Emerging investigator series: Quantification of Multiwall Carbon Nanotubes in Plant Tissues with Spectroscopic Analysis

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Environmental Significance

The increasing production and application of carbon nanotubes (CNTs) for industrial and consumer products will lead to continuous accumulation of CNTs in soils, which can reach a concentration with concerns for plant uptake and human exposure in the future. To the other side, several studies demonstrated positive effects of CNTs on plant growth, with a great potential for agricultural application. To manage the environmental risk and application of CNTs requires information about their concentration in environmental media, such as agricultural plants. We have developed a method for rapid quantification of CNTs in agricultural plants by coupling digestion with ultraviolet-visible (UV-Vis) spectroscopy. The method was efficient to quantify both pristine (p-) and carboxyl functionalized (c-) multiwall CNTs (p/c-MWCNTs) in the leaf, stem, and root tissues of lettuce. This rapid quantification method will be useful for understanding fate and transport of carbonaceous nanomaterials in environmental media and managing their application to secure sustainable nanotechnology.

Emerging investigator series: Quantification of Multiwall Carbon Nanotubes in Plant Tissues with Spectroscopic Analysis

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Abstract

If agricultural plants are exposed to carbon nanotubes (CNTs), they can potentially take up CNTs from the growing media and translocate them to different tissues. In addition, agricultural application of CNTs recently attracted increasing attention, as they could promote germination, enhance crop yield, and exert other benefits. For evaluating the environmental effects of CNTs and optimizing their agricultural application, it is essential to quantify CNTs in plant tissues. In this study, pristine (p-) and carboxyl functionalized (c-) multiwall CNTs (MWCNTs) were extracted from plant tissues by a sequential digestion with nitric acid (HNO_3) and sulfuric acid (H_2SO_4). The extracted MWCNTs were stabilized with a nonionic surfactant Triton X-100 and analyzed with ultraviolet-visible (UV-Vis) spectroscopic analysis to measure the concentration of MWCNTs in plant (lettuce) tissues. MWCNT concentration was linearly correlated with the absorbance at 800 nm. Detection limit for p- and c-MWCNTs was achieved at 0.10-0.12, 0.070-0.081, 0.019-0.18 μg/mg for leaf, stem, and root, respectively. The developed method was applied for lettuce (*Lactuca. sativa*, cv. Black Seeded Simpson) hydroponically grown with 5, 10, 20 mg/L of p-MWCNTs and c-MWCNTs in the culture solution. We detected 0.21 ± 0.05 – 4.57 ± 0.39 μg/mg p-MWCNTs and 0.20 ± 0.17 - 0.75 ± 0.25 μg/mg c-MWCNTs in lettuce roots, positively correlated with the dose of CNTs in solution. We have developed a method for rapid quantification of CNTs in plant tissues using a widely-accessible technique, which can enable reliable analysis of CNTs in plant tissues and provide critical information for evaluating the environmental implications and managing agricultural application of CNTs.

Introduction

Wide application of carbon nanotubes (CNTs) in consumer products, composite materials, and biomedical usage has led to their rapidly increasing production.[1] Global CNT market is expected to reach 8.1 billion dollars by 2024.[2] As CNTs are presumably persistent, these carbonaceous nanomaterials will be accumulated in water and soil upon the release from manufactured products during all the stages of their life cycles. Agricultural plants can potentially take up and translocate CNTs from soil to their different tissues, e.g. leaves, flowers, and fruits,[3-5] which raised concerns about the ecological and human health risk caused by CNTs. To the other side, CNTs can be applied in agriculture to enhance growth of agricultural crops and promote delivery of pesticides/fertilizers. For instance, application of CNTs in soils enhanced the flower and fruit production of tomatoes.[3,6,7] Information about CNT concentration in plants is crucial for understanding the ecological and human health risk caused by CNTs and improving their agricultural application. However, the quantitative information about CNT uptake and translocation in plants is sparse mainly due to technical difficulties for quantifying CNTs in biological tissues.

For quantifying CNTs in biological tissues, previous studies have examined various methods, including programmed thermal analysis (PTA), near infrared spectroscopy, Raman spectroscopy, inductively coupled plasma mass spectroscopy (ICP-MS), thermogravitational analysis (TGA), microwave-induced heating methods, and application of ^{14}C-labelled CNTs.[5, 7-11] Interferences from the background biological tissues and low concentrations of CNTs challenged their quantification.[11] The removal of background biological tissues requires prolonged digestion and extensive purification of samples.[8,9] Spectroscopic analysis has been used to quantify aqueous phase concentrations of multiwall CNTs (MWCNTs) using the

absorbance at wavelength of 500, 530, 550, 600, and 800 nm.[12] Linear relationship was observed between the applied CNTs concentration and UV-Vis absorbance obtained at these wavelengths.[13-16] Extinction coefficients were similar for CNTs with different diameters or structures.[16] Potential application of spectroscopic analysis for quantification of CNTs in biological samples can be attractive, as the instruments are widely accessible and easy to operate and the analysis is rapid, although the interferences of background materials can be challenging. In addition, formation of CNTs aggregates regulated by their surface properties and aqueous chemistry conditions can influence the quantification of CNTs in aqueous phase, and it is crucial to suspend CNTs homogeneously.[10, 17]

