



Phenanthrene binding by humic acid–protein complexes as studied by passive dosing technique



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ARTICLE INFO

Article history:

Received 30 May 2013

Received in revised form

9 August 2013

Accepted 12 August 2013

Keywords:

Dissolved organic matter
Hydrophobic organic contaminants
Atomic force microscopy
Hydrophobic interaction
Complexation

ABSTRACT

This work investigated the binding behavior of phenanthrene by humic acids (HA-2 and HA-5), proteins (bovine serum albumin (BSA)), lysozyme and pepsin), and their complexes using a passive dosing technique. All sorption isotherms were fitted well with Freundlich model and the binding capability followed an order of HA-5 > HA-2 > BSA > pepsin > lysozyme. In NaCl solution, phenanthrene binding to HA-BSA complexes was much higher than the sum of binding to individual HA and BSA, while there was no enhancement for HA-pepsin. Positively charged lysozyme slightly lowered phenanthrene binding on both HAs due to strong aggregation of HA-lysozyme complexes, leading to reduction in the number of binding sites. The binding enhancement by HA-BSA was observed under all tested ion species and ionic strengths. This enhancement can be explained by unfolding of protein, reduction of aggregate size and formation of HA-BSA complexes with favorable conformations for binding phenanthrene.

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1. Introduction

Dissolved organic matter (DOM), a complex and heterogeneous mixture of different classes of organic molecules, is ubiquitous in natural aquatic environments and soil environments as well. It plays a key role in the solubility enhancement of hydrophobic organic contaminants (HOCs), and is important to the long-range transport of HOCs (Lam et al., 2007; Pan et al., 2007). Humic acid, a major component of DOM, has been widely investigated regarding sorption and transport of HOCs (Pan et al., 2008; Polubesova et al., 2007). Protein is another type of DOM (maybe also part of HA fraction), which is pertinent to the biogeochemical cycling of elements in aquatic and soil environments (Tomaszewski et al., 2011). In wastewater effluent, proteins were found to be a major component of organic matter (Westgate and Park, 2010; Dignac et al., 2000). However, only very few studies have addressed the binding and sorption of HOCs on proteins (Zhang et al., 2010).

Proteins were observed in all HA fractions extracted from marine samples (Mecozzi and Pietrantonio, 2006), emphasizing that HA-protein complexes are widely present in the environment. Previous study has reported that HA could encapsulate positively charged protein molecules and form layer-by-layer encapsulation of protein due to electrostatic attraction (Tomaszewski et al., 2011). When

solution conditions (e.g., pH, ionic strength) are altered, hydrophobic interaction could be dominant by overcoming electrostatic attraction drives (Li et al., 2012). Furthermore, natural organic matter isolated from seawater has revealed that polymerization and de-polymerization reactions may occur during HA-protein complex formation (Mecozzi et al., 2009). Therefore, it is likely that binding of HOCs to HA-protein complexes will be deviated from that of their individual constituents due to such complexation reactions. However, there is no information available on this topic. In addition, most of previous studies focused on the interaction of HAs with positively charged proteins (Li et al., 2012; Tan et al., 2008; Tomaszewski et al., 2011), related investigations on neutral or negatively charged protein are limited. Therefore, in this study, three globular proteins, bovine serum albumin (BSA), lysozyme and pepsin with different isoelectric points (IEPs) and sizes were employed.

HOC binding to organic matter has been investigated using several techniques such as membrane dialysis (Pan et al., 2007; Yamamoto et al., 2004), batch with filtration (Jabusch and Swackhamer, 2005) and solid-phase micro-extraction partitioning (Kopinke et al., 2001; Zhang et al., 2012). However, experimental difficulties were encountered by using these techniques due to their limitations (van der Heijden and Jonker, 2009). Membrane dialysis and batch with filtration methods require large molecular weight organic matter, and HOCs could be adsorbed on membrane or filter, while solid-phase microextraction method requires accurate poly(dimethylsiloxane)-water coefficient of HOCs. Recently, we applied a passive dosing technique in a sorption study at a range of

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analyte concentrations for the first time (Zhao et al., 2012). This passive dosing technique is based on the partitioning of HOCs from a preloaded silicone to a sorbent-contained aqueous solution (Gouliarmou et al., 2012; Birch et al., 2012), which could overcome the disadvantages of the above mentioned techniques. Therefore, passive dosing technique was used to investigate the binding of HOCs to HA-protein complexes. This technique is based on the equilibrium partitioning of analytes from a pre-loaded silicone to an aqueous solution or suspension. By using this technique, the concentration of free analyte in the HA-protein solutions is tightly controlled.

