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# Aggregation Kinetics and Mechanism of Humic Acid Reaction with Cs<sup>+</sup> and Co<sup>2+</sup> Metal Ions Using Batch Techniques

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**Abstract:** Humic substances have a potential role in the fate and transport of toxic metal ions in the environment due to their colloidal characteristics and abundant surface functional groups. Batch techniques (DLS, EPM, FT-IR and fluorescence EEM) were developed to assess the aggregation mechanisms of humic acid (HA) reacting with Cs<sup>+</sup> or Co<sup>2+</sup> electrolyte ions in this work. The kinetic experimental results indicated that a much lower Co<sup>2+</sup> ion concentration (0.03–1.50 mmol/L) induced rapid aggregation of HA compared to that of Cs<sup>+</sup> (3.0–15 mmol/L), and the divalent Co<sup>2+</sup> ion was far more effective in enhancing HA aggregation than monovalent Cs<sup>+</sup>. The aggregation kinetics of HA were also found to be pH-dependent, and a much lower pH condition (pH 5.0) caused more rapid aggregation (the largest hydrodynamic diameter of ~3000 nm) compared to those at pH 7.0 (the largest hydrodynamic diameter of ~2000 nm). Positively charged metal ions in the solution can lower the electrostatic repulsive force between HA molecules through charge neutralization, thus leading to the rapid aggregation of HA aggregates. Furthermore, the carboxylic and phenolic groups on the HA surface were also involved in the aggregation reaction to form inner complexes and accelerate the aggregation process.

Keywords: humic acid; aggregation; metal ions; kinetics; spectrum analysis

## 1. Introduction

In many cases, most of the organic matter found in natural waters is derived from natural sources. Humic acid (HA), as an important component of natural organic matter, is very widespread in many environments (soil, sediment, surface water and ground water) [1–3]. HA has colloidal properties and reactive surface groups such as hydroxyl, methoxy and amide that can form complexes with the inorganic or organic components of many pollutants [4]. Many research works have been published about the aggregation behaviors of HA in the natural environment. Alvarez-Puebla et al. found that the stability and aggregation behaviors of HA seem to be more affected by the ionization of the phenolic groups than by carboxylic acid groups due to the generation of H-bonds [5]. Terashima et al. reported that micelle-like aggregation and the interfacial adsorption of HA were substantially facilitated in the acidic region [6]. HA also contains metal-binding functional groups (carboxyl, phenol, amino, etc.) that influence precipitation kinetics by forming dissolved complexes with metals and lowering the mineral saturation index [7,8]. The presence of HA in soil and water environments has a great influence on the fate and transport of contaminants and pollutants. Further research is encouraged to obtain knowledge of the interaction mechanisms between HA and metal ions, which is critical for researchers to predict the chemical reactivity, environmental fate and behaviors of inorganic and/or organic pollutants in natural waters [9].

The influence of different environmental conditions, such as pH, ionic strength, background electrolytes, inorganic/organic matter and other parameters concerned with the



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**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). physical situation of the system, on the interactions of HA with other chemicals has been studied extensively [10–12]. Computational molecular modeling analysis performed by Kalinichev et al. indicated that Ca<sup>2+</sup> ions were associated with 35–50% of the HA carboxylic groups at near-neutral pH and pointed to a strong preference for the stability of bidentate-coordinated contact ions [13]. Omar et al. reported that the anionic charges carried by aquatic humic substances were found to play a major role in the aggregation and disaggregation of ZnO nanoparticles. Additionally, environmental aquatic concentration ranges of humic acids largely modified the stability of aggregated or dispersed ZnO nanoparticles [14]. However, for polyethylene glycol terephthalate (PET) nanoparticles, recent research results confirmed that the aggregation of PET nanoparticles was significantly inhibited by the presence of HA in an aqueous environment [15]. Significant new advances have been made recently in the understanding of the colloid aggregation process of HA due to the presence of metal ions and other materials. However, the mechanism of the occurrence and development of these aggregation phenomena is still not fully understood.

