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Development of a novel methodology for *in vivo* quantification of N/O/S-containing polycyclic aromatic hydrocarbons located on the epidermis of mangrove roots using graphene quantum dots as a fluorescence quencher

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ABSTRACT

A novel approach for *in vivo* determination of typical N/O/S-containing PAHs located on the epidermis of mangrove roots was developed using graphene quantum dots (GQDs) as a fluorescence quencher. The decreasing fluorescence intensity from GQDs was attributed to the amount of N/O/S-containing PAHs introduced onto the epidermis of mangrove roots. The linear ranges of the proposed method were 10.3–980 ng g^{-1}, 9.5–1350 ng g^{-1} and 7.8–1200 ng g^{-1} for DBF, DBT and CAR located on the epidermis of *K. obovata* roots, respectively. This method was also shown to be valid for quantifying the N/O/S-containing PAHs on the root epidermis in the presence of heavy metal (10 mmol L^{-1}) and dissolved organic matter (1 mg L^{-1} C). Moreover, the death rates of epidermal cells were almost unchanged ($p > 0.05$) after acquiring the fluorescence spectra, which is superior to the previously reported LITRF method with which the cell death rates increased to 42.6%.

1. Introduction

Parent polycyclic aromatic hydrocarbons (PAHs) are of particular concern due to their carcinogenicity, mutagenicity and toxicity to biota (Salvo et al., 2016; Shen et al., 2013). Some recent studies have shown that the N/O/S-containing PAHs possess toxicities that are comparable to those of their parent compounds (Cachada et al., 2016; Andersson and Achten, 2015). In addition, unlike the parent PAHs commonly investigated, important factors in the determination of N/O/S-containing PAHs combined with root epidermal cell walls/membranes may also include π-π interactions, electrostatic interactions and steric stabilization, and thus need special investigation (Sun and Zhou, 2016; Chen et al., 2015).

Root uptake of PAHs has long been considered an important pathway for human exposure through the food chain (Gao and Zhu, 2004). To explore the uptake mechanisms, considerable efforts have been made to investigate the PAHs' uptake coefficients (K_f) and their relationships with the compositions of roots (mainly the functional groups of lipids and carbohydrates) (Naidoo and Naidoo, 2016; Zhang and Zhu, 2009). Nevertheless, Jiao et al. confirmed that portions of PAHs are retained on the epidermis of roots, and only approximately 60% of the PAHs actually enter into the root; their analysis used a sequential extraction ($CaCl_2$, methanol and ASE extraction) combined with GC–MS (Jiao et al., 2007) and made it necessary to re-determine the K_f values and re-evaluate the roles of composition on root uptake of PAHs. Therefore, an accurate determination of PAHs located on the epidermis of mangrove roots was critical to an improved understanding of the root uptake and further translocation processes.

However, the sequential extraction method destroys not only the original form of the PAHs but also the root compositions (lipid/carbohydrates/protein) and cell membranes, which affect the uptake extent of PAHs and their locations (epidermis, xylem and so on) (Chen and Schnoor, 2009; Li and Ma, 2016; Zhan et al., 2015). In other words, the results acquired by the sequential extraction method cannot represent the retention of PAHs in the epidermis of intact and living roots. Currently, as far as we know, there exists no PAH quantification method that meets the requirements of *in vivo* determination of the retention of PAHs on the epidermis of roots.

As PAHs have high fluorescence quantum yields, both the solid surface fluorescence (SSF) and fiber-optic fluorescence (FOF) spectroscopic methods have been established to determine the PAHs adsorbed on leaf surfaces *in situ* (Wang et al., 2008; Chen et al., 2011). As they are

Abbreviations: LITRF, laser-induced nanosecond time-resolved fluorescence spectra; DBT, dibenzothiophene; CAR, carbazole; DBF, dibenzofuran; TPLCSM, two-photon laser confocal scanning microscopy; HM, heavy metal; DOM, dissolved organic matter

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limited by the sensitivity of the fluorescence instruments, these methods are not suitable for investigating the location and transportation of trace concentrations of PAHs on mangrove leaves. With the application of laser-induced fluorescence (LIF) spectroscopy, the low sensitivities that hampered SSF and FOF methods were resolved by Sun et al., as the detection limit of the LIF method is lower by a factor of 10–30 (Sun et al., 2013; Sun et al., 2016). This method has been subsequently used to investigate the depuration of PAHs adsorbed onto the leaf surfaces of living mangrove seedlings.

Unfortunately, compared with the mangrove leaf, the strong auto-fluorescence of mangrove root epidermal tissues combined with the relatively low fluorescence signals of PAHs results in a low signal-to-noise ratio (S/N), rendering the LIF method useless for *in situ* monitoring of the PAHs located on the epidermal tissues of mangrove roots (Cheng et al., 2015). To suppress the background fluorescence signal, the *in situ* laser-induced nanosecond time-resolved fluorescence (LITRF) spectroscopic method was established in our previous reports (Li et al., 2017; Li et al., 2015), with which the S/N values improve by a factor of 3–4 for fluorene adsorbed onto the epidermal tissues of mangrove roots. However, further studies showed that the optimal excitation wavelength for the LITRF method determination of nearly all the PAHs was in the UV range (250–350 nm), and the high photon energy of UV light could result in the production of hydroxyl radical (·OH) and thereby cause negative effects on many aspects, including changing the normal physiological functions of the root cells, which will further induce changes in the locations of PAHs on root tissues (Verdaguer et al., 2017; Robson et al., 2015; Le Gall et al., 2015).

