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ECM Protein Nanofibers and Nanostructures Engineered Using Surface-initiated Assembly

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Abstract

The extracellular matrix (ECM) in tissues is synthesized and assembled by cells to form a 3D fibrillar, protein network with tightly regulated fiber diameter, composition and organization. In addition to providing structural support, the physical and chemical properties of the ECM play an important role in multiple cellular processes including adhesion, differentiation, and apoptosis. *In vivo*, the ECM is assembled by exposing cryptic self-assembly (fibrillogenesis) sites within proteins. This process varies for different proteins, but fibronectin (FN) fibrillogenesis is well-characterized and serves as a model system for cell-mediated ECM assembly. Specifically, cells use integrin receptors on the cell membrane to bind FN dimers and actomyosin-generated contractile forces to unfold and expose binding sites for assembly into insoluble fibers. This receptor-mediated process enables cells to assemble and organize the ECM from the cellular to tissue scales. Here, we present a method termed surface-initiated assembly (SIA), which recapitulates cell-mediated matrix assembly using protein-surface interactions to unfold ECM proteins and assemble them into insoluble fibers. First, ECM proteins are adsorbed onto a hydrophobic polydimethylsiloxane (PDMS) surface where they partially denature (unfold) and expose cryptic binding domains. The unfolded proteins are then transferred in well-defined micro- and nanopatterns through microcontact printing onto a thermally responsive poly(N-isopropylacrylamide) (PIPAAm) surface. Thermally-triggered dissolution of the PIPAAm leads to final assembly and release of insoluble ECM protein nanofibers and nanostructures with well-defined geometries. Complex architectures are possible by engineering defined patterns on the PDMS stamps used for microcontact printing. In addition to FN, the SIA process can be used with laminin, fibrinogen and collagens type I and IV to create multi-component ECM nanostructures. Thus, SIA can be used to engineer ECM protein-based materials with precise control over the protein composition, fiber geometry and scaffold architecture in order to recapitulate the structure and composition of the ECM *in vivo*.

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Video Link

The video component of this article can be found at http://www.jove.com/video/51176/

Disclosures

The authors declare that they have no competing financial interests.

Keywords

Bioengineering; Issue 86; Nanofibers; Nanofabrics; Extracellular Matrix Proteins; Microcontact Printing; Fibronectin; Laminin; Tissue Engineering; poly(N-isopropylacrylamide); Surface-Initiated Assembly

Introduction

The extracellular matrix (ECM) in tissues is composed of multifunctional proteins involved in physical and chemical regulation of multiple cell processes including adhesion, proliferation, differentiation, and apoptosis[1–3]. The ECM is synthesized, assembled, and organized by cells and the constituent protein fibrils have unique compositions, fiber size, geometries and interconnected architectures that vary with tissue type and developmental stage. Recent work has demonstrated that the ECM can provide instructive cues to guide cells to form engineered tissues[4], suggesting that recapitulating the ECM in terms of composition and structure might enable the development of biomimetic materials for tissue engineering and biotechnology applications.

A number of fabrication methods have been developed to engineer polymeric scaffolds that can mimic aspects of the ECM in tissues. For example, electrospinning and phase separation have both demonstrated the ability to form porous matrices of fibers with diameters ranging from tens of micrometers down to tens of nanometers[5–7]. Both techniques have also shown that highly porous matrices of nanofibers can support cell adhesion and infiltration into the scaffold[8]. However, these approaches are limited in the possible fiber geometries, orientations and 3D architectures that can be created. Electrospinning typically produces scaffolds with either randomly oriented or highly aligned fibers whereas phase separation produces scaffolds with randomly oriented fibers. There are also limitations on the materials, with researchers typically using synthetic polymers, such as poly(ε-caprolactone)[8] and poly(lactic-co-glycolic acid)[9], that are subsequently coated with ECM proteins to promote cell adhesion. Natural biopolymers are also used, including collagen type I[10], gelatin[11], fibrinogen[12], chitosan[13], and silk[14], but represent only a small subset of the proteins found in native tissue. Most tissues contain a larger milieu of ECM proteins and polysaccharides including fibronectin (FN), laminin (LN), collagen type IV and hyaluronic acid that are difficult or impossible to fabricate nanofibers using existing methods.

