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Complexation between Hg(II) and biofilm extracellular polymeric substances: An application of fluorescence spectroscopy

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ABSTRACT

The three-dimensional excitation emission matrix (EEM) fluorescence spectroscopy was employed to investigate the interaction of extracellular polymeric substances (EPS) from natural biofilm with Hg(II). The EEM spectra demonstrated that EPS with molecular weight over 14 kDa had two protein-like fluorescence peaks. The fluorescence intensity at both peaks was strongly dependent on the solution pH in the absence and presence of Hg(II), with the maximal fluorescence intensity at neutral pH. Fluorescence of both protein-like peaks was significantly quenched by Hg(II). The values of conditional stability constants ($\log K_a$ = 3.28–4.48) derived from modified Stern–Volmer equation are approximate to those for humic substances and dissolved organic matter (DOM), indicating that fluorescent components in EPS have strong binding capacity for Hg(II). Our findings suggest that EPS from biofilm is a class of important organic ligands for complexation with Hg(II) and may significantly affect the chemical forms, mobility, bioavailability and ecotoxicity of heavy metals in the aquatic environment.

1. Introduction

Extracellular polymeric substances (EPS), produced by many microorganisms in natural and artificial environments, are mainly composed of polysaccharides, protein, uronic acids, humic substances and lipids [1]. EPS play important roles in cell adhesion, formation of microbial aggregates, and sheltering microbial cells from harsh environments [2–4]. It was reported that EPS accounted for over 90% of organic substances in the biofilm [5]. EPS are ubiquitous in the aquatic environments and its concentration in the mud flat sediment was up to 441.5 mg kg^{-1} sediment [6].

Being poly-anionic in nature, EPS are able to form the organo-metal complexes by electrostatic interactions with multivalent cations [7]. Furthermore, EPS have a great complexation capacity for heavy metals because they contain a variety of functional groups including hydroxy, carboxyl, phosphoric amine, hydroxyl and amidocyanogen [8–12]. Chen et al. [13] noted that more than 90% of the heavy metals removed by biofilm were attributed to the binding capacity of EPS. Merroun and Selenska-Pobell [14] reported that EPS of *A. ferrooxidans* play a major role in the binding of U from aqueous solutions due to the coordination between the organic phosphate groups and U [15].

The availability and mobility of heavy metals in the environments should be strongly influenced by their complexation with EPS due to their ubiquitous presence and great metal binding capacity. Despite the importance of EPS in the aquatic environments, little information is available in this aspect. Studies on the interaction between EPS and heavy metals were mostly focused on the EPS from activated sludge, granular sludge and artificial biofilms of bioreactor [8–12]. On the other hand, the complexation between dissolved organic matter (DOM) and heavy metals has been extensively studied [16–19]. But researchers did not pay enough attention to examine the interaction of EPS with metal ions as well as its potential impacts on the species and fate of heavy metals.

Mercury, one of the most toxic heavy metals, is ubiquitously present in the environments [20]. Mercury exists in various forms in the aquatic environment and its toxicity, bioavailability and transfer into food chain depend on its various chemical forms [21]. In addition, mobilization of mercury is mostly influenced by its complexation with organic ligands [22]. However, it is still unclear how EPS affect the speciation and mobility of Hg in the aquatic environments.

The fluorescence excitation emission matrix (EEM) spectroscopy is a versatile, simple, rapid and sensitive method for

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studying fluorescent organic compounds [23]. This technique provides important information on fluorescence intensity and fluorescent functional groups for understanding the dynamics and chemical nature of organic compounds in aqueous media [23]. EEM spectroscopy has been successfully applied to investigate the DOM and its interaction with metals [24,25]. Since protein and humic substances are the key components that show fluorescence properties in the EPS, EEM spectroscopy can be employed to study the chemical properties of EPS [5] and the complexation of EPS with Hg(II).

The objective of the present study was to investigate: (1) the fluorescence properties of EPS from natural biofilm in the aquatic environments, and (2) the complexation between EPS and Hg(II) under various pH conditions using EEM spectroscopy.

