Biopolymerization-driven self-assembly of nanofiber air-bridges†

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Several proteins, including actin and fibrin, polymerize *in vivo* to form nanometre diameter fibers. These processes can be duplicated *in vitro* using only the essential enzyme and protein precursors. These same protein solutions are directed to self-assemble into oriented arrays of air-bridges using only the crude operation of hand brushing them over textured micron-scale surfaces. The creation of these suspended structures could be used as nanomechanical elements in various sensors and actuators, and their fabrication by this rapid directed self-assembly method would be useful, especially during the early phases of prototype device development. The fabrication method extends earlier studies (Harfenist *et al.*, *Nano Lett.*, 2004, 4, 1931) in which an organic polymer dissolved in a volatile solvent forms nanofiber air-bridges through a combination of capillary force driven thinning of liquid bridges and evaporation driven solidification of the polymer solution. However, in the current study polymerization is initiated when a monomeric solution of soluble protein is brushed over the micro-textured surface. When fibrinogen solutions are brushed over a thrombin primed surface, or monomeric actin over a KCl primed array, fiber air-bridges are formed, sometimes reaching diameters as small as 16 nm. The uniformity in diameter of one hand-brushed array of 358 parallel fibrin air-bridges was 36.4 nm (6.8 nm standard deviation), with no more than 3 broken fibers. The fibrin bridges are shown to be both highly elastomeric and adhesive through demonstration of the construction and stretching of a three point bridge using a micromanipulator. Also the brush-on method produced ordered arrays of suspended fibrin membranes, which sometimes were anchored perpendicular to the vertical sidewalls of the textured surface and other times were anchored parallel to the sidewalls. The demonstration of air-bridge formation by biopolymerization suggested that air-bridges might also be formed during initiated polymerization of organic monomers. Even though the monomer of norbornylene has a much smaller molecular weight than fibrinogen, hand brushing of norbornylene in toluene with Grubbs' catalyst resulted in the self-assembly of fiber air-bridges as small as 4 nm diameter over nearly 6 microns length.

Introduction

In living systems there are a number of proteins, including actin and fibrin, that self assemble into long nanometre diameter filaments. The processes can be remarkably fast and robust as in the case of actin polymerization that drives locomotion of single cell organisms and in the case of fibrinogen polymerization into a fibrin scaffold that captures platelets as part of the blood-clotting sequence. These materials have been the subject of numerous biophysical studies on their formation processes,[1,2] structure[3] and mechanical properties.[4,5] We are interested in using biopolymerization-driven self-assembly for the bottom-up fabrication of well organized arrays of nanostructures. We are especially interested in assembling suspended air-bridges that are easily accessible for mechanical properties testing and that could serve as key elements in nanomechanical sensors and actuators.

These structures can be compared with tissue-engineering scaffolds, which are often made from polymers[6] and biopolymers[7] by electrospinning. Normally the fiber of an electrospun fiber mat crosses itself randomly, though improved organization has been achieved by synchronizing the rotation of the collector plate or drum with precession of the fiber as it is ejected from the spinneret.[8]

A much higher degree of organization has been demonstrated by the directed self-assembly of polymeric liquids into fiber air-bridges that span the raised areas of a micron-scale textured silicon or glass substrate.[9] In this procedure a bead of polymer dissolved in a volatile solvent is hand applied to the substrate in a single-pass brushing motion (see Fig. 1). Surface tension drives the liquid membrane to first break up into liquid threads and then into narrow fibers through capillary thinning. Solvent evaporation drives solidification, which enables stable fibers to form prior to capillary break up of the thread. Fibers are typically oriented in the direction of brushing and their ends are often found below the top surface of the substrate, anchored on the sidewalls of substrate pillars.

Instead of brushing a liquid bead over the substrate, fibers have also been formed by dipping the raised portions of the substrate into a solution and then removing the substrate by a tilting motion that sets up a dewetting flow away from the

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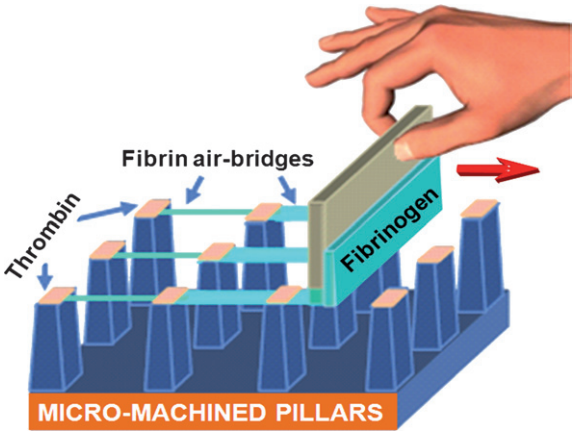

Fig. 1 Directed self-assembly of air-bridges by brushing on of a liquid over a textured substrate that has been primed with a polymerization initiator. The illustration specifically shows a primed array of thrombin initiator converting a brushed-on solution of fibrinogen into solidified fibrin. The paper also presents actin polymerization initiated by a primer of KCl, and norbornylene polymerized by priming the pillar array with Grubbs' catalyst. For the original brush-on approach,[9] a single solution of solvated polymer was brushed over an unprimed array with the fibers solidifying through evaporation alone.

