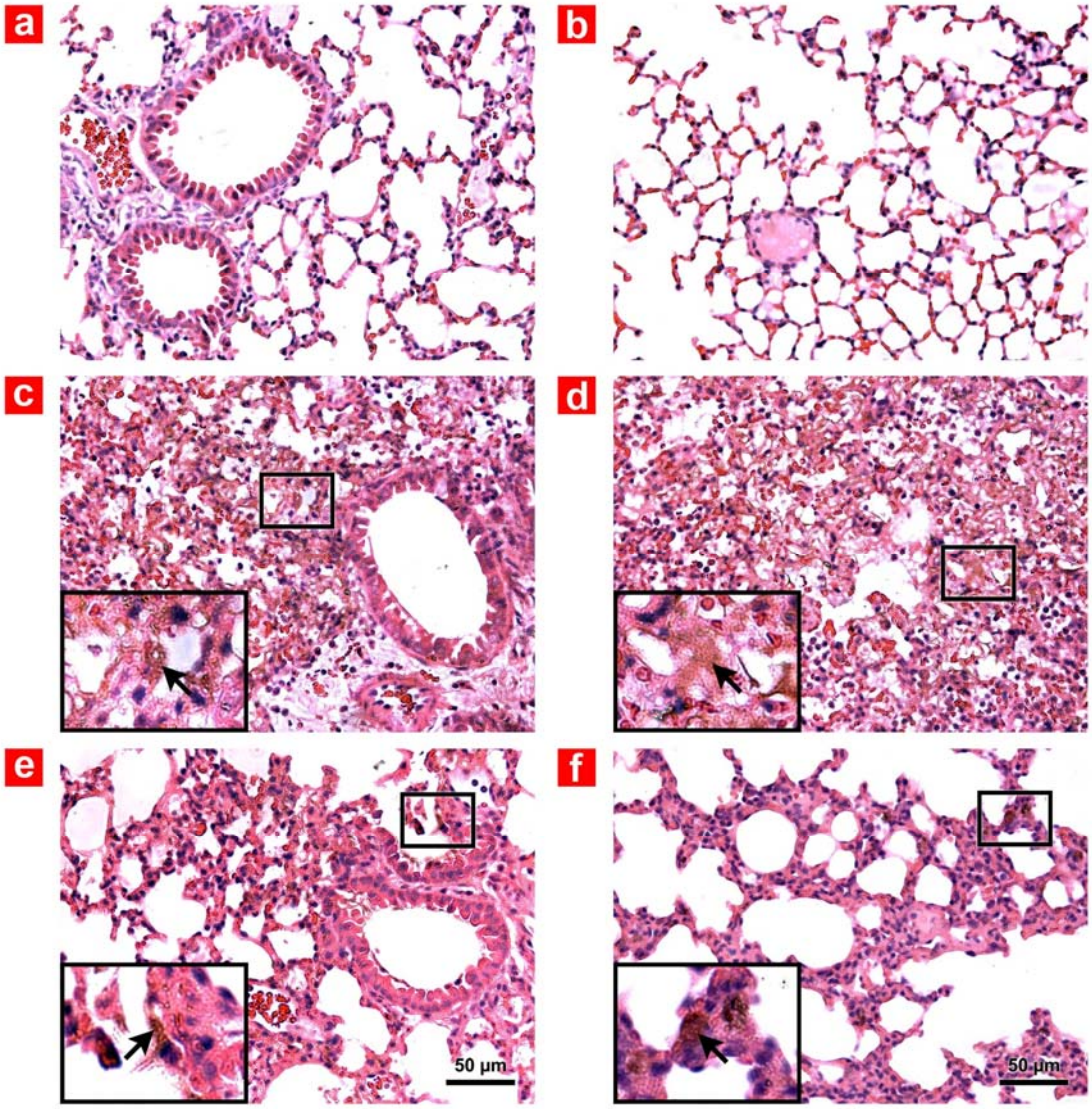
Supplementary Figure 2: **Clearance of NGO from the lungs of mice.** The hematoxylin-eosin staining of lung tissues of mice instilled with 10 mg/kg NGO at 3 months is shown. There is an obvious accumulation of alveolar macrophages laden with NGO in the bronchial lumen. The black arrows indicate NGO. The image is shown at 400× magnification with a 50 μm scale bar.