Therefore, the objectives of this work were to investigate i) binding of HOCs to individual HAs and proteins using the passive dosing technique; ii) binding of HOCs to HA-protein complexes; and iii) HA-protein interactions using different approaches and the associated binding mechanisms on HA-protein complexes. Phenanthrene, a three-ring polycyclic aromatic hydrocarbon, was used as a model HOC pollutant in this work. We hope that the information from this investigation will provide new insight into the binding behavior of HOCs by dissolved sorbents, as well as their transport and fate in aquatic and soil environments.

2. Materials and methods

2.1. Materials

Two structurally different HAs used for this experiment were obtained after sequentially extractions of Amherst peat soil (MA, USA). The detailed extraction procedure was described in our previous study (Kang and Xing, 2005; Wang et al., 2011a). The second (HA-2) and fifth (HA-5) HA fractions with different hydrophobic properties were selected for our experiments. Properties of HA-2 and HA-5 were shown in Table S1, Table S2, and Fig. S1. Three proteins, BSA, chicken egg white lysozyme, and pepsin were purchased from Sigma Aldrich. Selected physicochemical properties of these three proteins are listed in Table 1.

Poly(dimethylsiloxane) elastomer kit (Silastic® MDX4-4210) which contains a silicone pre-polymer and a catalyst was purchased from Dow Corning Co. Unlabeled and ¹⁴C labeled (8.2 μCi/μmol) phenanthrene was obtained from Sigma–Aldrich Chemical Co. The molecular weight, water solubility (25 °C) and octanol–water partition coefficient of phenanthrene are 178.2 g/mol, 1.29 mg/L and 4.57, respectively (Wang et al., 2011b; Yang et al., 2006).

2.2. Preparation of passive dosing vials and phenanthrene loading


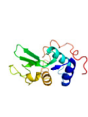
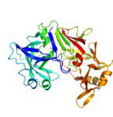
For preparation of passive dosing vials, the silicone pre-polymer and catalyst were mixed (10:1, w/w), and 600 ± 5 mg of this silicone mixture were added into 20 mL vials. Then, the vials were kept at 4 °C for 48 h, subsequently 25 °C for 72 h and 110 °C for another 72 h to complete the curing. The vials were rinsed with methanol (HPLC grade, Fisher) for three times (each time at least 12 h) to remove impurities and oligomers in the silicone. Ultrapure water was then added into the vials three times to remove the remaining methanol. After the cleaning procedure, the passive dosing vials were wiped with lint-free tissue.

Passive dosing vials were loaded by partitioning of phenanthrene from methanol into silicone according to the method described in our previous study (Zhao et al., 2012). The detailed procedure is further described in the Supporting information. After loading, the phenanthrene solution was discarded and 20 mL of ultrapure water was amended into the vials to remove the remaining methanol in silicone. All vials were shaken for 24 h to reach equilibrium (Birch et al., 2010) and the concentration of free phenanthrene in water was measured. This procedure was repeated three times to obtain the constant C_{free} in the water (Zhao et al., 2012). Finally, the water was discarded and the silicone in vials was wiped with lint-free tissue. Passive dosing vials with different C_{free} s were obtained by adding different concentrations of phenanthrene loading solutions (20–2005 mg/L). C_{free} in each procedure was measured by mixing 1.6 mL solution with 4.0 mL Ultima Gold XR cocktail (Perkin Elmer) for liquid scintillation counting (Beckman, LS6500).

2.3. Phenanthrene binding/sorption experiment

HA samples (HA-2, HA-5) were dissolved with a minimum volume of 0.5 M NaOH solution. The HA stock solution was then diluted to 50 mg/L (pH 7.0) using NaCl background solution (50 mM NaCl and 200 mg/L Na₂S₂O₃) for the binding experiment (Eriksson et al., 2004). BSA, lysozyme and pepsin solutions were also prepared in the same background solution with the final concentrations of 200, 3000 and 5000 mg/L, respectively. After the adjustment of pH to 7.0 with minimum volume of HCl and NaOH solutions, each of these solutions (20 mL) was added into the passive dosing vials which were already loaded with different concentrations of

Table 1
Selected physicochemical properties of BSA, lysozyme and pepsin.