In this study, spectroscopic analysis was developed for quantification of pristine (p-) and carboxyl functionalized (c-) MWCNTs in lettuce (*Lactuca sativa*, Bionda Ricciolina) tissues. The interferences of background tissues were minimized by a sequential digestion, and detection limit of CNTs in plant tissues was determined. The rapid extraction and analysis of MWCNTs were conducted by reducing the digestion time and using optimized preparation process for analyzing the samples in aqueous phase. Finally, the developed method was applied to quantify MWCNTs in lettuce hydroponically grown with CNTs in the culture solution. We have performed quantitative analysis of CNTs in plant tissues with programmed thermal analysis (PTA),[18] however, it requires special equipment for PTA, which is not widely accessible and could limit its application. In this work, optical analysis coupled with digestion was developed for quantification of CNTs, which is widely accessible and can potentially enable rapid quantification of CNTs in environmental matrix.

Materials and Methods

Materials. Research-grade p- and c-MWCNTs were purchased from Nanocyl (http://www.nanocyl.com/product/). The average diameter and length of the studied MWCNTs is 9.5 nm and 1.0 μm, respectively. More information about the MWCNTs can be found in previous publications, and their major physicochemical properties are listed in Table S1 (Supporting information (SI)).[19, 20] Concentrated nitric acid (HNO_3) (15.8 M) and sulfuric acid (H_2SO_4) (18.4 M) were purchased from EMD Millipore (Boston, MA) and VWR (Wayne, PA). Nonionic surfactant Triton X-100 (TX-100) was purchased from VWR (Wayne, PA).

Preparation of MWCNTs suspension. For spectroscopic analysis of original and digested CNTs as well as spiking of lettuce tissues, suspension of MWCNTs was prepared by adding 2.0 mg MWCNTs to 2.0 mL of 2.0 mg/mL TX-100 solution (made with doubly deionized water (DDW) (18.3 MΩ·cm)) and sonicating the solution for 30 minutes (Branson Ultrasonic 2510, 100 W at 40 kHz).

Digestion and extraction of c/p--MWCNTs in plant tissues. Eight-week-old lettuce (*Lactuca sativa*, Bionda Ricciolina) plants were purchased from a local nursery (Sparks, Nevada). The plants were washed with DDW and separated to leaves, stems, and roots, and dried in an oven at 80 ˚C for 12 hours. The dried plant tissues were ground and sieved with a 60-mesh (< 0.25 mm) sieve. Partial samples were spiked with MWCNTs by adding pre-determined amount of MWCNTs suspension to the dried lettuce tissue powders to achieve the concentration of 125-600 μg MWCNTs/g lettuce tissues. The lettuce tissues with MWCNTs were subject to the sequential digestion, developed in our recent study.[18] In brief, an aliquot (1 mL) of HNO_3 (15.8 M) was added to ~20.0 mg of leaf, stem, or root tissues in 15.0 mL Corex glass centrifuge tubes. The centrifuge tube was placed inside the Corex digestion tube containing 15.0 mL DDW in a digestion chamber for 5 hours of digestion at 60 ˚C. After the digestion, 5.0 mL DDW was

added to the digested samples, and samples were centrifuged at 3000 rpm for 10 minutes. The precipitates were subject to the secondary digestion, for which 0.3 mL H_2SO_4 (18.4 M) was added to the residues from HNO_3 digestion, and samples were set for 3-hour digestion at 60 ˚C. As soon as digestion was finished, 5.0 mL of DDW was added to the extract and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded from the tubes leaving 0.5 mL in the tubes with the precipitates. After the slurries were neutralized with 0.2-0.3 mL concentrated NH_4OH, it was centrifuged at 3000 rpm for additional 10 minutes, and the supernatant was discarded. 1.0 mL of nonionic surfactant added into the precipitate and vortexed for 1-5 seconds to have homogeneous suspension of digested c/p-MWCNTs and/or lettuce tissue residues. To analyze the impact of digestion on the analysis of original MWCNTs and background contribution of lettuce tissues, MWCNTs suspension and lettuce materials without MWCNTs were also subject to the same digestion.

Spectroscopic analysis. For spectroscopic analysis, the suspension of MWCNTs prepared with TX-100 or suspension obtained after the digestion was analyzed with an Evolution 260 BIO UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). Absorbance spectra of aqueous phase suspension (1.0 mL) of original p-MWCNTs and c-MWCNTs were obtained. A full scan of aqueous phase of TX-100 (2.0 mg/mL), and TX-100-aided suspension of p-MWCNTs and c-MWCNTs were obtained at 200-1000 nm in quartz cuvettes (SI, Figure S1 and S2). The featureless spectra of p-MWCNTs and c-MWCNTs were observed for TX-100 aided suspension of both MWCNTs. Previous studies have used the absorbance at 800 nm for the quantification of CNTs in aqueous phase.[13, 14] The final absorbance of the prepared CNTs suspension or digestion suspension was measured at 800 nm. To keep the absorbance value at 0.2-1, the suspension was diluted with TX-100 (2.0 mg/mL) solution.[15]