Due to the rapid development of the industry and the associated production of toxic waste, especially heavy-metal ions, there is an increasing awareness that the leakage of toxic metal ions into the natural environment can cause long-term environmental problems [16,17]. The disposal of highly contaminated industrial wastewaters containing heavy-metal ions can lead to a very big imbalance in the ecosystem due to their adverse effects on aquatic life, human beings and animals [18]. In the safety assessment of toxic metal ion disposal, it is extremely important to assess the migration behavior of metal ions in an aquatic environment. The migration behavior of toxic metal ions under various conditions has been studied extensively, and is an important parameter for assessing the performance of a geological repository [19–21]. Recent research found that colloids in the liquid phase act as mobile vehicles to enhance the migration of metal ions and affect the reduction process of pollutants in four ways: they enhance dissolution, increase adsorption, compete for surface active sites, and act as an electron transfer medium in ground water [22]. Therefore, the presence of HA colloid in an aquatic environment may significantly modify the transport behavior of metal ions because they have a strong tendency to complex the metal ions [22,23]. The interaction processes between HA and metal ions, such as complexation or aggregation, have a great impact on the transport and environmental fate of metal ions in nature [24–27]. Additionally, comparative analysis of the main physico-chemical conditions, such as solution pH, ionic strength, the presence of a competing metal ion and metal ion concentration, has shown different complexation mechanisms between HA and metal ions [28]. Considerable efforts have focused on the influence of HA on the transport of metal ions in the natural environment, but still, no conclusive generalization has been drawn, primarily because of the complexity of the interactions involved.

With the rapid development of industry, substantial amounts of metal ions in industrial wastewaters may be released into the environment. Among these metal ions, Cs<sup>+</sup> and  $Co^{2+}$  are common metal ions that are present in industrial wastewaters [29,30]. HA has substantial chelating properties for metal ions, and can therefore considerably affect the migration behavior of metal ions in the soil, water and ocean. Therefore, owing to their abundance and toxicity, they were chosen as representatives to study the properties of HA and the potential role of colloidal materials in enhancing the migration of metal ions in the natural environment. The aggregation behaviors of HA due to the presence of common cations has been commonly studied in previous research. However, so far, most studies concerning HA aggregation have been conducted under equilibrium or near-equilibrium conditions, and the effects of the combined factors, such as solution pH, cation type, cation valence and electrolyte concentrations, on HA aggregation are not thoroughly understood yet. The specific objectives of this work were as follows: (1) dynamic light scattering (DLS) measurement was used to explore the aggregation kinetics and hydrodynamic diameter changes in HA aggregates; (2) a variety of techniques, including electrophoretic mobility (EPM), fluorescence excitation-emission matrix (EEM) and Fourier transformed infrared (FT-IR), were applied to elucidate the mechanism of metal ion–HA interactions; (3) the attachment efficiency of HA aggregates was measured under combined experimental conditions. The present study will offer a contribution to the understanding of the effect of HA on the transport and fate of metal ions in the natural environment.

## 2. Materials and Methods

## 2.1. HA Solution and Reagents

Commercial HA was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and purified by following the method by Wang et al. [31]. HA stock solutions were prepared by dissolving the purified HA in Milli-Q water. ACS-grade electrolyte (CsNO<sub>3</sub> and Co(NO<sub>3</sub>)<sub>2</sub>) stock solutions were prepared and filtered using 0.1-µm alumina syringe filters before use. All solutions used in this work were adjusted to pH  $7.0 \pm 0.1$  or  $5.0 \pm 0.1$  using NaOH and HNO<sub>3</sub> as needed.

### 2.2. DLS, EPM, EEM and FT-IR Spectroscopic Measurements

The hydrodynamic diameter of HA aggregates was monitored at 25 °C using DLS (Malvern Zetasizer NS, Malvern, UK) of the incident light ( $\lambda$  = 663 nm) at 90° [32].

The EPM of the HA aggregates was analyzed using a Malvern Zetasizer Nano ZS90 instrument at different Cs<sup>+</sup> or Co<sup>2+</sup> concentrations as a function of pH (pH 5.0 and 7.0) at 25 °C. At least three replicate measurements were taken for each solution chemistry.

