Single molecule force spectroscopy study of γ-polyglutamic acid by using atomic force microscopy

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ABSTRACT

γ-PGA is a versatile multi-functional biopolymer possessing much useful biological functionality. Each biological function is associated with specific properties related to various forms of γ-PGA. However, to date, knowledge about the molecular mechanism to control specific structures of γ-PGA under a certain environmental conditions are unknown. In this study, we used single molecule force spectroscopy (SMFS) method to characterize the structure of γ-PGA in two environmental conditions. A water environment or 0.5 M MgCl₂ salt solution was used to observe the effect of metal ion on γ-PGA structure at room temperature. The obtained results revealed that γ-PGA exists branched and/or cross-linked structure of γ-PGA in water or MgCl₂ salt solution.

Key words: γ-PGA, single molecule force spectroscopy (SMFS), atomic force microscopy (AFM).

1. INTRODUCTION

Poly-gamma-glutamic acid (γ-PGA) is an unusual anionic, biodegradable, edible and nontoxic biopolymer. It is one of the three available poly amino acids present in nature. Ivonovics and Bruckner discovered γ-PGA in a capsule of Bacillus anthracis in 1937 [1, 2]. Much research has been done since then on γ-PGA production using various methods. Researchers have also found γ-PGA in a Japanese traditional food, “natto”, produced by Bacillus natto [3]. Now it is widely accepted that any bacteria containing the Bacillus genus can produce γ-PGA in large amounts for different purposes. For example, some bacteria use the released γ-PGA for the aggregation of toxic metals to protect themselves from hostile environments [4]. On the other hand, peptidoglycan bound γ-PGA acts as a source of glutamate to help bacteria under starvation conditions [5].

Different types of physiological functions of γ-PGA in bacterial cells have inspired scientists to use it in widespread applications such as food processing/additives [3], pharmaceuticals [6], healthcare [7], water treatment [8] and in other fields [9]. But the specific applications and unique biological functionalities of this bio-polymer are strongly dependent on its structural characteristics [10]. Structurally, γ-PGA is made of D-and L-glutamic acid residues as shown in Figure 1. The α-amino group of one residue is attached to the γ-carboxylic group of another residue to form peptide bond [7]. In the polypeptide backbone of γ-PGA strong intramolecular hydrogen bonds exist between the carbonyl group of one γ-peptide bond and the amino group of the other γ-peptide bond [11]. Such a strong intramolecular hydrogen bond possibly forms a tightly compact structure of γ-PGA. Depending on the environmental conditions such as pH, polymer concentration and ionic strength it possibly undergoes a conformation change from one state to another [12]. The conformation features of γ-PGA at the single molecular
level have not been clarified yet. Thus until now it remains unclear and in little confusion. Hence the complete understanding about the structural characteristics at the single molecular level is crucial in addressing the relevant questions and developing high performance \(\gamma\)-PGA bio-materials.

Over the last few years the behavior of a single polymer chain has been demonstrated extensively by using atomic force microscopy (AFM) based single molecule force spectroscopy (SMFS). It has opened a way to reveal the structure of single polymer molecules on piconewton scale [13-114]. For example, SMFS measurement of poly(ethylene glycol) and poly(vinyl alcohol) in different environment conditions have revealed that hydrogen bonding is the governing factor for their helical structure in water [15, 16]. Their force induced spectra revealed fingerprint information for better understanding of their conformation state as well as elasticity of the polymer molecules. In this study we use a SMFS approach for studying intermolecular and intramolecular interactions of \(\gamma\)-PGA in a water environment and a 0.5 M MgCl\(_2\) salt solution. Our aim is to observe the possible conformation change of \(\gamma\)-PGA in presence the of MgCl\(_2\) salt solution at room temperature.

2. EXPERIMENT

All the experiments were carried out on a custom-built single-axis atomic force microscope. In each experiment we used commercially available V-shaped Si\(_3\)N\(_4\) AFM cantilevers (MLCT, Bruker, Santa Barbara, CA) with a sharp tip (radius of curvature in the range of 20 to 60 nm) at the end of a soft cantilever. The spring constant was calibrated in solution using a Thermal Tune method. All SMFS experiments were performed at room temperature. Details of the SMFS experiment have been described elsewhere [16-18]. In brief, an AFM tip approached the sample surface until contact occurred. Then the tip was kept there to interact with the polymer molecule. The polymer molecules on the sample substrate adsorbed on the AFM tip by non-specific interactions like van der Waals, ionic and dipole-dipole interactions. Thus a bridge of the polymer molecule is formed between the tip and the substrate. Then the polymer molecule was then elongated when the tip separated from the substrate. The elongation of the polymer resulted in the deflection of the cantilever tip. The deflection of the cantilever as well as the elongation of the polymer molecule were recorded and converted to force-extension curves.

Here, we have used research grade poly-\(\gamma\)-glutamic acid from Wako Chemical Company. The average molecular weight of the polymer is between \(2 \times 10^5\) to \(5 \times 10^5\). Since \(\gamma\)-PGA is insoluble in water, it is dissolved to a concentration of 1 mg/mL in 5 M aqueous solution of urea. For a typical experiment, an appropriate amount of \(\gamma\)-PGA solution was deposited onto a clean glass surface and allowed to dry in a desiccator for around 30 mins. Then the substrate was successively rinsed using 1M urea solution and millipore water to remove loosely absorbed \(\gamma\)-PGA and then immediately used for force-extension measurements. Force curves were measured in pure water or in 0.5 M MgCl\(_2\) salt solution.

