Article

Molecular-size distribution (MSD)-dependent fluorescence quenching of humic substances by complex formation with Eu(III) for different fluorophores

Shuji Tamamura [1,*], Seiya Nagao [1] and Masayoshi Yamamoto [1]

[1] L Low Level Radioactivity Laboratory, INET, Kanazawa University, Wake, Nomi, Ishikawa 923-1224, Japan. Tel: +81-761-51-4440

Abstract

Fluorescence quenching of Pahokee peat fulvic acids (FAs) and humic acids (HAs) by complexation with Eu(III) was studied using high-performance size exclusion chromatography (HPSEC) with fluorescence detection. This study was performed in a 0.01 M Tris-HCl buffer solution at pH 8.0, mobile phase of HPSEC, and focused on molecular-size distribution (MSD) for four types of fluorophores of the Pahokee FA and HA at excitation (Ex)/emission (Em) wavelength of 309–315 nm/429–430 nm for Type I, 255 nm/439 nm for Type II, 439 nm/514 nm for Type III, and 300 nm/514 nm for type IV. In order of decreasing molecular size, three peaks labeled 1 (830–910 Da), 2 (830–770 Da), and 3 (690–750 Da), respectively, were identified in the HPSEC chromatograms of the Pahokee FA and HA samples for the Types I and II fluorophores. On the other hand, only peak 1 was distinct in the chromatograms for Types III and IV fluorophores. Regardless of the fluorophore type, fluorescence intensities of peak 1 were markedly quenched by 65–90% when Eu(III) was added to the mobile phase, whereas the intensity of peak 3 was quenched by only 10–20% in both the FA and HA samples. These results indicate that the fluorescence quenching of FAs and HAs by complexation with Eu(III) was strongly affected by MSD of humic substances.

Keywords: Humic substances; Molecular-size distribution; Fluorescence quenching; High-performance size-exclusion chromatography; Eu(III)

Introduction

Humic substances are widely distributed and abundant in organic matter found in soil, sediment, and natural waters (Thurman, 1985; Stevenson, 1994). These substances are classified into fulvic acids (FAs) and humic acids (HAs) that are soluble and insoluble at pH $\leq$1, respectively, and humin that is insoluble at any pH values (Thurman, 1985). Humic substances show polydispersity, with the average molecular weight of HAs being higher than that of FAs (Hayase and Tsubota, 1985; Stevenson, 1994; Perminova et al., 2003). The polydispersity of humic substances has been measured by high-performance size-exclusion chromatography (HPSEC), revealing the molecular-size distribution (MSD) (Chin et al., 1994; Peuravuori and Pihlaja, 1997; Perminova et al., 2003). For the purpose of characterization, the MSD of humic substances with fluorescence detection has also been determined (Artinger et al., 1999; Nagao et al., 2003; Sierra et al., 2006), signifying the presence of fluorescent moieties (fluorophores) in humic substances (e.g., Senesi, 1990).

Humic substances enhance the mobility of toxic metals owing to their strong complexing abilities with metals in an aquatic system (Kim et al., 1992, 1994; Pourret et al., 2007). Consequently, a number of studies concerned with these complexing abilities with evaluation of the stability constants have been conducted (e.g., Kim and Czerwinski, 1996; Kinniburgh et al., 1999; Shin et al, 2001; Tipping,

* Corresponding author. Present address: 5-3 Sakae-machi, Horonobe-cho, Teshio-gun, Hokkaido 098-3221, Japan. Tel: +81-1632-9-4112; Fax: +81-1632-9-4113; Email: shuji-tamamura@h-rise.jp

2002). In some studies, fluorescence quenching techniques have been utilized (Cabaniss, 1992; Lakshman et al., 1993; Lin et al., 1995; Shin et al., 2001; Saito et al., 2002; Chung et al., 2005; Kumke et al., 2005). However, fluorescence quenching dependence on the MSD of humic substances with fluorescence detection has seldom been investigated. This study may contribute to better understanding of the mechanisms for fluorophore-dependent fluorescence quenching of humic substances by complexation with metals (Shin et al., 2001).

