Biopolymerization-Driven Self-Assembly of Nanofiber Air-Bridges

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ABSTRACT

Many proteins polymerize *in vivo* to form nanometer diameter fibers, including actin and fibrin. These processes have been duplicated *in vitro* using only a few enzyme and protein precursors. In this study we consider the application of filament-forming proteins to three dimensional fabrication of nanofiber air-bridges. The formation of the fibers in three dimensions is driven by a process that is generally described as directed self-assembly, where user-defined initial conditions define the evolution of the self assembly process. In the process reported here a protein precursor in solution on an applicator (e.g. the edge of a microscope cover slip) is gently brushed by hand over an array of vertical pillars that are micron in diameter and tens of microns tall. The macroscopic brushing dynamics and the shape of the pillar array direct the assembly of protein solutions into nanostructures. Fibrin fibers, as small as 22 nm have been realized by this technique. Even smaller 16 nm diameter fibers were obtained for initiated polymerization of actin monomers.

Keywords: Protein polymerization, fibrin, thrombin, fibrinogen, actin, nanofibers, NEMS

1 INTRODUCTION AND BACKGROUND

This study is motivated by the robust abilities of biological materials to self assemble into filaments. For instance blood clots rapidly form upon the start of bleeding from a new wound. A clot is a mass of platelets enmeshed in a scaffold of fibrin filaments. When bleeding starts, the enzyme thrombin in the plasma is activated which converts soluble fibrinogen into insoluble fibrin that rapidly polymerizes forming a three dimensional mesh of fibrin filaments.

The growth of actin filaments drives cell locomotion, including chemotaxis. Each actin monomer includes a molecule of ATP. Under normal physiological conditions ATP-ADP conversion induces ligation of one monomer to the next, resulting in a step-wise polymerization [1,2]. The process is regulated by many factors *in vivo*. The reaction requires physiological levels of ions such as potassium, calcium, and magnesium. *In vitro*, addition of ions can be used to initiate polymerization of purified actin.

Three dimensional microscale and nanoscale scaffolds of biological materials such as collagen, chitosan, fibrin and actin are of great interest for applications in tissue engineering and drug delivery, as well in fundamental investigations of the biophysical properties of these materials [3]. While existing processes for fabricating polymeric fibers, including modified textile processing technologies, phase separation [4] and electro-spinning [5] can produce nanoscale fibers, and these fibers can be roughly aligned, they do not produce a degree of order that would be needed to fabricate custom nanodevices or *nano-electro-mechanical systems* (NEMS).

Suspended nanofibers, as small as 10 nm diameter, have been formed by simply hand brushing onto micron scale corrugated surfaces solutions of polymer dissolved in volatile solvents [6,7], similar to the brushing operation illustrated in Figure 1. The diameter of the polymer fibers in these reports depends upon the solution viscosity, evaporation rate and surface tension. This report highlights a modification of this process, where soluble proteins are induced to aggregate or biopolymerize into oriented nanofiber air-bridges, in most cases, and into air-suspended membranes, in a few cases.

2 METHODS AND MATERIALS

The fabrication method consists of priming an array of microscale pillars with an appropriate initiator. In the case

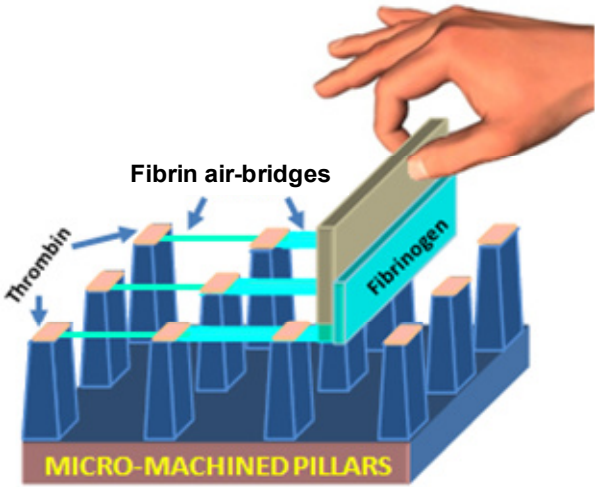

Figure 1. Directed self-assembly of biopolymer air-bridges.

of fibrin air-bridges, a thrombin solution is first brushed over the array, followed by bushing a solution of fibrinogen (as in Fig. 1). For actin fibers the array is primed with a solution of KCl that initiates polymerization when a solution of actin is brushed over the array in 1-2 seconds. The applicator sheet is typically a microscope cover slip or the very flexible plastic backing material on parafilm. In both cases the solution of the monomer is brushed over the array before the primer solution dries out.