Fluorescence EEM spectra were measured using the spectrofluorometer Hitachi F-7000 FL (Tokyo, Japan) with a  $1 \times 1$  quartz cuvette. The scanning excitation (EX) and emission (EM) were gathered from 300 to 500 nm and from 250 to 450 nm at 5 nm increments, respectively. The spectra were recorded at a scan rate of 1200 nm min<sup>-1</sup> using EM and EX slit bandwidths of 5 nm [33].

FT–IR (Nicolet-6700, Thermo Nicolet, Waltham, MA, USA) was used to analyze the changes in the functional groups of  $HA-Cs^+$  and  $HA-Co^{2+}$  aggregates in the range of 650-4000 cm<sup>-1</sup>. Each spectrum was obtained through 256 scans.

## 2.3. Aggregation Kinetics and Attachment Efficiency ( $\alpha$ ) Calculation

DLS measurement was used to investigate the aggregation kinetics of HA under various solution conditions: monovalent (Cs<sup>+</sup>) or divalent (Co<sup>2+</sup>) metal ions with 50 mg/L HA. For the aggregation experiments, a certain amount of electrolyte stock solution (Cs<sup>+</sup> or Co<sup>2+</sup>) was introduced into the HA solution to obtain the desired metal ion concentration in a vial. The mixture was vortexed for 3 s at room temperature, then, immediately introduced into the light scattering unit to start the DLS measurement. The hydrodynamic diameter of the aggregation changes was monitored at 5 s intervals and required a time period of 0.5–2 h to ensure an adequate increase in the particle size, which allowed us to obtain more accurate information on the aggregation kinetics.

The aggregation attachment efficiency ( $\alpha$ ) was used to characterize the aggregation kinetics of HA aggregates, and it was calculated by normalizing *K*a by the aggregation rate constant measured under diffusion-limited (fast) conditions [34]:

$$\alpha = \frac{1}{W} = \frac{Ka}{Ka_{,fast}} = \frac{\frac{1}{N_0} (\frac{d < D_h > (t)}{dt})_{t \to 0}}{\frac{1}{N_0, fast} (\frac{d < D_h > (t)}{dt})_{t \to 0, fast}}$$
(1)

$$Ka \propto \left(\frac{d < D_{\rm h} > (t)}{dt}\right)_{t \to 0} \tag{2}$$

where *W* is the stability ratio, *K*a is defined as the aggregation rate,  $D_h$  is the hydrodynamic diameter of the aggregates and  $N_0$  is the concentration of HA [35].

## 3. Results and Discussion

## 3.1. Aggregation Kinetics of HA in the Presence of Cs<sup>+</sup> or Co<sup>2+</sup> Metal Ions

The relationship between the hydrodynamic diameter of HA and the aggregation time under different concentrations of  $Cs^+$  and  $Co^{2+}$  metal ions at pH 5.0 and 7.0 are presented in Figure 1. At low metal ion concentrations (Cs<sup>+</sup>: pH 5.0, 3.0 and 5.0 mmol/L; Co<sup>2+</sup>: pH 5.0, 0.03, 0.05 and 0.07 mmol/L; Co<sup>2+</sup>: pH 7.0, 0.10 and 0.15 mmol/L), no obvious HA aggregation can be observed due to the addition of metal ions in the solution, and the hydrodynamic diameter maintains a constant value of 180 nm. The addition of low concentrations of metal ions in the HA solution has negligible effects on the stability of the HA aggregates. However, as the metal ion concentration further increased, the hydrodynamic diameter of HA aggregates gradually increased with the aggregation time after adding metal ions in the HA solution, except  $Cs^+$  at pH 7.0. Taking the  $Cs^+$  metal ion (Figure 1A, pH 5.0) as an example, when the concentration of the  $Cs^+$  was 7.0 and 7.5 mmol/L, the hydrodynamic diameter of the HA aggregates began to increase slowly at the aggregation time of 2000 s, and finally reached a maximum of 210 and 240 nm, respectively, over enough time (4500 s). However, once the  $Cs^+$  concentration exceeded a certain value of 8.0 mmol/L, the hydrodynamic diameter of the HA aggregates changed significantly, increasing sharply from 238 to 620 nm with increasing  $Cs^+$  concentration. Moreover, at pH 7.0 (Figure 1B), with the increase in  $Cs^+$  concentration, the hydrodynamic diameter of the HA aggregates also increased obviously at given Cs<sup>+</sup> concentrations; however, the hydrodynamic diameter kept a constant value (5.0 mmol/L, 187 nm; 7.0 mmol/L, 189 nm; 10 mmol/L, 224 nm; 15 mmol/L, 258 nm; 20 mmol/L, 305 nm; 25 mmol/L, 375 nm and 30 mmol/L, 375 nm) through the whole aggregation time, and no obvious increasing trend can be observed during the whole aggregation process.

