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# Simultaneous Presence of Diverse Molecular Patterns in Humic Substances in Solution

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The chemical and structural nature of humic substances (HS) is the object of an intense debate in the literature involving two main theoretical positions: the classical view defending the macromolecular pattern, and the new, more recent, view proposing a supramolecular pattern. In this study, we observe that both molecular patterns are present in different whole humic systems in solution. We also identify these molecular patterns with a specific fraction of HS. Thus, the HS family formed by the gray humic acids studied presented a clear macromolecular pattern, whereas the HS family formed by the fulvic acids studied presented the coexistence of supramolecular assemblies and individual molecules. The third HS family studied, the brown humic acids, presented both the macromolecular pattern and the supramolecular pattern. We also find that molecular aggregation−disaggregation has a strong influence in the fluorescence pattern of HS, thus indicating that the current concepts of HS structure derived from fluorescence studies need revision.

## Introduction

The adequate comprehension of the molecular and structural properties of humic substances (HS) is of major importance to better understand a great number of chemical and biological processes occurring in natural ecosystems, such as the soil dynamics of metals and xenobiotics, the development of plants and microorganisms, or carbon sequestration. However, the molecular pattern of HS still remains unclear and is the object of an intense current debate in the literature. Thus, a number of authors propose that humic substances are macromolecules,[1−4] linear polyelectrolytes, that can form stable aggregates under specific conditions of pH and ionic strength (IS). Other authors, however, propose that humic substances are supramolecular assemblies formed by stable molecular aggregates of relatively simple molecules joined together by weak attractive forces.[5−8] In some way, this discussion might be considered partially artificial since the different HS studied normally corresponded to extracted, isolated, and purified samples subjected to chemical conditions diverse from those occurring in natural ecosystems. However, it may be assumed that the molecular patterns presented by these HS samples under controlled experimental conditions would be representative of those corresponding to the native HS in their natural environment.

In a previous study, we reported that the behavior of different humic systems in solution as a function of pH and IS suggested the possible coexistence of macromolecules and supramolecular assemblies in these molecular systems.[9] In this previous study, we explored the different molecular patterns in the considered HS through the study of their molecular behavior under specific experimental conditions of pH, solvation, and IS. Thus, molecular aggregation−flocculation was related to a macromolecular pattern, whereas molecular disaggregation was linked to the presence of supramolecular assemblies.[9]

The aim of this study is to identify these different molecular patterns with specific fractions of HS. To this end, we investigated molecular association (aggregation, disaggregation, and flocculation) in specific fractions of selected HS. Molecular fractionation was carried out according to the different solubility of the molecules under specific conditions of pH and IS: we considered the gray humic acids (GHA), the brown humic acids (BHA), and the fulvic acids (FA). Molecular association in the different HS fractions was studied through the analysis of the molecular size distribution changes induced by the acidification with acetic acid and further realkalinization with sodium hydroxide of the samples (AcOH treatment).[6] These molecular size distribution changes were analyzed by using ultrafiltration (UF),[10−14] transmission electron microscopy (TEM),[15,16] confocal laser scanning microscopy (CLSM), and synchronous fluorescence spectroscopy (SFS).[17] Likewise, the structural features of the HS fractions studied were determined using ^{13}C NMR and elemental analysis.

## Materials and Methods

**HS Samples.** The humic substances selected for the study were a lignite humic acid obtained from China, a young brown coal (leonardite) humic acid obtained from the Czech Republic, a leonardite IHSS standard humic acid, a humic acid obtained from Aldrich, and two IHSS standard fulvic acids (Suwannee River fulvic acid, SRFA, and Waskish Peat fulvic acid, WPFA). The main molecular properties of the selected HS samples and their fractions are presented in Table 1.

The different humic substances were fractionated in three main molecular fractions:[4] the molecular fraction insoluble at acid pH and soluble at alkaline pH but insoluble at neutral pH and high IS (GHA); the molecular fraction insoluble at acid pH but soluble at alkaline pH and also at high IS (BHA); and, finally, the molecular fraction soluble at acid pH and at both low and high IS (FA).