To address this challenge, we have focused our research efforts on mimicking the way cells synthesize, assemble and organize ECM protein fibrils in their surroundings. While the specific fibrillogenesis process varies for different ECM proteins, typically a conformational change in the ECM protein molecule is triggered by an enzymatic or receptor-mediated interaction, which exposes cryptic self-assembly sites. Here we use FN as a model system to better understand the fibrillogenesis process. Briefly, FN homodimers bind to integrin receptors on the cell surface via the RGD amino acid sequence in the 10th type III repeat unit. Once bound, the integrins move apart via actomyosin contraction and unfold the FN dimers to expose cryptic self-assembly sites. The exposure of these FN-FN binding sites enables the FN dimers to assemble into an insoluble fibril right on the cell surface[15]. Work in cell-free systems has demonstrated that cryptic FN-FN binding sites can be revealed

through unfolding using denaturants[16] or surface tension at an air-liquid-solid interface[17–19]. However, the FN fibers created by these techniques are restricted to specific fiber sizes and geometries and are typically bound to a surface.

Here we describe an approach termed surface-initiated assembly (SIA) [20] that overcomes these limitations by utilizing protein-surface interactions to create free-standing insoluble nanofibers, nanofabrics (2D sheets) and other nanostructures composed of single or multiple ECM proteins (Figure 1). In this process, ECM proteins are adsorbed from a compact, globular conformation in solution and partially denatured (unfolded) onto a patterned, hydrophobic polydimethylsiloxane (PDMS) stamp. The ECM proteins are then transferred in this state onto a thermally responsive poly(N-isopropylacrylamide) (PIPAAm) surface through microcontact printing[22]. When hydrated with 40 °C water the PIPAAm remains a solid, but when cooled to 32 °C it passes through a lower critical solution temperature (LCST) where it becomes hydrophilic, swells with water and then dissolves, releasing the assembled ECM nanostructures off of the surface. The SIA method provides control over the dimensions with nanometer-scale precision. By controlling key parameters such as composition, fiber geometry, and architecture, it is possible to recapitulate many properties of the ECM found *in vivo* and to develop advanced scaffolds for tissue engineering and biotechnology applications.

Protocol

1. Fabrication of Master Mold Using Photolithography

1. The ECM protein nanofibers, nanofabrics and nanostructures to be fabricated are first designed using Computer Aided Design (CAD) software. This CAD file is then transferred to a photomask. The type of photomask will depend on the resolution of the features; with a transparency-based photomask adequate for feature sizes down to ~10 μm. Smaller features <10 μm will require a chrome on glass photomask. All of the nanofibers and nanostructures presented here were fabricated using a transparency-based photomask, and thus were nanometer-scale in thickness but not lateral dimensions.

   Note: It is important to distinguish which regions of the photomask will be dark (prevent UV light to pass through) and which will be transparent (allow UV light to pass through) as this, along with the type of photoresist (positive or negative), will dictate the final topography of the master mold.

2. To begin fabrication of the master mold, dehydrate a 4" silicon wafer by placing it on a hotplate set to 150 °C for 15 min.

3. Center the wafer on the vacuum chuck of a spin-coater. Pour SU8-2015 negative photoresist onto the middle of the wafer and continue pouring in concentric circles until about two thirds of the wafer is covered.

   Note: Keep the bottle of SU8 close to the wafer when pouring to minimize the formation of bubbles.

4. Program the spincoater as follows:

- Spread cycle: 500 rpm with an acceleration of 100 rpm/sec for 10 sec.
- Spin cycle: 4,000 rpm with an acceleration of 100 rpm/sec for 30 sec.

Note: This spincoating recipe will form a photoresist layer that is ~10 μm in thickness. By changing the spinning speed or the SU8 formulation, the thickness can be adjusted.

5. Soft bake the wafer by placing it on a hotplate set to 95 °C for 3 min.
6. Expose the wafer with UV light through the photomask for a total dosage of 140 mJ/cm^2.

   Note: SU8 is a negative photoresist therefore regions where UV light is able to pass through the photomask will remain after developing and become raised features on the master mold.
7. Post exposure bake the wafer by placing it on a hotplate set to 95 °C for 4 min.
8. Develop the wafer by placing it in SU8 developer for 3 min. After 3 min, rinse the wafer with isopropyl alcohol. If a white film is produced during the rinsing, the wafer is not fully developed and it should be placed back in the developer for another 30 sec. Rinse again with isopropyl alcohol. Repeat this process until a white film does not form during the isopropyl alcohol rinsing.
9. Dry the wafer in a stream of nitrogen and place in a 150 mm petri dish to protect from dust.