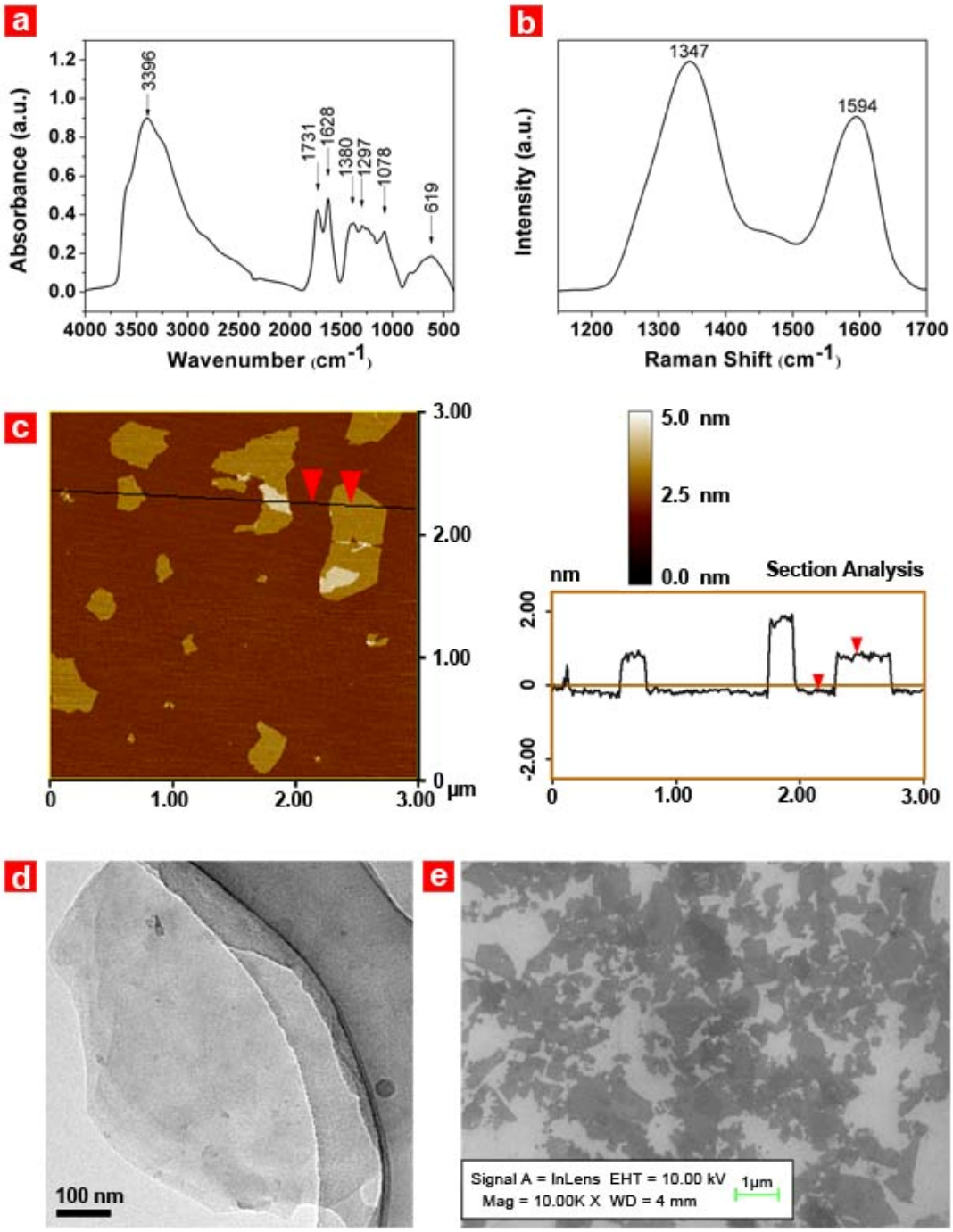
Supplementary Figure 3: **Dispersion and time-related histopathological characteristics of NGO in the lungs of mice. a-b**: 0 hour. **c-d**: 24 hours. **e-f**: 48 hours. **g-h**: 72 hours. **i-j**: 1 week. The black arrows indicate NGO. Different airway levels corresponding to bronchi (**a,c,e,g,i**) or alveolar spaces (**b,d,f,h,j**) are shown at 400× magnification with a 50 μm scale bar, and insets are shown at 1000× magnification.



