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# Preliminarily Investigating the Polymorphism of Self-organized Actin Filament *in Vitro* by Atomic Force Microscope

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**Abstract** With the atomic force microscope (AFM), we preliminarily investigated the large-scale structure of actin filaments formed in low concentration protein solution (5 μg/ml) via self-organization without the presence of any F-actin dynamic interfering factors (such as phalloidin) *in vitro*. It was found that the G-actin could be polymerized into ordered filamentous structures with different diameter from the slimmest filament of single F-actin to giant filament in tree-like branched aggregates. The observed polymerized actin filaments, to which our most intense attention was attracted, was discretely distributed and showed obvious polymorphism distinctly different from those in the presence of phalloidin or actin binding proteins (fimbrin, gelsolin, etc.) in previous experiments. Latter structures were mainly composed of single F-actin and/or multifilaments clearly consisting of several single F-actin. The experimental results clearly demonstrated that non-interference with the F-actin intrinsic dynamics in self-organizing could lead to the polymorphism of actin filamentous structures, and further analysis implied that the disturbance of normal F-actin dynamics by many factors could prevent the emergence of structural polymorphism, more often than not, give rise to formation of specific structures instead and different interference would bring about various particular structures under certain conditions.

**Key words** actin; self-organization; filamentous structure; polymorphism; atomic force microscope

Actin is a major structural component of eukaryotic cytoskeleton and exists in monomer G-actin and filamentous F-actin. G-actin consists of 375 amino acid residues with molecular weight 43 kD and is a highly conserved protein expressed in most living organisms [1,2]. Actin monomer can be polymerized into long right-handed double helical F-actin induced by $Mg^{2+}$, $K^+$, $Na^+$, and ATP [3,4]. F-actin can interact with an array of cytoplasmic proteins to meet different biological functions under various conditions *in vivo* [5]. Actin not only can take part in myosin-based motility as an essential component [6,7], but also can alone mediate motility through controlled polymerization [8] or gel-sol transitions [9].

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In recent years, more and more researches revealed that actin was involved in more extensive and complicated biological activities than we thought before, such as cell adhesion, signal transduction, modulation of ion channel, and so on [10–15]. So the molecular structure and physiological functions of actin have been being important subjects of research in cell and structural biology. Not only has the atomic structure of G-actin been solved in more than one crystal form [16–20], but also atomic models of the F-actin have been proposed based on fiber X-ray diffraction [21,22] and electron microscopy [23–25]. These studies help us understand the actin functions under various conditions, the essential correlation between its functions and structures, and the role of actin structural diversity in their functional versatility *in vivo* [26–29]. In the recent decades, the polymorphism in actin bundles [5,30,31], paracrystals [32,33], crystalline sheet [34,35], cross-linked networks [36,37], and F-actin assembly [38,39] have been reported. However, little attention has

been paid to the structural versatility *in vivo* or the structural polymorphism of polymerized actin filaments by self-organization without any interference with F-actin dynamics by any factors, such as chemical reagents and microfilament stabilizing reagents (phalloidin, etc.) that were widely employed in actin researches or any other actin-bind proteins involved in controlling F-actin dynamics. In fact, investigation on the structural polymorphism of actin filaments and polymerization dynamics in self-organizing could play a fundamental role in profoundly expounding the formation of actin structural diversity via self-assembly dynamic process modulated by many factors in living cells and in turn, revealing its functional versatility, and also have great theoretical implication in exploring the molecular dynamics of proteins and their polymerized structures in self-organizing.

In this research, we preliminarily investigated the large-scale structure of actin aggregates by self-organization in simple thermodynamic environment (with only necessary reagents) near to physiological conditions *in vitro* with atomic force microscope (AFM) which had been widely applied in actin researches [40], focusing on the high order structure of actin filaments and the filamentous structure polymorphism. It is observed that actin could form different types of filamentous structure in absence of any F-actin dynamics interfering factors in F-buffer. Under the experimental conditions, actin could form large-scale ordered structure of long filaments with different diameters in tree-like branched aggregates. The polymerized filaments are distinctly different from those formed in the presence of F-actin dynamic interfering factors such as phalloidin [41], gelsolin [38,39], or fimbrin [31] in previous experiments, in which the observed actin filaments are mainly composed of the single F-actin or multifilament aggregates (actin bundles, raft, etc.) clearly constituted of several single actin filaments, and the high order filamentous structures are rarely seen.