Plant cultivation and application of the developed method. Lettuce (*Lactuca. sativa*, cv. Black Seeded Simpson) was grown hydroponically with MWCNTs in the culture solution, and the plants were harvested after three weeks to quantify MWCNTs in leaf, stem, and root tissues. The plants were treated with p-MWCNTs or c-MWCNTs of 5, 10, 20 mg/L. Briefly, lettuce seedlings were grown in the greenhouse under natural conditions (30/15 °C (day/night), 15−63% daily relative humidity, natural light) for four weeks. The four-week-old healthy seedlings of similar size were used in the exposure experiments with TX-100 suspended p-MWCNTs and c-MWCNTs. An amount of 10% Hoagland solution (Sigma-Aldrich Hoagland No.2) was used as the medium (pH adjusted to 6.2−6.5) containing either p-MWCNT or c-MWCNT at 0, 5, 10, or 20 mg/L in amber vials. An air pump was used to continuously aerate the solution, and Hoagland solution was added upon needs to compensate the evapotranspiration loss.

After three weeks of culture, the plants were harvested and separated into leaf, stem, and root tissues. The plants were rinsed three to five times with DDI water upon harvest. The root tissues were sonicated for five minutes in DDI to remove the external MWCNTs sorbed on the root surface. The tissues were dried in oven at 80 °C for 12 hours and stored at 4 °C. Using the method developed in this study, the dried tissues were digested and analyzed with UV-Vis spectroscopy for quantification of uptake and translocation of MWCNTs.

Results and Discussion

Stability of MWCNTs suspension and calibration curve. An absorption peak for TX-100 (2.0 mg/mL) was observed at 276 nm, followed by featureless spectra at 300-900 nm (SI,

Figure S1). In comparison, both p-MWCNTs and c-MWCNTs (12 μg/mL) suspension with TX-100 did not show any additional peaks at 200-900 nm (SI, Figure S2). Similarly, featureless spectra of surfactant- and humic substance-stabilized single wall CNTs (SWCNTs) and MWCNTs at 200-1200 nm were observed by previous studies.[11, 17] Following published methods,[21,22] we have made power-law regression for wavelength-dependent absorption of MWCNTs using equation (1) (SI, Figure S3):

$$\mathrm{A} = C\lambda^{-AAE} \quad (1)$$

where A is absorption, C is a constant, λ is wavelength, AAE is Ångstrom exponent. Ångstrom exponent for absorption was 0.60 and 0.68 for p- and c-MWCNT, indicating their similar optical properties as natural black carbon.[21-23]

For quantification of MWCNTs, following published research, absorbance at 800 nm was used for the quantification of p-MWCNTs and c-MWCNTs concentrations prepared with TX-100 solution.[13, 14] A linear relationship was obtained between the surfactant-calibrated absorbance at 800 nm and the concentrations of p-MWCNTs (y=0.014x+0.0044, $R^2 = 0.99$, $p < 0.01$; Or Abs_{800}=0.014 C_{CNT} +0.0044, C_{CNT} is the concentration of p-MWCNT, Abs_{800} is the absorbance at 800 nm) and c-MWCNTs (y=0.015x+0.0037, $R^2 = 0.99$, $p < 0.01$; Or Abs_{800}=0.015C_{CNT} +0.0037, C_{CNT} is the concentration of c-MWCNT) (Figure 1). This result implied that the absorbance at 800 nm can be used for quantification of p- and c- MWCNTs suspended with TX-100. Hyung et al.[13] suspended MWCNTs with dissolved organic matter, and they found a linear relationship between the absorbance at 800 nm and the concentration of MWCNTs (1.0-7.0 μg/mL) in the suspension. The absorbance of CNTs has been attributed to the π electrons present in the benzene rings of CNTs.[16] The extinction coefficient of p-MWCNTs

and c-MWCNTs was calculated to be 0.0035 and 0.0038 mL μg^{-1} cm^{-1}, comparable to the reported values of 0.0046-0.0054 mL μg^{-1} cm^{-1}.[16]

Digestion of plant tissues and spectroscopic analysis. Lettuce tissues were digested sequentially with HNO_3 and H_2SO_4 to minimize the influences of plant biomass on the spectroscopic analysis of MWCNTs. The spectra for all the digested plant tissues showed that the absorbance gradually decreased from 300 nm to 700 nm, reaching a stable baseline at 700-800 nm with minimum absorbance of 0.006-0.005 (SI, Figure S4). Such observation was similar to other reported spectra for lignin extracted from various plants.[24, 25] The extracted lignin showed a peak absorption at 340 nm, and baseline at 500-1100 nm.[25] As another important component of plant tissues, extracted hemicellulose and cellulose have even lower absorption compared to lignin.[26] Absorbance at 800 nm for the digested plant tissues followed the order of leaf ≥ root > stem (Figure 2). There was no significant difference between the absorbance of leaf and root tissues for the most samples ($p > 0.05$). In comparison, the absorbance of the stem tissues was significantly lower than that of leaves and roots ($p < 0.05$). The absorbance obtained for ~20.0 mg samples was 0.022 ± 0.0080, 0.0080 ± 0.0020, and 0.015 ± 0.0060 for the digested leaf, stem, and root tissues, respectively.