In this context, HPSEC with fluorescence detection was used to reveal MSD-dependent fluorescence quenching of humic substances. The dependence was compared for different types of fluorophores. Both a FA and a HA were used for comparisons. The excitation–emission matrix (EEM) spectra of FA and HA were measured to determine fluorophore excitation (Ex) and emission (Em) wavelengths. Quenching experiments by using EEM were also performed to compare the quenching experimental results by HPSEC. Eu(III) was selected as the quenching agent, because Eu(III) models trivalent actinides such as Am(III) and Cm(III), thereby assisting nuclear waste safety assessment studies (Moulin et al., 1992; Kim and Czerwinski, 1996).

Materials and Methods

Humic substances

Peat FA (standard FA, Pahokee peat II, 2S103F) and HA (reference HA, Pahokee peat, 1R103H) were purchased from the International Humic Substances Society (2008) and used without further purification. Stock solutions were prepared by dissolving 12.5 mg of each sample in 25 mL ultrapure water. To reduce the ash content of the solutions (Underdown, 1981), the solutions were filtered through a 0.45-μm hydrophilic PTFE filter (ADVANTEC, Tokyo, Japan) without pre-treatment of the filters. The FA and HA solutions (10.0 mg L^{-1}) were prepared by diluting the stock solutions with the HPSEC mobile phase (0.01 M Tris-HCl buffer at pH 8.0 in 0.01 M NaCl solution) and used for complexation experiments with Eu(III).

Complexation experiments

A 1.0×10^{-3} M Eu(III) stock solution was prepared by dissolving Eu(III) nitrate hexahydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a 0.01 M HNO_3 solution. The Eu(III) stock solution was subsequently added to 9 mL of the 10.0 mg L^{-1} solutions of FA and HA in the HPSEC mobile phase to obtain a final Eu(III) concentration of 7.5×10^{-6} M. After shaking the solutions overnight at 25°C in the dark using a shaking apparatus with automatic temperature adjustment, the solutions were filtered through 0.45-μm hydrophilic PTFE filters and stored for subsequent quenching studies by HPSEC. In the HPSEC system for the samples containing Eu(III), Eu(III) was added to the mobile phase to a final concentration that was the same as that of the samples.

Fluorescence analyses

The EEM spectra were measured using a F-4500 fluorescence spectrometer with a 150 W ozone-free xenon lamp (Hitachi, Tokyo). The ranges of the Ex/Em wavelengths were 200–500 nm/ 350–550 nm, with 5 nm intervals (Nagao et al., 2001). The photomultiplier voltage was set to 700 V. To correct for baseline drift, the EEM spectrum of quinine sulfate (10 μg L^{-1} in 0.05 M sulfuric acid) was measured at 2 h intervals during the sample measurements. The normalized fluorescence intensity of the samples by the quinine sulfate at Ex/Em = 345 nm/455 nm was termed as the relative fluorescence intensity (RFI) with a quinine sulfate unit (QSU). The EEM spectra were not corrected for the inner filter effects due to the relatively low concentrations (~10 mg L^{-1}) of the humic substances in the samples (Plaza et al., 2006). The EEM spectral measurements were conducted in duplicate.

HPSEC analysis

The HPSEC system consisted of a L-6000 pump (Hitachi), an L-5020 column oven (maintained at 30°C; Hitachi), and a 7125 injection value with a 20 μL sample loop (Rheodyne, DEX Health & Science, WA, USA). Both a L-2420 ultraviolet–visible (UV–Vis) detector (Hitachi) and an L-7485 fluorescence detector (Hitachi) were used. The separation was performed with a stainless steel gel permeation chromatography (GPC) column (Hitachi GL-W530: 30 cm × 10.7 mm ID) packed with a water-soluble polyacrylate gel resin. A guard column packed with the same materials (5 cm × 10.7 mm ID) was used to protect the GPC column. According to the industrial manufacturer's specifications, the

approximate upper exclusion limit was 50 kDa (calibrated with pullulan). The mobile phase was 0.01 M Tris-HCl buffer at pH 8.0 in a 0.01 M NaCl solution (Nagao et al., 2003). A flow rate of 1 mL min^{-1} was maintained. The molecular weight calibration for the HPSEC system was performed using five polystyrene sulfonate sodium salt standards (Polymer Standard Service-USA, Amherst) with molecular weights of 1.10, 3.61, 6.53, 14.9, and 32.9 kDa. The void and total volumes of the column were 6.9 mL and 22.8 mL, respectively, as determined using blue dextran and acetone, respectively.