2. Materials and methods

2.1. EPS extraction from biofilm

Natural alga-bacterial biofilm sample was collected from a pond at the Institute of Geochemistry, Chinese Academy of Sciences. Immediately after collection of the biofilm, EPS was immediately extracted by centrifugation [11,26]. This EPS extraction method would not cause cell lysis [26]. The biofilm was thoroughly rinsed with high-purity Milli-Q water (greater than 18.2 MΩ resistance) at least for ten times. The dissolved EPS were separated by centrifugation at 20,000 × *g*, 4 °C for 20 min [27]. The supernatants were filtered through 0.22 μm acetate cellulose membranes and then dialyzed with membrane with a molecular weight cut-off of 14 kDa for 24 h. Since EPS are mainly composed of high molecular weight polymeric substances, high molecular weight cut-off dialysis membrane was used in order to remove the low molecular weight substances. The EPS solution after dialysis was immediately lyophilized and stored at −20 °C until for use.

2.2. Fluorescence measurements

The EEM spectra of the EPS solution were recorded with a fluorescence spectrophotometer (F-4500, HITACHI, Japan). The EEM spectra were collected at 5 nm increments over an excitation range of 200–400 nm, with an emission range of 250–550 nm by every 2 nm. The excitation and emission slits were set to 5 and 10 nm of band-pass, respectively. The scan speed was 1200 nm min^{-1}. The Milli-Q water blank was subtracted from the sample's EEM spectra and EEM data was processed using the software SigmaPlot 2000 (Systat, US). All the experiments were conducted in triplicate and the mean values were used.

2.2.1. Influence of the solution pH on the fluorescence properties of EPS/Hg(II) complexes

The lyophilized EPS was reconstituted in Milli-Q water at 7.5 mg L^{-1}. The pH of the sample solution was adjusted to desired pH by addition of 0.1 M HCl or 0.1 M NaOH. The EEM spectra of EPS with various pH values in absence and presence of 3.0 mg L^{-1} Hg(II) were recorded. For EPS–Hg(II) complexs, EPS solution in the presence of 3.0 mg L^{-1} Hg(II) was mixed using a magnetic stirrer for 15 min prior to fluorescence measurement.

2.2.2. Fluorescence quenching titration

10 mL of EPS solution (7.5 mg L^{-1}) in a 25 mL beaker was titrated with the incremental additions of 5 μL 0.1 M Hg(II) at room temperature. After each addition of Hg(II), the solution was mixed using a magnetic stirrer for 15 min. Fluorescence quenching titrations of EPS by Hg(II) under various pH conditions (4, 6 and 8) were conducted, respectively. The solution pH was kept constant during the titration.

2.3. Chemicals and chemical analysis

$HgCl_2$ of analytical grade was used and the Hg(II) stock solutions were prepared in Milli-Q water. Total organic carbon (TOC) was used to represent EPS concentration and measured using a TOC analyzer (TOC 5000, Shimadzu, Japan).

3. Results and discussion

3.1. Fluorescence characterization of EPS

Two fluorescence peaks (peaks A and B) were identified in the EEM spectra of EPS at pH 4–9. The EEM spectra of EPS in the absence and presence of Hg(II) were exemplified in Fig. 1. The peak A was detected at Ex/Em = 275–280/328–334 nm and the peak B at Ex/Em = 220–230/322–336 nm. Protein-like fluorophores have two excitation wavelengths at 220–230 nm and 270–280 nm in EEM spectra [8,23,28]. Since proteins are the main components in EPS [29], peaks A and B could be assigned to protein-like fluorescence. The peaks A and B could be further identified as tryptophan-like substances [8,23,28,30–32]. The similar peaks were found in EPS produced by activated sludge in earlier studies [33,34]. However, the peak position of two peaks in this study showed a blue shift, particularly in emission wavelengths compared with the previous studies [33,34]. It is interesting to note that there was

