Alignment of filamentous proteins and associated molecules through confinement in microchannels

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A technique has been developed to study the structure and interaction of aligned filamentous proteins by confining them in surface-treated silicon microchannels. The micron-size channels induce the semiflexible biopolymers with comparable or larger persistence lengths than the channel width to naturally align parallel to the channel in solution, which facilitates structural studies by x-ray diffraction and optical imaging techniques. As a model system, we investigated the cross-linking of filamentous actin (F-actin) with the bundling protein α-actinin in the microchannels. Synchrotron x-ray diffraction and fluorescence microscopy were used to confirm that F-actin, when bundled in the device, conforms to the alignment of the channel geometry. © *2004 American Institute of Physics*. [DOI: 10.1063/1.1830682]

Small angle x-ray scattering (SAXS) is widely used to investigate the structure and interaction of proteins and protein assemblies in solution because it can probe large length structures under physiological conditions. For example, filamentous proteins, such as actin and microtubules, interact with bundling proteins, e.g., α-actinin and tau, respectively, to form supramolecular structures that range in size from tens to hundreds of nanometers. These protein assemblies are difficult to crystallize and therefore must be studied in the solution state. SAXS data from biomolecules are generally isotropic, due to orientational averaging found in solution samples. This averaging diffuses the diffraction intensities and reduces the signal-to-noise ratio in the data. By introducing orientational ordering in the sample, i.e., aligning the molecules, it is possible to dramatically improve the diffraction signal statistics. Furthermore, using aligned samples simplifies interpretation of the diffraction data because the longitudinal and transverse scattering components will be clearly separated.

Traditional methods to induce alignment in protein solutions include using shear flow and magnetic fields;[1] the former relies upon the asymmetric shape of the molecule whereas the latter takes advantage of the anisotropic magnetic susceptibility in the molecule. Both methods require a fairly large amount of sample and often do not induce sufficient alignment. This letter presents a technique in which the molecules are aligned by confinement in arrays of microchannels. We note that many long chain, rod-like biopolymers have persistence lengths in the micron range. For example, the cytoskeletal proteins intermediate filament, F-actin, and microtubule have persistent lengths of approximately 1 μm, 10 μm, and 2 mm, respectively. It is therefore possible to induce alignment simply by confining these macromolecules in a microstructure that would only allow the molecule to extend in one direction. An additional important advantage of using the microfabricated channels is that only a very small amount of sample is required. Furthermore, using planar semiconductor techniques, a large number of devices can be fabricated on a single wafer enabling combinatorial structural studies.

We demonstrated this method by confining F-actin molecules in microchannels fabricated on a silicon substrate, linking the molecules into a supramolecular structure using α-actinin. The resulting protein–protein complex was analyzed using both SAXS and fluorescence microscopy. F-actin is a rod-like protein and is typically 1–10 μm long and 8 nm in diameter, with a persistence length of ~10 μm. α-actinin is a rod-like protein possessing actin binding sites at each end, thus acting as the network cross-linker.[2,3] It has been shown that in solution F-actin and α-actinin form a network of bundles.[4] A section of the bundle is represented in Fig. 1(a), which shows the F-actin rods and α-actinin cross-linkers. The network consists of bifurcated and overlapping bundles forming a three-dimensional isotropic network

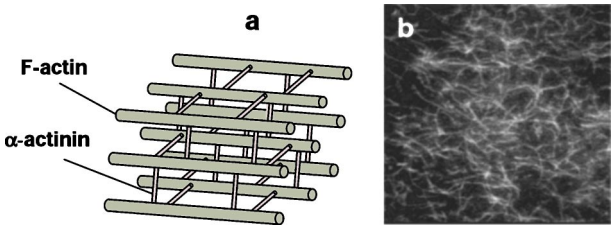

FIG. 1. (Color online) (a) Schematic drawing of the F-actin/α-actinin bundle structure. The F-actin and α-actinin form a 2D distorted square lattice. (b) Fluorescence optical micrograph of 0.1 mg/ml actin bundled with 0.1 mg/ml α-actinin showing the network of bundles.