## Materials and Methods

### Materials

G-actin was purified from bovine skeletal muscle following the method of Spudich and Watt [42]. A single band was observed on SDS-PAGE gel with no evidence of contamination by other proteins in the final extract. G-actin was stored in G-buffer at 4 °C for following use. Tris-base and Tris-HCl were products of Promega; $Na_2ATP$ and DTT were purchased from Sigma-Aldrich Co.. Other chemical reagents of analytic grade were made in China. The $Si_3N_4$ tip of AFM was model NP, NANOPROBE™.

### Solution preparation

G-buffer and F-buffer were prepared according to traditional method with a little modification for AFM assay. G-buffer contained 2 mM Tris-HCl (pH 7.5), 0.2 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM ATP; F-buffer contained 5 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, and 1 mM ATP. All freshly prepared solvents and solutions were passed through 0.22 μm filter in order to remove particles.

### Preparation of protein specimen

1 ml purified G-actin extract solution was accurately diluted to 100 ml with F-buffer, the final concentration of G-actin was 5 μg/ml. The prepared sample was incubated for polymerization at 37 °C for 30 min. Transfer 5 μl G-actin solution onto the surface of freshly cleaved mica that was already soaked by F-buffer with micropipette. After the polymer sunk for 15 min, the excess droplet was carefully sucked, and the specimen was naturally dehydrated.

### AFM imaging

The prepared sample, without any physical and chemical treatment in order to keep the sample in a polymerizing state near to physiological conditions, was immediately observed under AFM (CSPM-2000we, Ben Yuan Nano Instrument Ltd.) in contact mode at room temperature (the humidity was 30%; the main operation parameters I.G., P.G., HVS, and Ref were “125”, “125”, “open” and “–0.25”, respectively). The atomic force microscopic images were captured and saved in *.bmp format for future analyses, and the ruler unit was nanometer (nm) in all AFM images. All experimental operations above were carried out under super-clean conditions to avoid contamination.

## Results

In this research, it was found that G-actin could be polymerized into long filaments with different morphologies in various scales, from simple single F-actin to complicated filaments with multiple order branches, which showed distinct structural polymorphism and were significantly different from those formed in the presence of phalloidin [41], gelsolin [38,39] or fimbrin [31] under the relative more complicated thermodynamic conditions.

### Branch structure of actin filaments

The tree-like filament seemed to be composed of multiple-order helix filament with branches, which actually indicated the possible constructed manner of high order actin filaments by self-organization (Fig. 1), and the branch order could determine the filament diameter. Thicker filament could also be made in this way. It was preliminarily inferred that the giant filament should be made from single F-actin via non-covalent interaction among microfilaments such as van der Waals force, static electronic force, hydrogen bond, and so on.

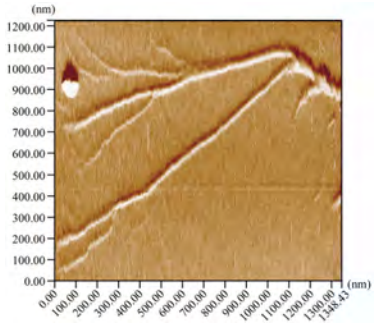

**Fig. 1 Tree-like branch structure with fractal feature**

In much larger scales, the branch filaments could wind one another and form new filament, and some could branch once again [Fig. 2(A)]. It was also observed that many stacked branch structures clustered together, which might be produced in the process of specimen preparation [Fig. 2(B)]. In another image, filaments winding one another form a complicated radial branched structure like a filamentous joint that could come from the cooperative action of polymer sinking and interaction among branched filaments [Fig. 2(C)].

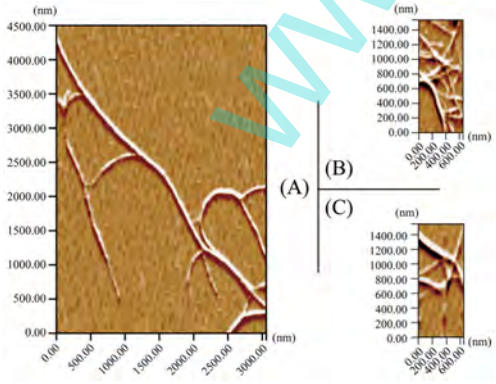


**Fig. 2 Complicated branch structures**

(A) Branch filaments could wind one another and form new filament, and some could branch once again. (B) Stacked branch structures clustered together. (C) Radial branched structure.

### Random coil structure of actin filaments

Many filaments existed in clustered distribution states (Fig. 3). It was thought that the high density of condensed filaments throngs have had homology with the branch filament structure in origin, and they were probably the resultants in different phase of polymerization, and so had different topological conformations.

