method using hybrid markers and HPLC analysis" were performed as described below.

### Novel method using hybrid markers and HPLC analysis

After adding 0.125 μg/ml B(ghi)P and 25 μl acetonitrile solution (Figure 1f), the solutions were stirred for 15 min to produce MWCNT solutions with actual concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml. The solutions were then centrifuged, and the supernatants were removed. After addition of 0.5 ml D.W., the resultant solutions were stirred and then centrifuged again. After removal of the supernatants, addition of 0.5 ml acetonitrile, and stirring (Figure 1g), the adsorbed B(ghi)P in MWCNTs was extracted to acetonitrile and filtered (Figure 1h) for HPLC analysis. Chromatography was performed using an Acquity UPLC system (Waters, Milford, MA, U.S.A.) coupled to a fluorescence detector FLR (Waters). Eluates were analyzed quantitatively by monitoring at fluorescent wavelengths of 294 nm for excitation and 410 nm for emission with 5 ml aliquots of extract injected onto a 1.7 mm C18 100 × 2.1 mm I.D. Acquity BEH column (Waters) (Figure 1i). The mobile phases were acetonitrile : methanol : distilled water =75 : 20 : 5. The peak of the injected sample was detected at about 1.3 min. An eluent flow rate of 0.5 ml/min was used for all analyses.

## Results and discussion

By treating the lungs removed from rats with formalin solution (10% neutral buffered formalin) and Clean 99-K200$^{R}$ (C99) in advance, the lungs could be rapidly dissolved (within 30 min) (Figure 2a,b and c). With regard to the analytical method, it was clear that for extraction of MWCNT from the lungs, dissolution with C99 is markedly faster after immersion in formalin compared to dissolution with C99 only. Scanning electron microscope (SEM) observation of centrifuge sediments of lung-dissolved solution, however, revealed MWCNT surrounded by undissolved components (Figure 2d), mainly connective tissue. The addition of concentrated sulfuric acid to the sediment led to the complete dissolution of sediment components other than MWCNTs, and as a result of subsequent filtration, MWCNTs were isolated as the only residue (Figure 2e).

MWCNTs, with clean smooth surfaces due to alkali (C99) and acid (sulfuric acid) treatments, were dispersed by ultrasonic vibration, and then B(ghi)P was adsorbed for 15 min, followed by centrifugation and removal of the supernatant. Finally, the marker was desorbed with acetonitrile in organic solvent (Figure 3a), and examined by HPLC. Chromatograms of MWCNT with B(ghi)P revealed a peak at 1.3 min (Figure 3b), no peak being observed with MWCNT alone (Figure 3c); this confirmed that untreated MWCNT had no B(ghi)P on their surfaces. Ten measurements were made to generate a calibration curve of MWCNT, the relationship between the concentration and area being shown in Figure 3d. Repeated generation of calibration curves using this method gave consistently similar values. The lower quantitation limit yielded was 0.2 μg. The correlation coefficient was 0.9991, confirming the linearity and reliability of the calibration

