

Quantitative Analysis of Biopolymer Conformation Using Atomic Force Microscopy: Copper-Induced Conformational Changes in Poly- γ -glutamic Acid

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Biopolymers, such as poly- γ -glutamic acid (PGA) can be used to remove copper and other metals from wastewater. However, the molecular-level interactions between metal ions and biopolymer molecules are poorly understood. We used atomic force microscopy (AFM) to probe and quantify the conformational changes induced in PGA by the addition of copper to the aqueous solution. PGA from solutions with or without copper was adsorbed onto mica surfaces, and the mica was imaged using tapping-mode AFM. In pure water, PGA chains aligned end-to-end, so that chain aggregates rather than single chains were observed. The average number of chain segments was 4.8 ± 2.6 per aggregate. The contour length of the individual segments was 325 nm, with a polydispersity (n) of 1.22. If we considered the aggregate of chains as a super-molecule, then the average contour length of the aggregate molecules was 2929 nm ($n = 1.21$). For either the individual chains or the aggregated chains, the persistence length was an extremely high percentage of the contour length (30-50%), since the molecules were practically rigid rod structures that exhibited no bending. When copper was added to the suspending solution, (0.05 mg/L), the contour length was nearly unchanged from that of the individual PGA segments in water (320 nm, $n = 1.35$), but the shape of the molecule was much more flexible, with a persistence length of 34 nm (~10% of the contour length). It appears that this conformational change in the macromolecule helps to remove more metal from an aqueous solution. Such conformational changes at the single-molecule and molecular aggregate level can be quantified with the combination of AFM and statistical image analysis.

Keywords Atomic Force Microscopy, AFM, poly-gamma-glutamic acid, PGA, polysaccharide conformation, biopolymer, image analysis

1. Introduction

Biopolymers are molecules which comprise and are produced by biological cells, including bacteria, fungi, and plant and animal cells. Biopolymers produced by bacteria and other microorganisms have a wide range of applicability in industrial problems. For example, biopolymers are used as food emulsifiers, thickeners, pharmaceutical products, and biosorbents. Several environmental applications rely on biopolymers. For example, biopolymers can be added to toxic waste streams to bind with heavy metal contaminants, thus cleaning the water. The biopolymer poly- γ -glutamic acid (PGA) has shown great promise for its ability to remediate copper-contaminated wastewater as part of a polymer-enhanced diafiltration system [1]. Although the metal-binding abilities of PGA and other biopolymers have been demonstrated [2-4], detailed studies of the conformational changes the polymer undergoes in this process have not been performed. Understanding the interaction between the metal and the polymer will help in the design of better environmental remediation processes.

An analysis of the conformational changes in biopolymers can be performed through a combination of atomic force microscope (AFM) imaging, image analysis, and statistical techniques. The contour length, end-to-end distance, persistence length, and height of the polymer chains can be quantified. Imaging techniques, such as electron microscopy and atomic force microscopy (AFM), have been used to study the physicochemical properties of several biopolymers [5-8]. When used in concert with careful sample preparation techniques, physical properties including conformation, flexibility, and molecular weight can

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be accurately measured [5, 9-11]. AFM studies are performed by spraying, depositing, or adsorbing the biopolymer from solution onto a solid substrate, most often mica. The samples are often imaged in non-contact or tapping-mode AFM under ambient conditions.

Tapping-mode AFM represents the ideal technique for this investigation since the biopolymers can be safely imaged without the need for coating the molecules or a pre-treatment, such as filtration. Although the molecules are imaged under ambient conditions, the mica and the biopolymers retain their water of hydration, allowing a more accurate determination of the molecular properties than if samples were dried or placed under vacuum.

In this study, we investigated and quantified the conformational changes underwent by an environmentally relevant biopolymer, PGA, in the presence or absence of a copper solution.