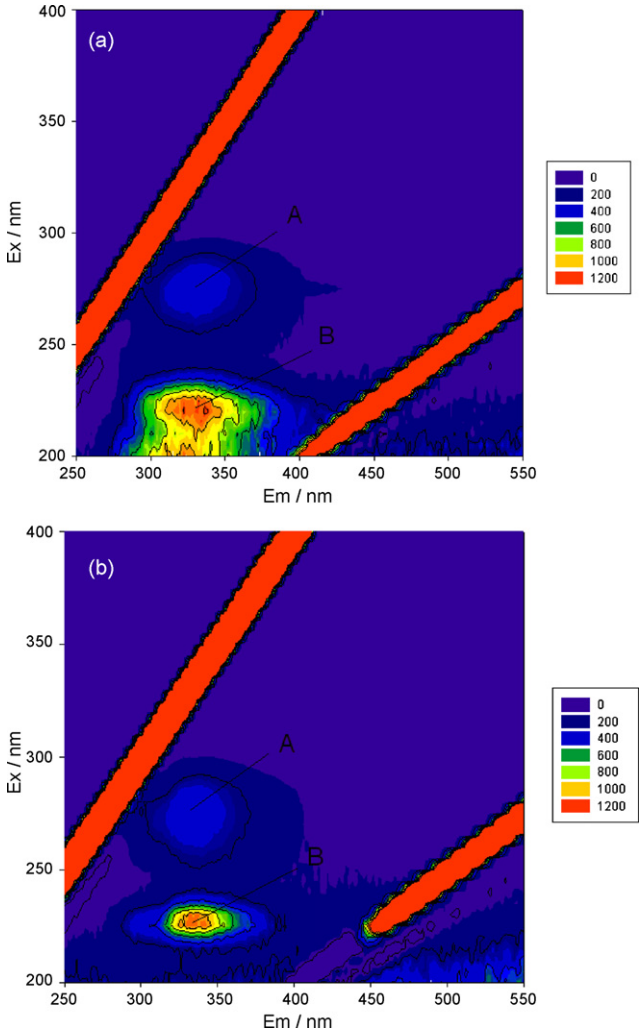

Fig. 1. Typical three-dimensional fluorescence EEM spectra of EPS at pH 6 in the absence (a) and presence (b) of 3.0 mg L^{-1} Hg(II).

Table 1
Peak position of fluorophores reported in the literature.

Peak		Fluorescence type	Reference
Ex (nm)	Em (nm)		
275	310	Tyrosine-like, protein-like	[25]
275	340	Tryptophan-like, protein-like	[25]
265–280	293–313	Tyrosine-like, protein-like	[17]
275–285	336–351	Tryptophan-like, protein-like	[17]
225	340–350	Protein-like	[27]
280–285	340–350	Protein-like	[27]
220–230	340–350	Aromatic protein	[28]
270–280	340–350	Soluble microbial by-product-like	[28]
270–280	320–350	Protein-like	[30]
275–280	328–334	Tryptophan-like, protein-like	In this study
220–230	322–336	Protein-like	In this study

no humic acid-like fluorescence observed in this study, which is in disagreement with earlier studies [27,28]. The possible reason is that the molecular weight of humic acids in EPS was less than 14 kDa and they were not retained in the EPS solution after dialysis in this study [35]. The peak positions of protein-like fluorophores found in DOM and EPS were summarized in Table 1. It was found that the fluorescence peaks of protein-like substances were located at Ex/Em = 270–280 nm/290–350 nm (peak A) and 220–230/322–350 nm (peak B) (Table 1).

3.2. Influence of pH on fluorescence of EPS

It is showed that fluorescence intensities of the peaks A and B in EEM spectra were strongly influenced by pH changes in the absence and presence of 3.0 mg L^{-1} Hg(II) in the samples (Fig. 2). The maximal fluorescence intensities of both peaks were observed under neutral conditions and their intensities often decreased as the solution pH increased or decreased (Fig. 2). Alteration of fluorescence by pH changes might be a result of the change in the molecular orbit of the excitable electrons, which could be resulted from ionization of the fluorophores or functional groups in the molecule due to pH changes [36]. Alternatively, Patel-Sorrentino et al. [37] suggested that the change in the fluorescence with pH changes could be related to the modifications in the macromolecular configuration of the organic compounds. More rigid structures emit stronger fluorescence. For example, the configuration of humic substances at higher pH becomes linear and some fluorophores, which coiled at lower pH, were uncoiled and then emit fluorescence, thereby increasing the overall fluorescence intensity [38]. This explanation might be in agreement with our study. The fluorescence of protein-like substances was mainly due to the presence of aromatic amino acid residues (tryptophan, tyrosine and phenylalanine). In our study, some of protein-like fluorophores in the EPS that might be masked under acidic and basic conditions were uncoiled under neutral conditions, therefore, the maximum fluorescence intensity was found under neutral conditions [38].