In the case of the  $Co^{2+}$  metal ion, a similar experimental phenomenon can be observed in Figure 1C,D. However, the concentration of  $Co^{2+}$ -induced HA aggregation was much lower than that of Cs<sup>+</sup>, as presented in Figure 1. Additionally, the divalent metal ion (Co<sup>2+</sup>) shows a greater impact (the largest hydrodynamic diameter of 3000 nm at pH 5.0) on HA aggregation than that of the monovalent metal ion (Cs<sup>+</sup>) (the largest hydrodynamic diameter of 620 nm at pH 5.0). In addition to the metal ion concentration, the metal ion type also had significant influence on HA aggregation [36]. This phenomenon has also been observed during the other metal (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, or Mg<sup>2+</sup>)-induced aggregation processes of HA [15]. High-valent metal ions were capable of neutralizing more negative charges on the HA than monovalent metal ions, and thus, led to a much larger aggregate size of HA in the solution [15,31].

The aggregation kinetics of HA were also found to be pH dependent. In the case of  $Cs^+$  and  $Co^{2+}$  metal ions, a much lower metal ion concentration ( $Cs^+$ : 3.0–15 mmol/L,  $Co^{2+}$ : 0.03–1.50 mmol/L, respectively) at pH 5.0 (Figure 1A,C) induced a rapid aggregation of HA aggregates compared to that at pH 7.0 (Cs<sup>+</sup>: 5.0–30 mmol/L, Co<sup>2+</sup>: 0.1–3.0 mmol/L, respectively) (Figure 1B,D). Additionally, a much higher hydrodynamic diameter of HA aggregates can also be obtained at pH 5.0. Wei et al. studied the stability and aggregation behaviors of illite colloids in an aquatic environment and found similar experimental results in their work. The mean hydrodynamic size of the illite colloids increased as the pH decreased, and the aggregation process of the colloids was also favorable at a low pH [37]. As reported by other researchers, a large number of reactive surface groups such as carboxyl, hydroxyl, methoxy and amide can be found on the HA surface, and the dissociation degree of these functional groups can be significantly influenced by the solution pH [38]. At acidic conditions (pH 5.0 in this work), the formation of hydrogen bonds between the protonated acidic functional groups of HA holds the molecules together, because hydrogen bonds provide fairly strong linkage since they involve a gain in energy from 10 to 20 kJ mol<sup>-1</sup> in comparison with van der Walls bonds [39]; thus, a certain number of metal ions in a low concentration range would induce rapid aggregation of HA molecules and form large aggregate sizes. However, under neutral or alkaline conditions (such as pH 7.0 in this work), the dissociation of the surface hydroxyl groups on the HA surface and the release of

protons from the adjacent hydroxyl groups in the solution would prevent the formation of hydrogen bonds and could leave the surface molecules more susceptible to detachment [33], leading to a relatively lower aggregation rate.



**Figure 1.** The hydrodynamic diameter changes in HA aggregates (50 mg/L) as a function of metal ion concentration under different pH conditions. ((**A**):  $Cs^+$  ion with different concentrations at pH 5.0; (**B**):  $Cs^+$  ion with different concentrations at pH 7.0; (**C**):  $Co^{2+}$  ion with different concentrations at pH 5.0; (**D**):  $Co^{2+}$  ion with different concentrations at pH 7.0).