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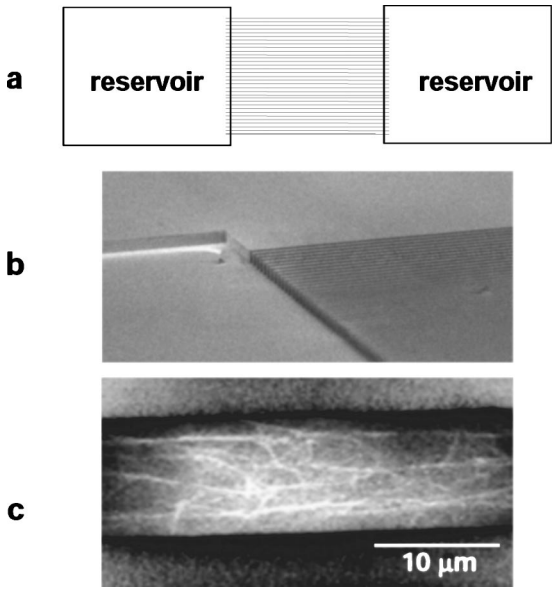

FIG. 2. (a) Schematic layout of the microchannel device. (b) SEM image of a section of the device; (c) fluorescence micrograph of 0.1 mg/ml actin bundled with 0.1 mg/ml α-actinin inside a single 10-μm-wide $\times$10-μm-deep-channel. The bundles are predominantly aligned parallel to the channel.

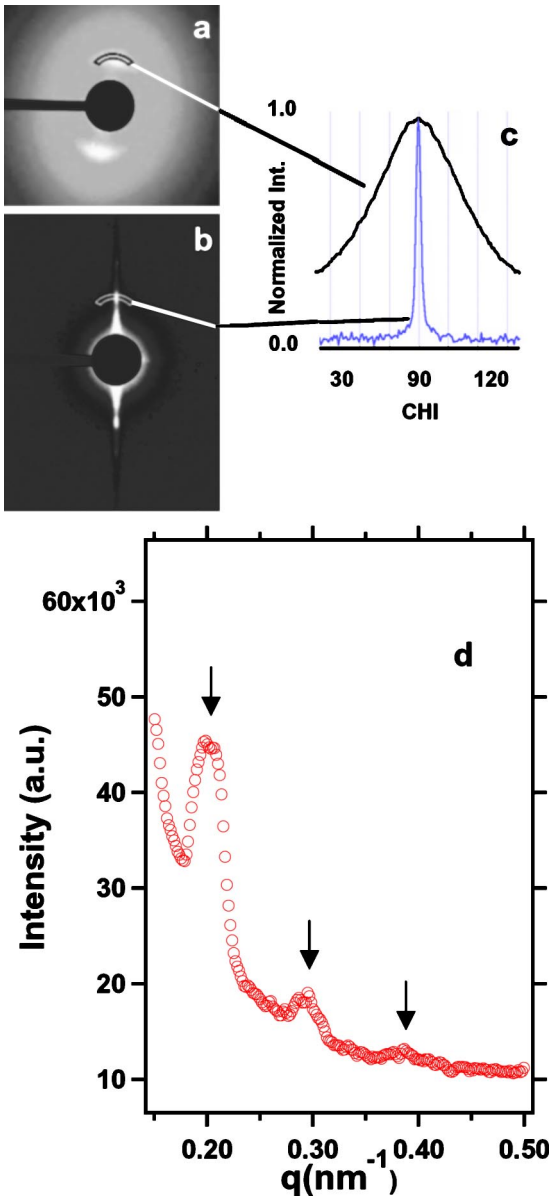

FIG. 3. (Color online) (a) 2D SAXS data from a bulk sample (in a quartz capillary) of the F-actin/α-actinin complex. The diffuse ring of scattering intensity at $q=0.19$ nm^{-1}, resulting from the F-actin/α-actinin structure shown in Fig. 1, shows partial alignment in the sample. (b) 2D SAXS pattern from an F-actin/α-actinin sample prepared in the microchannel device. The diffraction peaks are strongly aligned along the meridional direction, which is perpendicular to the channel. (c) Azimuthal (χ) scans taken at the first-order peak position from the two diffraction patterns demonstrate the dramatically increased alignment in the sample prepared in the microchannel. (d) q scan taken along the oriented diffraction data in (b) shows three peaks (marked by arrows) which index to the distorted square lattice of the F-actin/α-actinin bundle.