Our study demonstrated that the fluorescence intensities of both peaks after addition of Hg(II) at all pH values were often lower than in samples without addition of Hg(II), indicating that the quenching of fluorescence occurs due to complexation between EPS and Hg(II). In addition, the effect of pH on fluorescence of both peaks in the presence of Hg(II) showed the similar trend to that in the absence of Hg(II). The significant fluorescence quenching of EPS by Hg(II) (Figs. 2 and 3) indicates that EPS has strong binding capacity for Hg(II) and some fluorescent organic ligands may participate in the complexation of EPS with Hg(II). This complexation can be more understandable from fluorescence titration experiments under various pH conditions (Fig. 3).

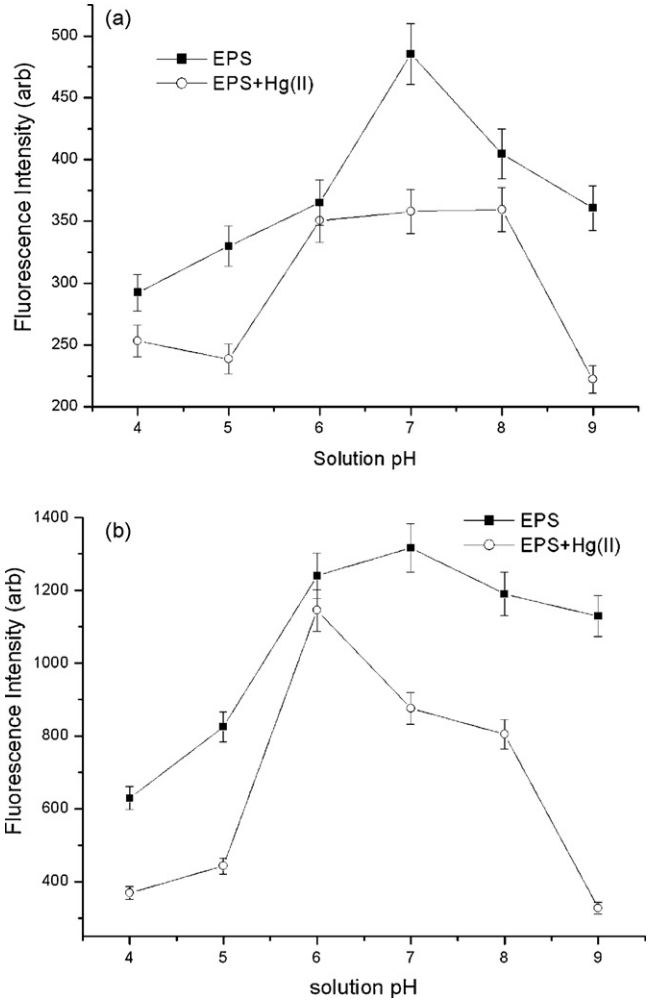

Fig. 2. Influence of solution pH on the fluorescence intensities of peaks A (a) and B (b) in the absence and presence of 3.0 mg L^{-1} Hg(II). Values represent mean ± SE of three independent measurements. Bars indicate standard errors.

3.3. Fluorescence titration of EPS with Hg(II)

It is showed that fluorescence of peaks A and B was significantly quenched by Hg(II) (Fig. 3). At pH 4, fluorescence of both peaks was largely quenched by about 300 μM Hg(II). At pH 6, fluorescence of the peak B was largely quenched by about 300 μM Hg(II) while over 500 μM Hg(II) was required to quench the fluorescence of the peak A. More Hg(II) was needed for quenching of fluorescence of both peaks at pH 8.

Fluorescence quenching data are usually analyzed using Stern–Volmer equation [39].