2. Making the PDMS Stamps

1. Prepare the PDMS prepolymer by combining the elastomer base and curing agent in a 10:1 w/w ratio. Typically 80 g of base and 8 g of curing agent are used to ensure there is sufficient PDMS to cover the master mold in a 1 cm thick layer.
2. Mix and degas the PDMS using a centripetal mixer set to the following:
   - Mix: 2,000 rpm for 2 min
   - Degas: 2,000 rpm for 2 min.
3. If a mixer is unavailable mix the PDMS by hand for 10 min using a 10 ml serological pipette. Degas the mixture by placing it in a vacuum desiccator for 30 min to remove bubbles.
4. Pour enough PDMS prepolymer over the master mold (patterned silicon wafer) to form a 1 cm thick layer. Cure the PDMS by baking at 65 °C for 4 hr or at room temperature for 48 hr.
5. Once cured, The regions containing the patterns can be cut out to form the PDMS stamps. To distinguish the feature side from the backside of the PDMS stamp, cut a notch out of one of the corners on the backside of the stamp.

3. Microcontact Printing of ECM Patterns

1. Clean 25 mm diameter glass coverslips by sonication in 95% ethanol for 1 hr and then dry in a 65 °C oven.
2. Prepare the PIPAAm solution by dissolving PIPAAm in 1-butanol at a concentration of 10% (w/v, typically 1 g in 10 ml).
3. Center a glass coverslip on the vacuum chuck of the spincoater and pipette 200 µl of the PIPAAm solution so that the entire glass surface is covered.
4. Spincoat the coverslip at 6,000 rpm for 1 min.
5. Clean the PDMS stamps by sonication in 50% ethanol for 30 min and then dry under a stream of nitrogen.

   Note: Drying and subsequent steps should be performed in a biosafety cabinet to maintain sterility for applications where the ECM nanostructures will be used with cells.
6. Coat the patterned surface of each PDMS stamp with 200 µl of the protein solution, typically 50 µg/ml in sterile distilled water for FN. Incubate for 1 hr at room temperature.

   Note: This coating volume is for a 1.5 cm^2 PDMS stamp and will need to be adjusted depending on the size of the PDMS stamp, the ECM protein used and the concentration of the ECM protein in solution.
7. Wash the PDMS stamps in distilled water to remove excess protein and dry thoroughly under a stream of nitrogen.

   Note: Any water left on the stamp will trigger the premature dissolution of the PIPAAm coating on the coverslip and prevent proper protein transfer.
8. For sterile fabrication, place the PIPAAm-coated coverslips inside a closed petri dish and sterilize using UV exposure, 45 min under the UV light in a biosafety cabinet is sufficient. If sterility is not required this step can be omitted.
9. Perform microcontact printing by placing the feature side of the PDMS stamp in contact with the PIPAAm-coated coverslip. If required, use forceps to tap lightly on the back of the stamps to remove any air bubbles and ensure uniform contact.
10. After 5 min, peel off the PDMS stamp from the coverslip.
11. At this stage, additional ECM proteins can be patterned to create more complex and multicomponent structures. Up to 3 printings have been verified to work with this process, and more may be feasible.

4. Release of ECM Nanofibers and Nanostructures

1. Place the patterned PIPAAm coated coverslip in a 35 mm petri dish and inspect the pattern fidelity using phase contrast microscopy. Depending on the pattern, a CCD camera may be necessary to resolve the features of the pattern. Fluorescence

microscopy can also be used to inspect the pattern provided the ECM proteins are fluorescently labeled.

2. Add 3 ml of 40 °C distilled water to the petri dish and allow the water to gradually cool.

3. The dissolution of the PIPAAm layer and the release of the ECM protein patterns can be monitored using phase contrast microscopy. If the application does not permit the use of optical techniques, the release can be monitored by measuring the solution temperature. Typically, the water is cooled to room temperature, well below the LCST of PIPAAm (32 °C), to ensure the ECM protein nanostructures have been released.

4. After release, the nanofibers, nanofabrics and other nanostructures are floating in water. To use them for further applications they need to be manipulated. The exact approach will depend on the experimental objective and may include steps such as immobilizing onto another surface, moving with a micromanipulator system or embedding in a hydrogel.