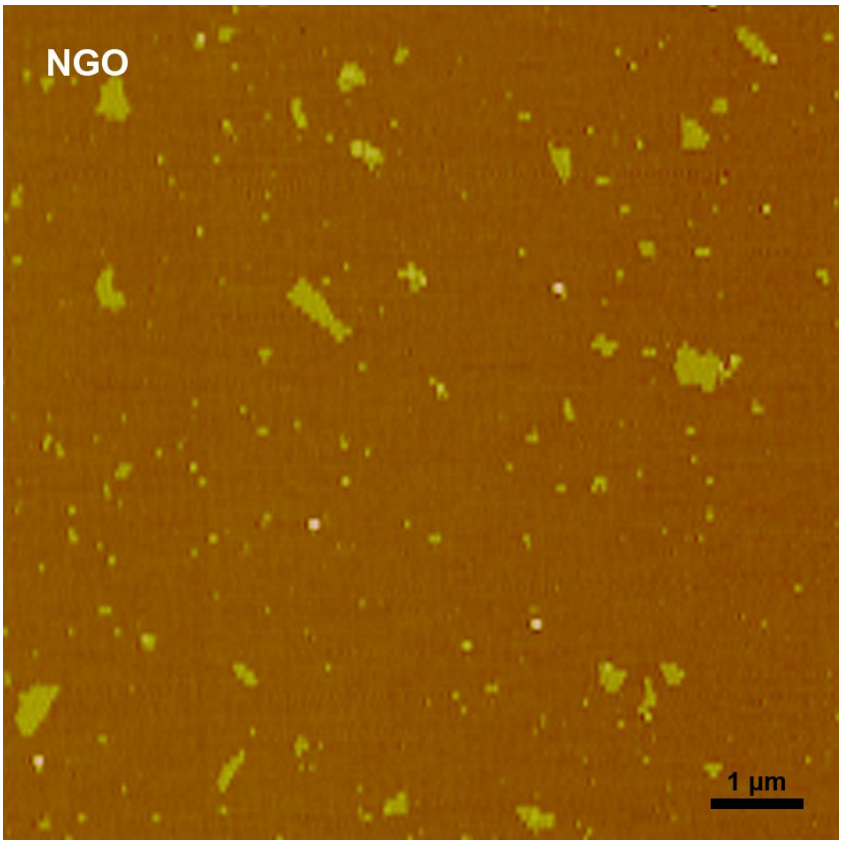
Supplementary Figure 4: **Dosage-related histopathological characteristics of NGO in the lungs of mice. a-b**: Control. **c-d**: Milli-Q water. **e-f**: 1 mg/kg NGO. **g-h**: 5 mg/kg NGO. **i-j**: 10 mg/kg NGO. The black arrows indicate NGO. Different airway levels corresponding to bronchi (A, C, E, G, J) or alveolar spaces (B, D, F, H, J) are shown at 400× magnification with a 50 μm scale bar, and insets are shown at 1000× magnification.



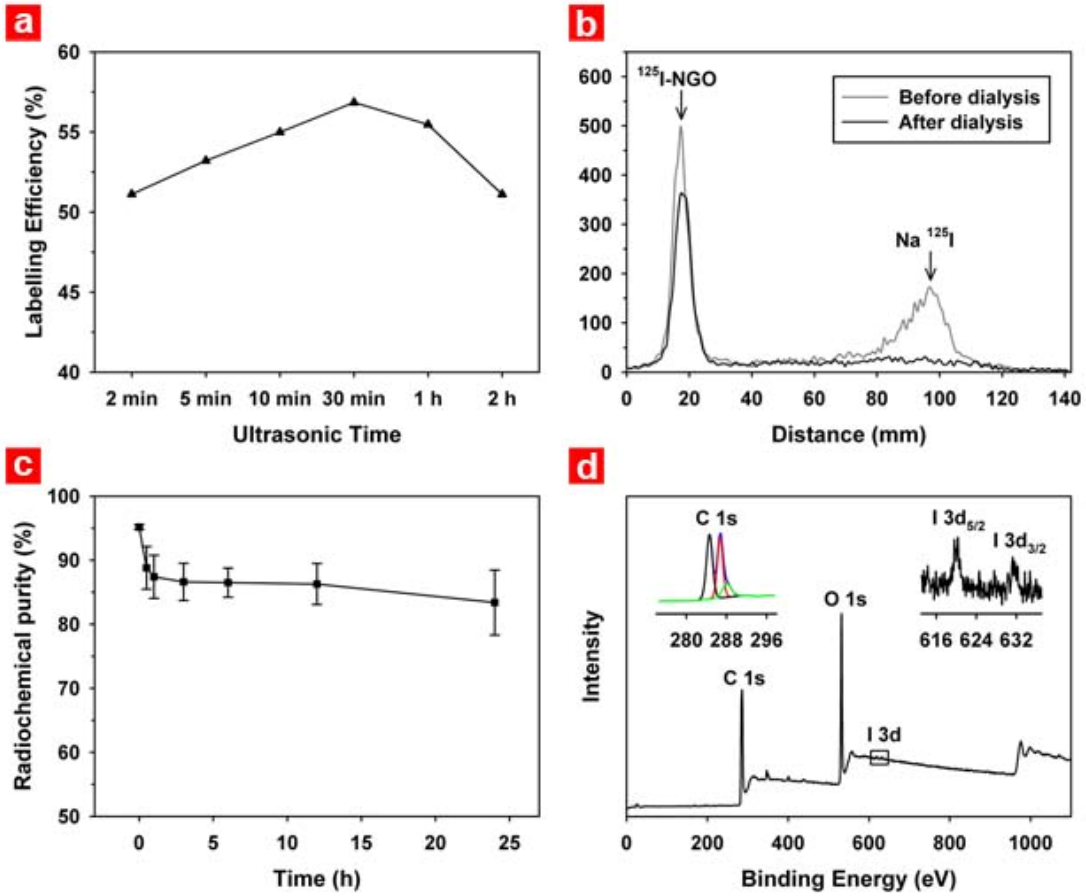
Supplementary Figure 5: **Effect of dexamethasone on NGO-induced histopathological characteristics in lungs of mice at 24 hours. a-b**: Milli-Q water group. **c-d**: NGO group. **e-f**: NGO+DEX group. The black arrows indicate NGO. Different airway levels corresponding to bronchi (**a,c,e**) or alveolar spaces (**b,d,f**) are shown at 400× magnification with a 50 µm scale bar, and insets are shown at 1000× magnification.