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensity in the absence and presence of quencher, respectively. Parameter k_q is an energy transfer rate (L mol^{-1} s^{-1}), while τ_0 refers as to lifetime of fluorescence(s), and [Q] is the concentration of the quencher. The typical plot of F_0/F versus [Q] is expected to be a straight line with intercept of 1 on the y-axis and slope equal to K_{SV}, the Stern–Volmer constant [40,41].

The fluorescence titration of EPS by Hg(II) at pH 4, 6 and 8 was subjected to the Stern–Volmer analysis, respectively. All plots of F_0/F versus [Hg] at various pH values clearly showed substantial concave-down features (Fig. 4), which are different from the typical linear feature of Stern–Volmer curve. According to Zhao and

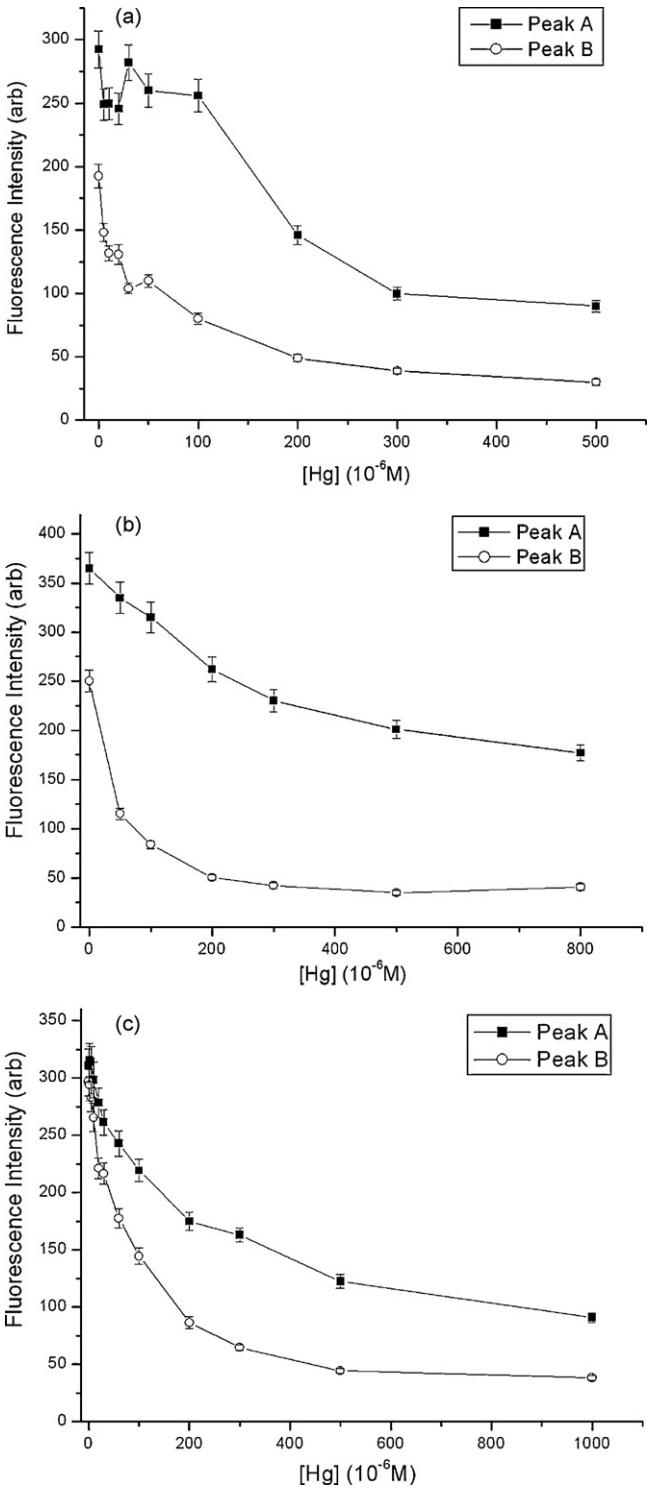

Fig. 3. Fluorescence quenching of EPS titrated with a Hg(II) solution under various pH conditions. (a) At pH 4, (b) at pH 6, and (3) at pH 8. Values represent mean ± SE of three independent measurements. Bars indicate standard errors.