Representative Results

SIA is capable of engineering ECM protein nanofibers with precise control over fiber dimensions. To demonstrate this, arrays of FN nanofibers with planar dimensions of 50 × 20 μm were patterned onto a PIPAAm coated coverslip (Figure 2A). Upon release, the fibers contracted because they were under an inherent pre-stress when patterned on the PIPAAm surface (Figure 2B). Analysis of the FN nanofibers revealed they were monodisperse pre-release with an average length of 50.19 ± 0.49 μm and width of 19.98 ± 0.17 μm (Figure 2C). Despite contracting appreciably, FN fibers post-release were still monodisperse with an average length of 14.15 ± 0.92 μm and width of 2.65 ± 0.32 μm (Figure 2D). Atomic force microscopy provided a higher resolution perspective of the fiber dimensional changes associated with the SIA release process. Notably, fibers pre-release had a uniform thickness of ~5 nm (Figure 2E) whereas fibers post-release had a heterogeneous thickness on the order of several hundred nanometers (Figure 2F).

Using the SIA process it is possible to engineer a variety of ECM protein nanostructures with tunable size, shape, and composition (Figure 3). For example, FN nanofibers initially 20 μm in width and 1 cm in length were patterned onto a PIPAAm-coated coverslip. Upon cooling and PIPAAm dissolution the nanofibers were released forming long threads (Figure 3A). Further, because the pattern is defined by the surface topography of the PDMS stamp used for microcontact printing, it is possible to engineer complex ECM protein nanostructures. As proof-of-concept, we created multi-armed FN stars that retained their general shape after release (Figure 3B). Interestingly, the arms of the FN star contracted like the nanofibers but the body of the star maintained its size. While FN is important, we also wanted to demonstrate that SIA works with other ECM proteins such as LN and that multiple ECM proteins can be incorporated into the same structure. For example, we engineered a 2-D nanofabric composed of orthogonal and interconnected nanofibers of FN and LN in a square lattice array (Figure 3C). Pre-release the FN nanofibers were 20 μm wide

and the LN nanofibers were 50 μm wide. Upon release both types of nanofibers contracted but the overall interconnectivity and square lattice structure was maintained. These results demonstrate that SIA can be used to engineer ECM materials with a variety of compositions and structures.

Instances of failed SIA of ECM nanofibers are shown in Figure 4. One cause is improper release of an incomplete pattern due to poor transfer of ECM protein to the PIPAAm surface during microcontact printing (Figure 4A). The presence of holes, irregular edges, and other defects will create nanofibers and nanostructures that are incomplete and prone to breakage and fragmentation upon release. Rapid dissolution of PIPAAm can also cause poor pattern fidelity after release (Figure 4B). For example, using room temperature 20 °C DI water already below the LCST of PIPAAm instead of 40 °C water will cause the PIPAAm to rapidly swell and dissolve. This can cause two problems; (i) the rapid expansion can rip apart some ECM nanofibers and (ii) rapid expansion can cause disruption of the pattern arrangement after release.

Discussion

The SIA method presented here mimics cell-mediated matrix assembly and enables the engineering of ECM protein nanofibers and nanostructures with tunable size, organization and composition. While not identical to cell-generated ECM, SIA creates ECM composed of nanoscale protein fibrils[20] that undergo reversible folding/unfolding during mechanical strain[21] and can bind cells[20]. This provides a unique capability to build ECM protein materials that recapitulate many properties of the ECM found *in vivo*. For example, ECM nanofibers can be fabricated in specific lengths with a level of control infeasible with other techniques. We demonstrate the ability to create arrays of monodisperse nanofibers (Figure 2) with precise control of length and width pre-release (Figure 2C) and post-release (Figure 2D). These ECM nanofibers can be any length, as demonstrated by fabricating FN nanofibers in ~1 cm lengths (Figure 3A). In contrast, other fabrication techniques such as electrospinning and phase separation can create nanofibers but not with the precise control of length, producing mostly continuous fibers. These techniques are also limited in the fiber geometries and organization within a scaffold. SIA can be used to build ECM nanostructures with arbitrary planar geometry, such as a star (Figure 3B), and in 2-D sheets with arbitrary control of fiber organization, such as a nanofabric (Figure 3C). Further, other fabrication methods typically use synthetic materials or only a limited number of natural ones such as chitosan and fibrin. In comparison, SIA enables the assembly of nanofibers and nanostructures composed completely of ECM proteins such as FN and LN, which cannot be fabricated with these other methods.