#### 3.2. EPM and Attachment Efficiency Studies

To determine the effects of solution pH and metal ion concentrations on the electrokinetic properties of HA aggregates, the EPM of HA was studied, and the results are shown in Figure 2A,B. The experimental results indicated that the HA aggregates were negatively charged over the entire metal ion concentration range studied, and solution pH had a great influence on the electrokinetic properties of the HA aggregates. At pH 5.0, the EPM of the HA aggregates was negative (Cs<sup>+</sup>:  $-3.02 \times 10^{-8}$  to  $-1.69 \times 10^{-8}$  m<sup>2</sup>/Vs; Co<sup>2+</sup>:  $-1.74 \times 10^{-8}$  to  $-0.92 \times 10^{-8}$  m<sup>2</sup>/Vs), and became more negative (Cs<sup>+</sup>:  $-3.21 \times 10^{-8}$  to

 $-1.23 \times 10^{-8} \text{ m}^2/\text{Vs}$ ;  $\text{Co}^{2+}:-1.84 \times 10^{-8}$  to  $-0.61 \times 10^{-8} \text{ m}^2/\text{Vs}$ ) as the pH was increased to 7.0 over a wide range of Cs<sup>+</sup> and Co<sup>2+</sup> concentrations; this confirmed the presence of numerous functional groups in the HA structure and the strong dependence of EPM on solution pH [40]. Additionally, one can also find that with increasing metal ion concentrations, the EPM of HA aggregates also became less negative due to an increase in charge–screening or charge–neutralization effects [8].



**Figure 2.** EPM and  $\alpha$  of HA (50 mg/L) aggregates as a function of Cs<sup>+</sup> or Co<sup>2+</sup> concentration at pH 5.0 and 7.0 (measurements were taken at 25 °C). ((**A**): EPM changes of HA in the presence of Cs<sup>+</sup>; (**B**): EPM changes of HA in the presence of Co<sup>2+</sup>; (**C**):  $\alpha$  changes of HA in the presence of Cs<sup>+</sup>; (**D**):  $\alpha$  changes of HA in the presence of Co<sup>2+</sup>).

The attachment efficiencies of HA aggregates are presented as a function of metal ion concentrations at pH 5.0 and 7.0 in Figure 2C,D. The changing trends of the  $\alpha$  of HA aggregates, due to the addition of either Cs<sup>+</sup> or Co<sup>2+</sup> to the solution, are remarkably similar. In order to characterize the variation tendency of the  $\alpha$  of HA aggregates, the observed aggregation behavior was divided into two regimes: (1) In regime I, with the increase in metal ion concentrations, the  $\alpha$  of the HA aggregates increased gradually from ~0 to the maximum value ( $\alpha = 1$ ) at Cs<sup>+</sup> = 15.2 and 24.6 mmol/L, and Co<sup>2+</sup> = 1.825 and 2.65 mmol/L, at pH 5.0 and 7.0, respectively. Correspondingly, the EPM of the HA aggregates increased from  $-3.02 \times 10^{-8}$  ( $-1.74 \times 10^{-8}$ ) to  $-1.69 \times 10^{-8}$  ( $-0.92 \times 10^{-8}$ ) m<sup>2</sup>/Vs and  $-3.21 \times 10^{-8}$  ( $-1.84 \times 10^{-8}$ ) to  $-1.23 \times 10^{-8}$  ( $-0.61 \times 10^{-8}$ ) m<sup>2</sup>/Vs for Cs<sup>+</sup> (for Co<sup>2+</sup>)

at pH 5.0 and 7.0 in the metal ion concentration range, respectively. At the inflection points, the  $\alpha$  value reached the maximum value of 1, and accordingly, the EPM increased to certain values. These experimental data showed that the addition of positively charged metal ions in the solution could neutralize the negatively charged HA surface and lower the electrostatic repulsive force between HA molecules, thus leading to the rapid aggregation of HA aggregates [41]. (2) in regime II, the  $\alpha$  almost remained constant (~1), which meant the aggregation rate of the HA aggregates reached a maximum at high metal ion concentrations (Cs<sup>+</sup> > 15.2 mmol/L and Co<sup>2+</sup> > 1.82 mmol/L at pH 5.0, and Cs<sup>+</sup> > 24.6 mmol/L and Co<sup>2+</sup> > 2.65 mmol/L at pH 7.0), and the  $\alpha$  was unaffected by a further increase in the metal ion concentration. At high metal ion concentrations, the repulsive energy barrier between the HA molecules was completely screened and resulted in diffusion-limited aggregation (DLCA), as confirmed by the lower EPM (Figure 2A,B) of the HA aggregates [42].