shown in Fig. 1(b), as observed by fluorescence microscopy with labeled F-actin. The bulk F-actin/ α-actinin bundles are generally randomly oriented; leading to powder averaging of the x-ray diffraction data.[4] It was expected that by forming the bundle structure inside a micron-width microchannel one would obtain an aligned sample, which can enhance the x-ray diffraction signal.

The microchannel device was fabricated on a silicon substrate using the standard semiconductor processing methods photolithography and deep reactive ion etching (DRIE). The layout of the device is schematically shown in Fig. 2(a). It consists of an array of channels connected to two fluid reservoirs. By using a plasma enhanced RIE tool developed for silicon micromachining, it was possible to etch deep, vertical, high aspect features. The design of the device is "molecule specific" since the width and depth of the channels is scaled with the persistence length of the molecule to be studied. For devices that were used to confine F-actin, the channel dimensions are $5-10$ μm (depth) $\times 5-10$ μm (width) $\times 1500$ μm (length) and the two reservoirs are 1600 μm $\times$ 1600 μm. The pattern was replicated to produce a large number of devices on a single silicon wafer. A scanning electron microscope (SEM) image of a section of the fabricated device is shown in Fig. 2(b). The biopolymers in the microchannel are typically confined in two dimensions by the width and the depth. When the channel depth becomes much larger than the persistence length of the molecule, the confinement becomes one-dimensional.

The biological application of the device requires the surface of the microchannel to be resistant to protein adsorption while maintaining a hydrophilic characteristic necessary for filling.[5] These properties were achieved by oxidizing the silicon surface in a mixture of hydrogen peroxide and sulfuric acid (75:25 v/v), then grafting a PEG-silane layer to the surface. The PEG-silane resists protein adsorption while keeping the surface relatively hydrophilic for a few weeks.[6] After the surface treatment; the reservoir is filled with a glycerol:water mixture (90:10 v/v) using standard pipetting techniques. The glycerol solution acts as a wick to keep the protein solution inside the channels from drying completely.

A micromanipulator/microinjector system mounted on an optical microscope was used to fill the channels with F-actin and α-actinin. The system allowed us to control the X,Y,Z movements of a micropipette using stepping motors while providing the ability to apply a specific injection pressure to the protein solution in the micropipette tip. Each channel was filled individually with a F-actin solution (0.1 mg/ml) under a constant flow of humid air. After the channel was filled the humidity was turned off and the solu-

tion in the channels would evaporate, but due to the glycerol solution in the reservoir, complete evaporation was not achieved. (At this point we had a thin layer of highly concentrated actin still in solution. Without the glycerol, complete evaporation was observed.) A new micropipette was then filled with a solution of α-actinin (0.1 mg/ml) and injected into the channel to mix with the F-actin. The humidity-rich environment was maintained after this second fill. The filled device was sealed by either a thin piece of oxidized polydimethly[siloxane] (PDMS) or by a thin piece of polyimide film. The PDMS sealed device was used for fluorescence microscopy experiments due to its optical clarity. Figure 2(c) shows a fluorescence microscopy image of a single channel, filled using the above-noted method (F-actin was labeled with a fluorescent dye). It is apparent that the bundles have a preferred direction of orientation. Channels sealed using polyimide film were used in x-ray transmission experiments because of the low attenuation.