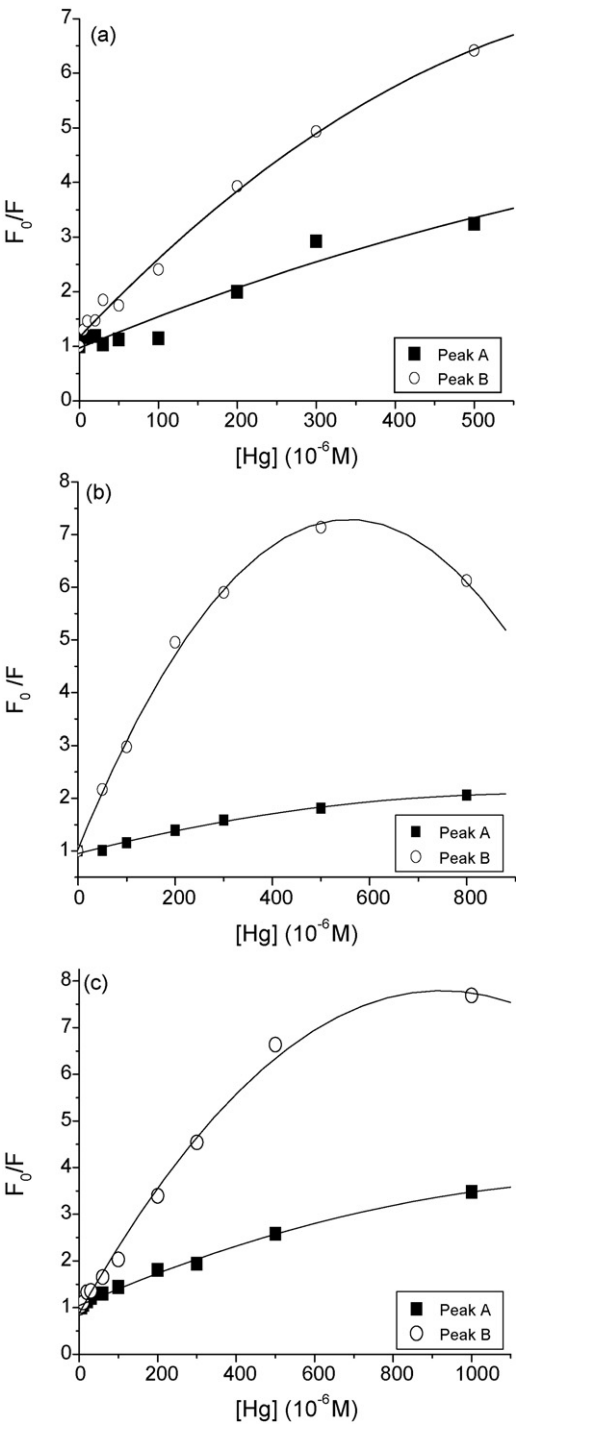

Fig. 4. Stern–Volmer plots of fluorescence emission quenching of EPS titrated with a Hg(II) solution under various pH conditions. (a) At pH 4, (b) at pH 6, and (3) at pH 8.

Nelson [41], a concave-down response is usually reflective of the existence of two fluorophore populations at an individual peak. One population is accessible to the quencher and the other is not accessible to the quencher, indicating that only the florescence from the accessible fluorophore has the potential to be quenched. The Stern–Volmer curve thus deviates from linearity. Therefore, the modified Stern–Volmer equation [42,43] was used to depict the fluorescence quenching. In the modified equation, both the quenchable fluorophores and the quencher inaccessible fluorophores were considered. Following Lakowicz [40], the total fluorescence (F_0) fluorophores in the absence of quencher, F_0, equals to F_{0a} and F_{0b}.

$$F_0 = F_{0a} + F_{0b} \quad (2)$$

where F_{0a} is the fluorescence of the fluorophore moieties that can complex with quencher and F_{0b} is the fluorescence of the inaccessible fluorophore moieties.