The wavelength for the UV–Vis detector was set at 280 nm for all samples. The Ex/Em wavelengths of the fluorescence detector were chosen on the basis of the fluorophore Ex/Em wavelengths in the EEM spectra of the FA and HA samples used. The chromatograms were measured in duplicate.

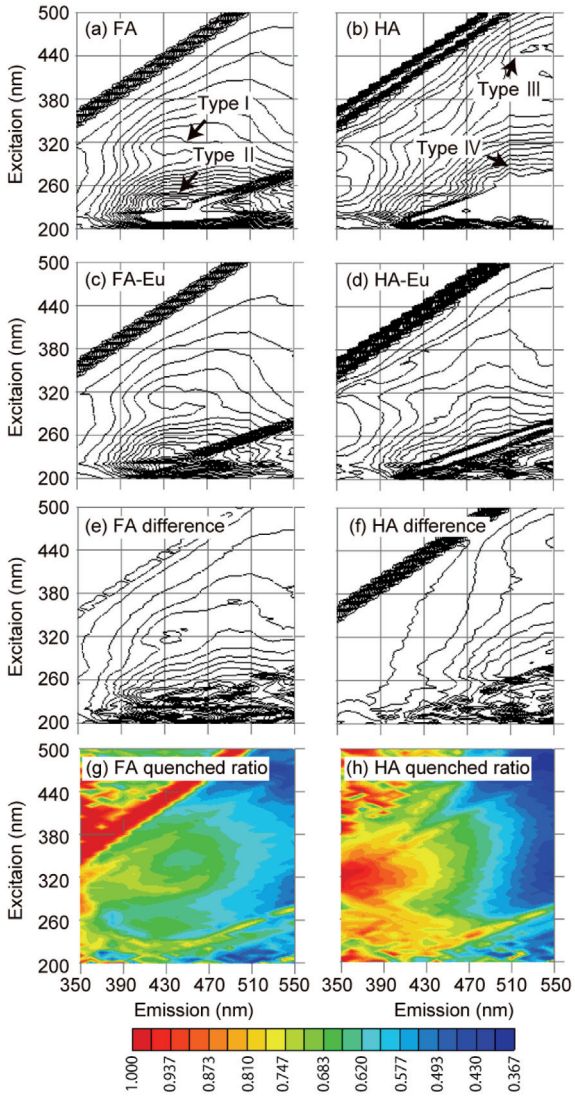

Fig 1. EEM spectra of Pahokee peat FA (a) and HA (b) in the HPSEC mobile phase (tris-HCl buffer at pH 8.0), and those in the presence of 7.5×10^{-6} M Eu(III) (c and d). The difference spectra between (a) and (c) and between (b) and (d) are illustrated in (e) and (f), respectively. The RFIs in the presence of Eu(III) divided by those in the absence of Eu(III) for the FA and HA are illustrated in (g) and (h). The intervals of the counter lines in (a), (c), and (e) are 1.81 QSU and those in (b), (d), and (f) are 0.50 QSU, with maximum QSU of 32.6 and 10.1 for the FA and HA samples, respectively.

Results and Discussion

EEM spectra

Figure 1 shows the EEM spectra of the Pahokee FA and HA in the presence and absence of Eu(III). Two broad peaks in the FA spectrum were attributed to different fluorophores, referred to as Type I (Ex/Em = 315 nm/430 nm) and Type II (Ex/Em = 255 nm/439 nm). In the HA spectrum, there were also two broad peaks at longer emission wavelengths, which were designated Type III (Ex/Em = 439 nm/514 nm) and Type IV (Ex/Em = 300 nm/514 nm). Table 1 summarizes the RFIs for each fluorophore. Although the peaks for the Types III and IV fluorophores were not recognized in the FA spectrum, the RFIs of FA corresponding to those fluorophores were measured for comparison. Similarly, the RFIs of HA corresponding to Types I and II fluorophores were measured. Despite the absence of Types III and IV fluorophores in FA, the RFIs of FA at Types III

Table 1 RFI of Pahokee peat FA and HA

Sample	RFI[a]			
	Type I	Type II	Type III	Type IV
FA	14.2±0.2	24.0±0.1	3.8±0.1	12.6±0.3
HA	3.5±0.1	5.4±0.0	3.8±0.0	7.7±0.2

[a] Measured for 10 mg L^{-1} FA and HA in the HPSEC mobile phase (tris-HCl buffer at pH 8.0). Although Types III and IV fluorophores were not recognized in the EEM spectra of the FA sample (see Figure 1), RFIs were calculated for comparison. Similarly, RFIs of HA for Type I and II fluorophores were calculated. The excitation/emission wavelengths (nm) for Type I fluorophore were 315/430 for FA, and 309/429 for HA. Those for Type II, III, and IV fluorophores for FA and HA were 255/439, 439/514, and 300/514, respectively. Errors are ranges of duplicated data.