2. Materials and Methods

The PGA used in the present study was a gift from Prof. DiIorio in the Department of Biology and Biotechnology, Worcester Polytechnic Institute. The PGA was prepared by cultivation of *Bacillus licheniformis* in 200 ml of PGA production medium. The medium consisted of (g/L): L-glutamic acid, 50.0; citric acid monohydrate, 50.0; glycerol, 80.0; NH₄SO₄, 8.63; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.5; CaCl₂·2H₂O, 0.15; MnSO₄·H₂O, 0.08. The culture was inoculated with 5 L of production medium in a bioreactor mixed at 500 rpm. Oxygen supplementation was used for when the dissolved oxygen ratio dropped to <10% of the saturation value. The resulting product was harvested when the solution viscosity reached a steady level (~ 4 days). To lower the pH and decrease the viscosity, concentrated HCl was added. The solution was diluted using 0.1 M citric acid monohydrate (added to reduce the concentration of the HCl from the previous step) and centrifuged to remove bacterial cells. An ultrafiltration membrane was then used to remove any divalent cations from the supernatant. In order to maintain a constant conductivity, the filtrate was diafiltered against deionized water. The resulting material was then freeze-dried in order to be stored. Purified PGA was found to have a maximum yield of 53 g/L.

Atomic Force Microscopy. Atomic force microscopy (Digital Instruments Nanoscope III Multimode) was performed under ambient conditions in tapping mode using TESP tips (Digital Instruments, spring constant = 2-100 nN/m, as reported by manufacturer). Images were collected of PGA with and without copper in solution. For each condition, an analysis was made of 50 macromolecules. Images were digitized and processed using the Fovea Pro 1.0 plug-ins for Adobe Photoshop 6.0. Each image was converted to grayscale and the contrast was enhanced before the threshold was set. After setting the threshold, a "close" filter was applied and the data was skeletonized, or converted to the width of one pixel. The pixelized data was opened in Sigma ScanPro 5.0, where the contour length and x and y coordinates of each data point in the chain were measured after calibration of the image size. From this raw data, the mean-square end-to-end distance and contour length were calculated.

The degree of polydispersity in the polymer sample was quantified as the ratio of the weight-average contour length (L_w) to the number-average contour length (L_n), as

$$\text{polydispersity index (n)} = \frac{L_w}{L_n} \quad (1)$$

where

$$L_n = \frac{\sum_i n_i L_i}{\sum_i n_i} \quad (2)$$

and

$$L_w = \frac{\sum_n n_i L_i^2}{\sum_n n_i L_i} \quad (3)$$

3.1 Chain Statistics

To make a quantitative analysis of chain flexibility, the persistence lengths were compared for the polymer under various chemical conditions. A statistical analysis of the change in tangent direction along the polymer contour length was calculated using techniques developed by Frontali et al. [12]. The persistence length calculation is based on the following assumptions:

1) the probability density function of the angle between consecutive links of the chain is Gaussian, and can be represented by

$$P(\theta(\ell)) = \left(\frac{L_p}{2\pi\ell} \right)^{1/2} \exp\left(-\frac{L_p\theta^2}{2\ell} \right) \quad (4)$$

where θ is the bend angle, ℓ is the distance between segments, and L_p is the persistence length of the polymer;

2) the observed two-dimensional conformation is obtained by permitted deformations, and is not a projection of the three-dimensional structure;

3) the adsorbing surface does not alter the local rigidity of the polymer chain.

Frontali et al. [12] showed that the odd moments of the distribution (eq. 2) are equal to zero, and for the even moments,

$$\langle \theta^2(\ell) \rangle = \frac{\ell}{L_p} \quad (5)$$

and

$$\langle \theta^4(\ell) \rangle / \langle \theta^2(\ell) \rangle^2 = 3 \quad (6)$$

The definition of the second moment (eq. 3) was used to calculate L_p , while eq. 6 was applied to check if the first assumption was valid.

To check assumptions two and three, a calculation was made to determine whether the polymer adopted an equilibrium conformation on the surface. For polymer chains that have reached equilibrium with the surface, their conformation will correspond to a 2D conformation rather than a 3D projection. The end-to-end distances corresponding to 2D and projected conformations are given by [13]

$$\langle R_{ee} \rangle_{2D}^2 = 4L_p L \left(1 - \frac{2L_p}{L} \left(1 - e^{-\frac{L}{2L_p}} \right) \right) \quad (7)$$

and

$$\langle R_{ee} \rangle_{\text{proj}}^2 = \frac{4}{3} L_p L \left(1 - \frac{L_p}{L} \left(1 - e^{-\frac{L}{L_p}} \right) \right) \quad (8)$$