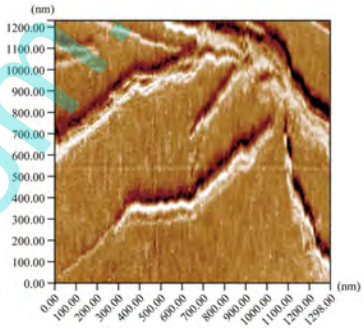

**Fig. 3 Random coil filaments cluster**

### Various long actin filaments with different diameters

Long filament with different diameter was formed from F-actin in different way, and could be easily observed. Possibly the side interaction among the microfilaments could played a key role in the forming of this high order structure, which remained unknown and needed further researches.

*Structural analysis of single actin filament* The pitch of the helix of single filament was variable, with published value around 36 nm, and the width was 5–7 nm or so [23, 27,43–46]. In our experiment, single F-actin was judged according to the structural parameters. The slimmest filament was similar to the single F-actin in size, with 7.31 ± 1.21 nm in width and 35.64±3.43 nm in pitch (mean ± SD, $n$=8), which indicated that such filament was a single F-actin (Fig. 4). The result also demonstrated that the AFM tip was so sharp that it brought no obvious widen effect, which was generally produced when the tip curvature radius was near to the size of the examined sample.

*High order structure of actin filaments with much thicker diameter* Some polymerized filaments had much larger diameter than that of the single F-actin (Fig. 5), which

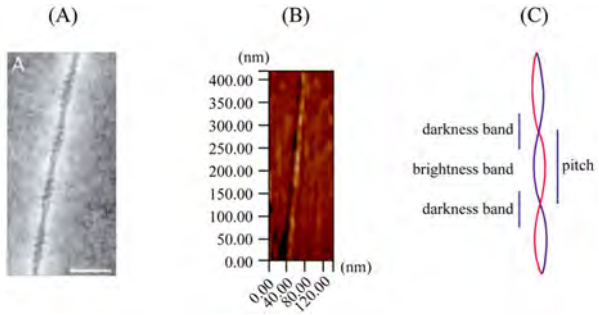


**Fig. 4 Structural analysis of single F-actin**

(A) A negatively stained STEM image of single F-actin using annular dark-field detector, the scale bar was 20 nm [27]. (B) AFM image of the single F-actin. (C) A simplified model of single F-actin was used to illustrate the AFM image.

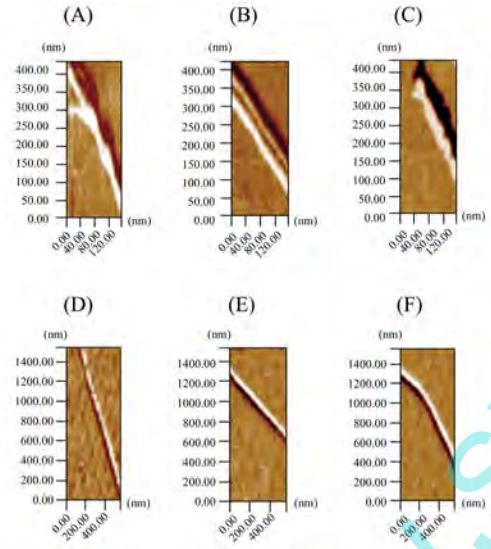


**Fig. 5 Thicker single filament with different diameter**

(A) The branch filament could be constituted of two enlaced slimmer filaments, forming the branch in the joining point of another filament. (B) Two filaments were aligned side by side, and there might exist certain interaction between them, which could be the early step for further assembly of filaments. (C) Filament with short fork structure at the end was also likely composed of much thinner filaments winding with each other. (D–F) Filaments had much larger diameters than the single F-actin.

suggested that the structure of actin filaments formed in process of self-organizing was far more different and complicated than those in the presence of phalloidin or actin binding proteins, in which the polymerized filaments were mainly made of single F-actin or/and simple multifilament. Although it is clearly that the high order filaments are identically made from F-actin, the structural constitution manner remains unclear and waits to be solved.