**Acidification−Realkalinization Process Involving Acetic Acid (AcOH Treatment).** This treatment has two main effects on the size distribution of complex molecular systems. On the one hand, it causes disaggregation through the solvation of the molecules with AcOH at acid pH and the consequent disruption

**TABLE 1: Humic and Fulvic Acid Characterization**[a]

| | %C | %H | %N | 0−100 ppm (aliphatic) | 100−160 ppm (aromatic) | 160−190 ppm (carboxyl) | 190−230 ppm (carbonyl) |
|---|---|---|---|---|---|---|---|
| | | | | $^{13}$C NMR regions (%) | | | |
| AGHA | 56 | 4.1 | 2.6 | 72.5 | 21.0 | 4.34 | 2.17 |
| LGHA | 69 | 6.8 | 0.7 | 76.9 | 15.4 | 0.80 | 6.92 |
| ZGHA | 55 | 4.0 | 0.5 | 62.1 | 26.7 | 1.86 | 9.32 |
| ChGHA | 58 | 2.7 | 0.7 | 10.2 | 75.1 | 3.48 | 11.2 |
| ABHA | 45 | 2.5 | 0.8 | 38.2 | 49.6 | 11.8 | 0.38 |
| LBHA | 38 | 1.9 | 0.6 | 30.4 | 55.6 | 3.95 | 10.0 |
| ZBHA | 43 | 2.5 | 0.4 | 41.3 | 45.9 | 3.31 | 9.50 |
| WPFA | 54 | 4.2 | 1.1 | 44.2 | 37.2 | 13.7 | 4.87 |
| SRFA | 53 | 4.4 | 0.8 | 59.5 | 20.8 | 15.5 | 4.17 |

[a] AGHA: Aldrich gray humic acid. LGHA: IHSS leonardite gray humic acid. ZGHA: Czech soil gray humic acid. ChGHA: Chinese lignite gray humic acid. ABHA: Aldrich brown humic acid. LBHA: IHSS leonardite brown humic acid. ZBHA: Czech soil brown humic acid. WPFA: Waskish Peat fulvic acid. SRFA: Suwannee River fulvic acid.

**TABLE 2: Ultrafiltration of Aldrich Humic and Waskish Peat Fulvic Fractions**

| % mass | GHA | GHA$_{AcOH}$ | BHA | BHA$_{AcOH}$ | BHA$_{NaCl}$ | FA | FA$_{AcOH}$ | FA$_{NaCl}$ |
|---|---|---|---|---|---|---|---|---|
| >0.45 µm | 26 | 59 | 0.2 | 0.5 | 2.0 | 1.9 | 3.4 | 6.7 |
| 100 kDa to 0.45 µm | 14 | 2.4 | 0.1 | 30 | 27 | 1.5 | 2.4 | 1.6 |
| 50−100 kDa | 33 | 11 | 26 | 1.7 | 5.5 | 17 | 0.5 | 1.8 |
| 10−50 kDa | 6.7 | 1.0 | 6.3 | 0.2 | 8.2 | 33 | 3.1 | 6.7 |
| 5−10 kDa | 20 | 12 | 58 | 6.6 | 31 | 26 | 11 | 25 |
| <5 kDa | 0.5 | 15 | 9.6 | 61 | 23 | 14 | 63 | 48 |
| <1 kDa | | | | | | 3.0 | 17 | 9.9 |

of relatively weak intermolecular forces.[6,9] On the other hand, this treatment is accompanied by an important increase in IS (up to 2 M), which can cause the aggregation−flocculation of macromolecules.[10] To assess whether the variations in molecular size distributions and fluorescence properties caused by AcOH treatment are due to disaggregation or to a reduction in size linked to high IS values, we also studied the size distribution of the different HS alkaline systems subjected at high IS using NaCl (2 M).

