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A. Ikai

Tokyo Institute of Technology

R. Osterberg

Swedish University of Agricultural Sciences

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ATOMIC FORCE MICROSCOPY OF HUMIC ACIDS

A. Ikai and R. Österberg^{1,*}

Laboratory of Biodynamics, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama, Japan

¹Department of Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden

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Abstract

Atomic force microscopic (AFM) images of humic acids show discrete, globular particles, where particles of the order of magnitude 100 to 300 nm dominate the image fields; the humic acids had been grown to a steady state at pH 5.0. The AFM data are consistent with our previously reported small-angle neutron scattering (SANS) study done under similar conditions. In further agreement, the cluster-cluster interactions shown in our previous SANS study may have their counterparts in closely interacting particles appearing as twin particles in the AFM images.

Key Words: Atomic force microscopy, humic acids, particles, size, cluster-cluster interaction.

Introduction

Humic acids, which form the important interphase between the biosphere and inorganic material, play a key role in the food chain by being mainly responsible for the release of nutrients to the plant kingdom {see, for example, the reviews by Kononova (1966) and Hayes *et al.* (1989b)}. Numerous attempts have been made to analyze the structure of humic acids but, so far, there is only a limited amount of knowledge about their size and shape (*cf.* Hayes *et al.*, 1989a) as well as about their molecular structure (*cf.* Schulten and Schnitzer, 1993). Previously, we reported that small-angle neutron scattering (SANS) from humic acid solutions obey a power law, revealing that the particles in solution have a fractal structure with a fractal dimension (\underline{D}) of 2.35 ± 0.05 . The particle size varied within the range 100-320 nm, depending on the source and method of preparation (Österberg *et al.*, 1993, 1994, 1995; Österberg and Mortensen, 1992, 1994a). Titrations indicated that the aggregation process of humic acids involves both protons and electrons (Österberg *et al.*, 1995; Wahlberg, personal communication), although the detailed mechanism of aggregation is unknown. Current models such as Witten-Sanders' diffusion limited aggregation ($\underline{D} = 2.5$) and cluster-cluster aggregation ($\underline{D} = 1.78$), apply only under certain conditions, for instance, at low temperature $\leq 9^\circ\text{C}$ (Österberg *et al.*, 1994). The humic acid system is kinetic in the sense that it is not at a thermodynamic equilibrium, and the "end" stage of the polymerization process varies due to the source and method of preparation (Österberg and Mortensen, 1994a). For example, humic acids prepared by a gentle method from brown soil yield a steady-state, where large particles remain in the solution even after storage for several months. In addition, we observe interactions of large clusters and gel formation (Österberg and Mortensen, 1994a). In an attempt to further analyze cluster size and cluster-cluster interactions, we have now studied humic acids with an independent method: atomic force microscopy (AFM). This method has proven to be successful when applied to various biological specimens, such as

*Address for correspondence:

Ragnar Österberg

Department of Chemistry,

Swedish University of Agricultural Sciences

Box 7015, S-750 07 Uppsala, Sweden

Telephone number: 48-18-671544

FAX number: 48-18-673476

cell surfaces (Bonfiglio *et al.*, 1992), DNA (Yang *et al.*, 1992) and bacteriophages (Ikai *et al.*, 1993); for a review, see Hansma and Hoh (1995). Here, we report direct observation of humic acid particles using the AFM method; the results are in good agreement with our earlier SANS studies (Österberg and Mortensen, 1992, 1994a,b; Österberg *et al.*, 1995).

Materials and Methods

Humic acids

Humic acids were prepared from A1 horizon, brown soil taken from a meadow outside Uppsala, Sweden (*cf.* Österberg *et al.*, 1994). The soil was extracted with 0.05 M pyrophosphate buffer pH 7.0 (Buffer A) in an argon atmosphere at $30 \pm 0.5^\circ\text{C}$. After centrifugation, the supernatant was treated with cold 6 M HCl on an ice bath until the humic acid precipitated. The precipitate was recovered via centrifugation at 4°C and then dissolved in Buffer A. After adjusting the resulting solution to pH 7 and separating a minor precipitate via centrifugation, the procedure involving precipitation with HCl followed by dissolution in Buffer A was repeated thrice. The final humic acid sample obtained was stored in Buffer A at -80°C ; elementary analysis gave a C/N molar ratio of 11.5. The elementary analysis of C was also used for the determination of the humic acid concentration and then the concentration of C in dry humus was set to 50% (*cf.* Lindqvist, 1982).