## Statistical analysis of branch angle and filament diameter

The frequency distribution of branch angle and filament diameter has been analyzed with SPSS 10.0 for windows (SPSS Inc.). It was found that the branch angle was mainly in the scope of 40–65 degree with an average value of 52.08±9.42 degree [Fig. 6(A)], and diameter of filaments approximately varied from 7 nm to 90 nm, and mainly distributed in the scope of 20–50 nm with an average value of 36.61±18.07 nm [Fig. 6(B)]. The frequency distribution demonstrated that the aggregated filaments had

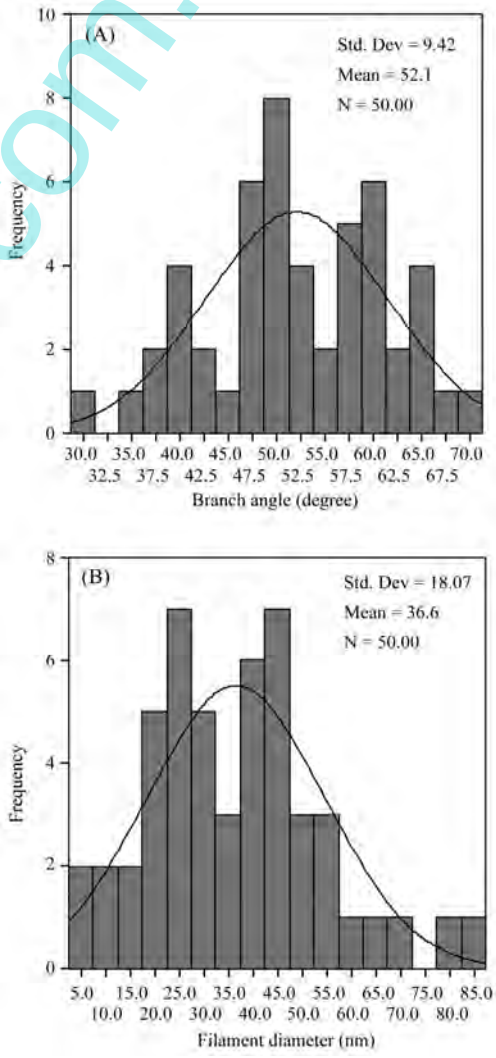


**Fig. 6 Frequency distribution of branch angle and filament diameter**

Both the frequencies showed distinct characteristics of normal distribution, which suggested that they could be the indirect reflection of "the rule of minimal energy" in actin self-organization.

dominant distributed scopes in branch angle and filament diameter, which implied that the filament structure in these scopes was easier to form than that out of these scopes. In fact, the polymerized filament structure should also conform to "the rule of minimal energy" when formed in self-organization, so it is very possible that the filaments in the dominant scopes stand at much lower energy level, thus more stable. Whereas those out of the dominant scopes could have higher energy level, thus less stable and harder to form, so as to result in less magnitude.

## Discussion

In previous researches, the tree-like actin filamentous structure has never been observed *in vivo* and the structural polymorphism of actin filaments *in vitro* has also not been paid much attention. Considering the huge difference in thermodynamic environment between *in vivo* and *in vitro*, the phenomena should not be difficult to be understood. Further careful analyses revealed that, in many experiments on F-actin structure exploration *in vitro* before, the F-actin stabilizing reagents, generally phalloidin, were usually employed to stabilize the structure of polymerized filamentous actin for the benefit of analysis on its stationary structure. Although the stabilizing reagent could provide convenience in these studies and help to clarify the structure of single actin filament, it can also interfere with the normal dynamics of actin polymerization through inhibiting the depolymerization process. Furthermore, it could prevent actin from bringing about high order structure and structural polymorphism, which accidentally cause us to ignore the polymorphism of actin filament by self-organization *in vitro* for a long period that, in fact, should be paid enough attention as well.

The present experimental results demonstrate that the high order structural polymorphism of actin filament can be easily produced in process of undisturbed self-organization *in vitro*, which is propelled by its inherent thermodynamic properties (which refer to the integrated physical and chemical characters including the native conformation, molecular weight, owned net charges and their distribution, biomechanics features, residue distribution on molecular surface, and so on) essentially determined by its amino acid sequence. Together with previous research results [31,38,39,41,47], further analysis can find that the intact non-linear dynamics of F-actin is the necessary condition in formation of the structural polymorphism, from which it is inferred that the destruction of the normal F-actin dynamics could lead to emergence of particular structure and different interference with the dynamics could give rise to different specific structures. Obviously the insight can perfectly explain the structural diversity of polymerized actin producing in the living cells.