AcOH treatment was carried out as follows: Solutions of 0.8 g L$^{-1}$ of organic carbon (OC) of each of the samples were prepared in deionized Milli Q water. The pH was fixed at 10 with NaOH, and IS at 0.015 M with NaCl. The solutions were divided into two portions: one of them was directly used in the analytical determinations with no further treatment (alkaline samples: GHA, BHA, and FA), and the other was acidified to pH 2 with acetic acid, left 30 min to stabilize, realkalinized to the initial pH (pH 10) with 12.5 M NaOH, and left an average of 2 h before starting the analytical studies. In the case of BHA and FA, a fraction of the alkaline samples were treated with NaCl to reach an IS of 2 M.

**Physicochemical Characterization of HS Samples.** *Elemental Analysis.* The carbon, hydrogen, and nitrogen contents of the lyophilized samples were analyzed in duplicate by a LECO CHN 900 analyzer. The oxygen content was determined by difference.

*$^{13}$C NMR.* Solid-state $^{13}$C NMR spectra were obtained on a Bruker Avance AV-400WB (9.4T) spectrometer at 100.47 MHz using the cross-polarization magic-angle spinning (CPMAS) technique, with a spinning speed of 12 kHz, 90° pulse width, 30-ms acquisition time, and 4.0-s delay.

*Ultrafiltration.* The samples of the different humic fractions used in the study were fractionated using a UF cell (Amicon model 8200) under inert atmosphere (N$_2$). Five UF membranes (Millipore) of molecular weight cutoff (MWCO) of 100 [poly-(ether sulfone)], 50 [poly(ether sulfone)], 10 [poly(ether sulfone)], 5 [poly(ether sulfone)], and 1 (cellulose) (only for FA) kDa were used. First, the solutions were filtered through the membrane with the largest pore size, and the process was halted

when the volume of the retained solution had been reduced to 5−10% of the initial solution volume. The filtrate was introduced in the UF cell and filtered through another membrane of lower MWCO. This operation was repeated for each membrane. The mass percentages of the different fractions were evaluated by measuring absorption differences at 400 nm between consecutive filtrates during the process. The calibration made previously showed a high correlation between the OC content (measured by TOC) and the absorption at 400 nm ($r^2 = 0.93$, $n = 20$).

*Transmission Electron Microscopy.* TEM images were obtained using an electron microscope (EM 10 CR, Zeiss equipment). Perforated support-covered grids were coated with a thin film of Formvar 15/95 (Fluka). After that, the grid was immersed in chloroform to dissolve the film of plastic, leaving only a carbon film covering the grid. A drop of 20 µL of sample was deposited on the grid, and afterward a drop of 2% phosphotungstic acid was added as a negative staining solution. The excess was drained off, and the grid was allowed to dry before observation in the TEM.

*Confocal Laser Scanning Microscopy.* A confocal laser-scanning microscope (Zeiss LSM 510 META) and a Plan-Apochromat 63×/1.4 oil objective were used for confocal imaging experiments. A small volume of sample solutions (at its initial concentration) was deposited onto slides, which were excited at 488 nm with an argon laser. Emission intensity was recorded at 505 nm. Data were processed using Zeiss LSM Image Browser software (version 3.5.0.223).

*Fluorescence Spectroscopy.* Synchronous fluorescence spectra were performed on a Perkin-Elmer LS50B fluorescence spectrophotometer, with the same conditions described in Peuravuori et al.[17]