Table 2 Quenched percentage for each fluorophore (Types I–IV) for Pahokee peat FA and HA by complexation with Eu(III)

Sample	Fluorophore type (Ex/Em, nm)	Quenched percentage (%)[a]		
		Peak 1	Peak 2	Peak 3
FA	Type I (315/430)	65±5	36±5	12±1
	Type II (255/439)	60±2	39±3	11±1
	Type III (439/514)	88±1	43±1	10±2
	Type IV (300/514)	87±3	45±1	9±3
HA	Type I (309/429)	72±3	47±2	14±3
	Type II (255/439)	68±2	50±3	19±4
	Type III (439/514)	73±1	60±2	51±6
	Type IV (300/514)	75±1	60±1	42±1

[a] Quenched percentages were calculated for each peak in HPSEC chromatograms with fluorescence detection (Figures 3 and 4). The errors are ranges of duplicated data.

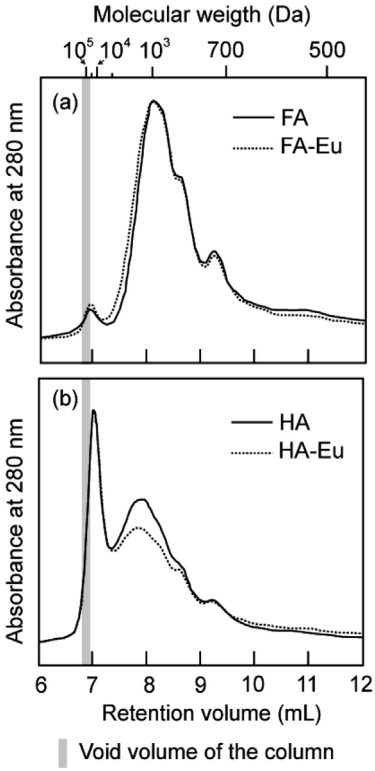

Fig 2. HPSEC chromatograms detected at 280 nm for10 mg L^{-1} Pahokee peat FA (a) and10 mg L^{-1} Pahokee peat HA (b) in the absence and presence of Eu(III). The mobile phase was tris-HCl buffer at pH 8.0 with Eu(III) concentration of 7.5×10^{-6} M for the Eu(III) -contained samples. Molecular weight scale is based on the calibration curve drawn with standard polystyrenesulfonate compounds. Void volume (6.9 mL) is designated in the figure. Total volume of the column was 22.8 mL.

and IV fluorophores were comparable to those for HA, signifying the superior fluorescent properties of the Pahokee FA over the Pahokee HA.

By adding Eu(III), the emission intensities from the Types I and II fluorophores in Pahokee FA (Figure 1a) decreased with a slight blue-shift (i.e., a shift to a shorter wavelength) in the peak positions (Figure 1c), and those from Types III and IV fluorophores in Pahokee HA (Figure 1b) decreased with a definite blue-shift (Figure 1d). The RFIs of the FA and HA for Types I and II fluorophores in the presence of Eu(III) divided by those in the absence of Eu(III) (I/I_0) (quenched ratio) were approximately 0.7–0.8, whereas the ratios for Types III and IV fluorophores (longer emission wavelengths) were approximately 0.6 for FA and 0.5 for HA (Figures 1g and h). Preferable quenching of fluorophores in humic substances at longer emission wavelengths by adding Eu (III) was also observed by Nagao et al. (2001), Chung et al. (2005), and Kumke et al. (2005). The mechanism for the preferable quenching at longer emission wavelengths as well as the blue-shift of the fluorophore will be discussed in connection with the quenching experimental results by HPSEC.