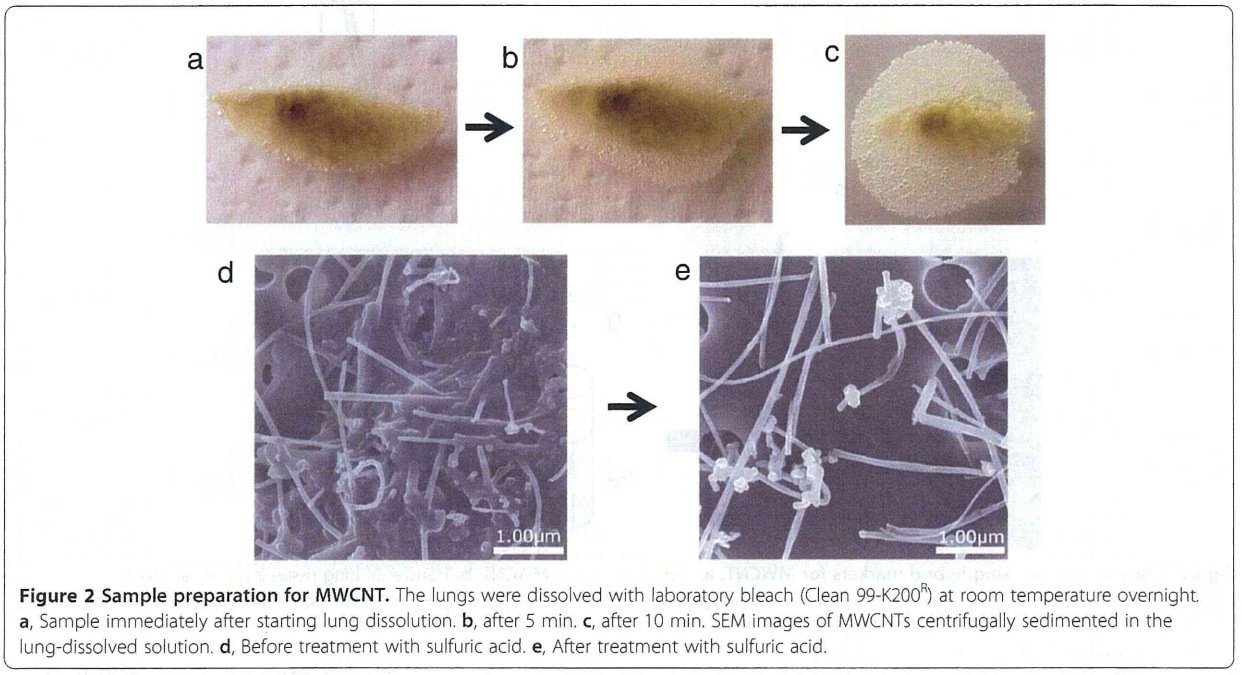


**Figure 2 Sample preparation for MWCNT.** The lungs were dissolved with laboratory bleach (Clean 99-K200$^{R}$) at room temperature overnight. **a**, Sample immediately after starting lung dissolution. **b**, after 5 min. **c**, after 10 min. SEM images of MWCNTs centrifugally sedimented in the lung-dissolved solution. **d**, Before treatment with sulfuric acid. **e**, After treatment with sulfuric acid.

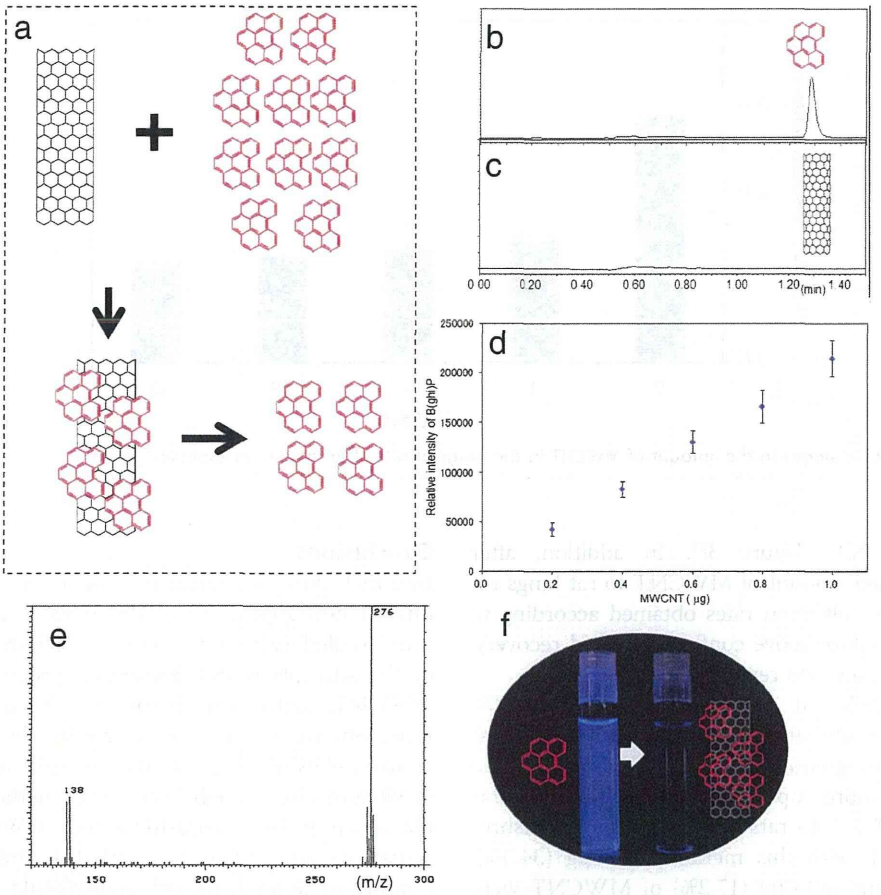


**Figure 3 Adsorption and desorption of the marker (B(ghi)P). a**, Processes of adsorbtion onto the surface of the MWCNT and desorption with organic solvent. **b**, HPLC chromatogram of MWCNTs with markers. **c**, HPLC chromatogram of MWCNT without markers. **d**, Calibration curve of MWCNT. **e**, GC-MS mass spectrum of MWCNT with the marker. **f**, Quenching effects of MWCNT with marker adsorption.

curve. To confirm that B(ghi)P was adsorbed onto the surfaces of MWCNT, B(ghi)P-adsorbed MWCNT were directly introduced into a mass spectrometer, and the mass spectrometry measurement revealed peaks at m/z = 276 and m/z = 138, coincident with the molecular ion peak (m/z = 276) and a fragment peak (m/z = 138) of B(ghi)P (Figure 3e). Pale-blue fluorescence was observed upon UV lamp irradiation of B(ghi)P dissolved in TW solution while such fluorescence was not seen with B(ghi)P solution containing MWCNTs, due to the quenching effects of B(ghi)