raised edge that is somewhat similar to the flows driven by brushing.[10]

The tilting method has been used to form air-bridges of DNA.[10] Also, suspended polymer air-bridges have been used as sacrificial templates to pattern air-suspended capillaries of polymers, glass and metals.[11] Composite nanomaterial–polymer air-bridges have been formed similarly, and the polymer has been sacrificed through thermal decomposition to leave air-bridges of carbon nanotubes, monolayer sheets of graphene and inorganic nanowires.[12]

This current report on using the materials of fibrin and actin for directed self-assembly of nanostructures was motivated by our atomic force microscopy (AFM) measurements related to mechanical characterization of freshly drawn blood cells. During one measurement in which a special constant diameter AFM tip[13] was repeatedly inserted and retracted from the plasma, a nanofiber was found to be forming. Images of the fiber that formed together with repeated AFM force–distance (*F*–*D*) scans taken as the fiber formed are presented in the ESI†.

We assume that the fiber is due to the formation of fibrin from fibrinogen. Under physiological conditions and temperature (37 °C) soluble fibrinogen converts to insoluble fibrinogen upon activation by the enzyme thrombin. However, these fibers were formed at room temperature so it is possible that evaporation is instead causing saturation of the plasma solution and aggregation of fibrinogen. To confirm that fibrin itself can be induced to form nanoscale diameter fibers, rather than perform a composition analysis on this small quantity of material that was mixed with the numerous other constituents of blood, we instead chose to assemble fibers by the brush-on method using purified extracts of fibrinogen and thrombin (as illustrated in Fig. 1). The extracts when processed at the physiological temperature form fibers (and other nanostructures) in a matter of seconds. Various combinations of brush-on applications that include fibrinogen, thrombin and the cross-linking enzyme factor XIIIa are used to help distinguish between simple aggregation due to drying and actual polymerization (as summarized in Table 1 and discussed in detail below).

As will be shown, during brush-on fibrin does indeed polymerize from monomers of (albeit, high molecular weight 340 kDa) fibrinogen, as do actin filaments from monomeric G-actin. Several novel suspended membrane structures are also observed to form. These results for fibrin and actin further suggest that organic monomers might also be brushed on followed by rapid polymerization and the formation of stable fibers, and this possibility is also experimentally demonstrated below.

Materials and methods

Materials

The fibrin precursors were human fibrinogen (340 kDa depleted of plasminogen, fibronectin and factor XIIIa, product no. 448), α-thrombin (37 kDa, product no. 470HT) and the cross-linking agent factor XIIIa (320 KDa, product no. 413a) all obtained from American Diagnostica (Stamford, CT). Samples were prepared with and without factor XIIIa. The supplied vial of powder when reconstituted in 2 ml deionized water gives a 10 mg ml^{-1} fibrinogen solution at pH 7.4 in 20 mM HEPES, 100 mM NaCl, 5 mM EDTA and 7.5% Trehalsoe. The solution is heated in a bath at 37 °C until a clear solution is observed. A concentration of 5 NIH units per ml of the thrombin is mixed in equal volume with 20 mM $CaCl_2$. Factor XIIIa was obtained 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 is 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 per ml activity (where 1 unit corresponds to the activity of 1 ml of pooled normal human plasma.) The factor XIIIa solution is only mixed into the fibrinogen solution immediately before brushing. A brief evaluation of fiber drawing with half the manufacturer recommended concentration of fibrinogen resulted in smaller

Table 1 Nanostructures resulting from brush-on of combinations of the principal constituents of fibrin

Process type	Brush pillars with solutions of	Resulting structures	Mechanical properties
1	Thrombin	No bridges form	N/A
2	Fibrinogen or Fibrinogen + factor XIIIa	Sparse array of fiber bridges	Fibers dissolve instantly in buffer
3	Thrombin followed by Fibrinogen	Arrays of: ∥ fiber air-bridges, ∥ and ⊥ fiber bridges, trampolines, vertical membranes	Strain to break fiber: up to 150% in buffer, up to 10% dry in air
4	Thrombin followed by Fibrinogen + factor XIIIa	Arrays of: ∥ fiber air-bridges, ∥ and ⊥ fiber bridges, trampolines, vertical membranes	Strain to break fiber: up to 350% in buffer, up to 10% dry in air

diameter fibers, with most of the fibers being broken. At one-tenth the concentration no fibers were observed, even when the thrombin concentration was increased 5-fold. As a result, all experiments were performed with the manufacturer recommended concentrations given above.

G-actin monomer (43 KDa, product no. A3653) 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 β-mercaptanol and 0.2 mM $CaCl_2$. The supplied vial of powder when reconstituted in 1 ml of deionized water gives a 1 mg ml^{-1} G-actin solution. The G-actin is then mixed 1 : 1 (by volume) with a solution containing 50 mM KCl and 2 mM $MgCl_2$. A 0.6 M KCl solution is used as the array primer that initiates polymerization of the actin.