3. RESULTS & DISCUSSION

In this study, we used a worm like chain (WLC) model [18] for fitting the force-distance curves. The WLC model explains the relationship between force and extension considering the bending energy of polymer chain as

\[
F(x) = \frac{k_B T}{L_p} \left[ \frac{1}{4} \left( 1 - \frac{x}{L_c} \right)^2 - \frac{1}{4} + \frac{x}{L_c} \right]
\]

Where \(F(x)\) is the force at the distance of \(x\), \(k_B\) is the Boltzman constant, \(T\) is the absolute temperature, and \(L_p\) and \(L_c\) are the persistence length and the contour length, respectively.

In a typical experiment we obtained large number of force-distance curves of \(\gamma\)-PGA in water or 0.5 M MgCl\(_2\) salt solution. All of the force profiles in each measurement environment exhibit the general deformational characteristics. Initially the force increases gradually with the extension of the polymer molecule and then rapidly drops to zero upon detachment of the \(\gamma\)-PGA from the cantilever tip. Three force profile of \(\gamma\)-PGA in water environment are shown in the Figure 2.
Extended length ($L_{\text{max}}$) and the corresponding detachment force ($F_{\text{max}}$) for each force profiles are different. This is possibly due to two factors: (i) $\gamma$-PGA molecules are randomly dispersed on the glass substrate (ii) the contour length of the extended polymer chain is different as it is impossible to specify the exact location for binding of cantilever tip along the dispersed polymer chain. As a result, extension ($L_{\text{max}}$) and detachment force ($F_{\text{max}}$) vary for each force profile. The obtained force profiles of different contour length were then normalized at a certain pulling force to prove the stretching of a single polymer chain during measurement. Figure 3 shows the normalized force curves of the force profiles from Figure 2. All of the normalized force curves obtained from water environment are almost completely superimposed. This phenomenon suggests that single polymer chain of $\gamma$-PGA has maintained a uniform elasticity in the water environment.

In order to see the effect of metal ions on the structure of $\gamma$-PGA, we performed the single chain stretching experiment in 0.5 M MgCl$_2$. As shown in Figure 4, it represents the force profiles of $\gamma$-PGA in 0.5 M MgCl$_2$ salt solution environment. Their corresponding normalized force curves in are shown in Figure 5.

For comparison, the extended length and corresponding detachment force of $\gamma$-PGA in two different environments are shown in Table 1. In water $L_{\text{max}}$ values varies in the range of 80 nm to 90 nm and the detachment force $F_{\text{max}}$ in the range of 356 pN to 572 pN. In 0.5 M MgCl$_2$ salt solution environment $L_{\text{max}}$ values were recorded in the range of 224 nm to 272 nm and the value of $F_{\text{max}}$ ranges 348 to 476 pN.

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<th>$L_{\text{max}}$ (nm)</th>
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<td>0.5 M MgCl$_2$ salt solution environment</td>
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According to the molecular weight of the $\gamma$-PGA used in this study, the theoretical value of the contour length is in the range of 900 nm to 2200 nm. However, the experimentally obtained extended lengths in both measurements

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environment are found significantly smaller than these values. It suggests that this difference is reflecting the compact structure due to the -branched and/or cross-linked polymers of \(\gamma\)-PGA in water or MgCl\(_2\) salt solution. When the compact structure of the polymer chains is extended during pulling, the experimental value of the contour length will be significantly small compared to the theoretical one. Furthermore, we found that the \(L_{\text{max}}\) value of \(\gamma\)-PGA in MgCl\(_2\) is higher than in water. The larger \(L_{\text{max}}\) could be due to the bridging effect of Mg\(^{2+}\) ions between two carboxylic groups in the single polymer backbone. Mg\(^{2+}\) ion forced a part of the polymer chain possibly to hold folded structure. Even with the same “apparent” polymer length in the beginning of stretching, fully extended length of the polymer with a folded structure will be much longer than that of unstructured, disordered polymer. It suggests that the observed contour length is increased in presence of salt solution.

Here we have normalized the force curves of two different conditions to compare the flexibility of the polymer chain-(see Figure 6). It is interesting to see that the normalized curves of two different measurement environments are almost completely superimposed each other as shown in Figure 7.

![Figure 6: Force curves of gamma PGA in water as well as in 0.5 M MgCl\(_2\) salt solution.](image)

![Figure 7: A plot of force vs normalized extension of force curves from figure 6. Normalization was done at 0.21 nN.](image)

No significant difference is observed in the normalization curves from the low force region to the high force region. It means that the single chain elasticity of \(\gamma\)-PGA remains same even in the presence of 0.5 M MgCl\(_2\) salt solution. The structure of \(\gamma\)-PGA may not significantly change due to the presence of the 0.5 M MgCl\(_2\) salt solution.

4. CONCLUSION

We have studied the single chain mechanism of \(\gamma\)-PGA at room temperature in two different environment conditions using the SMFS technique. Force measurement was performed with or without of Mg\(^{2+}\) ion. The stretching curves of \(\gamma\)-PGA under different measurement environments are well fitted by worm-like chain models and normalization curves were superimposed. This information suggests that \(\gamma\)-PGA possess special structure which is slightly affected by the presence of MgCl\(_2\) salt solution at room temperature. Further study on the behavior of \(\gamma\)-PGA at different environment conditions is underway in our laboratory. We believe that studying the interaction of \(\gamma\)-PGA with metal ions at the single molecular level is crucial to developing more bio-available compounds of this bio-polymer.

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