The Derjaguin-Labdau-Verwey-Overbeek (DLVO) theory defines the critical coagulation concentration (CCC) value as the minimum electrolyte concentration that balances the attractive and repulsive potential energies between colloidal particles, and it is also considered one of the most important parameters for evaluating the particles' aggregation and sedimentation [43]. The related CCC values are all marked in the intersections in Figure 2C,D. The CCC values at the  $\alpha$  inflection points were 15.2, 24.6 mmol/L for Cs<sup>+</sup> and 1.82, 2.65 mmol/L for Co<sup>2+</sup> at pH 5.0 and 7.0, respectively. The CCC values were also found to be highly dependent on the solution pH in this work. The CCC values obtained at pH 5.0 to induce HA aggregation were much lower than those at pH 7.0 ( $Cs^+ = 15.2 \text{ mmol/L}$  and  $Co^{2+} = 1.82 \text{ mmol/L at pH 5.0}, Cs^+ = 24.6 \text{ mmol/L and } Co^{2+} = 2.65 \text{ mmol/L at pH 7.0}.$ The correlation between the CCC values and solution pH was also observed for graphene oxide and hematite particles in the presence of metal ions [34,43], which may be attributed to the protonation and deprotonation processes of the surface functional groups. In addition, the CCC values obtained were 15.2 mmol/L for Cs<sup>+</sup> and 1.82 for Co<sup>2+</sup> at pH 5.0, and the concentration of the metal ions needed to induce HA aggregation was inversely proportional to the metal ions' valence state. Divalent  $Co^{2+}$  displayed a more aggressive influence on the EPM and  $\alpha$  of HA in the aqueous solution than monovalent Cs<sup>+</sup>. A behavior predicted by the rule of Schulze and Hardy was observed, according to which the higher the valence of the cations, the higher the charge–screening capability of the cations [44]. Furthermore, we also considered other interaction mechanisms besides the higher charge-screening capability of high valence metal ions, which will be discussed in detail in the following sections.

#### 3.3. FT-IR Spectral Analysis

The FT–IR spectra of HA and HA aggregates obtained at pH 5.0 and 7.0 at several selected points are presented in Figure 3. As presented in Figure 3A, pronounced peaks that belong to the functional groups of HA are located at: ~3370 cm<sup>-1</sup> (–OH stretching in protonated carboxylic acids); ~1624 cm<sup>-1</sup> (C = C stretching in aromatic rings); ~1390 cm<sup>-1</sup> (–OH deformation, C–O stretching of phenolic –OH, and COO<sup>-</sup> antisymmetric stretching); and 1038–1050 cm<sup>-1</sup> (C–O and OH stretching in alcohols) [45].

The presence of Cs<sup>+</sup> and Co<sup>2+</sup> metal ions in HA solution significantly modified the HA spectra in the intensity of the COOH and OH bands, as presented in Figure 3B,C. In the cases of Cs<sup>+</sup> = 3.0 mmol/L at pH = 5.0, Cs<sup>+</sup> = 5.0 mmol/L at pH = 7.0, Co<sup>2+</sup> = 0.03 mmol/L at pH = 5.0 and Co<sup>2+</sup> = 0.10 mmol/L at pH 7.0 (no HA aggregation occurred under these conditions, as presented in Figure 1), the typical bands of functional groups that appeared in the HA molecules showed no significant variation in either intensity or location, as presented in Figure 3B,C; this indicates that the addition of low concentrations of metal ions in HA solution has little influence on HA aggregation and molecule changes. However, as metal ion concentration further increased, for example, in the cases of Cs<sup>+</sup> = 14.0 mmol/L at pH = 5.0, Cs<sup>+</sup> = 25.0 mmol/L at pH = 7.0, Co<sup>2+</sup> = 1.3 mmol/L at pH = 5.0 and Co<sup>2+</sup> = 0.90 mmol/L at pH 7.0 (obvious HA aggregation can be observed in Figure 3), as presented in Figure 3B,C, the intensity of the band at ~3370 cm<sup>-1</sup> decreased; this indicates that the complexation