An alternate way to quantify polymer rigidity is to characterize the Flory exponent for the system. Polymer chains in solution can be modeled according to the self-avoiding walk (SAW) analysis that describes the behavior of real chains on a lattice. The chain is represented by a random walk subject to the constraint that the walk can never intersect itself. SAW analysis relates the end-to-end distance to the size of the polymer by [14]

$$R_{ee} \propto N^{\nu} \quad (9)$$

where N is the number of monomers and ν is the Flory exponent. N can be replaced by the contour length for a linear polymer, since the number of monomer units per unit length would be constant. By taking the log of both sides of the equation, a linear plot of R_{ee} vs. L_c can be used to determine the Flory exponent, ν . The value of this exponent provides information on polymer-solvent interactions.

3. Results and Discussion

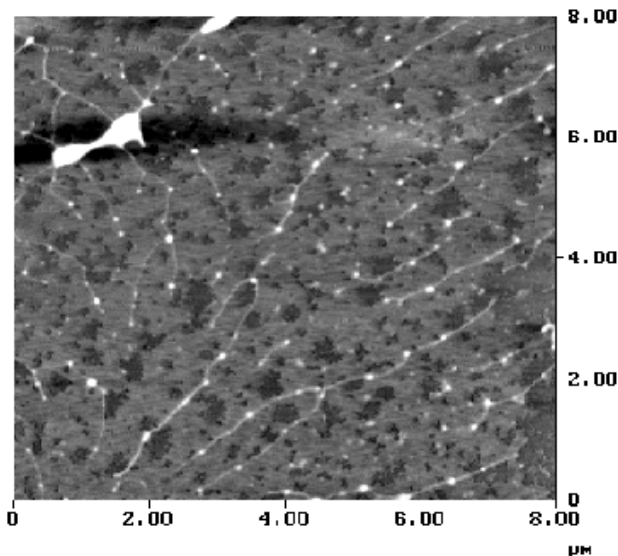


Fig. 1 PGA chains adsorbed onto mica from a solution of pure water (i.e. no copper present in solution).

Figure 1 is a representative tapping-mode AFM image of PGA chains adsorbed onto mica. The biopolymers are highly extended and stretch out on the mica surface. This is due in part to charge-charge interactions. Mica has a negative charge, and PGA will have negative charges along its subunits. There appears to be substantial intrachain or intramolecular repulsion, causing the biopolymers to stretch as much as possible to reduce these repulsions.

Quantitative analyses were made of the biopolymer properties. The contour and persistence lengths of the biopolymers were calculated based on statistical image analysis. For PGA in water, the average contour length was 2929 nm (Table 1). The persistence length of PGA in water was extremely high, approximately 30% of the contour length. The molecules are almost rigid rods in this case. The measured end-to-end distance of the polymers was closer to the 2D representation, indicating that equilibrium adsorption did occur, rather than non-equilibrium kinetic trapping of the molecules on the mica. However, the estimate of the contour length did not seem realistic based on previous studies of this fermentation-produced PGA [1]. Careful examination of the AFM images reveals the presence of intersection points that are likely the joining of individual chain segments. All of the PGA aggregates were reanalyzed so that the properties of the individual segments could be obtained.

When the individual segments were considered, the contour length was greatly reduced, to 352 nm ($n=1.22$; Table 1). The average number of chain segments observed was 4.8 ± 2.6 per aggregate. The persistence length remained a very high percentage of the contour length. The 2D representation of the molecules was again closer to the experimentally measured end-to-end distance. Therefore, we can conclude that PGA in water is a very rigid molecule, with segments of ~ 350 nm. These segments associate with one another when on the mica surface.

Table 1. Summary of Chain Properties

	PGA in water, aggregates of multiple chains	PGA in water, individual segments	PGA with copper present in solution
L_c [nm]	2929	352	320
L_p [nm]	769	147	35
$\langle R_{ee} \rangle$ [nm]	2734	341	187
$\langle R_{ee} \rangle_{proj}$ [nm]	1494	207	115
$\langle R_{ee} \rangle_{2D}$ [nm]	2232	294	187

Upon adding copper to the solution, the conformation of PGA was greatly altered. This could be easily viewed via tapping-mode AFM images (Figure 2).