Norbornylene monomer (94.16 Da) is polymerized by the well known ring-opening metathesis polymerization (ROMP) using 2nd generation Grubbs' catalyst (Sigma-Aldrich) as the initiator.[14] Norbornylene monomer in powder form (Sigma-Aldrich) is dissolved in anhydrous toluene to a concentration of 0.3 g ml^{-1}. An initiator solution is prepared by mixing the Grubbs' catalyst to a concentration of 3 mg ml^{-1} in anhydrous toluene. The solution was mixed in a nitrogen atmosphere to avoid oxidation of the catalyst.

Different micro-structured surfaces are used as mechanical supports for the self-assembled nanostructures. High-resolution arrays of vertical pillars were fabricated by deep reactive ion etching of photo-patterned silicon. Lower resolution substrates were made using a diamond dicing saw to cut grooves into glass substrates. Also, inexpensive transmission electron microscope (TEM) grids were used as substrates for a few of the experiments.

Methods

The fabrication of nanofiber air-bridges consists of priming the microstructured pillar arrays with an enzyme or catalyst followed by brushing the array with a protein or monomer solution (Fig. 1). The applicator used for brushing can be a thin sheet such as a glass or microscope cover slip; however, to minimize the risk of breaking microscopic pillars on the substrate, a flexible plastic sheet (which is the backing material from a roll of parafilm) of ~40 μm thickness is used. All brushing operations reported apply a 1 cm long bead (of volume between 2 and 5 μl) to the edge of the applicator. In some experiments the drop is applied by pipette and in others the applicator is dipped into a large drop on a glass slide. Generally, a uniform bead is easier to obtain by dipping of the applicator. Loading the applicator with the solution, by either method, takes about 5 s. This applicator is gently brushed by hand over the top of the pillar array (which is secured to a microscope slide by double-sided tape) at speeds ranging from 1 to 10 mm s^{-1}. The monomer (on a second applicator) is applied within 5 to 10 s after the initiator is brushed on. All solutions, substrates and applicators are maintained at room temperature except the reservoir of fibrinogen solution, which tends to aggregate over long times at room temperature.

Alternatively, it is also possible to perform the substrate-tipping method to form fibers, as reviewed in the Introduction.[10] A few experiments with fibrin produced quite similar results as performed by brushing. However, this method was avoided because dipping the primed pillar into a solution of fibrin polymerizes the entire solution. With the brush-on method a much smaller volume of solution is used.

Scanning electron microscopy. A Supra 35VP SEM in high vacuum mode was used for imaging the air-bridges. The SEM operating system includes dimensional measurement software, which enables direct measurement of the diameters of the fibers.

Micromanipulator. A micromanipulator (Cascade Microtech DCM 210 Series) with eyepiece and video microscope viewing was used to manipulate individual fibrin fibers in air and under buffer in order to evaluate elastic and adhesive properties of the fibers. The manipulator tips were tapered tungsten probes of 25 μm tip radius (Cascade Microtech tungsten PTT-24/4-25).

Resulting fibrin structures

Numerous applications of fibrinogen were performed resulting in oriented fiber air-bridges and membranes formed both parallel and perpendicular to the substrates. This section presents several of the resulting patterns along with confirmation of the requirement for using a thrombin initiator for mechanical integrity of the suspended structures and a discussion of the processing conditions that affect which type of structures form.

Fibrin nanofiber air-bridges

Brushing the fibrinogen over a thrombin-primed substrate at speeds in the range of 5 to 10 mm s^{-1} produces arrays of parallel fibers (~380 nm diameter) along the brushing direction (Fig. 2(A)) in some parts of the substrate and in other parts of the substrate (Fig. 2(B)) the fibers, now of greater diameter (~700 nm) are found both along and perpendicular to the brushing direction. The fibers are also anchored lower on the pillar than in Fig. 2(A). Based on these two differences with Fig. 2(A) we believe that the local brushing speed was slower in the area of Fig. 2(B), which led to the liquid thread wetting further down the pillar and to less liquid draining in the orthogonal direction, which enabled the perpendicular fibers to resist capillary break up.

Even though the processing conditions change in some way over the array leading to variations in the structures that assemble, there can be a surprisingly long range order produced, even when the liquids are hand applied to the substrate. This is well illustrated by the array of fibers in Fig. 3. The air gap between pillars is ~1 μm. The sidewall roughness is quite apparent and is due to limitations in the reactive ion etching process. Even with this coarse substrate the brush-on method produces a well ordered and uniform diameter parallel array of fibrin fibers, as shown in Fig. 3(A). Casual visual examination of SEM images of the entire sample suggests that this degree of order and uniformity is maintained over an area of 111 × 128 μm. The ESI†includes an annotated image of a 48 × 62 μm area of this same sample. It shows 361 air gaps in the brushing direction. Only three gaps, as annotated on the image, contain broken fibers and two of these were observed to break when the SEM scan was focused at high magnification on these fibers. (Extended exposure to electron beams does appear to induce breakage of fibrin, as well as many other synthetic polymers.)