The critical step in the SIA process is the thermally-triggered release from the PIPAAm surface. To ensure proper release, there are a few key steps that should be considered. First, after the microcontact printing step, the fidelity of the transferred ECM pattern should be inspected either using phase contrast microscopy or fluorescence microscopy (if the ECM proteins are fluorescently tagged). If there are any defects in the protein pattern on the PIPAAm surface, then the subsequent release will produce nanostructures that contain these defects (as observed in Figure 4A). If a PDMS stamp repeatedly produces protein patterns

with defects, it is probable that the micropatterned side of the PDMS stamp has been scratched or contains defects and a new stamp and potentially a new master mold should be made. In addition, care should be taken when hydrating the protein patterned, PIPAAm-coated coverslip. The water should be at or near 40 °C and be allowed to cool slowly. If the water is not warm enough or cools too rapidly, the PIPAAm will swell and dissolve too quickly causing the ECM protein nanofibers and nanostructures to be potentially torn apart from the forces and haphazardly released such that any structural organization resembling the dry, pre-released state will be lost (Figure 4B).

ECM protein nanofibers, nanostructures and nanofabrics assembled using SIA have many applications in biomechanics, tissue engineering, and biotechnology. For example, recent evidence indicates that the mechanical properties of ECM protein fibrils governs their biological functionality[23]. SIA is ideally suited to create ECM protein nanofibers in purified form for mechanical analysis. To perform these experiments, released ECM protein nanofibers can be manipulated using precision micropositioners and then stretched. Deravi *et al* have recently used this approach to demonstrate that FN nanofibers can withstand up to 8-fold extensions and that they undergo elastic, plastic, and strain-stiffening regimes with increasing strain[21]. In tissue engineering, ECM protein based scaffolds in the form of gels (*e.g.*, collagen type I and fibrin) or decellularized tissues[4] are widely used. Compared to these techniques, the advantage of SIA is the ability to engineer scaffolds with well-defined architecture in 1-D fibers and 2-D sheets (Figure 3C). For example, we have previously shown that cardiomyocytes can be seeded onto FN nanofibers to form functional strands of cardiac muscle[20]. This shows that the FN nanofibers are cell binding and that they can direct the anisotropic assembly of cells into aligned tissue structures. The 2-D nanofabrics can mimic the composition and laminar structure of the basement membrane for application in the engineering of epithelial and endothelial tissues. Further, we are developing new ways to deploy these ECM nanofibers and nanofabrics in 3-D by embedding them within hydrogel matrices. From the process described in this article, it is possible to use SIA to engineer ECM protein-based, nanostructured materials for a wide variety of applications.