## Results and Discussion

**Molecular Behavior of GHA Subjected to AcOH Treatment.** In the case of GHA, the UF study indicated that AcOH treatment caused a clear molecular coagulation−flocculation that was reflected in a marked increase in the molecular fraction
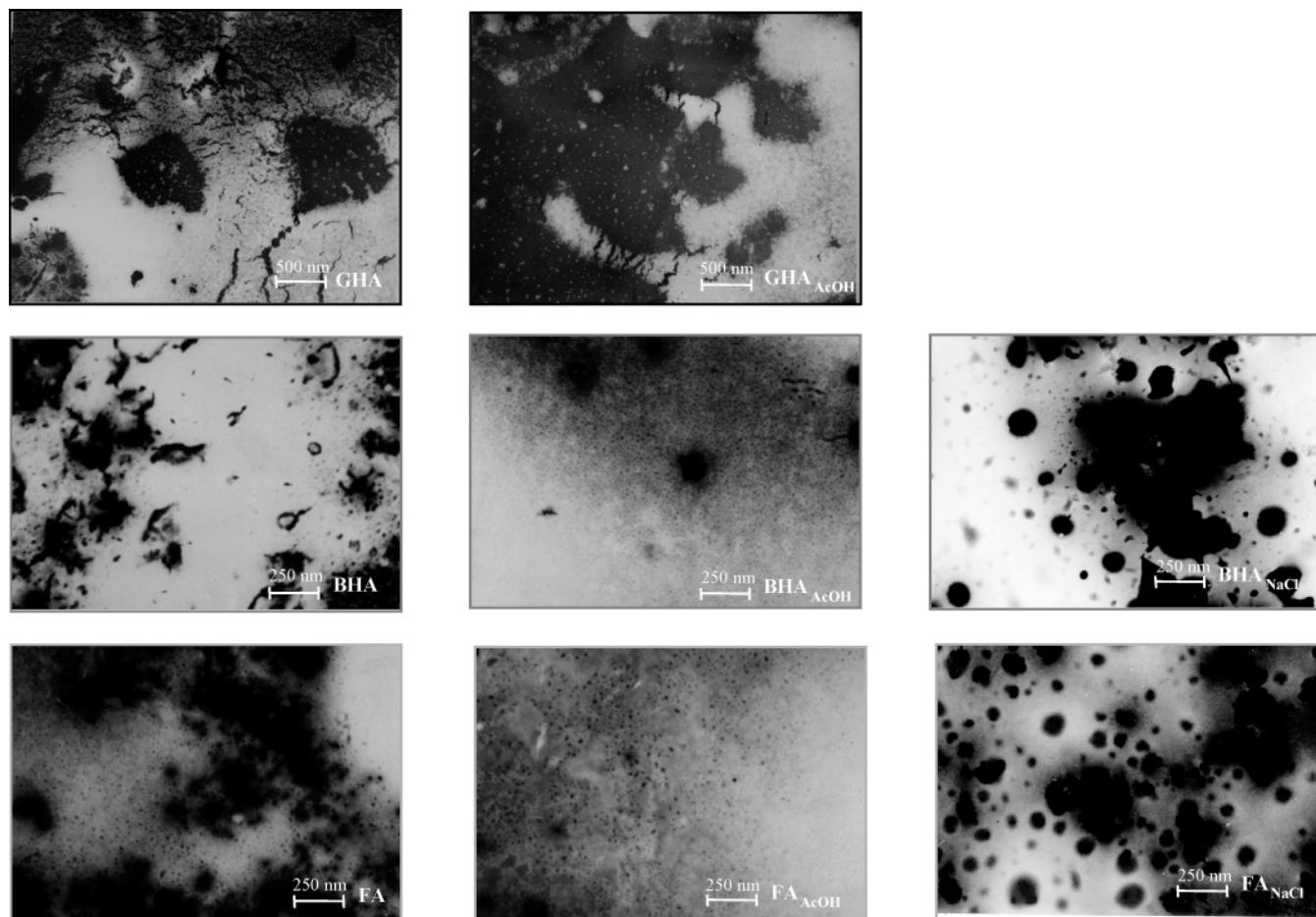
Presence of Diverse Molecular Patterns in HS

