



Using two-dimensional correlation size exclusion chromatography (2D-CoSEC) to explore the size-dependent heterogeneity of humic substances for copper binding[☆]



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ARTICLE INFO

Article history:

Received 6 March 2017

Received in revised form

13 April 2017

Accepted 13 April 2017

Available online 8 May 2017

Keywords:

Humic substances

Metal binding

Two-dimensional correlation spectroscopy (2D-COS)

Fluorescence quenching

Size exclusion chromatography (SEC)

ABSTRACT

Knowledge of the heterogeneous distribution of humic substances (HS) reactivities along a continuum of molecular weight (MW) is crucial for the systems where the HS MW is subject to change. In this study, two dimensional correlation spectroscopy combined with size exclusion chromatography (2D-CoSEC) was first utilized to obtain a continuous and heterogeneous presence of copper binding characteristics within bulk HS with respect to MW. HS solutions with varying copper concentrations were directly injected into a size exclusion chromatography (SEC) system with Tris-HCl buffer as a mobile phase. Several validation tests confirmed neither structural disruption of HS nor competition effect of the mobile phase used. Similar to batch systems, fluorescence quenching was observed in the chromatograms over a wide range of HS MW. 2D-CoSEC maps of a soil-derived HS (Elliot soil humic acid) showed the greater fluorescence quenching degrees with respect to the apparent MW on the order of 12500 Da > 10600 Da > 7000 Da > 15800 Da. The binding constants calculated based on modified Stern-Volmer equation were consistent with the 2D-CoSEC results. More heterogeneity of copper binding affinities within bulk HS was found for the soil-derived HS versus an aquatic HS. The traditional fluorescence quenching titration method using ultrafiltered HS size fractions failed to delineate detailed distribution of the copper binding characteristics, exhibiting a much shorter range of the binding constants than those obtained from the 2D-CoSEC. Our proposed technique demonstrated a great potential to describe metal binding characteristics of HS at high MW resolution, providing a clear picture of the size-dependent metal-HS interactions.

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1. Introduction

Humic substances (HS), ubiquitous in terrestrial and aquatic environments as major constituents of natural organic matter, are comprised of a polymorphous mixture of organic substances with a wide range of molecular sizes from hundreds up to ~100 kDa and different chemical moieties (i.e., functional groups) (Leenheer and Croue, 2003; D'Orazio and Senesi, 2009; Hur et al., 2009). HS play fundamental roles in aquatic ecosystems through the supply of carbon and nutrients to heterotrophs, electron shuttling, and metal complexation (D'Orazio and Senesi, 2009; Nebbioso and Piccolo, 2013). In particular, the interactions of HS with metals are

considered a critical factor in assessing the fate, mobility, and toxicity of metals in aqueous systems (Porcal et al., 2009; Chen et al., 2013). Under fixed solution chemistry, the affinity and the extent of metal binding, which are often represented by stability constants and the abundance of ligands, respectively (Plaza et al., 2006; Zhu et al., 2015), are heavily affected by HS structures and the chemical composition. Carboxylic and phenolic groups attached to HS are known to operate as effective binding sites/ligands for metals (Plaza et al., 2006; Steelman et al., 2013).

Due to the strong merits of fast, high sensitive, and cost-effective measurements, the fluorescence quenching titration (FQT) method has been widely used to probe metal binding affinity of HS. The parameters for the binding characteristics are estimated by employing either the Ryan-Weber model or the Stern-Volmer equation, which is primarily based on an assumption of 1:1 metal/ligand complex. Due to the HS heterogeneous structures, it is more reasonable to describe the metal binding characteristics as a

[☆] This paper has been recommended for acceptance by B. Nowack.

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distribution of the number of the ligand types (or binding sites) bearing different binding strengths rather than to present a single representative value for bulk HS (Luster et al., 1996). To date, much effort has gone into using the FQT method in resolving the heterogeneous presence of the binding sites, which requires the decomposition of a bulk HS into several distinctive constituents with respect to metal binding affinities. For example, Yamashita and Jaffé (2008) applied parallel factor analysis (PARAFAC) model, a linear spectral decomposition method, to obtain several fluorescent components with different metal binding properties from fluorescence excitation emission matrix (EEM) of a bulk HS sample. Wu et al. (2012) and Chen et al. (2013) separated a bulk HS into several different size fractions through ultrafiltration (UF) to explore molecular size-dependent characteristics of metal binding. However, it is noteworthy that, although the previous studies successfully revealed the presence of different metal binding sites within bulk HS, the methodologies used are very limited in providing a continuous distribution of the metal binding properties.