Sequential digestion of lettuce tissues with HNO_3 and H_2SO_4 facilitated the decomposition and removal of biomass. Our recent studies showed that digestion of plant tissues with HNO_3 reduced the biomass of leaf, stem, and root to 1-2% residual.[20] Further digestion with H_2SO_4 decreased the residual biomass to 0.02% of original values. The variation in the absorbance for residual materials of leaf, stem, and root could be due to their different contents of cellulose, lignin, proteins, and other compositions.[27] Lignin was more resistant to digestion and had higher absorption compared to cellulose and other components. Leaf and root in lettuce

had higher content of lignin than the stem, which can partially explain their relatively higher absorption after digestion.

Digestion and recovery of MWCNTs. Influences of sequential digestion on the spectroscopic analysis of p-MWCNTs and c-MWCNTs were examined. After the digestion, absorption spectra of MWCNTs were similar to those original, with an absorption peak at 276 nm presumably from TX-100 and featureless spectra after 300 nm (SI, Figure S5). A linear relationship was obtained between the amount of p-MWCNTs (R^2 = 0.98)/c-MWCNTs (R^2 = 0.99) (2.5-12.0 μg/mL) and the absorbance at 800 nm (Figure 3). And based on the linear regression for original MWCNTs, the recoveries of MWCNTs were calculated following equation 2 and 3:

$$C_{obs} = kAbs_{800} + b \quad (2)$$

$$R = \frac{C_{obs}}{C_{dig}} \quad (3)$$

where C_{obs} is the observed concentration of MWCNTs, Abs_{800} is the observed absorption at 800 nm, k and b are regression parameters obtained for original MWCNTs (Figure 1), R is the recovery, C_{dig} is the concentration of MWCNTs used for digestion. The recoveries of p-MWCNTs (55.2 ± 10.3%) are greater than those of c-MWCNTs (46.1 ± 6.2%) ($p < 0.05$). These recovery values for c-MWCNTs are comparable to those of MWCNTs after H_2SO_4 digestion in a previous study.[9] Doudrick et al. [9] observed that the recoveries of pristine MWCNTs were higher than functionalized MWCNTs. The relatively lower recoveries of c-MWCNTs compared to p-MWCNTs could be due to their higher degradation during the digestion with HNO_3 and H_2SO_4.[28] The recovery of CNTs can potentially be improved by alternative digestion using

specific enzymes for plant tissues removal, which is under our current research. Our results suggest that the UV-Vis absorbance can be applied to quantify the p-MWCNTs and c-MWCNTs after the digestion. The final suspension for UV-Vis spectroscopy was prepared by sequential digestion with HNO_3 (for 5 hours) and H_2SO_4 (for 3 hours) at 60 °C, followed by neutralization with NH_4OH and suspended with TX-100 (SI, Figure S6).

Digestion and quantification of MWCNTs in MWCNTs-spiked lettuce tissues. The spectra of digested MWCNTs-spiked lettuce tissues, showing absorption peak at 276 nm and featureless spectra at 300-900 nm, were similar to digested tissues without MWCNTs (SI, Figure S7). Upon subtraction of the background absorption from digestion residue of plant tissues, linear relationships were obtained between the absorbance at 800 nm and concentration of MWCNTs in the final digestion solution (Figure 4, SI, Table S2, S3, $R^2 > 0.94$). Consequently, there was also close regression between absorbance at 800 nm and the spiked p-MWCNTs concentrations in leaf ($R^2 = 0.99$), stem ($R^2 = 0.99$), and root ($R^2 = 0.95$). Based on the calculation using equations 1 and 2, the recoveries of p-MWCNTs from leaf, stem, and roots were 64.8 ± 17.0%, 39.2 ± 9.4%, and 68.6 ± 18.6%, respectively. The recoveries of p-MWCNTs from leaf and root are not significantly different ($p > 0.05$), when the value for stem was significantly lower than leaf and root ($p < 0.05$). The recoveries of p-MWCNTs in the presence of leaf and root tissues were higher than those for p-MWCNTs digested without plant tissues ($p < 0.05$). The digestion residue of plant tissues may protect MWCNTs from oxidation by the strong acid or affect the aggregation/precipitation of CNTs in case of spiked leaf and root tissues.

Linear relationships were also found between the absorbance at 800 nm and spiked concentration of c-MWCNTs (0.12-0.59 μg/mg) in leaf ($R^2 = 0.98$), stem ($R^2 = 0.97$), and root ($R^2 = 0.94$). The recoveries of c-MWCNTs were 51.9 ± 13.9%, 54.9 ± 10.7%, and 38.8 ± 12.7%

for leaf, stem, and root, respectively. There was no significant difference between the recoveries of c-MWCNTs from leaf and stem tissues ($p > 0.05$). In comparison, the c-MWCNTs recovered from roots tissues are lower than those from leaf and stem, and the control c-MWCNTs ($p < 0.05$). Recoveries of c-MWCNTs in leaf and root were much lower than p-MWCNTs, however, the value was higher for c-MWCNTs in stem than p-MWCNTs. Previous studies showed that the recovery of CNTs during the digestion depended on their ability to form aggregates.[8] The negatively charged c-MWCNTs likely had less efficiency to form stable aggregation due to the electrostatic repulsion between CNTs.