In the presence of quencher, only the F_{0a} will change and the observed fluorescence intensity will be provided to form the

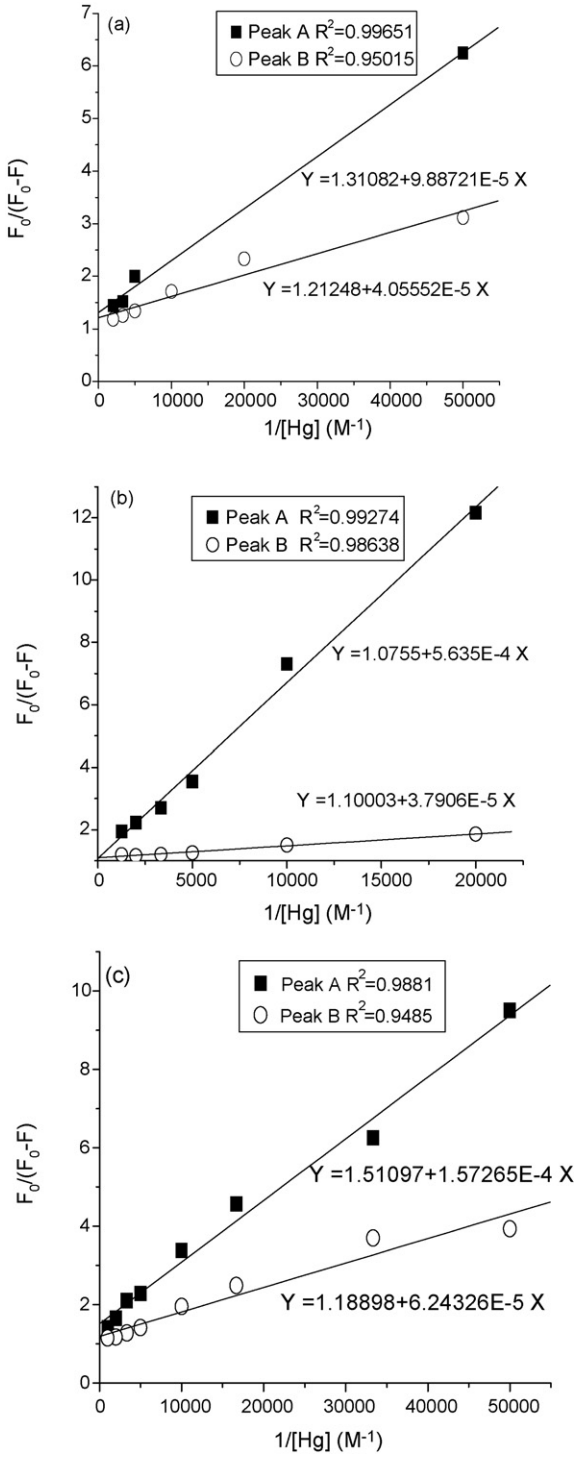

Fig. 5. Modified Stern–Volmer plots of fluorescence emission quenching of EPS titrated with a Hg(II) solution under various pH conditions. (a) At pH 4, (b) at pH 6, and (3) at pH 8.

modified Stern–Volmer equation to evaluate the complexation parameters, i.e. conditional stability constants and binding capacities [42,43]:

$$\frac{F_0}{\Delta F} = \frac{F_0}{(F_0 - F)} = \frac{1}{(f_a K_a [\mathrm{Hg}])} + \frac{1}{f_a} \quad (3)$$

$$f_a = \frac{F_{0a}}{(F_{0a} + F_{0b})} \quad (4)$$

Table 2
The calculated complexation parameters of peaks A and B under various pH conditions.

pH	Peak A			Peak B		
	K_a (M^{-1})	$\log K_a$	f_a	K_a (M^{-1})	$\log K_a$	f_a
4	13245.70	4.12	0.76	29832.35	4.48	0.83
6	1908.61	3.28	0.93	29023.75	4.46	0.91
8	9617.83	3.98	0.87	19045.33	4.28	0.84

where K_a is the Stern–Volmer quenching constant of the accessible fraction, f_a represents the fraction of the initial fluorescence, which is accessible to quencher, and [Hg] is the concentration of Hg(II). The parameters f_a and K_a can be obtained from Eq. (3).