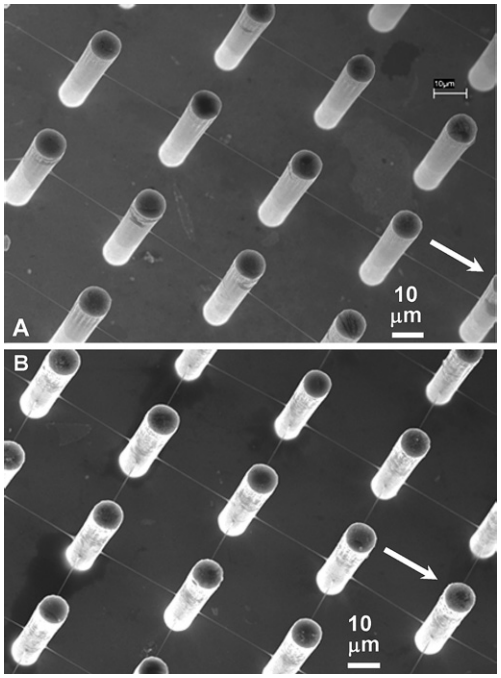

Fig. 2 Suspended arrays of (A) parallel aligned and (B) parallel and perpendicular aligned fibrin fibers obtained by brush-on. The pillars on the silicon substrate are 6 μm in diameter by 50 μm tall on a 34 μm pitch. Arrows are used in A and B (and all subsequent SEM images) to indicate the direction of brushing.

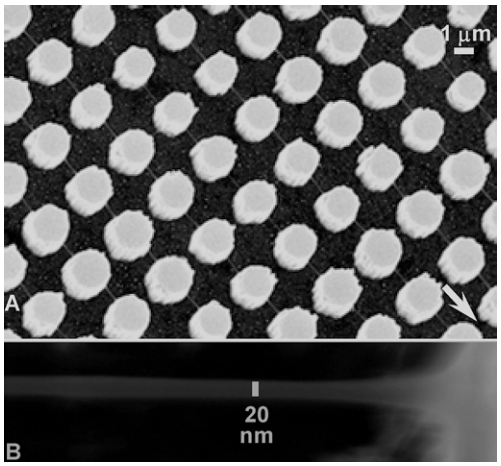

Fig. 3 Hand brushed nanofiber air-bridges. (A) A parallel array of fibers and (B) a single fibrin fiber from the same sample (including metal coating) that is the same thickness as the (vertically oriented) 20 nm scale bar. The coating is used to avoid breakage during SEM. No conductive coatings are on the sample in A.

Also, there are only two fibers, also annotated on the image, that form perpendicular to the brushing direction. Close-ups were taken of 33 randomly selected fibers from this area and measured using the SEM metrology software. The fibers have an average diameter of 36.4 nm with 6.8 nm standard deviation. Also individual fibers as small as about 20 nm (including the ~5 nm thick gold–palladium sputter coating) are found on the same sample (Fig. 3(B)). Note that this diameter represents the thickness of 2 to 3 fibrin monomers.[15]

Fibrin membrane air-bridges and septums

The brush-on method produced novel two-dimensional structures when fibrinogen was brushed at a somewhat lower rate (2 to 4 mm s^{-1}) than the rates used for producing the fibers. In some cases (Fig. 4), suspended membranes parallel to the substrate surface form that resemble trampolines. The anchoring fibers in Fig. 4(A) have diameters ranging from 320 to 530 nm. Side views of the membranes show that the edges are around 180 nm thick but it is unclear from these measurements if the central portion of membrane is the same thickness as the edge or if it is thinner. Membranes were also formed by brush-on over TEM grids. These membranes completely seal the openings in the grids and then can fracture during SEM viewing. The edge of these fractures revealed thicknesses as small as 168 nm.

Note that the array of membranes is not only periodically spaced over the pillar array, but the membranes do not form between every group of four neighboring pillars. Instead, the membranes form between alternate groups of four pillars. Equivalently, only two fibers (instead of 4 if there were no alternation) are anchored on each pillar. We assume that one factor in the formation of a membrane is the slow brushing speed, which tends to thin the liquid applied to the substrate at the same rate along and perpendicular to the brushing direction.