In order to remove metal ions and to minimize the degree of polymerization, the sample of humic acids was dialyzed first against ethylenediaminetetra-acetate at pH 7.0 and then against bidistilled water. Finally, the sample was dialyzed against an acetate buffer of pH 5.0 with 0.1 M ionic strength (NaCl). Before subjecting the sample to AFM, it was diluted 1:1000 with bidistilled water.

Atomic force microscopy

A Nanoscope III (Digital Instruments, Santa Barbara, CA) was used in Tapping (Digital Instruments registered) AFM mode and a 3700 scanning probe microscope (Seiko Instruments Co., Tokyo, Japan) in contact AFM mode was used in air at 20 to 23°C with a relative humidity of 40 to 50%. Pyramidal shaped commercial tips made of silicon nitride were used. About $5 \mu\text{l}$ of diluted humic acid solution ($\times 1/1000$) was placed on a freshly cleaved graphite surface, then dried under reduced pressure and finally subjected to AFM measurements within 60 minutes. Dilution was repeated when the particles of the dried sample were found to be too crowded for the visual inspection of AFM images.

Results

Figure 1 shows a stepwise magnification of the diluted sample from 1 to 500 in area size illustrating humic acid particles. Please note that, even at low magnification, discrete particles are clearly shown, although the particles tend to adhere to each other. The sizes of the humic acid particles observed by AFM show a significant distribution, although particles of the order of magnitude 100 to 300 nm dominate the image fields. It should be noted that the particles sizes shown by Figures 1 and 2 are those observed after changes have occurred due to evaporation and deformation. It is the high water content of the particles (according to the fractal dimension 2.35: > 90 volume% water), which results in an instability that leads to the deformation of the particles shown in Figures 1B and 1C, and to the relatively small height shown in Figure 1D. Also, the moving of the AFM tip over the particles may increase the flattening and deformation. As a result, the dimensions of the particles, except the height, have probably been somewhat smaller in solution. We also believe that most of the particles have had an essentially spherical shape in solution, which is common for fractal particles formed via random growth processes. This is probably also true for the single particle displayed in Figure 1D, which has the dimensions 230 nm \times 260 nm \times 50 nm. Using SANS data recorded from similar samples of humic acids with a fractal dimension of 2.35 (*cf.* Österberg and Mortensen 1992, 1994a; Österberg *et al.*, 1995), we may estimate the order of magnitude for the molecular mass of this particle to be 5,000 kDa. Then it is assumed that in solution a corresponding spherical particle exists and that its volume is essentially the same as that of the particle in Figure 1D. The calculation was done using the procedure and parameters described in Österberg *et al.* (1993).

As shown by Figure 2, AFM reveals the interaction of large clusters. Of particular interest is the group of three particles in Figure 2A, where two of the particles appear to be involved in cluster-cluster interactions with clusters of about the same size. One of these "twin" particles is displayed in Figure 2B. It is shown, especially for the solid image (on the right hand side of Fig. 2B) that the two clusters penetrate into the inner structures of each other. Since these two particles appear to be "squeezed" into each other rather than interacting with their outer surface, it seems likely that this interaction is not an artifact of evaporation but the result of interactions occurring in solution. Likewise, this solid image of Figure 2B reveals protrusions and indentations which are in agreement with the idea that the particles grow via the interactions of relatively large particles. However, caution should be exercised before any

