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Passive and active microrheology for cross-linked F-actin networks *in vitro*

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ABSTRACT

Actin filament (F-actin) is one of the dominant structural constituents in the cytoskeleton. Orchestrated by various actin-binding proteins (ABPs), F-actin is assembled into higher-order structures such as bundles and networks that provide mechanical support for the cell and play important roles in numerous cellular processes. Although mechanical properties of F-actin networks have been extensively studied, the underlying mechanisms for network elasticity are not fully understood, in part because different measurements probe different length and force scales. Here, we developed both passive and active microrheology techniques using optical tweezers to estimate the mechanical properties of F-actin networks at a length scale comparable to cells. For the passive approach we tracked the motion of a thermally fluctuating colloidal sphere to estimate the frequency-dependent complex shear modulus of the network. In the active approach, we used an optical trap to oscillate an embedded microsphere and monitored the response in order to obtain network viscoelasticity over a physiologically relevant force range. While both active and passive measurements exhibit similar results at low strain, the F-actin network subject to high strain exhibits non-linear behavior which is analogous to the strain-hardening observed in macroscale measurements. Using confocal and total internal reflection fluorescent microscopy, we also characterize the microstructure of reconstituted F-actin networks in terms of filament length, mesh size and degree of bundling. Finally, we propose a model of network connectivity by investigating the effect of filament length on the mechanical properties and structure.

1. Introduction

Cells sense, generate and respond to forces in their environment through cytoskeletal dynamics, and mechanical force plays important roles in fundamental cellular processes such as migration, cytokinesis and apoptosis [1–3]. Actin, one of the principal constituents of the cytoskeleton, contributes to the mechanical integrity of the cell and is involved in numerous cellular functions, organizing various microstructures according to functional demands [4,5]. Structural assembly of F-actin, critical in these processes, is regulated by over 100 actin-binding proteins (ABPs) [6,7]. Two major structures of F-actin organized by ABPs are the cross-linked network and the bundled filament. For example, the ABP filamin assembles filaments into three-dimensional orthogonal networks serving as a scaffold for cell motility and signaling [8,9]; in contrast, α-actinin at high concentration forms thick bundles contributing to structural stability of the cell, providing added mechanical strength [10,11]. Therefore, an understanding of cytoskeletal mechanical properties governed by dynamic interactions between actin and ABPs is essential for understanding cell mechanics and the associated biological phenomena.

Cell experiments have revealed that the cytoskeleton exhibits both elastic and viscous characteristics under applied stress [12,13]. Since it is difficult to accurately characterize the mechanical properties of the cytoskeleton in vivo due to active remodeling as well as the presence of numerous other, uncontrolled factors, *in vitro* experiments on reconstituted gels of F-actin have proven useful [14–19]. *In vitro* studies have characterized the viscoelastic properties of F-actin polymerized from purified actin in combination with various ABPs. Many of these measurements of mechanical properties have been performed using a bulk rheometer, which yields global properties of the F-actin matrix. Discrepancies have been observed, however, between these large length scale measurements and microrheometry using micron-scale beads [20]. These have been attributed to a variety of factors, including the non-uniform local stress field, different deformation modes [21], the formation of a depletion zone around the microbead [22,23] and other effects present when the bead is comparable in size to the characteristic dimensions of the actin mesh and individual actin filaments, both of which tend to be on the scale of one to several microns [24]. While this similarity of length scales complicates

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interpretation of the results of microrheometry, it also provides an opportunity to probe the local mechanical response and provide insight into the specific roles of ABP in mediating rheological behavior. Other *in vitro* experiments have demonstrated that actin gels stiffen with increasing strain up to a point, then rapidly soften as strain is further increased [15,25–29]. Actin networks under shear deformation exhibit an irreversible non-linear behavior, suggesting network remodeling and rupture of network bonds [26]. However, compressive force imposed on a dendritic actin network results in reversible stress softening, suggesting that it might be caused by a different mechanism such as filament buckling [27]. The mechanisms for both the increase and sudden fall in modulus remain a subject of debate. Although models to explain these findings of actin cytoskeleton have been proposed [19,27,30], observation of the network's response at the microscale will undoubtedly help elucidate the origin of this non-linear behavior.

Here we employ both passive and active microrheology to measure mechanical properties at the microscale using optical tweezers. Optical tweezers-based microrheology provides the advantage of high-precision force control in the range of 0.1–100 pN, while simultaneously monitoring the motion of the bead with nanometer resolution [31]. Although this technique has been used to measure viscoelastic properties of fd viruses and micellar solutions [32,33], its application to study F-actin networks has been limited [34]. In our passive approach, we track the motion of a thermally fluctuating microbead to estimate the frequency-dependent complex shear modulus of the F-actin network over a frequency range of 10^{-1} to 10^{4} Hz. For the active approach, we apply a sinusoidal driving force to an embedded microbead and monitor its response to obtain the viscoelastic properties of the network. In particular, microscale non-linear behavior of F-actin network is demonstrated by performing the active measurement at large deformation.