Previous work showed that the concentration of CNTs linearly governed their spectroscopic absorption, due to π electrons.[15, 16] In this study, our work demonstrated linear relationships between the concentration of digested p- and c-MWCNTs in lettuce leaf, stem, and root tissues and absorbance at 800 nm. After sequential digestion to remove plant biomass, the absorbance derived from π electrons in graphene sheets still obeyed linear response to the concentration of MWCNTs. The intactness of MWCNTs following strong chemical digestions has been shown by other studies using thermal and Raman analysis.[9, 20] As the dispersion and aggregation of MWCNTs can influence their absorption coefficient,[15, 30] the addition of nonionic surfactant can facilitate homogenous suspension and efficient quantification of digested MWCNTs. The recoveries of MWCNTs could also be influenced by the presence of functional groups on the MWCNTs and the type of the plant tissues. In general, p-MWCNTs had higher recoveries than negatively charged c-MWCNTs.

Considering the background absorption from residual plant tissues and recoveries of MWCNTs after the sequential digestion, the detection limits of the p-MWCNTs in leaf, stem, and root were determined to be 0.12, 0.081, and 0.019 μg p-MWCNT/mg plant tissues (SI, Text,

Table S4), respectively. The detection limit of c-MWCNTs was similar with the value of 0.10, 0.070, and 0.18 µg c-MWCNT/mg of leaf, stem, and roots tissues, respectively. The detection limit of MWCNTs with the value of 19-180 µg/g plant tissues was comparable to other methods such as TGA-MS, and PTA,[9, 29, 31] but higher than those obtained with microwave induced heating analysis.[6] The spectroscopic procedure developed in this study has additional advantage of wide accessibility and rapid analysis. The analysis of MWCNTs with the UV-Vis absorption at 800 nm will facilitate quick and easy detection and quantification of CNTs varying in surface chemistry. As previous studies showed that the extinction coefficients of CNTs are not interfered by the structure and diameter of CNTs,[16] our method can also be potentially applicable to a broader range of CNTs with varied size, diameter and structures. Raman spectroscopy, a complementary method to UV-Vis spectroscopy, could be applied for improving the detection limit of CNTs in plant tissues. The digestion of plant tissue by a single digestion step (HNO_3 digestion) was efficient to remove the interferences in the Raman signals from the plant tissues.[20] Using this single step digestion and Raman spectroscopy could help to avoid additional digestion step and enhance CNTs recovery.

Lastly not leastly, stable homogeneous suspension of MWNCTs was essential for its quantification (Figures 3 and 4). In previous studies, homogeneous suspensions of MWCNTs were achieved by treating CNTs with anionic, cationic, and nonionic surfactant and polyethylkene glycol (PEG), and centrifugation at high speed for several hours (~6 hrs) (Han et al., 2008; Liu et al., 2009). Digestion with strong oxidative reagents could possibly increase functionalization of MWCNTs and affect the suspension stability. However, the application of TX-100 with original and digested MWCNTs (original and spiked in plant tissues) showed the versatility of this nonionic surfactant for preparing homogeneous suspension, providing linear

calibration curves for the CNTs concentration varying in orders of magnitudes (Figures 3 and 4). This method can be useful for suspending CNTs or other carbonaceous nanomaterials for other analysis.

Analysis of MWCNTs in hydroponically cultured lettuce tissues. We have applied our method for quantification of multiwall carbon nanotubes (MWCNTs) in lettuce (*L. sativa,* cv. Black Seeded Simpson) hydroponically cultured with 5, 10, 20 mg/L pristine (p-) and carboxylic-functionalized (*c*-) MWCNTs. We detected 0.21 ± 0.05 – 4.57 ± 0.39 μg/mg p-MWCNTs and 0.20 ± 0.17 – 0.75 ± 0.25 μg/mg c-MWCNTs in lettuce roots, positively correlated with the dose of CNTs in solution (Pearson correlation coefficient $r = 0.98$, $p < 0.05$). The bioconcentration factor for root (C_{root}/C_{water}, with C_{root} and C_{water} representing concentration in root and culture solution, respectively) ranged 0.042-0.23 and 0.028-0.040 L/g for p-MWCNT and c-MWCNTs, respectively. In addition, concentration of p-MWCNTs in leaf (0-0.014 μg/mg) was also much higher than c-MWNCTs (below background) (SI, Figure S8), although still below the detection limit.