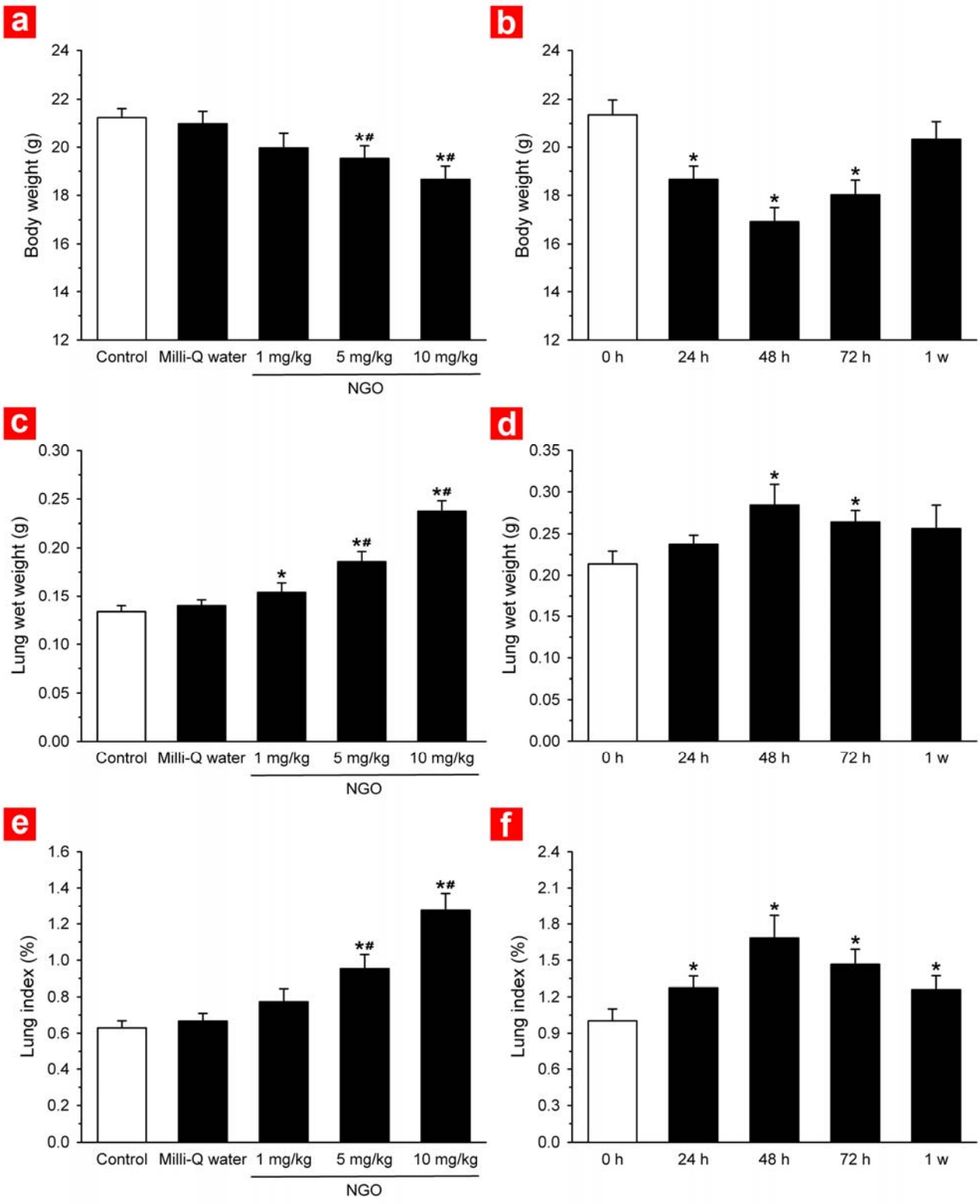
Supplementary Figure 6: **Characterization of NGO.** (**a**) The Fourier transform infrared spectrum of NGO. (**b**) Raman spectrum of NGO. (**c**) Height and corresponding phase tapping-mode AFM image of NGO dispersed in water after deposition onto freshly cleaved mica. (**d**) TEM image of NGO deposited onto a carbon-coated copper grid. (**e**) SEM image of NGO. The image was acquired from samples deposited onto a silicon ship.