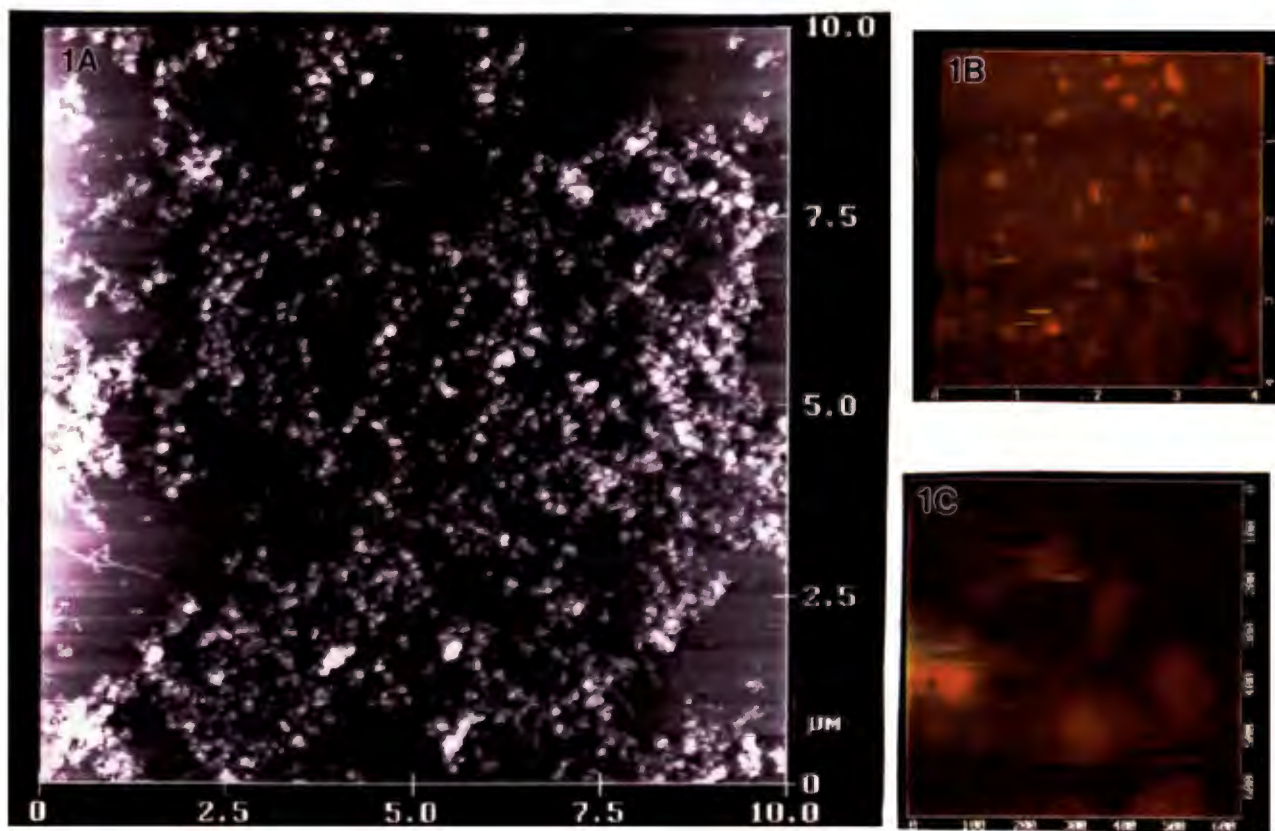


Figure 1. Atomic force microscopic images of humic acids (from a solutions of pH 5 diluted 1:1000 with distilled water) dried on a graphite plate. Imaging areas: (A) $10\ \mu\text{m} \times 10\ \mu\text{m}$, (B) $4\ \mu\text{m} \times 4\ \mu\text{m}$, (C) $650\ \text{nm} \times 650\ \text{nm}$ and (D) $448\ \text{nm} \times 448\ \text{nm}$. The linear "structures" seen at the lower left of Figure 1A are artifacts which arose during the drying procedure.

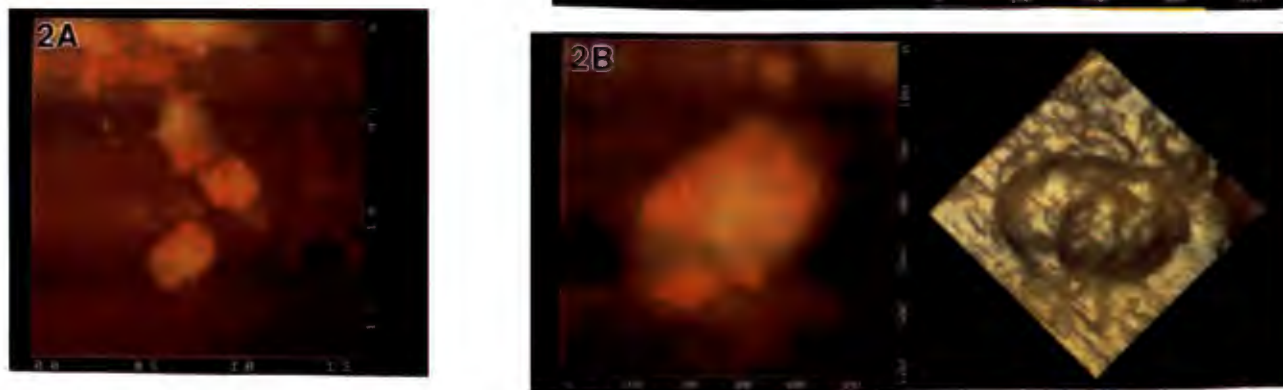


Figure 2. AFM images of humic acids showing cluster-cluster interactions. (A) Imaging area $1680\ \text{nm} \times 1680\ \text{nm}$. (B) Left. Image of single particle involved in cluster-cluster interaction on the area $608\ \text{nm} \times 608\ \text{nm}$. (B) Right. Solid height image of the particle on the left with the same magnification.