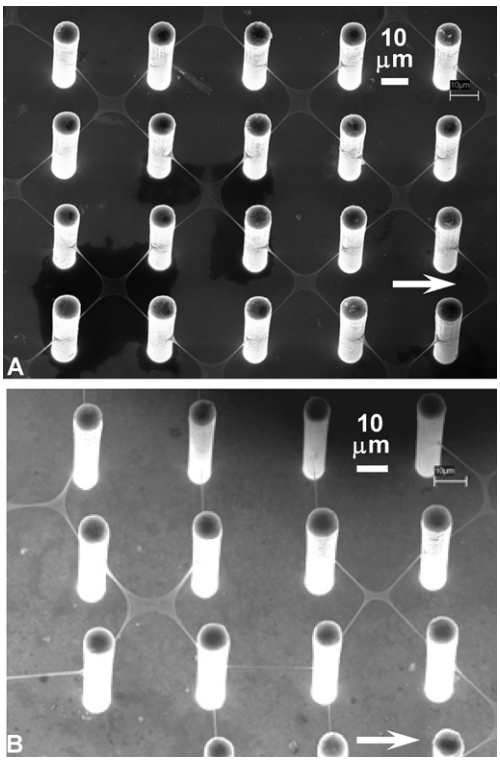

Fig. 4 Four point suspended membrane trampolines obtained by the brush-on method. SEM images of (A) an array of trampolines and (B) the zone at the edge of the trampoline arrays that switches between trampolines, fibers in the brushing direction and fibers perpendicular to the brushing direction.

It is possible that polymerization perpendicular to the brushing direction keeps the membrane from breaking up into fibers (as in Fig. 2).

The alternation pattern on this sample persists over an area of 296 × 227 μm. At the edge of this region is a transition zone (Fig. 4(B)) where the structures appear to randomly or chaotically switch between trampolines, fibers along the brushing direction and fibers perpendicular to the brushing direction.

At an even slower brushing rate than for the horizontal membranes (1 to 2 mm s^{-1}) arrays of vertical membranes having the appearance of septums form along and perpendicular to the brushing direction (Fig. 5.) The membranes in Fig. 5(A) are 6.6 μm in height and span 14 μm between the sidewalls of adjacent pillars. Top views of the membranes show that their edges are around 750 nm thick. The center of the membrane appears somewhat thinner, but due to low contrast of the SEM, it has not been possible to determine how thin the membranes might be. Fig. 5(B) shows a similar array of membranes between 30 μm tall vertical membranes spanning 10 μm gaps between adjacent rectangular pillars. The membranes in Fig. 5(B) developed vertical cracks while they were being imaged in the SEM. The ESI† provides an SEM image that demonstrates long range order of this array over an area of 550 × 400 μm (excluding the annotated boundary region where the membrane pattern ceases). Out of a total of 767 air gaps along the brushing direction there are 741 horizontal membranes. (Due to the perspective in this image, it was not possible to count the number of membranes in the perpendicular brushing direction.) The remaining defects include 4 fiber air-bridges and 13 trampolines (including the 4 trampolines found at the edge of the membrane pattern).

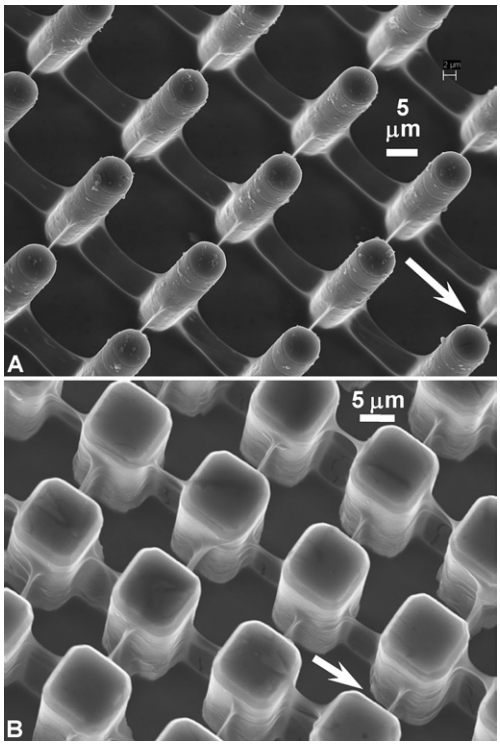

Fig. 5 Fibrin septums. Arrays of vertical membranes between (A) cylindrical and (B) square pillars.

In Fig. 5(A), especially, it appears that the membranes are anchored both to the sidewalls of the pillars and the plane that supports the pillars. At these slowest of brushing speeds it seems that the liquid thread between adjacent pillars formed far enough down the pillars that it touched and wet the substrate plane. The additional wetting to the plane caused capillary thinning both along and perpendicular to the liquid thread, which resulted in the formation of a stable membrane septum instead of a fiber.

Elastomeric and adhesive properties of fibrin air-bridges

Fibrin is a notable elastomer, being able to be strained up to 500% without breaking.[4] Fibrin also readily adheres to itself, which is not surprising given the non-covalent, but nonetheless strong bonds that form between fibrin monomers on polymerization. Using identical solutions as those used in producing the arrays shown above, large (from 1 to 2 μm) diameter fibrin fibers were individually fabricated across a 500 μm wide trench on a glass slide using a pipette tip (tip size ~750 μm) as a brush. The fibers were then mechanically stressed using the micromanipulator. Each fiber was fabricated by pipetting 1 μl of thrombin and 1 μl of fibrinogen into a single drop on one side of the trench. The same pipette is then used to drag a liquid thread (at a rate of 10 mm s^{-1}) from the droplet to the opposite side of the trench groove, resulting in the formation of a stable fiber air-bridge. The process is repeated along the length of the groove to produce an array of parallel fibers.