*J. Phys. Chem. B, Vol. 111, No. 35, 2007* **10579**



**Figure 1.** TEM of Czech gray humic acid fraction (GHA), brown humic acid fraction (BHA), and Suwannee River fulvic humic fraction (FA). Without treatment, acetic acid treated (AcOH), and sodium chloride treated (NaCl).
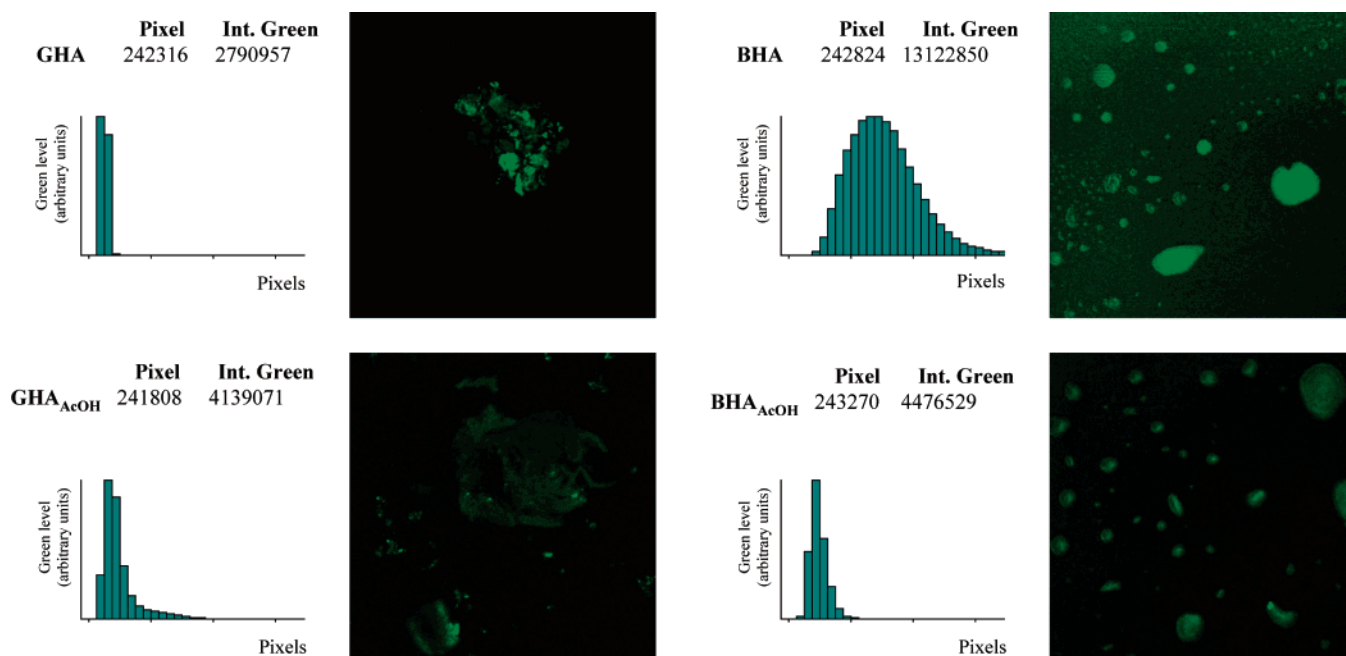


**Figure 2.** CLSM of Czech gray humic acid fraction (GHA) and brown humic acid fraction (BHA) without treatment and acetic acid treated (AcOH).

with sizes higher than 0.45 $\mu$m (from 11−63% to 40−88%) (Table 2 and Supporting Information Table SI1). This fact was also confirmed by the TEM and CLSM studies. Thus, both studies revealed that AcOH treatment of GHA samples was associated with the development of important flocculated aggregates (Figure 1 and Supporting Information Figure SI1a). Moreover, the CLSM study (Figure 2) showed the presence of at least two main types of aggregates with different relative concentrations of fluorophore groups: a population of higher aggregates (20−40 $\mu$m) that showed a relatively weak fluores-

**TABLE 3: Metal (Cu, Fe, Mn, and Zn) Distributions in the Different Fractions of HS[a]**

| sample | Cu | Fe | Mn | Zn |
|--------|-----|------|--------|--------|
| AGHA | 65 | 3991 | 61 | 60 |
| ABHA | 15 | 3192 | 16 | 10 |
| LGHA | 4.4 | 3085 | 0.2 | 6.0 |
| LBHA | 14 | 308 | <dl[b] | 4.0 |
| ZGHA | 21 | 8429 | 14 | 11 |
| ZBHA | 28 | 2974 | 3.6 | 3.2 |
| ChGHA | 4.8 | 3842 | <dl | <dl |
| WPFA | 14 | 130 | 1.7 | 12 |
| SRFA | <dl | 43.4 | <dl | 5.9 |