Size exclusion chromatograph

The HPSEC chromatograms with UV absorbance detection for the Pahokee FA and HA samples in the HPSEC mobile phase in the presence and absence of Eu(III) are shown in Figure 2. There was almost no difference in peak shape or intensity between FA and FA-Eu (Figure 2a), whereas the peak area of HA-Eu was depleted about 28% compared with the peak area of HA (Figure 2b). However, the depleted percentage of peak area with UV absorbance detection was much smaller than the quenched percentages in the chromatograms with fluorescence detection (see Table 2).

The chromatograms with fluorescence detection for Pahokee FA and HA samples in the HPSEC mobile phase in the presence and absence of Eu(III) are shown in Figures 3 and 4, respectively. Chromatogram peaks began to appear from the retention volume of 7.6 mL with florescence detection (Figures 3 and 4), whereas they began to appear at the void volume (6.9 mL) with absorbance detection, especially for the HA sample (Figure 2). The presence of "less-fluorescent" large molecular-size fractions of humic substances has also

been demonstrated by other studies (Chin et al., 1994; Lin et al., 1995; Fukushima et al., 2001; Saito et al., 2002). A higher fluorescence quantum yield from smaller molecular-size fractions of the HA sample may be attributed to the "rigidity" of the fractions, resulting in the decreased non-radiative transitions from the photo-excited state to the ground state (Fukushima et al., 2001).

In this study, we describe the Pahokee FA component detected by Ex and Em wavelengths for Type I fluorophore as Type I-FA. The chromatograms for Types I- and II-FA/HA showed three distinct peaks labeled 1, 2, and 3 from the large to small molecular-size region (Figures 3a, 3b, 4a, and 4b). For Types III- and IV-FA/HA, peak 1 was distinct, whereas peaks 2 and 3 were shoulders (Figures 3c, 3d, 4c, and 4d). The retention volumes for peaks 1, 2, and 3 were in the range of 8.1–8.6 mL, 8.6–8.9 mL, and 9.2–9.6 mL, respectively, for all types of the fluorophores in the FA and HA analyzed in this study. The corresponding molecular weights were 830–910 Da for peak 1, 770–830 Da for peak 2, and 690–750 Da for peak 3.

The chromatograms of the FA-Eu and HA-Eu in the mobile phase also exhibited three peaks (or shoulders), 1′, 2′, and 3′ (Figures 3 and 4), whose retention volumes were almost same as those for peaks 1, 2, and 3. The decrease in fluorescence intensities of all types of fluorophores in FA and HA samples in the presence of Eu(III) was larger for the larger molecular-size fractions (recognized by peak 1) than for the smaller molecular-size fractions (recognized by peak 3, or corresponding peak shoulder)