In other culture experiments, the concentrations of CNTs in plant tissues ranged from 0.001-0.085 μg/mg plant tissues.[7] Using C14-labeled MWCNTs, Zhao et al.[5] captured 0.001-0.077 μg MWCNTs/mg plant tissues in Arabidopsis, rice, maize, and soybean grown in a hydroponic condition containing 2.5 mg/L MWCNTs. The bioconcentration factor ranged 0.0004-0.031 L/g. Another study showed that the accumulation of single wall CNTs (SWCNTs) was 0-0.024 μg/mg in corn, grown in SWCNT applied soil.[7] Our method can be applied to unambiguously determine the concentration of CNTs in such culture experiments, without the need of radio-labelled materials and special equipment setup. The accumulation of CNTs

quantified in lettuce roots was similar to other reported values for CNTs in plant tissues, although we did not determine unambiguous translocation.

Based on the emission data, it was estimated that the concentration of CNTs in soils ranged 23-46 ng/kg, and for biosolid-applied soils, the concentration of CNTs can range up to 11 μg/kg.[32] Using the biological uptake factor determined in the recent studies,[7] the concentration of CNTs in agricultural plant can range up to 5 μg/kg, which was much lower than the detection limit of our method. The spectroscopic analysis can potentially be used to quantify the concentration of CNTs in cultured samples and provide critical information for evaluating their environmental effects and managing their application, although further investigations are needed to validate its application for natural samples and improve the detection limit.

Conclusions

A method using UV-Vis spectroscopic analysis coupled with sequential digestion has been developed for the quantification of p-MWCNTs and c-MWCNTs in lettuce leaf, stem, and root tissues. The digestion removed the plant biomass and facilitated extraction of MWCNTs. Using this method, the detection limits of p-MWCNTs and c-MWCNTs were achieved as 0.10-0.12, 0.070-0.081, 0.019-0.18 μg/mg for the leaf, stem, and root, respectively. Based on experiments for spiked lettuce tissues, the recovery of p-MWCNTs and c-MWCNTs ranged 39.2-68.6% and 38.8-54.9%, respectively, which can be potentially improved by alternative enzymatic digestion. This method is rapid and widely-accessible compared to other technologies such as programmed thermal analysis, and potentially can enable reliable quantification of CNTs in a larger amount of environmental samples. Using this method, we have quantified the

concentration of p-MWCNTs and c-MWCNTs to be 0.21 ± 0.05 – 4.57 ± 0.39 and 0.20 ± 0.17 – 0.75 ± 0.25 μg/mg in the root tissues of lettuce hydroponically cultured with CNT-spiked culture solution, respectively. The method can also be potentially used for quantification of MWCNTs in other environmental media to determine the environmental risk of CNTs and optimize their application.

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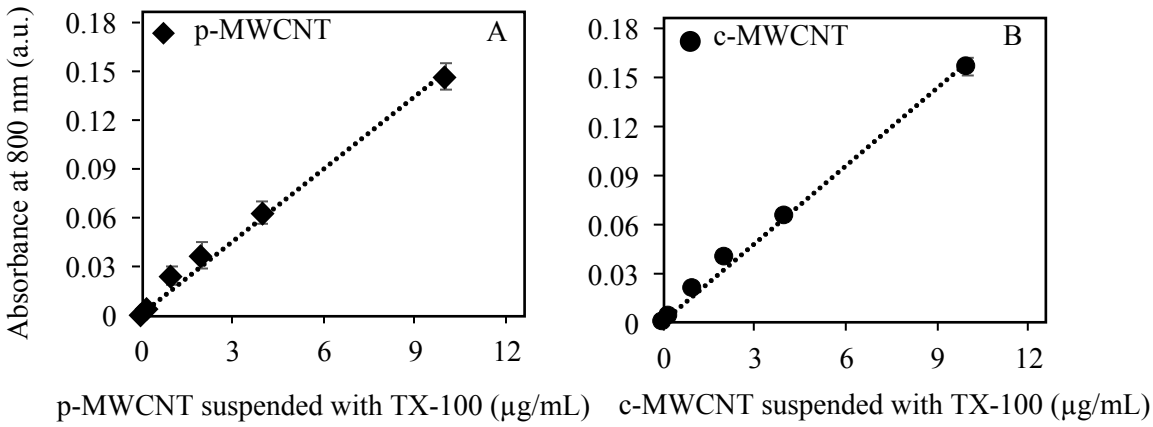

Figure 1. Calibration curves obtained for p-MWCNTs (2.5-12.0 μg/μL) (A) and c-MWCNTs (B) (2.5-12.0 μg/μL) prepared with a nonionic surfactant Triton X-100 (TX-100) (2.0 mg/mL). A linear relationship has been found for p-MWCNTs ($R^2 = 0.99$, $p < 0.01$) and c-MWCNTs ($R^2 = 0.99$, $p < 0.01$) for the concentrations of 0.2 to 10.0 (μg/mL). The error bars showed the replicates of 3 samples at each point.