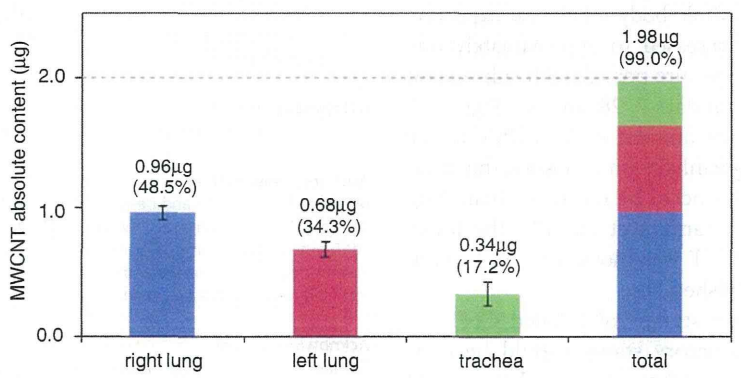


**Figure 4 Measurement of MWCNT in the lungs of rats administered by the intra-tracheal route.** Values are mean ± SD.

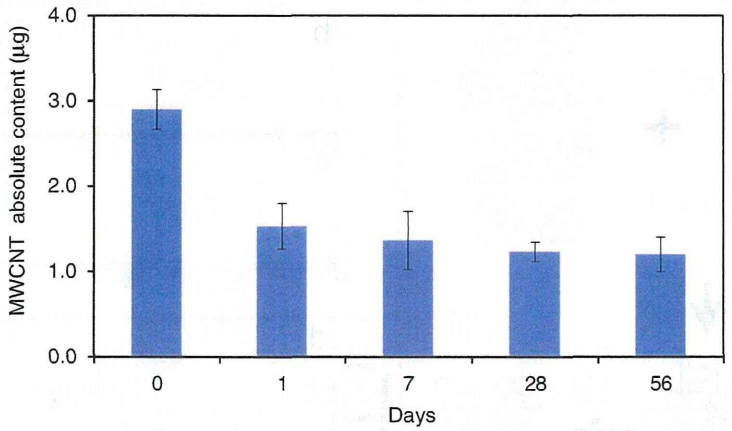


**Figure 5 Time-course changes in the amount of MWCNT in the lungs of rats after inhalation exposure.** Values are mean ± SD.

P-adsorbed MWCNT (Figure 3f). In addition, after addition of a defined amount of MWCNT to rat lungs removed in advance, collection rates obtained according to the analytical procedure above confirmed spiked recovery, measurement accuracy, and repeatability.

Recovery was 92.5% at approximately 0.4 μg, 93.0% at 1.0 μg, and 98.0% at 2.0 μg, demonstrating that MWCNTs in the lung could be measured accurately and precisely. Furthermore, upon intratracheal administration of 2 μg MWCNT to rats and subsequent measurement of MWCNT with this method, 0.68 μg (34.3%), 0.96 μg (48.5%), and 0.34 μg (17.2%) of MWCNT were collected from the right lung, left lung, and trachea, respectively (Figure 4). Although it was possible that MWCNTs remained in the trachea, the results showed that almost all the administered tubules could be recovered from the lungs and the trachea and that the amount could be appropriately measured despite a very low dose. The fact that the right lung was found to contain more MWCNTs is consistent with the larger size as compared to the left lung.

Following the method of Kasai et al. [19], when rats were exposed to MWCNTs by whole body inhalation exposure, the amounts of tubules decreased to approximately one half on day 1 of the post-exposure period, with subsequent further gradually decrease on days 7, 28, and 56 (Figure 5). As a result of measuring the amount of MWCNT in the left lung of rats after a single inhalation exposure, the absolute amount was actually found to be not more than 2 μg [19]. With the method of Tamura et al. [12], the lower quantitation limit of MWCNT was thought to be 1 μg, in contrast to the 0.2 μg established here.

In the future, correlation studies of inhaled CNTs in the lung and lung inflammatory status should be conducted as this is an important issue for which we could utilize this method in environmental toxicology.

## Conclusions

In conclusion, our novel method using hybrid markers offers a new approach for very accurate measurement of multi-walled carbon nanotubes. Because of the nature of the adsorption and desorption processes, weights of MWCNTs and marker levels directly correlate. Furthermore, our method here proved applicable to measurement of nanotubes in lungs of animals after administration in in vitro/in vivo models. The novel method using hybrid markers provides a platform to study MWCNTs with high sensitivity and versatility, with the ability to conduct repeated analyses. This technique should facilitate assessment of nano-bio interactions and carcinogenicity of nanotubes. Since the socioeconomic potential of MWCNT is two-sided (with both benefit and risk), comprehensive safety studies need to be conducted. For this reason, there is a further need to investigate the amounts of MWCNT accumulating in the lungs, the organ most impacted by inhalation of nanotubules.

### Abbreviations

B(ghi)P: Benzo[*ghi*]perylene; C99: Clean 99-K200$^{R}$; HPLC: High performance liquid chromatography; MWCNT: Multi-walled carbon nanotube; PAH: Polycyclic aromatic hydrocarbon; TW-mixture: 9.6% phosphate-buffered saline containing 0.1% Tween 80; SD: Standard deviation; SEM: Scanning electron microscope.

### Competing interests

The authors declare that they have no competing interest.

### Authors' contributions

MO and HY conceived and designed the experiments. MO, MY and MS performed the experiments. MO, TK, YU, SY and TN analysed the data. HO contributed toxicological information. MO and SF co-wrote the paper. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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