Different micro-structured surfaces are used as supports for the fibers. These arrays were obtained by micromachining of silicon, dicing grooves in glass and purchasing of TEM grids.

The fibrin precursors were fibrinogen and thrombin, both obtained from American Diagnostica (Stamford, CT). Samples were prepared with and without factor XIIIa which is a known crosslinker that increases the extensibility of fibrin. The fibrinogen solution used (10 mg/ml in 20 mM HEPES, 100 mM NaCl, 5 mM EDTA, 7.5% Trehalsoe, pH 7.4, Mol. Wt.~340,000D) derived from human plasma depleted of plasminogen, fibronectin and factor XIIIa. The solution was heated in a bath at 37 °C until a clear solution is observed. The solution is maintained at this temperature during the brushing step.

The thrombin used was specifically human alpha-thrombin (Mol. Wt ~ 37,000D) generated by the proteolytic activation of the zymogen prothrombin. A concentration of 5 NIH units/ml of the thrombin was mixed in equal volume with 20 mM $CaCl_2$.

Factor XIIIa derived from human plasma was obtained from American Diagnostica as a stock solution in 50% (v/v) glycerol/water with 0.5 mM EDTA. It was diluted 100 fold in 20 mM $CaCl_2$. The final solution was 1 µl of the diluted factor XIIIa mixed with 200 µl of the fibrinogen solution. The final concentration of factor XIIIa corresponds to 0.4 units/ml activity (where 1 unit corresponds to the activity of 1 ml of pooled normal human plasma.) The factor XIIIa is only mixed into the fibrinogen solution immediately before brushing.

G-actin (the monomeric form of actin) derived from bovine muscle was obtained from Sigma-Aldrich (St. Louis, MO) as a dried powder in 2 mM Tris, pH 8.0 containing 0.2 mM ATP, 0.5 mM ß-mercaptanol and 0.2 mM $CaCl_2$. This powder was reconstituted in 50 mM KCl and 2mM $MgCl_2$ at a concentration of 1 mg/ml.

A solution of 0.6 M KCl is used as the array primer that is used to initiate polymerization of the actin.

3 RESULTS AND DISCUSSION

As with the earlier polymer brush-on method [4,5] surface tension driven capillary thinning and wetting forces play a major role in driving large scale liquid films to transform into nanoscale fibers. The initiator, whether thrombin or potassium ions, induces polymerization. Within a second or less of the brushing, gel-like bridges form between the pillars due to breakup of the film and thinning of the resulting liquid threads. Without the polymerization the liquid would thin even more rapidly and break apart. Upon reaching a stable diameter the fibers can continue to dry in air or be immersed in buffer solution without breaking.

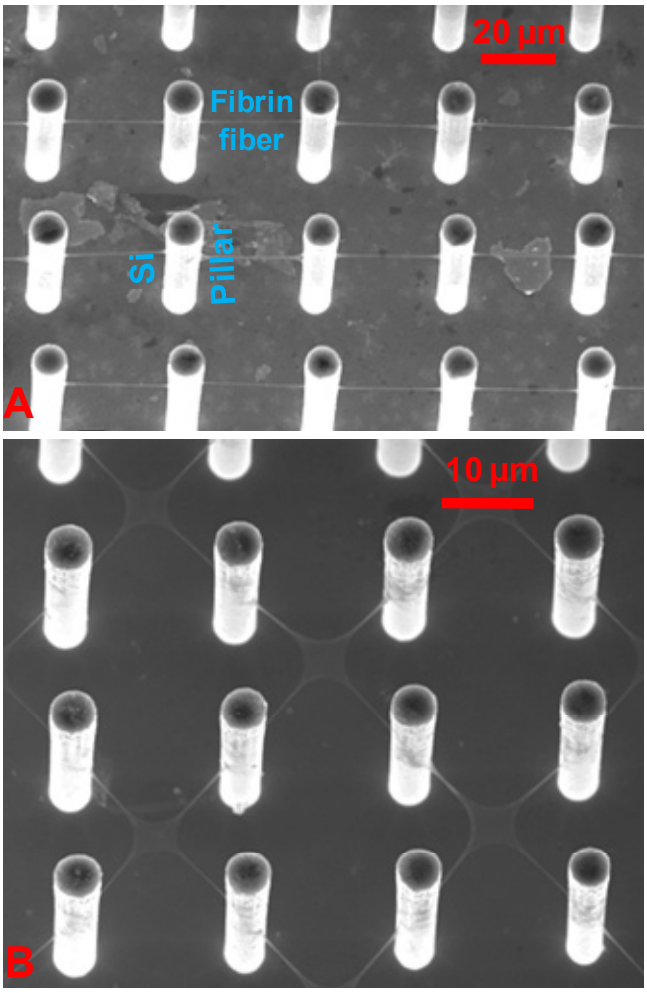

Figure 2. Suspended nanostructures of fibrin produced by the brush-on method. SEM image of (A) air-bridges and (B) air-suspended nanotrampolines.