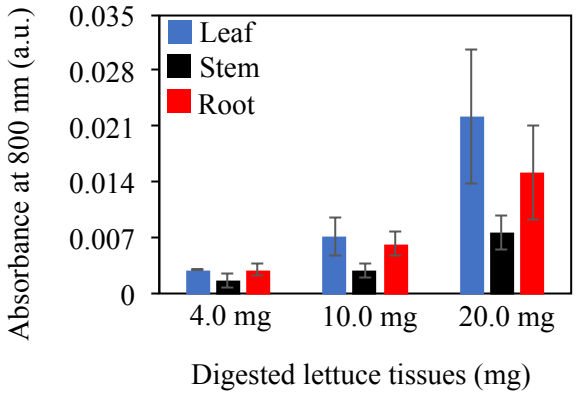

Figure 2. Spectroscopic absorbance of digestion residue of lettuce leaf, stem, and root. The residue was suspended with a nonionic surfactant TX-100 (2.0 mg/mL), and absorbance was measured at 800 nm. The error bars showed the standard deviations obtained from replicated samples. Three replicates were used for 4.0 mg and 10.0 mg, and six replicates were used for 20 mg.

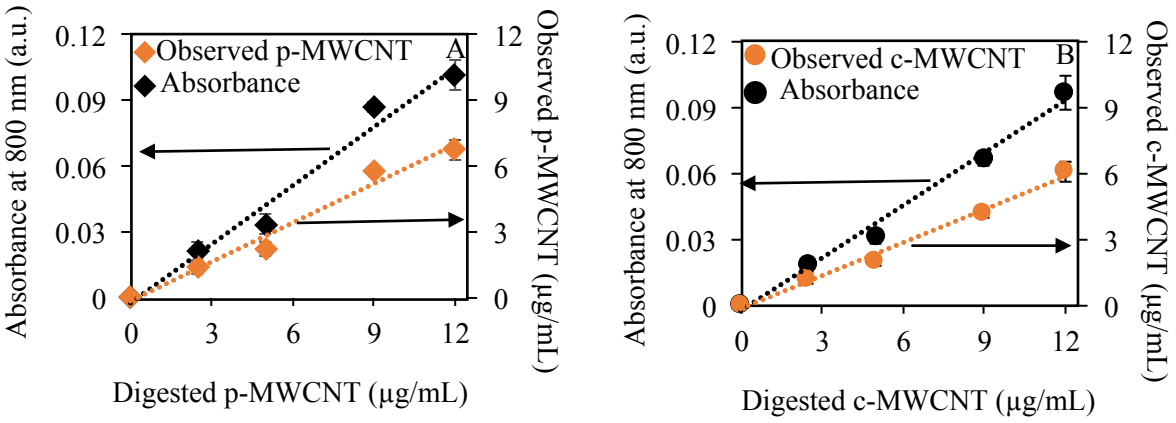

Figure 3. Calibration curves for the digested p-MWCNT (A) and c-MWCNT (B) based on the absorbance at 800 nm. MWCNTs were digested sequentially with HNO_3 (for five hours at 60 °C) and H_2SO_4 (three hours at 60 °C). A linear relationship with the absorbance was found for the concentrations of p-MWCNTs ($R^2 = 0.98$, $p < 0.01$) and c-MWCNTs ($R^2 = 0.99$, $p < 0.01$). The expected p-MWCNTs (A) and c-MWCNTs (B) were calculated based on the linear regression for original MWCNTs (equation 1 and 2, Figure 1). The error bars represent standard deviation from triplicates.