At present, the nucleation-condensation mechanism of F-actin polymerization dynamics based on "upper dimer" (UD) pathway has been commonly accepted, which is generally believed to involve three distinct steps: (1) fast G-actin activation; (2) rate-limiting nucleation; (3) moderately fast elongation conforming to a pseudo-first order kinetics [3,7,48]. Although there is a general agreement that dimerization of a significant fraction of G-actin was involved in F-actin dynamics, "lower dimmer" (LD) was unable to polymerize into F-actin filament by itself [49]. Hence it is thought that LD formation may represent an unproductive side reaction [27]. But Steinmetz *et al.* [27,50] recently demonstrates that LD, while being unproductive by itself, can add to growing F-actin filament via one of its subunit. It is revealed that LD can directly incorporate into the growing filaments in the presence of $K^+$ or divalent cation ($Mg^{2+}$, $Ca^{2+}$), thereby producing ragged filaments that frequently branch in the early stages of polymerization and the shift from LD to UD occurs approximately after several to 15 min [27,28,50]. Interestingly in the presence of phalloidin, no significant amount of LD can be detected during polymerization, and the phalloidin-induced polymerization pathway appears to be dominated by a simple nucleation-condensation mechanism [27,50]. According to the experiments [28,50], it is definitely confirmed that the UD- and LD-based pathway co-exist under the condition of undisturbed self-organization. On the basis of the novel discovery, a possible mechanism for the structural polymorphism of actin filaments via self-organization is put forward and schematically described in Fig. 7(A).

It is well known that the F-actin dynamic process can be modulated by many factors, such as icon concentration, ATP, and actin-binding proteins *in vivo* [1,3,4,47]. So it is naturally induced that actin can be kinetically assembled into various particular structures with the modulation of biomechanics properties, space conformations and topological features via interaction with related cytological proteins, and most important, with the regulation of the F-actin dynamics by icon concentration, ATP, and chiefly by actin-binding proteins [Fig. 7(B)]. Furthermore, it is self-evident that the actin-binding proteins could play a key role in controlling actin to form different structure (after all, reagents such as phalloidin etc. don't exist *in vivo*) and providing actin with functional versatility, and

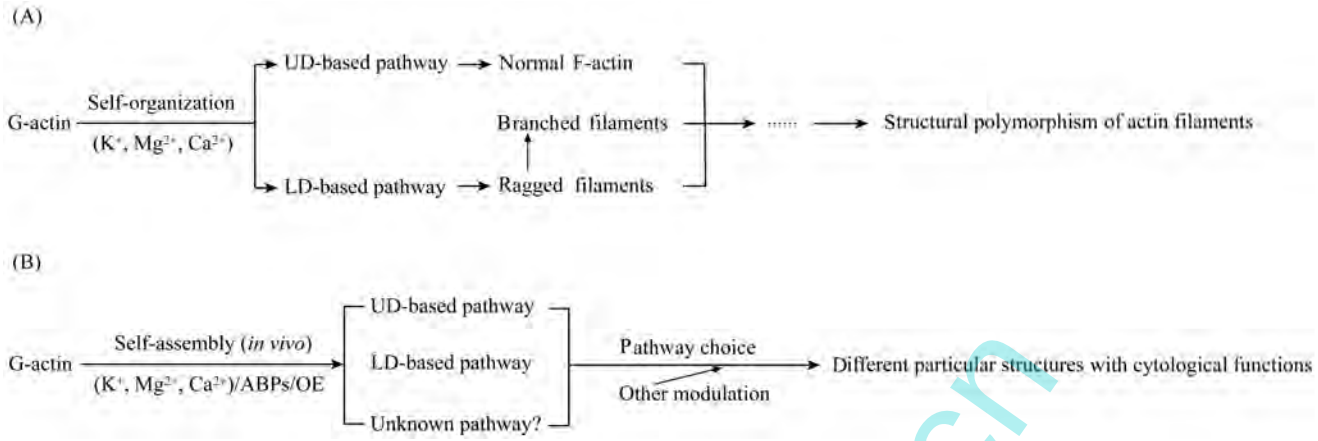


**Fig. 7 Schematically representation of actin polymerization**

(A) Self-organization without interference. (B) Self-assembly in complicated thermodynamic system. ABPs, the actin binding proteins; OE, other effectors; the symbol of "?" denoted the probability.

are thought to exert their functions mainly through affecting the polymerization dynamic mechanism or pathway and altering biomechanics properties, space conformations and topological features via direct or indirect interaction with actin in order to meet the demand of different cytological functions *in vivo*.

Although we qualitatively analyzed the possible mechanism for the structural diversity of actin *in vivo* based on previous and our current experiments, we didn't know the molecular dynamic process and regulatory details yet, and we would continue the exploration in the future. The resolution of the high order filament structure and measurement of their molecular mechanic features will be also an important and interesting subject in the following researches.

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