Two dimensional correlation spectroscopy (2D-COS) is a powerful mathematical tool offering a clear picture of spectral changes of a complex mixture upon external perturbations such as pH, temperature, and metal addition, over a wide range of spectral variables (Noda, 2016). It enables one to accurately identify the sequential order of the changes over the variable's range by comparing two types of maps (i.e., synchronous and asynchronous maps) generated by the 2D-COS. Previous studies have demonstrated many successful applications using 2D-COS for exploring the heterogeneous distribution of metal binding sites within bulk HS, in which the spectral responses to metal binding have been primarily measured by UV-Vis and fluorescence spectroscopy (Hur and Lee, 2011a; Yu et al., 2012; Chen et al., 2015; Wei et al., 2016) or Fourier transform infrared spectroscopy (Li et al., 2014a, 2014b; Chen et al., 2015). However, applying 2D-COS is not constrained to such spectroscopic tools and can be extended to other analyses such as chromatography (Izawa et al., 2001; Park et al., 2016). In this context, size exclusion chromatography (SEC) is very attractive in coupling the 2D-COS for studying metal-HS interactions because it provides high-resolution information on HS size continuum with minimal disturbance/damage to the inherent structures of HS (Romera-Castillo et al., 2014). Considering that the apparent HS molecular size could be easily changed with various physico-chemical and biological processes in natural and synthetic systems, in-depth knowledge about size-variant metal binding characteristics would be crucial in obtaining further insight into metal-HS interactions.

To our best knowledge, no studies were previously conducted to explore the molecular heterogeneity with respect to metal binding using the combination of 2D-COS with SEC, which is here referred to as two-dimensional correlation size exclusion chromatography (2D-CoSEC). The objectives of this study were (1) to test the feasibility of the 2D-CoSEC to characterize metal binding properties of two representative HS; (2) through the technique, to obtain a heterogeneous and continuum distribution of metal binding sites within bulk HS along the molecular sizes; and (3) to compare the 2D-CoSEC results with those of physically separated size fractions (i.e., UF fractions).

2. Materials and methods

2.1. Preparation of HS solutions and metal binding experiments

To represent two different HS with contrasting sources (i.e., soil-derived versus aquatic HS), Elliott Soil Humic Acid (ESHA) and Suwanee River Humic Acid (SRHA) were purchased from the

International Humic Substances Society (IHSS) and used for this study. Initial HS solutions were prepared by dissolving 40 mg of each sample in 1 L of Milli-Q water containing Tris-HCl buffer (0.01 M, pH 6.8) and NaCl (0.1 M) (i.e., the same solution chemistry as the mobile phase of the SEC system) (Tamamura et al., 2012). No precipitation was observed for the HS solutions. The basic information on the two HS is available at the IHSS web site (www.humicsubstances.org).

The HS solutions were filtered through a pre-washed 0.45 μm -membrane filter (Advantec, Japan) prior to metal binding experiments. Copper is used as a representative metal for this study because it has been long studied for metal-HS interactions and easily comparable with the previous literature. Copper stock solutions were prepared in different concentrations (0.2, 1.0, and 5.0 mM) by dissolving appropriate amounts of $\text{Cu}(\text{NO}_3)_2$ into Milli-Q water. The HS solution was transferred into polypropylene vials (10 mL), and the copper stock solutions were spiked into the vials to achieve the final copper concentrations varying from 0 to 100 μM (Hur and Lee, 2011b). The total volume was kept the same (10 mL). The vials were then placed on a shaker at 150 rpm for 15 min for equilibrium.

2.2. SEC analysis

The SEC system used for this study consists of a pump (1515 Isocratic pump, Waters), a fluorescence detector (FLD, Multi λ -fluorescence 2475, Waters), a UV detector (UVD, UV-visible 2489, Waters), and a protein-pak 125 column (Waters, 7.8 \times 300 mm) as major components. For the fluorescence detector, the excitation (Ex) and the emission (Em) wavelengths were set at 270 nm and 530 nm for ESHA, and 340 nm and 475 nm for SRHA, respectively, which were determined based on the highest peak locations observed in the measured fluorescence EEM of the bulk HS samples. Mobile phase is a NaCl solution (0.1 M) containing 0.01 M Tris-HCl buffer at pH 6.80 (Tamamura et al., 2012). The injection flow was maintained at 1 mL/min for the SEC system. The calibration for average-weight molecular weight (MW_w) was performed using five polystyrene sulfonate sodium salt standards (Polymer Standard Service-USA, Amherst) and acetone, each of which has the apparent weight-average MW (MW_w) value of 0.125, 1, 4.95, 6.53, 15.2 kDa, and 0.06 kDa, respectively. A good linear correlation between the retention times and the log MW_w was observed for the calibration curve ($R^2 = 0.998$). The MW_w values of HS samples either with or without copper were calculated from the SEC chromatograms using the following equation (Hur and Schlautman, 2003a):

$$MW_w = \frac{\sum_{i=1}^N (h_i MW_i)}{\sum_{i=1}^N h_i}$$

where h_i is the fluorescence intensity of the sample and MW_i is the equivalent MW from the calibration curve, both corresponding to an appropriate retention time, i .

2.3. 2D-COS application

To investigate detailed signal variations upon copper addition over a wide range of MW values, the 2D-COS was combined with the SEC chromatograms created by the fluorescence detector (i.e., 2D-CoSEC). 2D-COS typically generates two types of two-dimensional maps called synchronous and asynchronous maps, which were performed by using open-access software (2Dshige version 1.3, <http://sites.google.com/site/Shigemorita/home/2dshige>). In the synchronous map, auto peaks, appearing on the diagonal line, indicate the locations of spectral changes by the

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