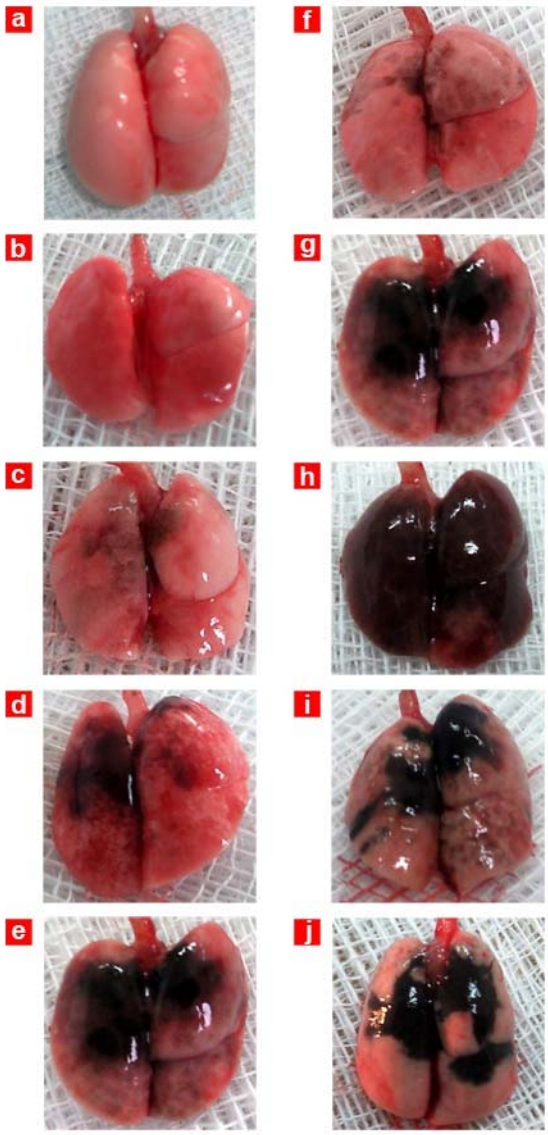
Supplementary Figure 7: **AFM images of NGO.** NGO was present as large sheets with a size range of 10-800 nm.



Supplementary Figure 8: **Synthesis of ^{125}I-NGO.** (**a**) The relationship between the labeling efficiency of ^{125}I-NGO and the ultrasonication time. (**b**) The effect of purification by dialysis. (**c**) The stability of ^{125}I-NGO in serum. (**d**) Representative XPS spectrum of ^{125}I-NGO. The inset illustrates that the binding energies of I 3d are 620.3 and 631.3 eV.



Supplementary Figure 9: **Evaluation of body weight (a-b), lung wet weight (c-d) and lung index (e-f)**. (**a,c,e**) Dosage-related response. * Values that differed significantly from the control group at $P < 0.05$; # Values that differed significantly from the Milli-Q water group at $P < 0.05$. (**b,d,f**) Time-related response. * Values that differed significantly from the 0 h group at $P < 0.05$. N=5 in each group. Values are presented as the mean ± SEM.



Supplementary Figure 10: **Morphological evaluation of lungs from mice.** (**a-e**) Dosage-related response. **a**: Control. **b**: Milli-Q water. **c**: 1 mg/kg NGO. **d**: 5 mg/kg NGO. **e**: 10 mg/kg NGO. (**f-j**) Time-related response. **f**: 0 hour. **g**: 24 hours. **h**: 48 hours. **i**: 72 hours. **j**: 1 week. The dorsal view shows the distribution of NGO (black region).

Supplementary Table 1: General experimental design of acute pulmonary toxicity of NGO