[a] AGHA: Aldrich gray humic acid. ABHA: Aldrich brown humic acid. LGHA: IHSS leonardite gray humic acid. LBHA: IHSS leonardite brown humic acid. ZGHA: Czech soil gray humic acid. ZBHA: Czech soil brown humic acid. ChGHA: Chinese lignite gray humic acid. WPFA: Waskish Peat fulvic acid. SRFA: Suwannee River fulvic acid. Concentration in milligrams per kilogram. [b] <dl = lower than detection limit.

cence and a population of smaller aggregates (0.5–2 $\mu$m) that showed more intense fluorescence. Likewise, CLSM images (Figure 2) suggest an aggregation dynamics consisting of the association of the smaller (more fluorescent) aggregates with the higher (less fluorescent) aggregates. Likewise, the CLSM study revealed a significant increase in fluorescence intensity in AcOH-treated GHA systems that could be associated with the increase in molecular size associated with aggregation. Thus, molecular aggregation could favor the interaction between fluorophores, which in turn is associated with a shift of maximum fluorescence to higher wavelengths.

The UF study also indicated that the AcOH treatment of GHA samples caused an increase in the molecular fraction lower than 5 kDa (from 0.4–2.2% to 6.6–23%), which might be the consequence of a disaggregating effect and/or a molecular size reduction caused by high IS. The presence of disaggregation processes was confirmed by the TEM study, which showed the presence of a minor population of relatively small molecules in AcOH-treated GHA samples (Figures 1 and SI1a). In any case, UF results indicated that disaggregation was clearly less important than aggregation in AcOH-treated GHA samples.

As to the chemical nature of the aggregates formed as the consequence of IS increase, a number of studies have shown that in macromolecular systems the increase in the background electrolyte decreases intermolecular and intramolecular repulsion forces favoring molecular contraction and the exclusion of solvating solvent molecules from the molecular domain. This decrease in solubility is also associated with the development of short-range intermolecular and intramolecular attractive electrostatic interactions, which give rise to coagulation or flocculation (precipitation).[2,18,19]

**Molecular Behavior of BHA Subjected to AcOH and NaCl Treatments.** In the case of BHA, the UF study revealed that the AcOH treatment caused both aggregating and, possibly, disaggregating effects (Tables 2 and SI1). The aggregating effect was reflected in the increase in the size fractions corresponding to 100 kDa to 0.45 $\mu$m and >0.45 $\mu$m, while the possible presence of a disaggregating effect was reflected in the marked increase in the size fraction lower than 5 kDa (from 1.9–9.6% to 25–61%). This conclusion was also supported by the results obtained in NaCl-treated BHA samples, which did not present significant increases in the size fraction lower than 5 kDa. The presence of both aggregation and disaggregation was confirmed by TEM. As can be observed in Figures 1a and SI1b, both molecular aggregates and disaggregated small molecules are present in BHA samples treated with AcOH. In NaCl-treated
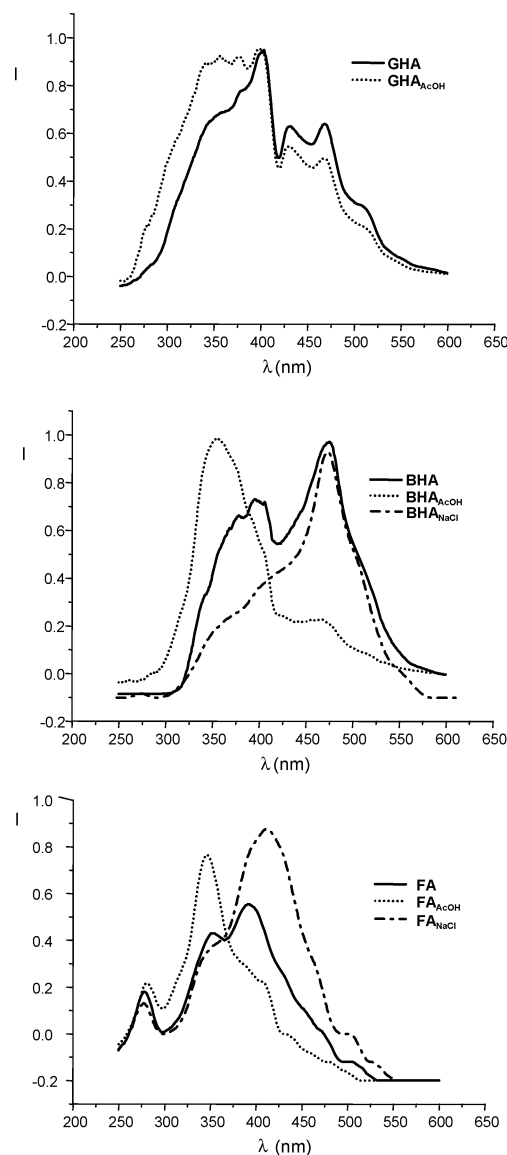