between the metal ions and -OH groups was involved in the aggregation process. Additionally, one can also find that the bands at ~1390 decreased obviously, especially in the presence of Co<sup>2+</sup> metal ions (Figure 3C), which indicates that the carboxylic and phenolic groups on the HA surface were involved in the aggregation reaction to form inner complexes and accelerate the aggregation process [46]. A previous study conducted by Ai et al. through molecular dynamic simulations also confirmed that inter-molecular bridging between metal ions and COO<sup>-</sup> groups on the HA surface played an important role in HA aggregation [47]. The spectrum changes in the HA molecules, as presented in Figure 3, provided proof for the interactions between HA and metal ions.

### 3.4. Fluorescence EEM Analysis

Figure 4 displays the three-dimensional EEM fluorescence spectra of HA (A and B) and HA aggregates (C, D, E, F, G, H, I and J) due to the presence of  $Cs^+$  and  $Co^{2+}$ metal ions at pH 5.0 and 7.0. In the absence of metal ions, each contour plot of HA at pH 5.0 and 7.0 had two main peaks corresponding to the fluorophores, i.e., peak 1 (EX/EM = 245-270 nm/425-460 nm) and peak 2 (EX/EM = 300-325 nm/410-450 nm), as marked in Figure 4A,B, respectively. The shapes of these spectra were broad, resulting from the overlapping of several peaks. According to the experimental data obtained by Liao et al. [48], peak 1 was attributed to carboxylic-like fluorophores, whereas peak 2 may be attributed to phenolic-like fluorophores [49]. The maximum value of the observed fluorescence intensity of peak 1 was always obviously higher than that of peak 2, suggesting abundant carboxyl functional groups on the HA surface. Compared to the fluorescence EEM spectra of HA (Figure 4A,B), the molecular structure of HA aggregates due to the addition of Cs<sup>+</sup> and Co<sup>2+</sup> metal ions changed obviously, as presented in Figure 4C–J, especially at high metal ion concentrations (E, F, I and J); moreover, the main fluorescence peaks showed an appreciable change in the large quenching effect of these metal ions on HA fluorescence. In the cases of C (Cs<sup>+</sup> = 3.0 mmol/L, pH = 5.0), D (Cs<sup>+</sup> = 5.0 mmol/L, pH = 7.0), G (Co<sup>2+</sup> = 0.03 mmol/L, pH = 5.0;) and H (Co<sup>2+</sup> = 0.10 mmol/L, pH = 7.0) at low metal ion concentrations, no significant HA aggregation was detected under these conditions, as shown in Figure 1. Prominent peaks (peak 1 and peak 2), without modifications in their spectral features, were observed for all of the HA aggregates compared to those of the original HA (Figure 4A,B), and the emission intensity of the two peaks suffered only slight decreases, as presented in Figure 5. However, at higher metal ion concentrations (E:  $Cs^+ = 14.0 \text{ mmol/L}$ , pH =5.0; F:  $Cs^+ = 25.0 \text{ mmol/L}$ , pH =7.0; I:  $Co^{2+} = 1.3 \text{ mmol/L}$ , pH = 5.0; and J:  $Co^{2+} = 0.90 \text{ mmol/L}$ , pH = 7.0), the metal ions' addition caused an obvious decrease in fluorescence intensities. The sharp peaks at around 440 (peak 1) and 430 nm (peak 2), with relative fluorescence intensities of ~2184 and ~2075 a.u., decreased sharply after a long aggregation time, especially for I and J in the presence of Co<sup>2+</sup> metal ions, which decreased from ~2184 and ~2075 a.u. to almost 0 a.u., as presented in Figure 5. Furthermore, it is noteworthy that the disappearance of the characteristic peaks of HA after a long aggregation time can also be observed in Figure 4I,J, indicating that HA has a strong ability for complexation to metal ions due to the presence of carboxyl and phenolic functional groups on its surface. The divalent Co<sup>2+</sup> metal ion was more effective in binding HA molecules than monovalent Cs<sup>+</sup>, which was consistent with the more aggressive influence of  $Co^{2+}$  metal ions on the EPM and  $\alpha$  of the HA, as presented in Figure 2. The different extents of the modifications observed can be ascribed to the different strength of binding between  $HA-Cs^+$  and  $HA-Co^{2+}$  as the aggregation process occurs [50]. Recent molecular dynamics simulations suggested that multivalent cations could react with the functional groups of the carboxylic and phenolic groups of HA, forming a bidentate complex during the aggregation process, but for monovalent cations, charge neutralization is the main mechanism of aggregation [13,51]. The FT–IR and fluorescence EEM spectrum analysis in this work also confirmed the simulation.