Figure 2A shows an SEM image of ~380 nm diameter fibrin air bridges oriented in the direction of brushing. Sometimes even smaller fibers are found in the cross direction, suggesting that the brushing actually encourages fibers to thin and breakup faster in the cross direction. Figure 2B shows another SEM image of fibrin membranes resembling trampolines suspended between four pillars. This additional result reinforces the idea that initially a liquid film is stretched over the pillar array and that this film breaks up into discrete structures. The array of trampolines is on a regular spacing which alternates

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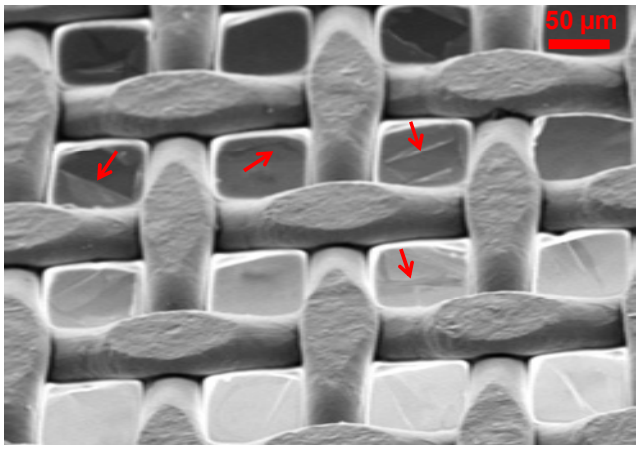

Figure 3. Free standing fibrin membranes on a TEM grid (SEM image). Arrows indicate breaks in the membrane due to handling-induced flexing of the mesh.

between the presence and absence of a suspended structure. .It is hypothesized that the membranes form as a result of the liquid having a longer time to break up (possibly due to a higher initial viscosity of a particular sample, a thicker initial film thickness, or reinforcement against breakup due to polymerization laterally to the direction of brushing.) It is also interesting to note that both types of structures can be found on different portions of the same sample. Regions have also been observed that show a chaotic alternation between both types of structures.

Complete membrane formation is demonstrated by brushing of thrombin and fibrinogen on an iron mesh TEM grid (Fig. 3). The dried membranes break easily with flexing of the grid during peeling it from an adhesive backing used to secure the grid during the brushing step. The arrows in the figure highlight breakage in the membranes. A few of the membranes shown are intact.

Fibrin air-bridges as narrow as 22 nm were found in experiments using an array of pillars of 1 µm diameter spanning a 1 µm air gap (Fig. 4B). This diameter represents the thickness of about 2-3 fibrin monomers as compared to the size of individual fibrinogen molecules (model shown in Figure 4A).

Even narrower air-bridges, down to 16 nm diameter were made by hand brushing of actin. In this case, the actin solution was brushed across a long, ~1.5 µm wide trench forming several fibers, with the smallest fiber found shown in Fig. 4C.

4 MECHANICAL ANALYSIS

Biological fibers and filaments found in animal cells and the extracellular matrix have widely varying compliance and tensile strength. Suspended fibers, isolated from the body, would be more accessible for mechanical evaluation. We have begun such studies on the fibrin fibers. Fibers spanning 500 µm gaps were used for these experiments. The diameters of the fibers are around 1.6 µm. The dried fibers were hydrated and submerged in a solution of 20 mM HEPES, pH 7.4. The fibers are manipulated with a micromanipulator probe station while being observed under its optical microscope. Extensibility of up to 150 % is observed for the fibrin fibers. Enhanced extensibility of up to 350 % is obtained upon addition of the crosslinking factor XIIIa. These extensibilities match with previous reports [9].