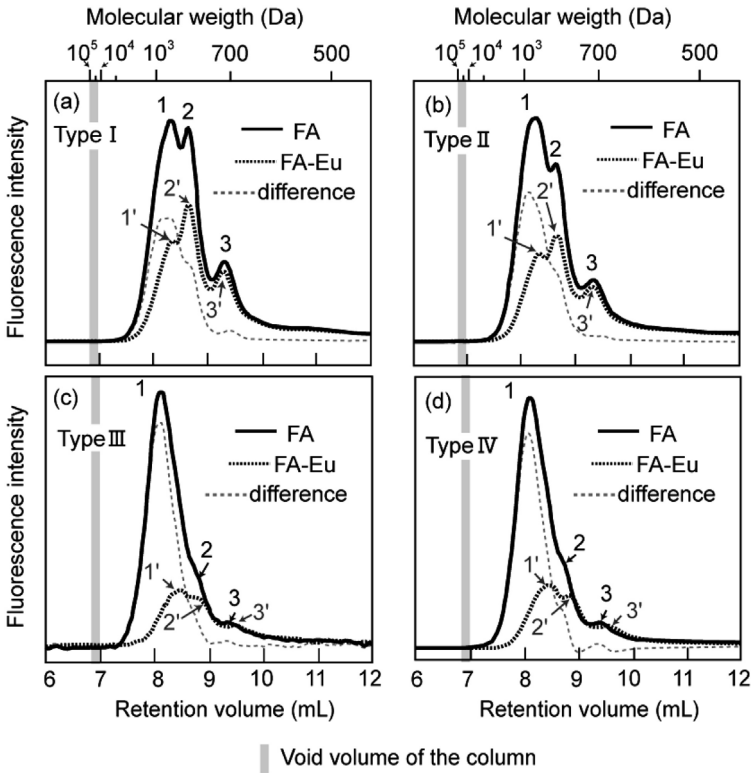

Fig 3. HPSEC chromatograms with fluorescence detection for 10 mg L^{-1} Pahokee peat FA in the presence and absence of Eu(III). (a) Type I-FA, (b) Type II-FA, (c) Type III-FA, and (d) Type IV-FA. The mobile phase was tris-HCl buffer at pH 8.0 with Eu(III) concentration of 7.5×10^{-6} M for the Eu(III)-contained sample. The vertical scale is identical among all the chromatograms. The excitation/emission wavelengths (nm) for Types I, II, III, and IV fluorophores were 315/430, 255/439, 439/514, and 300/514, respectively. The molecular weight scale is based on the calibration curve drawn with standard polystyrenesulfonate compounds. Void volume (6.9 mL) is designated in the figure. Total volume of the column was 22.8 mL.

Table 3 Quenched ratios of the Pahokee FA and HA samples by complex formation with Eu(III)

Sample	Fluorophore type (Ex/Em, nm)	Area ratio S/S_0[a]	RFI ratio I/I_0[a]
FA	Type I (315/430)	0.75±0.03	0.72±0.01
	Type II (255/439)	0.51±0.01	0.71±0.01
	Type III (439/514)	0.33±0.01	0.61±0.01
	Type IV (300/514)	0.35±0.05	0.61±0.00
HA	Type I (309/429)	0.54±0.02	0.79±0.01
	Type II (255/439)	0.50±0.07	0.76±0.00
	Type III (439/514)	0.33±0.05	0.50±0.00
	Type IV (300/514)	0.33±0.04	0.51±0.02

[a] The area ratios S/S_0 were calculated from the peak area of chromatograms with fluorescence detection (Figures 3 and 4). The RFI ratios I/I_0 were calculated from the EEM spectra in HPSEC mobile phase (tris-HCl buffer at pH 8.0). The subscript "0" associated with "S" and "I" indicates Eu(III)-free matrix of the samples. Errors are ranges of duplicated data.