**Figure 3.** FT–IR spectra of HA before (**A**) and after (**B**,**C**) interaction with  $Cs^+$  or  $Co^{2+}$  metal ion after long aggregation time at pH 5.0 and 7.0.



**Figure 4.** Fluorescence EEM spectra of HA and HA aggregates after long aggregation time due to the addition of Cs<sup>+</sup> or Co<sup>2+</sup> metal ion: (**A**) HA—pH 5.0; (**B**) HA—pH 7.0; (**C**) Cs<sup>+</sup> = 3.0 mmol/L, pH = 5.0; (**D**) Cs<sup>+</sup> = 5.0 mmol/L, pH = 7.0; (**E**) Cs<sup>+</sup> = 14.0 mmol/L, pH = 5.0; (**F**) Cs<sup>+</sup> = 25.0 mmol/L, pH = 7.0; (**G**) Co<sup>2+</sup> = 0.03 mmol/L, pH = 5.0; (**H**) Co<sup>2+</sup> = 0.10 mmol/L, pH = 7.0; (**I**): Co<sup>2+</sup> = 1.3 mmol/L, pH = 5.0; (**J**) Co<sup>2+</sup> = 0.90 mmol/L, pH = 7.0; HA = 50 mg/L.



**Figure 5.** Changes in the intensity of the emission spectra of peak 1 and peak 2: (A) HA—pH 5.0; (B) HA—pH 7.0; (C) Cs<sup>+</sup> = 3.0 mmol/L, pH =5.0; (D) Cs<sup>+</sup> = 5.0 mmol/L, pH =7.0; (E) Cs<sup>+</sup> = 14.0 mmol/L, pH =5.0; (F) Cs<sup>+</sup> = 25.0 mmol/L, pH =7.0; (G) Co<sup>2+</sup> = 0.03 mmol/L, pH =5.0; (H) Co<sup>2+</sup> = 0.10 mmol/L, pH =7.0; (I) Co<sup>2+</sup> = 1.3 mmol/L, pH =5.0; (J) Co<sup>2+</sup> = 0.90 mmol/L, pH =7.0; HA = 50 mg/L.

## 4. Conclusions

The present study provided an insight into metal-induced HA aggregation under different pH conditions. The divalent  $Co^{2+}$  with a much lower concentration showed a much stronger effect on HA aggregation compared to that of monovalent  $Cs^+$ . Additionally, a much lower pH condition (pH 5.0) also led to a relatively higher aggregation rate. Experimental data obtained through EPM and the attachment efficiency studies confirmed that the addition of positively charged metal ions to the solution could neutralize the negatively charged HA surface, thus leading to the rapid aggregation of HA aggregates. Divalent  $Co^{2+}$  also displayed a more aggressive influence on the EPM and  $\alpha$  of HA in the aqueous solution. FT-IR and fluorescence EEM spectrum studies indicated that the carboxylic and phenolic groups on the HA surface were involved in the aggregation reaction to form inner complexes and accelerate the aggregation process, but for monovalent metal ions, charge

neutralization is the main mechanism of aggregation. The results above may provide context for the environmental treatment of metal ions and have significant importance for environmental systems.

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