The modified Stern–Volmer plots of EPS titrations with Hg(II) at various pH values are shown in Fig. 5. All the titration data were well fitted with the modified Stern–Volmer equation (R^2 = 0.95–0.99). The parameters f_a and $\log K_a$ of fluorophores at peaks A and B at various pH values were calculated and summarized in Table 2. The f_a and K_a of tryptophan-like fluorophores of peak A varied more significantly with pH than that of peak B. The values of $\log K_a$ for complexation of EPS with Hg(II) were close to those for complexation of EPS with other metals [44–46]. For example, Guibaud et al. [44,46], using polarographic methods, determined the relative stability constants of EPS metal complexes, log(*K*), to be 1.54–3.35 for Cd, 0.45–1.28 for Pb, 3.0–4.4 for Cu and 2.6–3.0 for Ni, respectively. The values of f_a and $\log K_a$ of EPS–Hg(II) complex are also comparable with those of humic substances or DOM metal complexes [41,43,47], indicating that EPS may have the similar binding capacity for Hg(II) to humic substances and DOM.

At pH 6, the values of f_a for both peaks were highest, indicating that the fraction of fluorescent components accessible to Hg(II) was maximal under neutral conditions. This result also implies that the protein-like fluorophores in EPS are uncoiled under neutral conditions but masked under acidic and basic conditions. The changes in the values of $\log K_a$ for peaks with pH suggest that the fluorescent components of peak A are relatively weak in binding with Hg(II), whereas the complexation between fluorescent components of peak B and Hg(II) is relatively stable.

In our study, EEM spectroscopy analysis showed that the protein-like substances in the EPS had the strong ability to complex Hg(II). On one hand, protein has the anionic properties, which contributes to the electrostatic interaction with metals. On the other hand, infrared spectroscopy (IR) analysis showed that the amide groups of protein in the EPS are able to react with metals [11,45,48]. Polysaccharides are another important type of binding sites for metals [11,45,49]. Brown and Lester [49] showed that the hydroxyl groups of neutral polysaccharides and the carboxyl groups of anionic polysaccharide may be the metal cation binding sites. In addition to the proteins and polysaccharides, uronic acids, acidic amino acids and phosphate-containing nucleotides are also involved in binding with cations [7,14,15,50,51]. Merroun and Selenska-Pobell [14] reported that the organic phosphate groups of EPS of *A. ferrooxidans* played a major role in the binding of U from aqueous solutions although this bacterium contained small amount of phosphates. They also pointed out that other molecules such as nucleic acids may supply additional phosphate groups. Carboxylate, sulfates and/or phosphates in EPS from phytoplankton were also reported to be strong chelates for Th(IV) [50,51]. Although our study demonstrates that fluorescence EEM spectroscopy is a useful tool for interpretation of the interaction of EPS and metals, this technology is only applicable to the fluorescent components (e.g., proteins and humic acids) but not to other components that do not emit fluorescence, such as phosphate, sulfate and polysaccharide. In order to obtain comprehensive knowledge about the mechanisms involved in the complexation of EPS with metal,

other methods including infrared spectroscopy (IR), extended X-ray absorption fine structure (EXAFS) analysis, nuclear energy resonance (NMR) and transmission electron microscopy-energy-dispersive X-ray analysis (TEM-EDXA) need to be complementarily used.

4. Conclusions

This study shows that EEM spectroscopy is an effective tool for studying the chemical properties of EPS and its complexation with metal ions. The following conclusions can be made from this study:

(1) The EEM spectra of EPS originated from natural biofilm have two protein-like fluorescence peaks and their fluorescence intensities in the absence and presence of Hg(II) were strongly influenced by pH. The fluorescence of both protein-like peaks with and without addition of Hg(II) was strongest at neutral pH values, and fluorescence intensities decreased under either acidic or alkaline conditions.
(2) The fluorescence of both protein-like peaks was significantly quenched by Hg(II) at various pH values. The modified Stern–Volmer equation can well depict the fluorescence quenching titration. The values of conditional stability constants of EPS–Hg(II) complexes are similar to of those metal complexes formed by humic substances as well as DOM, indicating that fluorescent components in EPS have strong binding capacity for Hg(II).
(3) These findings suggest that the chemical forms, mobility, bioavailability and ecotoxicity of heavy metals are significantly influenced by EPS in the aquatic environment.

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