Synchrotron based SAXS experiments were carried out on bulk F-actin/ α-actinin complexes as well as on channel-aligned samples at the Stanford Synchrotron Radiation Laboratory (SSRL) Beamline 4-2 using a 0.3×0.3 mm beam at 11 keV. Figure 3(a) shows the diffraction pattern from the bulk sample contained in an x-ray capillary; the diffuse ring pattern at $q=0.19$ nm^{-1} corresponds to the spacing between parallel actin filaments (33 nm) set by the length of the α-actinin and the diameter of the F-actin.[4] The data showed weak alignment (anisotropy) probably due to centrifuging used to produce the sample. Figure 3(b) shows the diffraction pattern taken from a channel-aligned sample at normal incidence geometry with the length of the channel in the horizontal direction. The sharp peaks demonstrate strong F-actin alignment along the channel length. An azimuthal scan, taken at constant q corresponding to the first order peak, further demonstrates the degree of alignment in the sample [Fig. 3(c)]. A q scan taken vertically is shown in Fig. 3(d), which clearly shows three expected diffraction peaks from the distorted square lattice in the F-actin/α-actinin bundle structure.[4]

The usefulness of the microchannel as a generic system for orienting filamentous proteins for x-ray diffraction investigations hinges on the quality of the diffraction data when compared to a bulk sample. At normal incidence, the small sample thickness and the substrate absorption reduce the overall diffraction intensity when compared to the bulk. However, the signal to noise ratio is locally enhanced at the diffraction peaks, as shown in Fig. 3(c), due to the alignment of the filaments. Several strategies were adopted to improve the diffraction signal from the channel sample. First, a large number of channels were filled with the sample to increase the active area illuminated by the x-ray beam. Second, the Si substrate on the back of the channel area was thinned by etching to an overall thickness of ~50 μm, which significantly reduced attenuation. Furthermore, deep channels were used to increase the sample thickness and thus the volume of sample illuminated by the beam. These strategies allowed us to acquire useful data, but there are additional methods that can be explored to further enhance the signal. For example, high energy x rays (>15 keV) can be used for the experiment. This will cut down the absorption of the substrate dramatically while preserving most of the diffraction signal, which is only weakly dependent on photon energy. It is also possible, and in fact more desirable, to conduct measurements in the grazing-incidence reflection geometry, in which the effective sample thickness (hence the x-ray signal) will be enhanced, while eliminating substrate adsorption.

Ideally the microchannel samples should be used in conjunction with a micro-diffraction experiment in which the beam size would be comparable to the width of one channel. This would allow the microchannel device to be used as a combinatorial sample cell that can be scanned with the microbeam as a high throughput technique. The microchannels are compatible with standard cryogenic sample cooling systems, which can be used to reduce radiation damage in the thin film sample. In the F-actin experiment, we were able to collect useful data without cryogenic cooling and with no observable sample damage.

In summary, we have developed a technique to examine the structures formed by parallel, interacting rod-like molecules. By creating microchannels with length scales similar to the persistence length of the molecule, sample alignment can be achieved. The microchannel device allows for convenient optical observation along with the powerful analysis of x-ray diffraction. The micro-array induced alignment method can be applied to studying the structure and interactions of a wide range of filamentous biomolecules such as microtubules and intermediate filaments and their associated proteins.

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[1] T. A. Cross and J. R. Quine, Concepts Magn. Reson. **12**, 55 (2000).
[2] M. Imamura, T. Endo, M. Kuroda, T. Tanaka, and T. Masaki, J. Biol. Chem. **263**, 7800 (1988).
[3] R. K. Meyer and U. Aebi, J. Cell Biol. **110**, 2013 (1990).
[4] O. Pelltier, E. Pokidysheva, L. S. Hirst, N. F. Bouxsein, Y. Li, and C. R. Safinya, Phys. Rev. Lett. **91**, 148102 (2003).
[5] T. Pfohl, J. H. Kim, M. Yasa, H. P. Miller, G. C .L. Wong, F. Bringezu, Z. Wen, L. Wilson, M. W. Kim, Y. Li, and C. R. Safinya, Langmuir **17**, 5343 (2001).
[6] A. Papra, N. Gadegaard, and N. B. Larsen, Langmuir **17**, 1457 (2001).