A micromanipulator probe is then brought into contact with a single fiber bridge at its midpoint and the fiber is stretched laterally. The dry fiber (whether or not factor XIIIa is included in the solution) elongates by no more than 10% strain before breaking. Rehydrating the fibers by immersing the fibers in a pipetted drop of 20 mM HEPES buffer resulted in an elastomeric fiber that was strained up to 150% before breaking. When factor XIIIa was included in the fibrinogen solution, fibers were strained up to 350% before breaking. These extensibilities are comparable to those in previous reports.[4]

Fibrin's adhesiveness is demonstrated by using it to construct a more complex structure—an air-bridge anchored at three points. One of the submerged fibrin bridges (made without factor XIIIa) was contacted by a manipulator probe at its midpoint and pulled until it broke free from one side of the trench. The tip remained connected to the fiber midpoint, and over time the free end floated into contact with the probe and became adhered to it. Further pulling detached the fiber from the other side of the trench. The newly freed end of the fiber was then brought into contact with a second fibrin bridge resulting in the structure in Fig. 6(A). The structure is then pulled with increasing force in Fig. 6(B,C) without losing adhesion.

In Fig. 6(C), the adhered segment between the tungsten probe and the fiber that spans the groove is strained 33%. Fiber 1 is strained by 38% and upon removal of the force it returned instantly to its original unstressed shape. However, Fiber 2 remained at its stretched length when the force was removed. Apparently, when the fiber was broken free from the substrate it was strained beyond its tensile limit destroying its ability to recover.

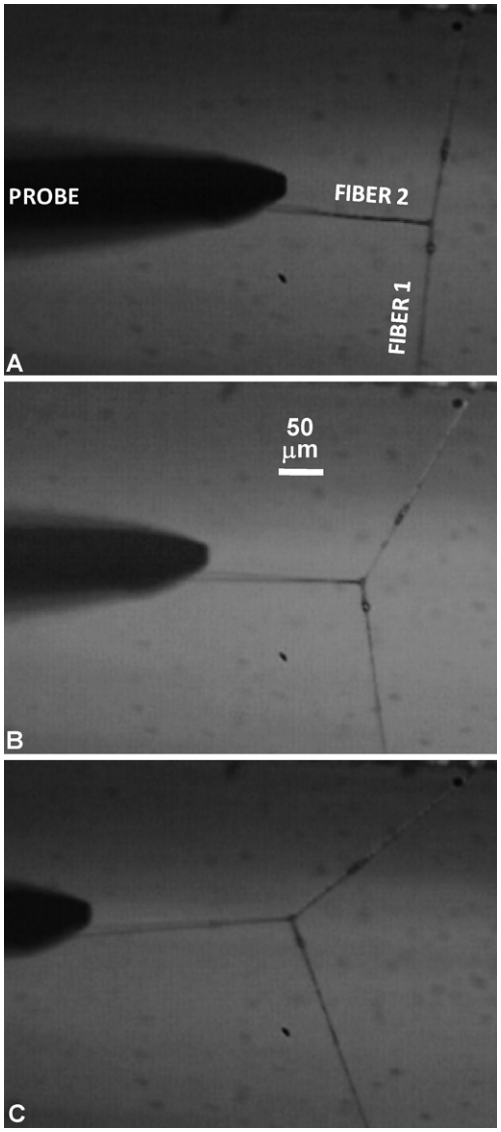

Fig. 6 A three point bridge constructed from two fibrin fibers as viewed through the micromanipulator's video microscope. Fiber 1, which spans the groove is (A) unstrained, (B) strained by 12% and (C) strained by 38% by continuous retraction of Fiber 2, which is attached to the tungsten probe.

Discussion of fibrin nanostructure formation

Our experiments with fibrinogen and thrombin extracts demonstrate that fibrin air-bridges form by polymerization initiated by the enzyme thrombin. The reasoning can be understood by review of Table 1. For Process 1, in which only thrombin is applied, no bridges appear after brush on. For Process 2, in which only fibrinogen is brushed on, air-bridges that are generally smaller and less uniform in diameter do appear. These dissolve instantly when the fibers are submerged in the buffer solution. We are led to conclude that these fibers solidify as a result of precipitation and aggregation of the fibrinogen as the liquid bridges dry. For Process 3 and Process 4 (which is Process 3 with the addition of factor XIIIa cross-linking enzyme) the fiber and the more complex membrane air-bridges form. Not only are these structures insoluble in the buffer, but they are both elastomeric and adhesive. Therefore, we conclude that either Process 3 or Process 4, both of which are initiated polymerizations, are responsible for the formation of the fibrin air-bridges. The reliable formation of the air-bridges is due in large part to the robust nature of biological processes.

There is an unresolved question as to the dynamics of the formation of the fibrin bridges. Our current experimental setups are incapable of observing the formation process due to limited abilities to monitor the high speed of fiber thinning at submicroscopic nanoscale dimensions. The air-bridges could form in at least two ways.