final conclusions are drawn about solution cluster-cluster interactions based on a study in the dry state.

Discussion

The previous sections have furnished support for the idea that dilute solutions of humic acids of pH 5, and grown to a steady state, involve discrete clusters, where particles of the order of 100 to 300 nm dominate the image fields (Fig. 1). The present AFM images also yield an excellent resolution of the humic acid particles (Figs. 1 and 2). Similar images of discrete particles, but with less resolution, have recently been obtained by transmission electron microscopy (TEM) (Drozd, 1986; Österberg and Drozd, unpublished results). It should be kept in mind that the soil source and the method of preparation might influence the particle size; for instance, harsher methods involving sodium hydroxide extractions appear to yield smaller particles (Österberg and Mortenson, 1992; Österberg *et al.*, 1993). This might be one reason that early studies using TEM and scanning electron microscopy under somewhat different conditions, showed smaller, less defined particles, 9-50 nm; however, their images also showed aggregates and conglomerates expanding into filaments or/and network (Chen and Schnitzer, 1976; 1989; Stevenson and Schnitzer, 1982; *cf.* Schnitzer and Kodama, 1975). These latter structures do not seem to have their counterparts among solution studies using SANS (Österberg and Mortenson, 1992, 1994a,b; Österberg *et al.*, 1995). The present AFM images, on the other hand, are in good agreement with these SANS studies: both AFM and SANS indicate that humic acid solutions grown to a steady state involve discrete globular particles. And, particles of the same order of magnitude as those observed by SANS (100-300 nm) appear to dominate the AFM image fields (Fig. 1), although some smaller particles also are shown. It should be noted that SANS strongly emphasizes the larger particles, since it yields the weight average squared. Furthermore, both methods indicate that cluster-cluster interactions are involved (see Fig. 2 and Österberg and Mortenson, 1994a).

Finally, from the resolution of the twin humic acid particle (see, e.g., Fig. 2B) showing protrusion and indentations, it is tempting to speculate that the humic acid clusters grow as a result of cluster-cluster interactions involving an ever increasing cluster size.

Acknowledgement

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Discussion with Reviewers

H. Hansma: What exactly are humic acids?

Authors: Humic acids constitute the major organic part of the soil, and aromatic carboxylic acids dominate their composition. Humic acids involve degradation products from the plant kingdom and to a certain extent from the animal kingdom. In the soil, these products undergo aromatization with release of oxygen, and there they are subjected to microbiological activity. Autocatalytic polymerization and aggregation occur in the presence of oxygen, iron(III) or manganese(IV).

H. Hansma: What does it mean that titrations indicate that both protons and electron are involved in the aggregation process?

Authors: We observe that protons and electrons are consumed in a certain pH range, where it is indicated that aggregation processes occur. The true meaning of this is not known, and we can only speculate that hydrogen bonds and free radicals are involved.

H. Hansma: What is the meaning of particle size in a fractal structure?

Authors: A particle has to be sufficiently large in order to yield a fractal structure, although particle size and fractal structure in general do not depend on each other. The pair correlation function which form the basis for the mathematical analysis of fractals is, of course, only valid up to the limit of the particle size. Its Fourier transform, which yields the scattering curve in the form of a power law (indicating scale invariance), should extend over at least one magnitude in order to consider the particles in solution to be fractals. As an example, a fractal unit of 0.5 nm would need a particle which is larger than 5 nm in order to be considered fractal.

H. Hansma: Why was graphite used as the substrate, since it is known to show artifacts in, for example, scanning tunneling microscopy (STM) of DNA, instead of mica?

Authors: We are aware of the study by Clemmer and Beebe (1991). However, these artifacts are on a much smaller scale than the objects observed in this study. As far as we know graphite does not cause any serious problem when used as substrate for AFM.

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