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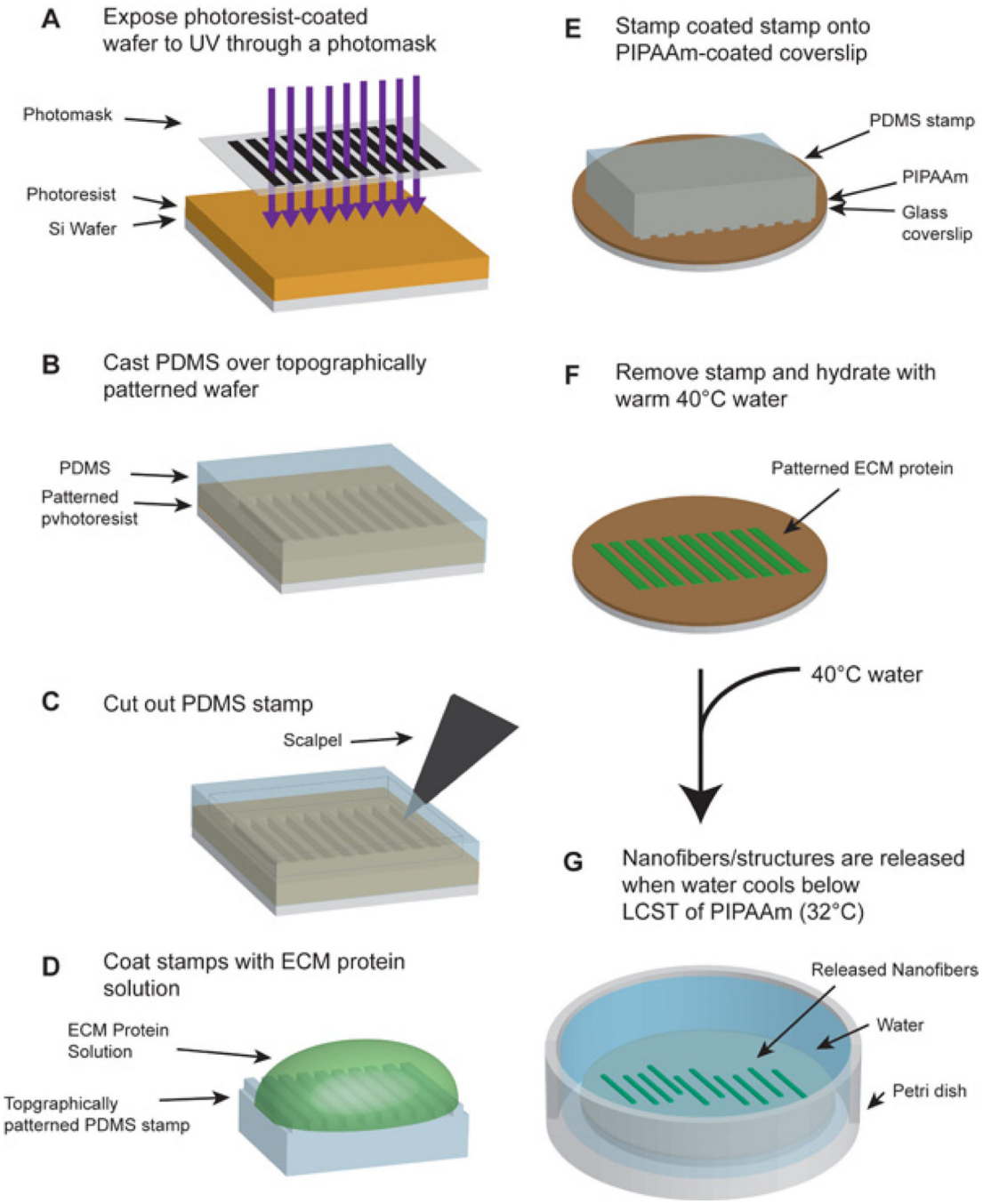

Figure 1. Schematic of the SIA process

(A) A silicon wafer is spincoated with SU8 negative photoresist and exposed to UV light through a photomask. Non-exposed regions are developed away leaving a topographically patterned master mold. **(B)** PDMS prepolymer is poured over the master mold and cured for 4 hr at 65 °C. **(C)** After curing, a PDMS stamp is cut out. **(D)** The stamp is then incubated with an ECM protein solution where the proteins adsorb to the stamp in a partially unfolded conformation. **(E)** The stamp is rinsed to remove excess protein, dried, and placed in conformal contact with a PIPAAm-coated glass coverslip. **(F)** The stamp is then removed

leaving behind patterned ECM protein on the PIPAAm coated coverslip, the pattern is dictated by the topography of the stamp. **(G)** The coverslip is then placed in a Petri dish and covered with 40 °C water and then cooled below the LCST of PIPAAm (~32 °C), which triggers the dissolution of PIPAAm and the release of assembled ECM protein nanofibers and/or nanostructures from the surface.