**Figure 3.** SFS of Aldrich gray humic acid fraction (GHA), brown humic acid fraction (BHA), and Waskish Peat fulvic humic fraction (FA). Without treatment, acetic acid treated (AcOH), and sodium chloride treated (NaCl).

samples, however, only the presence of molecular aggregates was observed (Figure 1). In this sense, CLSM studies also showed disaggregation in AcOH-treated samples reflected in the significant decrease in fluorescence intensity (Figure 2), which could be due to both molecular size decrease and the disappearance of interactions interfluorophores. In principle, these results indicate that in BHA both aggregation and disaggregation processes are present as a result of AcOH treatment.

Regarding the chemical nature of the forces involved in the molecular aggregates present in BHA, both hydrophobic effects and ionic effects could play an important role.[6] In this sense, other studies indicated that the importance of metal bridges in the formation of stable aggregates might be significant.[20,21] In our samples, we found significant concentrations of Fe in BHA, although these were lower than those of GHA (Table 3). The fact that in GHA the presence of stable aggregates was less important than in BHA suggests that Fe bridges could be an additional factor but probably not the main factor. In any case, a possible role of metal bridges in the formation of stable aggregates in BHA cannot be ruled out.
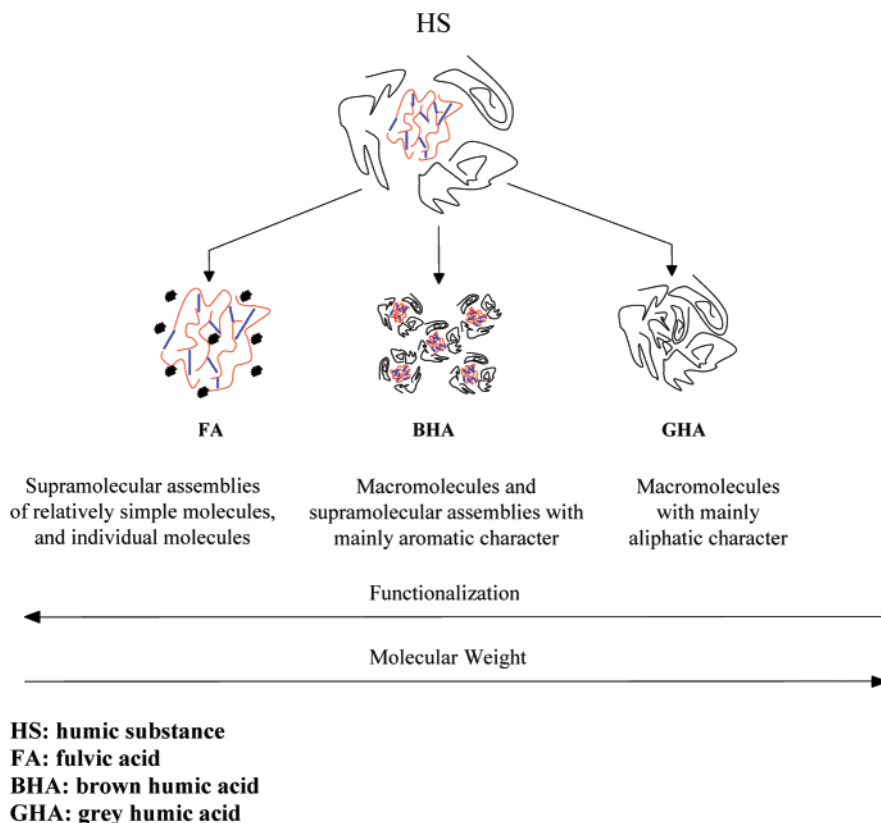
Presence of Diverse Molecular Patterns in HS

*J. Phys. Chem. B, Vol. 111, No. 35, 2007* **10581**



**Figure 4.** Main molecular patterns associated with the principal fractions of the studied humic substances in solution.