Protein	BSA	Lysozyme	Pepsin
Source	Bovine serum	Chicken egg white	Porcine gastric mucosa
Molecular weight (kDa)	68.0	14.3	35.5
Isoelectric Point (IEP) ^a	4.8	10.5–11.0	1.0
Geometrical dimensions ^b (nm)	9.5 × 5.0 × 5.0	4.2 × 3.0 × 3.0	4.5 × 5.0 × 6.6
S_n/S_p ^c	2.94	1.27	1.35
Adiabatic compressibility (10 ⁻⁶ cm ³ /g/bar) ^d	6.6 ± 0.5	1.3 ± 0.5	5.2 ± 0.5
Apparent specific volume (cm ³ /g) ^d	0.739	0.702	0.733
No. of amino acids ^e	607	147	386
Hydrophobic residues (%) ^f	42	35	35
Aromatic residues (%) ^f	11	10	11
Basic residues (%) ^f	23	25	9
Molecular shape ^g			

^a IEPs of proteins are taken from Imamura et al. (2008).

^b Geometrical dimensions of BSA, lysozyme and pepsin are from Jachimska et al. (2008), Tomaszewski et al. (2011) and Giussani et al. (2012), respectively.

^c S_n/S_p is the ratio of nonpolar surface area (S_n) and uncharged polar surface area (S_p), the data of lysozyme and pepsin are from Chalikian et al. (1996) while BSA from Courtenay et al. (2001).

^d Data are from Chalikian et al. (1996).

^e Numbers of total and individual amino acids are from Wolschin et al. (2005).

^f Percentages of hydrophobic, aromatic and basic residues are from Matsuura et al. (2006).

^g The models of proteins were produced using the program PyMOL (<http://www.pymol.org>).

phenanthrene. All vials were allowed to shake for 5 days at 25 ± 1 °C to attain equilibrium. During this binding process, silicone in the vial did not significantly adsorb HAs or proteins, thus, the aqueous phase concentration of HA or protein did not change in the vials (Fig. S2). Then 1.6 mL solution from the vial was mixed with 4.0 mL of cocktail and the total phenanthrene concentration (C_{total}) was measured using the liquid scintillation counter. For the vials which had precipitates, the samples were shaken evenly before the measurement. The presence of HAs or protein had no significant influence on the radioactive measurement (Fig. S3). The remaining solution was discarded and the vials were then quickly rinsed five times with background solution and wiped with lint-free tissue. Background solution (20 mL) was then added in these vials and the measured phenanthrene concentration equilibrated in the background solution was used as a surrogate for the C_{free} . This measured C_{free} was also the equilibrium aqueous concentration during the solubilization experiment. The concentration of bound phenanthrene on HAs or proteins was calculated by the difference between C_{total} and C_{free} .

For the HA-protein complex binding experiment, individual HA (HA-2 or HA-5) and protein (BSA, lysozyme or pepsin) were mixed in NaCl background solution, with final concentrations of 50 and 200 mg/L for HA and protein, respectively. “200 mg/L” was selected as the protein concentration because this is the minimum protein concentration used in the above binding experiments on individual proteins, and this concentration is reasonable according to the reported data in wastewater (Massé and Masse, 2000). The pHs of the mixture solutions were adjusted to 7.0 followed by transferring 20 mL of solution to the phenanthrene-loaded passive dosing vials immediately. After 5 day equilibration at 25 ± 1 °C, C_{total} of phenanthrene in the mixture solution was measured. The remaining solution was then discarded and the background solution was amended into the vials. The phenanthrene concentration in background solution was measured after shaking for 24 h.

To investigate the effect of ion species, binding experiment was also conducted in the CaCl₂ background solution (16.7 mM, the same ionic strength to the NaCl background solution). In view of the aggregation and precipitation of HAs at this CaCl₂ concentration, we further selected 2 mM CaCl₂ as the background solution, in which no precipitation was observed for either HA-2 or HA-5 (Fig. S4).

2.4. Determination of hydrodynamic diameter and zeta potential

Hydrodynamic diameter and zeta potential of the HAs, proteins, and HA-protein complexes were measured using a Particle Size Analyzer (90Plus, Brookhaven Instruments Co.). In brief, the HA-protein solution was prepared by

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