One possibility is that the fibrin filaments form, drift to the center of liquid bridge and produce an air-bridge spanning two pillars. Liquid surrounding the pillars drains to the large (compared to the fibrin bridges) pillars through capillary thinning, leaving a solid fibrin air-bridge behind.

A second possibility is that the fibrin filaments are distributed throughout the liquid thread. The filaments at some point form a network completely spanning the liquid thread. A resulting gel of large enough modulus can resist further thinning due to capillary forces. The thread can continue to thin from this point by evaporation. Then below a certain diameter the capillary force can overwhelm the gel modulus leading to a sudden thinning due to capillary forces.

The two models are similar enough that they could simultaneously be at play, or either one might occur for differences in fibrin concentration or thread diameters present in a particular experiment.

Self-assembly of other monomers into polymer nanostructures

Since fibrin as it is polymerizing can self-assemble during brush-on into organized nanostructures, it suggests the generalization that other materials undergoing polymerization could form similar structures during brush-on. One question is can other filamentary proteins undergoing polymerization form nanostructure air-bridges? A second question is can much lower molecular weight monomers form air-bridges as they are undergoing polymerization? We had assumed that the formation of air-bridges using organic monomers would be more challenging than using protein monomers because protein monomers are already large enough to be considered polymers. For individual proteins and increasingly as even a few protein monomers bind, the solution can become very viscous due to increasing chain entanglements. This increased viscosity then reduces the rate at which liquid threads thin and break up immediately following brush-on. For a much lower weight organic monomer the initial viscosity might be so low that the liquid thread breaks prior to the solution achieving adequate viscosity through polymerization. A limited set of experiments with actin and norbornylene indicate that other protein, as well as organic monomers undergoing polymerization can be patterned into air-bridges by brush-on.

Actin fibers

Hydrolysis of the ATP molecule in each G-actin monomer to ADP drives a stepwise ligation or polymerization of actin. In

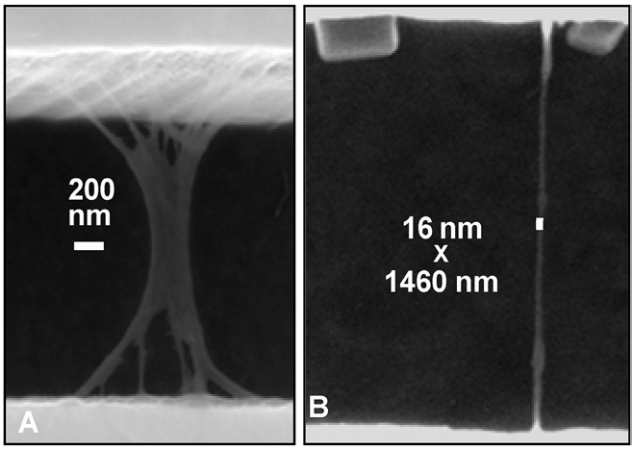

Fig. 7 Actin nanostructures produced following the sequential brush-on of KCl and actin monomer over a 1.46 μm wide groove in a silicon substrate.

solutions of actin, Mg and K ions at sufficient concentrations activate the polymerization process.[2] In our experiments the addition of a high concentration of KCl (see Materials and Methods) is used to initiate polymerization of actin. First, we prime an array of grooves in silicon of ~1.5 μm width with KCl. Then the actin solution is applied in a second brushing. Two representative structures produced in the same application are shown in Fig. 7. Note that neither the initiator nor the monomer solutions by themselves were observed to form stable fibers. In Fig. 7(A) an air-bridge forms that is 246 nm at its waist. The tapered waist is suggestive of the shape that would be expected due to capillary thinning. Therefore, it may be possible that this structure formed as a result of several individual actin fibers fusing together during simultaneous polymerization and thinning. Fig. 7(B) shows an isolated individual actin fiber that is 1.46 μm long by about 16 nm wide. The cubic structures are crystals of the dissolved salts originally in the solution.

Poly(norbornylene) nanostructures

Polymerization of norbornylene by Grubbs' catalyst is fast enough with the small volume of solution used that stable fibers do indeed form, as shown in Fig. 8. However, the individual initiator and monomer solution by themselves did not produce fibers. In Fig. 8(A) the fibers are 25 nm to 400 nm wide by 12 μm long. Note that these fibers, compared to the fibrin fibers, form at the top of the pillars. This result is consistent with the higher evaporation rate of toluene compared to water, which rapidly depletes the volume of solution available to wet the sidewalls. Fig. 8(B) shows an isolated set of parallel fibers formed between two pillars that are 12 μm long. A magnified view in Fig. 8(C) of one of these fibers shows that the diameter of this fiber, as measured by the SEM, is 4 nm over at least 6 μm of length. It is possible that during the thinning process a single membrane bridged the two pillars. If the membrane was not dry, perhaps the edges due to their increased surface area dried and solidified before the membrane solidified. The membrane between the edges then broke apart leaving solidified nanofibers. Alternatively, perhaps the membrane solidified and then broke apart due