**Molecular Behavior of FA Subjected to AcOH and NaCl Treatments.** In the case of FA samples, the UF study clearly showed that AcOH treatment caused a remarkable increase in the size fraction lower than 5 and 1 kDa (from 18−34% to 89−90%), thus suggesting the important presence of molecular disaggregation processes (Tables 2 and SI1). The results obtained in NaCl-treated samples, however, indicate that in the case of the WPFA the increase in the lower size molecular fractions might be emphasized by a decrease in molecular size caused by high IS. In the case of the SRFA, however, the disaggregating effect is clearer. The development of disaggregation processes was confirmed in the TEM study, which showed the presence of a dominant population of small molecules in AcOH-treated FA samples (Figures 1 and SI1c) but not in NaCl-treated FA samples (we did not obtain clear results in the CLSM study because of the low molecular size of FA samples). These results, therefore, indicate the predominant presence of stable aggregates in FA, as well as the possible presence of an important fraction of individual molecules.

**Effect of Aggregation−Disaggregation Processes on the Fluorescence Pattern of HS Samples.** As suggested by the CLSM study, these phenomena of aggregation and/or disaggregation experienced by the selected AcOH-treated HS fractions were also reflected in important changes in the SFS spectra. As can be observed in Figure 3, in the case of BHA, and to a lesser extent in FA, disaggregation caused by AcOH treatment was associated with a clear shift of maximum fluorescence intensities to lower (shorter) wavelengths ($\lambda$). As expected, this effect was less important in GHA. These results suggest that the fluorescence observed at longer $\lambda$ in the alkaline samples (nontreated with AcOH) of BHA and FA, but not so clear in GHA, may be caused by a singular interaction between fluorophores (i.e., phenolic groups) and is not directly related to true bond conjugation. These effects may be intramolecular (local effects associated with conformational changes) and/or intermolecular, associated with molecular association. Indeed, the results obtained in AcOH-treated samples suggest that FA and BHA might contain simpler conjugated structures than GHA. On the other hand, these results also suggest that the decrease in fluorescence intensity observed in the CLSM study (Figure 2), principally for BHA, may be mainly caused by a shift in the fluorescence intensity to lower wavelengths associated to disaggregation.

## Conclusions

In summary, this study suggests the presence of the following molecular patterns in the studied HS (Figure 4). In GHA, the clear molecular aggregation−flocculation associated with the increase in IS caused by AcOH treatment, along with the absence of significant disaggregation, suggests the main presence of macromolecules (macromolecular pattern). In the case of FA, however, the clear disaggregation caused by AcOH treatment and the absence of significant aggregation suggests the presence of stable aggregates (supramolecular assemblies) along with individual molecules (supramolecular pattern). In the third HS fraction studied, BHA and both the macromolecular pattern and the supramolecular pattern seem to coexist. Regarding the structural features of these main fractions, the studied GHA presented a dominant aliphatic character, whereas the BHA studied presented more aromaticity. On the other hand, UF studies suggested an order of average molecular weight of GHA > BHA > FA. Likewise, the elemental composition and ^{13}C NMR indicated a functionalization of FA > BHA > GHA (Figure 4).

Likewise, the results obtained in the SFS study also indicate that the current interpretation of HS fluorescence spectra needs revision.

**Supporting Information Available:** UF data (Table SI1), TEM images (Figures SI1a, SI1b, and SI1c), SFS spectra (Figures SI2a, SI2b, and SI2c), and solid-state $^{13}C$ NMR spectra (Figure SI3). This material is available free of charge via the Internet at http://pubs.acs.org.

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