We investigate the effect of ABPs on the mechanical properties of F-actin networks using both passive and active techniques. To correlate mechanical properties with structural geometry, both material properties and microstructure of the cross-linked F-actin network are probed as a function of ABP concentration. Confocal microscopy and total internal reflection fluorescent (TIRF) microscopy are used to visualize the F-actin networks organized with filamin, α-actinin and gelsolin. Unique features of F-actin networks polymerized with each ABP are visualized and quantified in terms of mesh size and degree of bundling. Average length of actin filaments is varied using gelsolin to investigate how the length of individual filaments alters network formation and its mechanical properties. While previous rheological measurements on entangled F-actin solutions have demonstrated that particle thermal motions are more constrained as the length of filament increases and as mesh size decreases [16,35], to our knowledge, no comparable measurements have been reported in cross-linked F-actin networks. Based on our measurements, we propose a model to explain how the length of individual actin filaments influences connectivity of the cross-linked network and its elasticity.

2. Materials and methods

2.1. Microspheres

Amino functionalized beads (2.73% solids, Polybead Amino Microspheres; Polysciences, Warrington, PA) 0.5 and 1 μm in radius, were coated with mPEG-NHS (5 kDa; Nektar, San Carlos, CA) to prevent protein absorption as described previously [36] with the following modifications. Stock beads (40 μl) were diluted with 200 μl of deionized water. This solution was spun down for 10 min at 14,000 rpm, supernatant removed and the bead pellet resuspended with 200 μl of methanol. Next, the bead solution was again centrifuged as described above, the supernatant removed and the bead pellet resuspended with 200 μl of 10 mg ml^{-1} PEG-NHS diluted in one part DMSO and four parts methanol. After gently mixing the bead solution for 2 h at room temperature, the beads were stored at 4 °C with continuous rotation to prevent aggregation by sedimentation. Beads were used within 6 months of preparation.

2.2. Reconstituted in vitro F-actin networks

Lyophilized actin monomers and α-actinin both from rabbit skeletal muscle were purchased from Cytoskeleton Inc. (Denver, CO). The activity and purity of actin were tested with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Polymerized actin filaments were separated from the non-polymerized G-actin by centrifugation at 100,000*g* for 40 min [37] and both supernatant and pellets were loaded on a 9% (w/v) PAGE gel. Protein bands stained with Coomassie blue showed that most of G-actin was polymerized into F-actin (Fig. 1A). Protein activity was confirmed by examining the geometry of polymerized actin filaments in the micrographs (Fig. 1B and C). Recombinant filamin-A was purified from Sf9 cell lysates [38] and recombinant human gelsolin is produced in *Escherichia coli* [39].

Actin monomers were diluted in fresh G-buffer (5 mM Tris–HCl, 0.2 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM ATP, pH 8.0) and incubated on

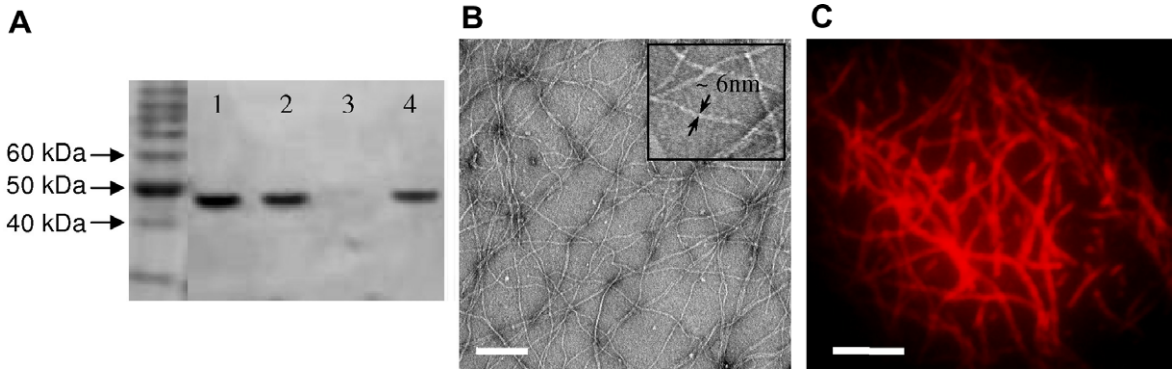

Fig. 1. Characrerization of actin. (A) Scanned image of the polyacrylamide gel. Lane 1, G-actin kept overnight on ice; lane 2, G-actin after centrifuge without polymerization; lane 3, supernatant after centrifugation of polymerized actin; lane 4, pellet after centrifugation of polymerized actin. Bands observed in lanes 1 and 2 confirmed that actin is in monomeric form in G-buffer. In contrast to lane 4, no protein band is observed in lane 3, suggesting that most of the G-actin monomers are polymerized into F-actin during polymerization. (B) Electron microscope image of F-actin which are negatively stained with 2% uranyl acetate (scale bar, 200 nm). Inset: the diameter of actin filament is measured to be approximately 6 nm. (C) TIRF microscopy shows that the length of polymerized actin filaments are varying over 20 μm (scale bar = 5 μm).