The quantitative analysis confirmed that adding copper to solution changed the conformational properties of the biopolymers (Table 1, Figure 3). The contour lengths of PGA in water, taken as individual segments, or PGA in copper-containing solution, were nearly equal. PGA in the copper solution had a contour length of 320 nm ($n=1.35$). This was not surprising since the intrinsic length of the molecules should not be altered by the presence of the metal ions. However, the persistence length was substantially decreased with copper present, as was the end-to-end distance. We also confirmed that the assumptions underlying the Frontali et al. analysis were applicable to this system, since the experimentally-measured end-to-end distances were closer to the 2D representations than the projected conformations [12, 15].

Fig 3

A final quantitative validation of this change in conformation was provided by calculation of the Flory exponent for each system (eq. 9; Figure 4). For an unperturbed polymer, this exponent would be 0.5 [14]. In a good solvent, some expansion of the chain is expected. PGA in water exhibited Flory exponents that were nearly one, indicating practically rigid rod structures.

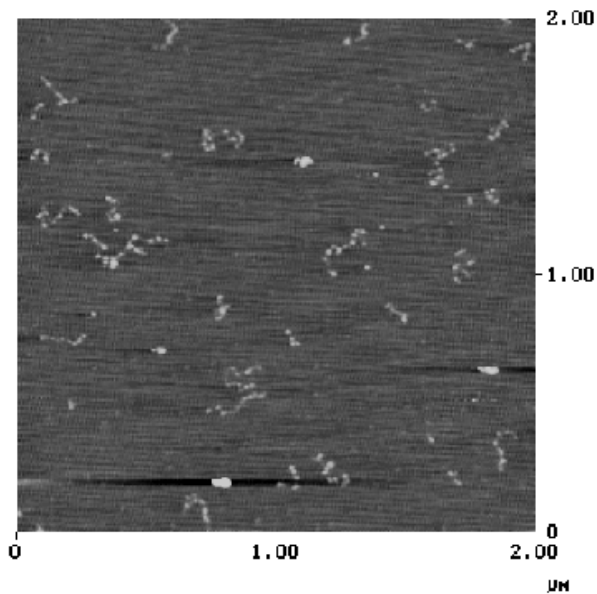


Fig. 2 Representative image of PGA chains adsorbed onto mica from a solution of water containing 0.05 mg/L copper.

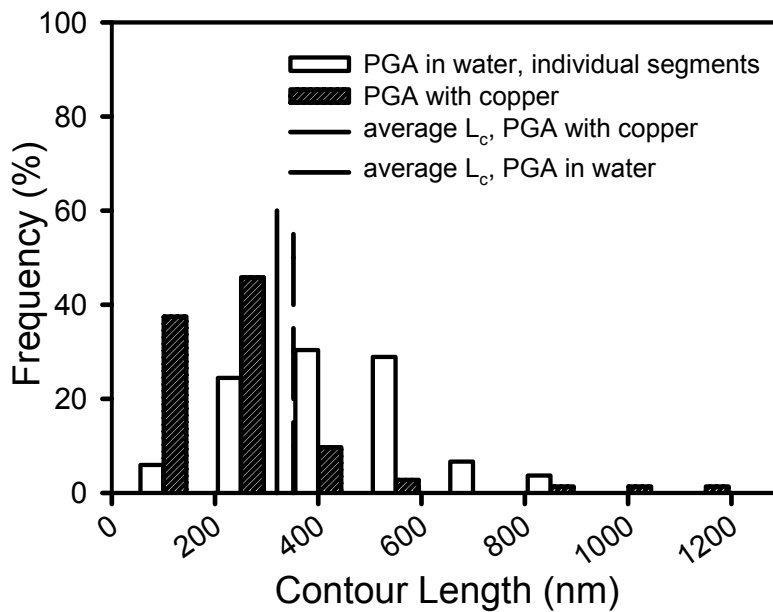


Fig. 3 Comparative histograms of contour length distribution of PGA chains, for PGA adsorbed onto mica. Dashed line represents PGA in water.

The Flory exponent for PGA adsorbed from a copper solution was reduced to 0.74. Although the PGA molecules in the presence of copper were more flexible than the molecules in water, the persistence length and Flory exponent still indicate that we have fairly rigid molecules compared to other kinds of polymers. Several biopolymers, including DNA and xanthan, have rigid structures [10, 15].