As the excitation wavelength of two-photon laser confocal scanning microscopy (TPLCSM) equates to a two-photon adsorption wavelength, this method, by using visible instead of UV light, minimizes the negative effects of the excitation light and realizes *in vivo* monitoring of the translocations and degradation of PAHs in microalga (Feng et al., 2017; Li and Chen, 2014; Niu et al., 2016). Nevertheless, the *in vivo* TPLCSM method, if not in combination with high sensitivity fluorescence spectroscopy, is unable to quantify the PAHs located on the epidermal tissues of mangrove roots (Tan et al., 2017).

Graphene quantum dots (GQDs), zero-dimensional luminescent carbon-based nanomaterials, have attracted widespread interest in the quantification of organic/inorganic substances, sensors and biosensors (Park et al., 2015; Benitez-Martinez et al., 2014; Ji et al., 2016). Among these fluorescence sensors, in theory, the quantification methods based on GQDs fluorescence quenching have the potential ability to avoid the detrimental effects on root for the longer excitation wavelength of GQDs (visible light). The objective of this study was, therefore, to use GQDs as a fluorescence quencher to achieve *in vivo* determination of PAHs located on the epidermis of mangrove roots. Dibenzothiophene (DBT), carbazole (CAR) and dibenzofuran (DBF) possess the same number of rings and were selected to represent S-containing, N-containing and O-containing PAH compounds, respectively.

2. Materials and methods

2.1. Reagents, plants and instruments

DBT, CAR and DBF with a purity of 99.9% were obtained from Sigma-Aldrich Co. Ltd. (UK). All of the other chemicals used in the study were analytical reagents (A.R.) obtained from Sinopharm Chemical Reagent Co. Ltd. (China). The GQDs used in this study were purchased from J&K Scientific Co. Ltd. (USA), with the particle diameter < 5 nm (the maximum excitation and emission wavelengths in aqueous solutions were 350 nm and 462 nm, respectively).

The hypocotyls of *K. obovata*, *A. marina* and *A. corniculata* were collected from the Yunxiao mangrove swamp located in the Zhangjiang River Estuary, southeast China, and were then cultivated in a sand bed for 12 months. The other cultural conditions were the same as previously reported (Li et al., 2016). The fluorescence spectrometer purchased from the Edinburgh Instruments Co Ltd. (FLS-980, UK) was used to acquire the fluorescence spectra of the GQDs retained on the epidermal tissues of mangrove roots. The parameters of this instrument are as follows: Excitation wavelength, 350 nm; Emission wavelengths, 400–550 nm; Slit width, 2.00 nm; Dwell time, 0.500 ns.

2.2. In vivo determination of the N/O/S-containing PAHs on the epidermis of mangrove roots

Healthy *K. obovata*, *A. marina* and *A. corniculata* with similar length roots were selected and then cleaned with tap water and Milli-Q water three times each to remove silt, sand and sediment. Similar to our previous report (Li et al., 2017), after the roots had been air-dried, the special zones, which include the divisional and elongation zones, were selected in the mangrove roots. Then, the mangrove root was immersed in 10 mL of a 100 mg mL^{-1} GQD solution for 30 min. To obtain calibration curves, different concentrations of the CAR, DBF and DBT acetone solutions were slowly introduced onto the special zones of mangrove root epidermis using a 50 μL transferpettor. Finally, the fluorescence spectra of the GQDs were acquired using the FLS-980, and the statistical results of nine measurements were used to calculate the calibration curves and the corresponding correlation coefficients.

2.3. Effects of heavy metals (HM) and dissolved organic matter (DOM) on the fluorescence quenching of GQDs

Several groups of N/O/S containing PAHs contaminated *K. obovata* roots were prepared in order to evaluate the effects of HM and DOM on the fluorescence quenching of GQDs by the method described in Section 2.2. Then, the contaminated roots were fully immersed into the aqueous solutions containing the inherent DOM (1 and 10 mg L^{-1} C) and HM (10 mmol L^{-1}). The inherent DOM that originated from mangrove sediment was collected, filtered and acidified by the same procedures described by Gao et al. (2007). The fluorescence spectra of the GQDs located on the epidermis of mangrove roots were acquired at 120 h using the proposed method.

2.4. Death of the epidermal cells caused by the excitation light of LITRF and the proposed method

To evaluate the impacts of the excitation light on the physiological status of the roots, the quantity of dead epidermal cells was determined by Evans blue staining, which only enters dead cells that have a freely permeable plasma membrane. The experimental procedures were similar to those reported by Mergemann et al. (Mergemann and Sauter, 2000). Briefly, the mangrove roots were first immersed into 2% (w/v) Evans blue for 3 min. The root samples were subsequently washed with tap water and Milli-Q water, three times each. Finally, wide-field fluorescence microscopy was used to examine the Evans blue staining patterns from which the death rates of mangrove root epidermal cells were calculated.

2.5. Statistical analysis

The mean and standard deviation of replicates were calculated and compared by one-way analysis of variance (ANOVA) tests. All statistical analyses were run using Origin version 9.1.

3. Results and discussion

3.1. The possibility of determining, in vivo, the N/O/S-containing PAHs on the epidermis of mangrove roots using the fluorescence spectra of GQDs

3.1.1. The implications of auto-fluorescence of root epidermis for the acquisition of GQD fluorescence spectra

Li et al. reported that the auto-fluorescence of woody root epidermis