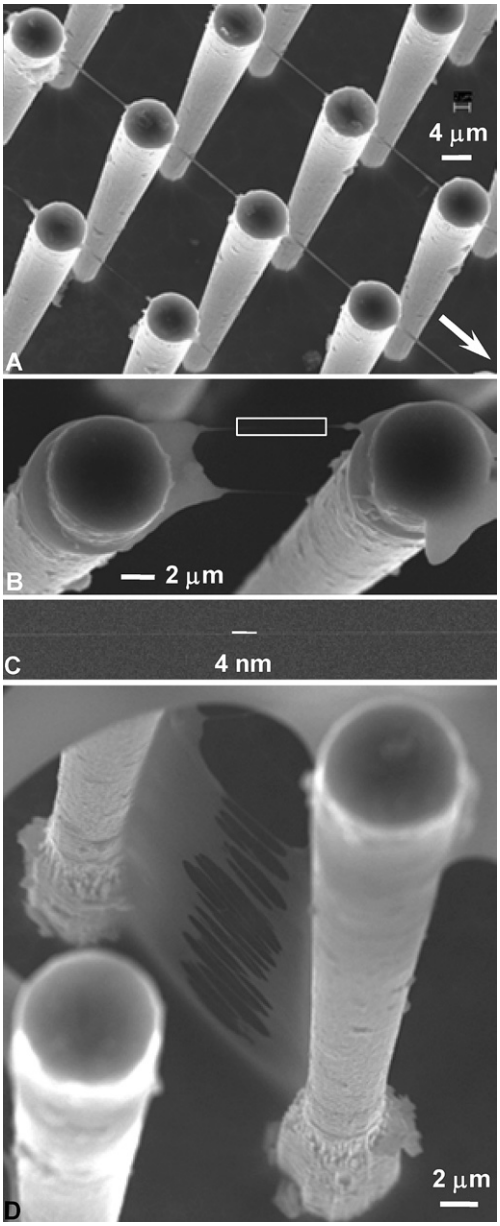

Fig. 8 Poly(norbornylene) air-bridges on a silicon pillar array. The pillars are 7 μm diameter with a gap between each pillar of 12 μm. (A) An oriented array of fibers of diameters from 36 × 50 μm. (B) Two fibers suspended between two pillars. (C) Magnified image of the area indicated in B. A line that is 4 nm wide is traced over the fiber. (D) A vertical membrane that appears to have broken up into numerous strings. The smallest fiber in D is 55 nm diameter.

to residual stress, which is known to be present in norbornylene polymerized by ROMP.[14] If this second formation process is in play, then other remnants of the broken membrane would be expected to be visible. In Fig. 8(D) we did see evidence of a membrane that had torn apart into numerous strings resembling the lacing of an old fashioned corset. Whatever the actual formation process might be, it is remarkable that intact fiber air-bridges of 4 nm diameter by 6 μm long can exist following manual brush-on.

Conclusions

Many biological processes are noted for being robust and well regulated. In the case of filamentary proteins, including fibrin and actin, we observed the robust self-assembly of macroscopic, hand-applied films of protein monomers into nanoscale air-bridges under the combined driving forces of polymerization and capillary thinning. Moreover, this process was found to be extendable to organic monomers undergoing initiated polymerization. This assembly method generalizes the earlier process of brush-on with polymers dissolved in volatile solvents, to admit monomers in the process of polymerizing. This subtle extension might be enough of a difference to lead to the richer set of well organized horizontal and vertical membrane air-bridges that were observed to form.

Further understanding of the dynamics of the formation of these structures might be developed by comparing differences in structure due to whether or not the polymer is initially anchored to and grows from the surface of the substrate, which is possible with a step-wise polymerization. For example, perhaps the step-wise ROMP process is possible both with an anchored[16] and a dissolved Grubbs' catalyst enabling the comparison of difference in air-bridge formation when the polymer only grows from a surface and when it grows in solution only eventually becoming adhered to the surface.

The ability to start the brush-on process with a monomer, rather than a polymer, has additional potential applications to rapid custom patterning of three-dimensional structures. Specifically, low enough molecular weight organic monomers could be evaporated and then locally condensed on hydrophobic patches arranged in a desired pattern on a three-dimensional substrate. A small brush or array of styluses treated with an appropriate initiator drawn between a monomer droplet and a second location could then create an array of air-bridges of even greater complexity than the periodic arrays presented above. Considering the problems (*e.g.* slow flow rates and clogging) associated with delivering ultrasmall volumes of polymers by nanodiameter capillaries, delivery by condensation of monomers might prove to be a reasonable alternative.

While proteins would not be viable for the proposed condensation-based patterning method, the ability demonstrated above to pattern proteins into highly flexible soft structures does support their use for applications as scaffolds capable of supporting individual live cells. Organized arrays of the types presented above might provide a more geometrically controlled environment for the study of cell–cell communication through the surrounding liquid medium.

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