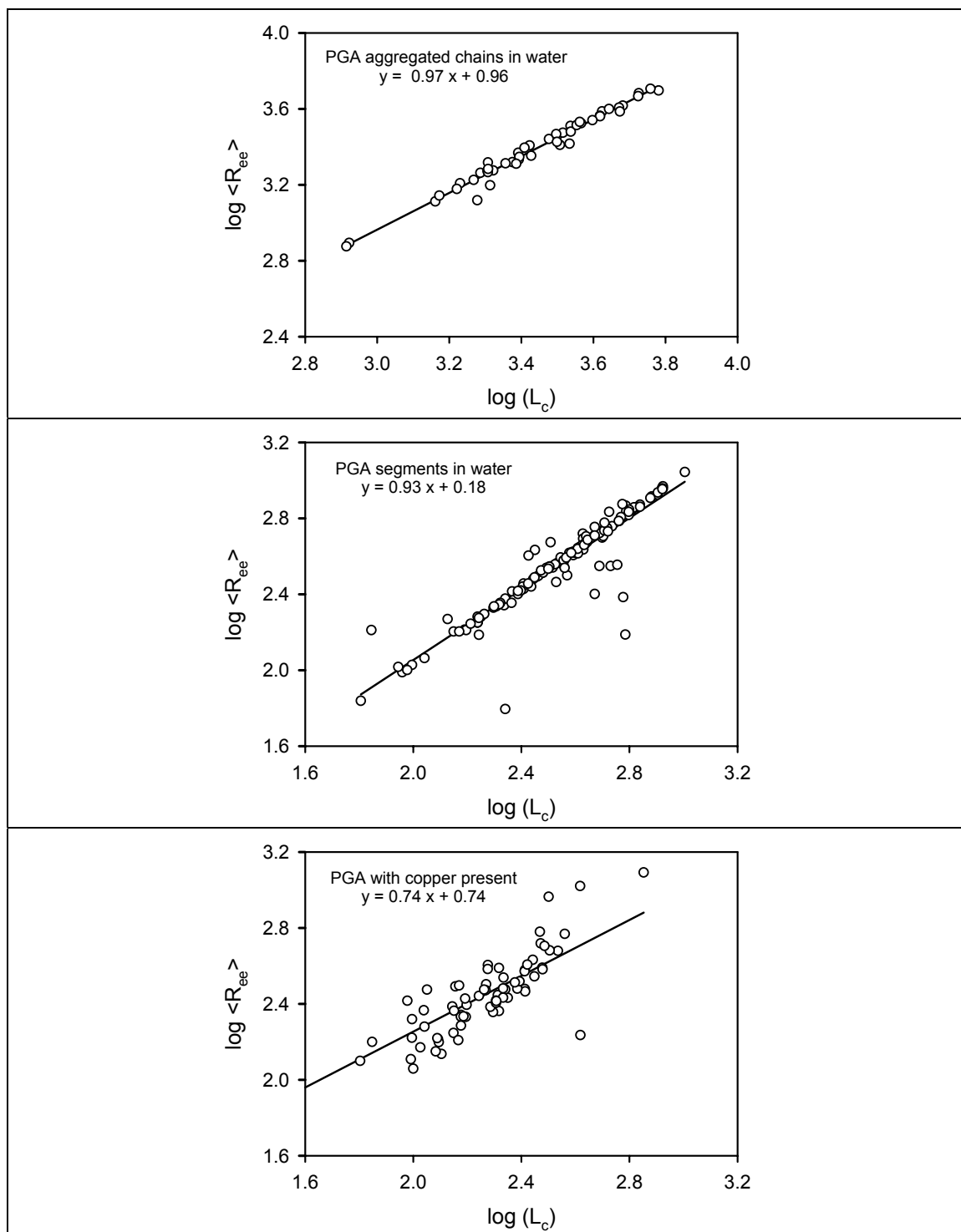


Fig. 4 Determination of Flory exponent for PGA chain segments or aggregates, in water or a copper solution.

4. Conclusions

A quantitative method of image analysis, based on tapping-mode AFM, was used to investigate the conformational changes of a biopolymer in the presence of metal ions. This type of biopolymer image analysis is useful to a number of scientific and engineering fields, especially in environmental engineering, biotechnology, food science, and pharmaceuticals.

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