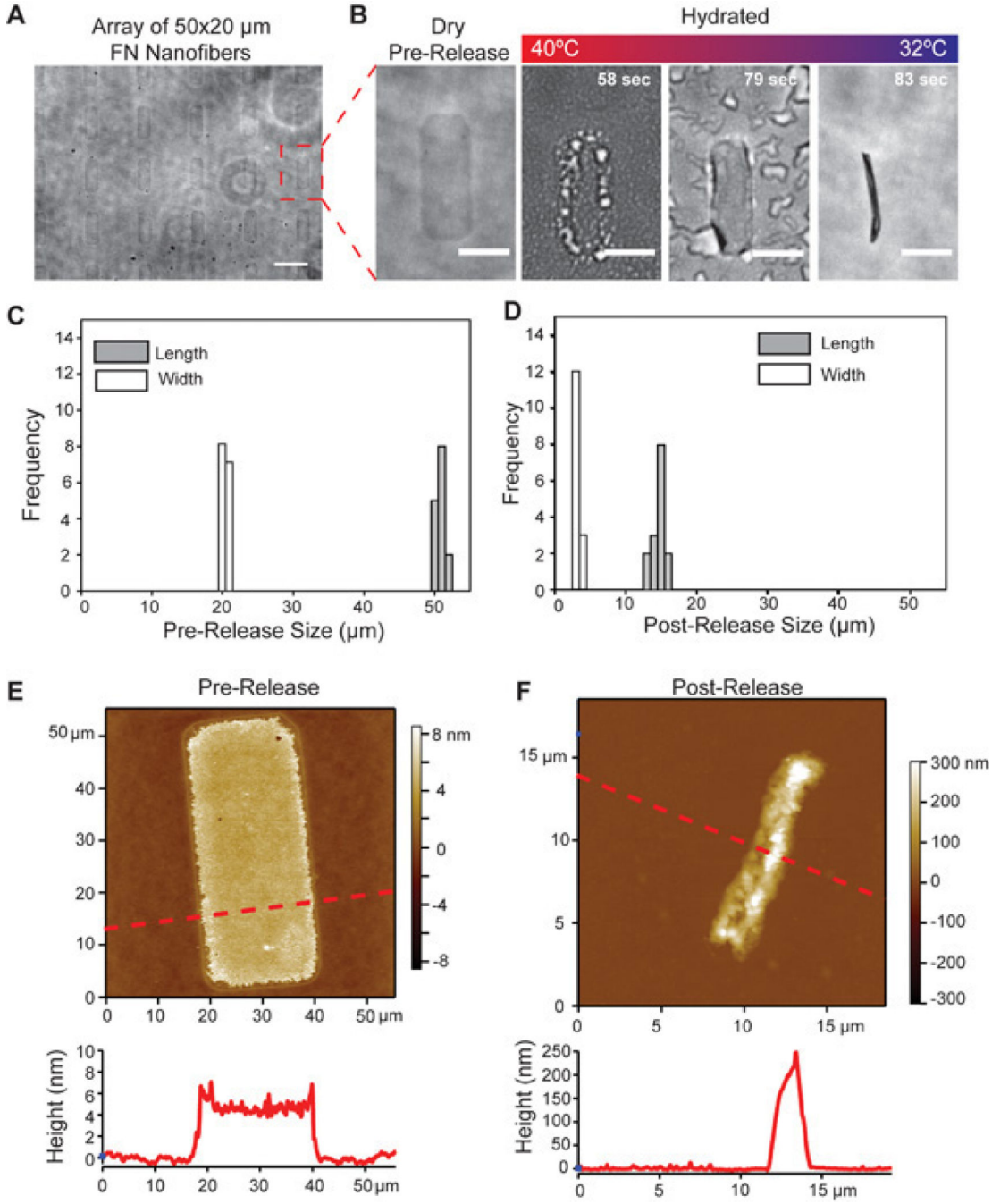

Figure 2. Using SIA to engineer populations of monodisperse nanofibers

(A) An array of FN rectangles, 50 µm in length and 20 µm in width were patterned onto the PIPAAm surface. **(B)** Addition of 40 °C DI water and subsequent cooling below the LCST of PIPAAm triggered the dissolution of PIPAAm and the release of the FN nanofibers. Upon release, the fibers contracted as they are under a pre-stress when patterned on the PIPAAm surface. **(C)** Analysis of nanofiber dimensions pre-release reveals the fibers are monodisperse with a length and width of 50.19 ± 0.49 µm and 19.98 ± 0.17 µm, respectively. **(D)** Upon release the nanofibers contracted but remained monodisperse with

post-release length and width of 14.15 ± 0.92 μm and 2.65 ± 0.32 μm, respectively. **(E)** AFM showed that the pre-release nanofibers were ~5 nm thick. **(F)** AFM of post-release nanofibers showed that the thickness had increased to several hundred nanometers while the length and width decreased. -Scale Bars are (A) 50 μm and (B) 10 μm.