Fibrin is known to be very sticky, which is one the reasons it aggregates so readily. Using the micromanipulator we pulled a fibrin fiber (made without factor XIIIa) to breaking and found the fiber attached to the end of the probe. The fiber was brought in contact with another suspended fiber resulting in the structure in Fig. 5A. The 3-point suspended

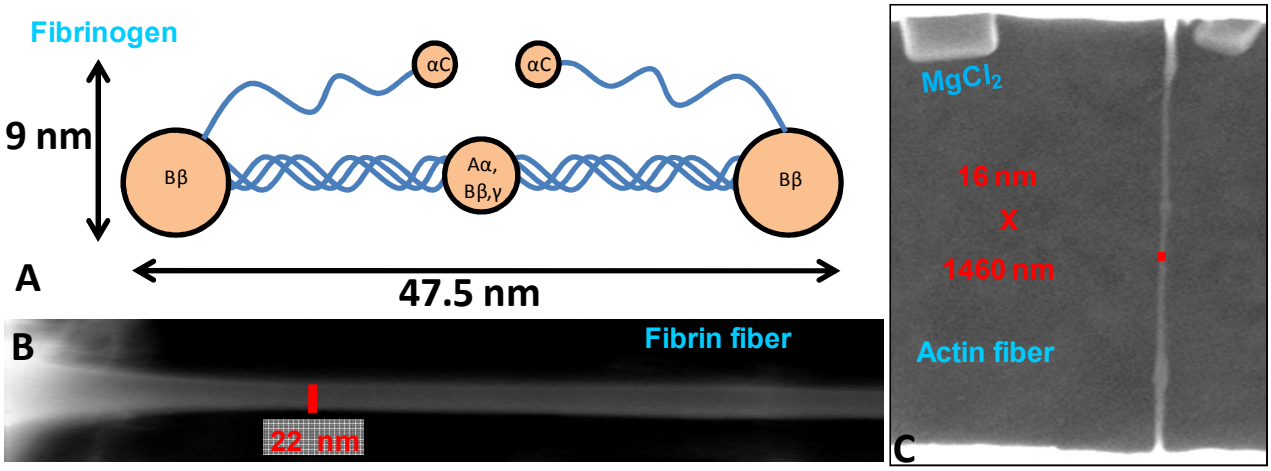

Figure 4. Hand brushed on filaments that approach molecular dimensions. (A) Model showing the dimensions of the fibrinogen molecule [8]. (B) SEM image of a hand-brushed fibrin fiber that is 22 nm in diameter and which spans a 1 µm gap, (C) SEM image of a suspended actin fiber 16 nm in diameter.

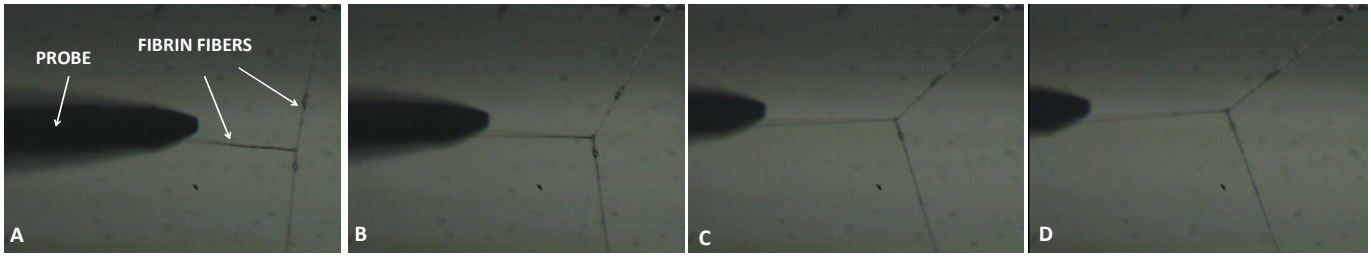

Figure 5. Stretching of two adhered fibrin fibers in HEPES buffer under a micromanipulator, with increasing strain (A-D).

structure is then pulled with increasing force in Fig. 5b-d without losing adhesion and straining the 500 µm fiber by 38 %. Upon removal of the force the fiber returned to its original unstressed shape instantaneously. If the strain is increased further the recovery time is no longer instantaneous, requiring a few seconds to recover. However, fibers made with factor XIIIa, even when strained to near breaking, recover instantaneously upon release of tension.

5 CONCLUSIONS

Biological materials exhibit exceptional properties that can be applied to nanodevice and NEMS fabrication. In this report we have shown that two well known protein systems can be induced to self-assemble into complex three dimensional arrays through biopolymerization, upon addition of an appropriate initiator. These materials are of great interest for their exceptional elastomeric properties. Furthermore, the resulting self-assembled structures provide complex three dimensional templates for the bottom up fabrication of even more complex three dimensional nanodevices.

ACKNOWLEDGEMENTS

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