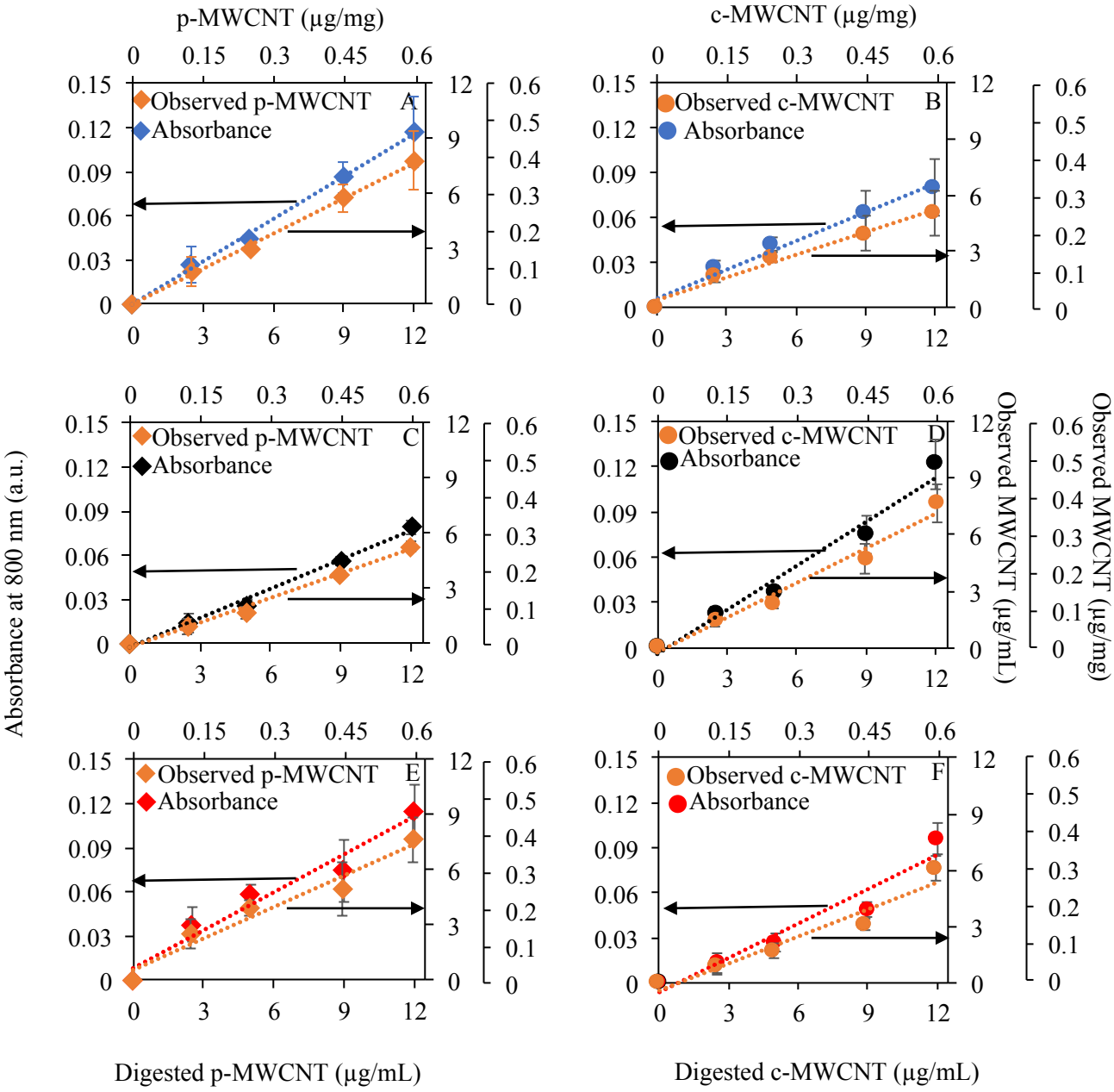
p-MWCNT (μg/mg)
c-MWCNT (μg/mg)
Observed p-MWCNT
Absorbance
A
Observed c-MWCNT
B
C
D
E
F
Absorbance at 800 nm (a.u.)
Observed MWCNT (μg/mL)
Observed MWCNT (μg/mg)
Digested p-MWCNT (μg/mL)
Digested c-MWCNT (μg/mL)

Figure 4: Calibration curves for the digested p-MWCNT- (A, C, E) and c-MWCNT- (B, D, F) spiked lettuce tissues based on the absorbance at 800 nm. The c/p-MWCNT-spiked lettuce tissues were digested sequentially with HNO_3 and H_2SO_4. The residue was suspended with TX-100 (2.0 mg/mL). Regressions ($R^2 > 0.94$, $p < 0.01$) were obtained for lettuce tissues spiked with p-MWCNTs and c-MWCNTs (SI, Tables S2 and S3). The expected p-MWCNTs (A, C, E) and c-MWCNTs (B, D, F) were calculated based on the regression for the original CNTs. The error bar represents the standard deviations derived from triplicates.

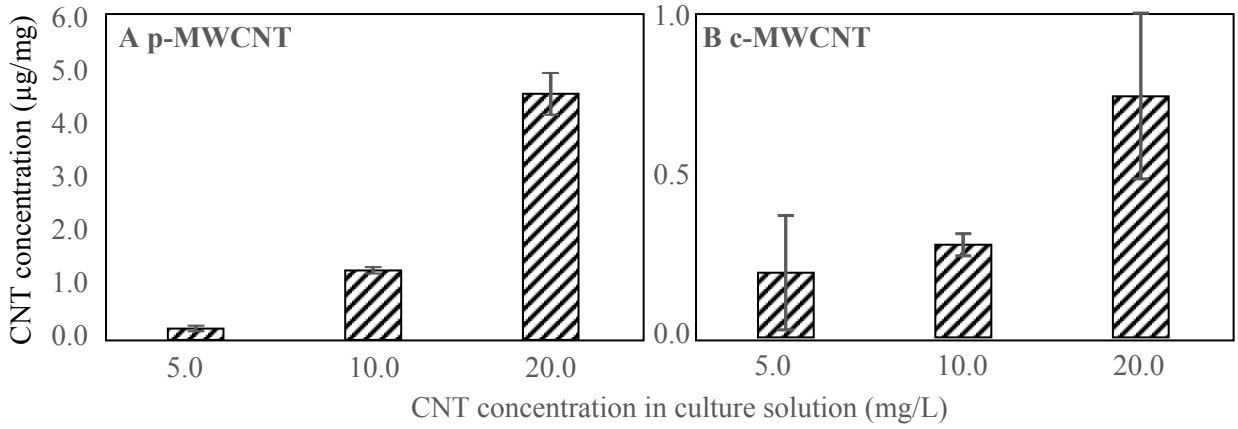

Figure 5. Concentrations of p-MWCNT (A) and c-MWCNT (B) in lettuce roots quantified by digestion coupled with analysis of UV-Vis absorbance at 800 nm. The plants were grown in greenhouse at hydroponic system containing 5, 10, and 20 mg/L CNT solutions.