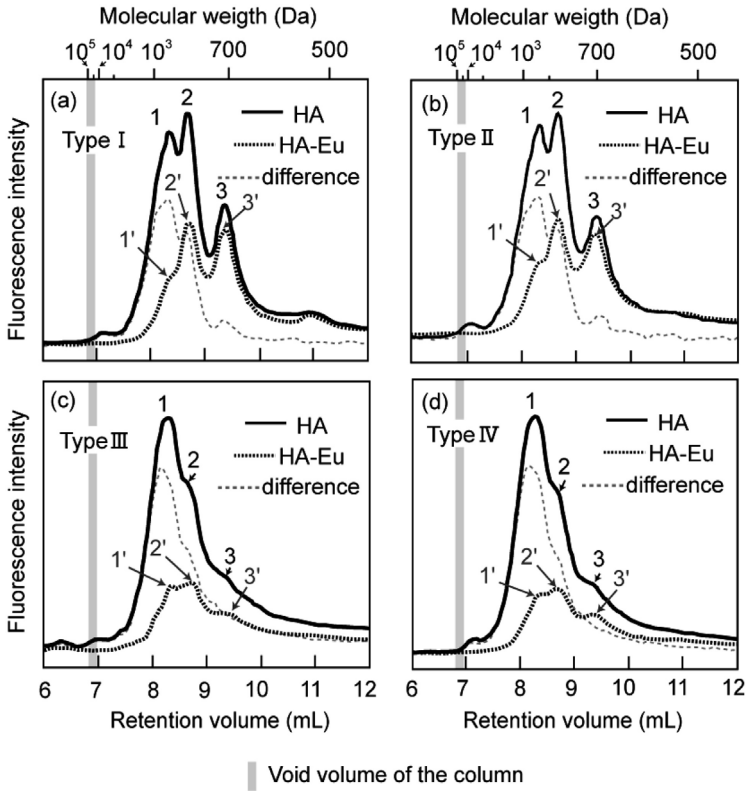

Fig 4. HPSEC chromatograms with fluorescence detection for 10 mg L^{-1} Pahokee peat HA in the presence and absence of Eu(III). (a) Type I-HA, (b) Type II-HA, (c) Type III-HA, and (d) Type IV-HA. The mobile phase was tris-HCl buffer at pH 8.0 with Eu(III) concentration of 7.5×10^{-6} M for Eu(III)-contained samples. The vertical scale is identical among all the chromatograms. The excitation/emission wavelengths (nm) for Types I, II, III, and IV fluorophores were 309/429, 255/439, 439/514, and 300/514, respectively. The molecular weight scale in the upper part of the figure is based on the calibration curve drawn with standard polystyrenesulfonate compounds. Void volume (6.9 mL) is designated in the figure. Total volume of the column was 22.8 mL

(Figures 3 and 4). As summarized in Table 2, the quenched percentages of peaks 1, 2, and 3 were approximately 90, 45, and 10%, respectively, for Types III- and IV-FA and approximately 75, 60, and 45%, respectively, for Types III- and IV-HA. Those values were approximately 60, 40, and 10% for Types I- and II-FA and 70, 50, and 15% for Types I- and II-HA, respectively.

Comparison of the experimental results by EEM and HPSEC

The quenched ratios by the chromatogram area (S/S_0) for the Pahokee FA and HA (Figures 3 and 4) were compared with the I/I_0 ratios for those in the EEM spectra in the HPSEC mobile phase (Figure 1) for each fluorophore (Table 3). The S/S_0 and I/I_0 ratios showed similar trends, the higher values for Types III and IV than for Types I and II fluorophores. On the other hand, the I/I_0 ratio was higher than the S/S_0 ratio by about 20% for each fluorophore, except for Type I-FA. In the HPSEC quenching experiments before sample injection into the HPSEC system, free Eu(III) concentrations should have been considerably lower than the added Eu(III) concentration of 7.5×10^{-6} M owing to the complexation with FA and HA. After sample injection, the FA and HA would have been further complexed with Eu(III) since the flowing mobile phase contained free Eu(III) at a concentration of 7.5×10^{-6} M. Further complexation of Eu(III) with FA and HA in the HPSEC system likely resulted in the lower S/S_0 ratios than the I/I_0 ratios.

Regulation of fluorescence quenching by MSD

Types III- and IV-FA/HA were mainly composed of larger molecular-size fractions (recognized by peak 1), while Types I- and II-FA/HA contained smaller molecular-size fractions (recognized by peak 3) that were resistant to quenching (Figures 3 and 4). This should be the preferable quenching mechanism for Types III and IV fluorophores over Types I and II fluorophores for the FA and HA in the EEM quenching experiments (Figure 1). The tendency of preferable quenching in longer emission wavelengths was even discerned within each fluorophore resulting in a blue-shift of the peak positions in the quenched spectra (Figure 1).

Regarding the tendency that larger molecular size fractions of humic substances is more liable to quenching, an electrostatic effect was proposed by Green et al. (1992). Further research is required to consider the differences in other factors, including binding site density (Lakshman et al., 1993; Fukushima et al., 1996; Pourret and Martinez, 2009;

Reiller et al., 2011), chemical structure (Rao and Choppin, 1995; Tombácz, 1999; Plaschke et al., 2004), and intramolecular electron transfer processes (Boyle et al., 2009), between larger and smaller molecular size fractions (Štamberg, 2003).

Conclusions

HPSEC with fluorescence detection was used to examine MSD-dependent fluorescence quenching for the Pahokee peat FA and HA by complexation with Eu(III). The marked MSD dependence was manifested by chromatographic comparison in the presence and absence of Eu(III) in the HPSEC mobile phase. Larger molecular size fractions (830–910 Da) in both the FA and HA were liable to quenching regardless of types of fluorophores. Preferable quenching in longer emission wavelengths for both the FA and HA in the presence of Eu(III) reflects the dominant constitution of the larger molecular size fractions with fluorescence detection.

Acknowledgments

We thank Dr. Motoki Terashima and Ms. Noriko Yoshida in our laboratory for their technical assistance. We also thank Professor Tsutomu Sato, Hokkaido University, for the geochemical code for Eu(III) speciation. We appreciate the assistance of Tomoyo Suzuki, Kanazawa University, in data management. This study was partly supported by the special committee of "Fundamental chemical research for the stable operation of a reprocessing plant and the management and disposal of TRU waste."

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