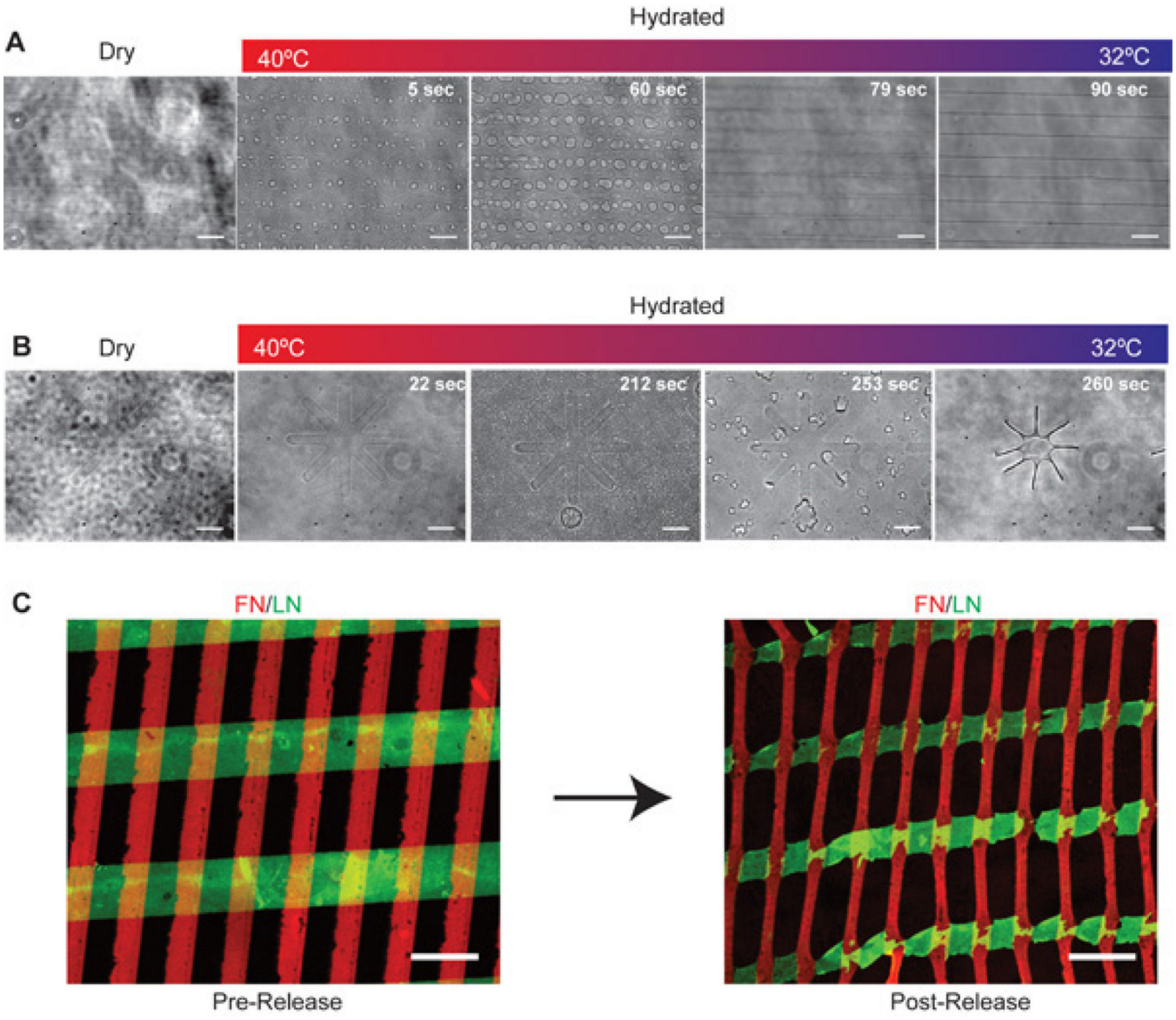

Figure 3. Using SIA to engineer ECM protein nanofibers and nanostructures with tunable size, shape, and composition

(A) FN nanofibers were patterned onto a PIPAAm-coated coverslip as 1 cm in length and 20 μm in width. Thermal release resulted in the SIA of long, intact FN nanofibers with a reduced width of ~3 μm. **(B)** An example of a more complicated multi-armed FN star, representative of the diverse nanostructures that can be created using SIA. Thermal release resulted in the contraction of the arms but not the central region of the star, where the arms joined together. **(C)** It is also possible to integrate multiple ECM proteins into the same structure. For example, orthogonal, interconnected 20 μm wide lines of FN (red) and 50 μm wide lines of LN (green) lines integrated into a 2D nanofabric were patterned and then released. Even after release, the pattern retained its initial geometry and interconnectivity. Scale bars are 50 μm.

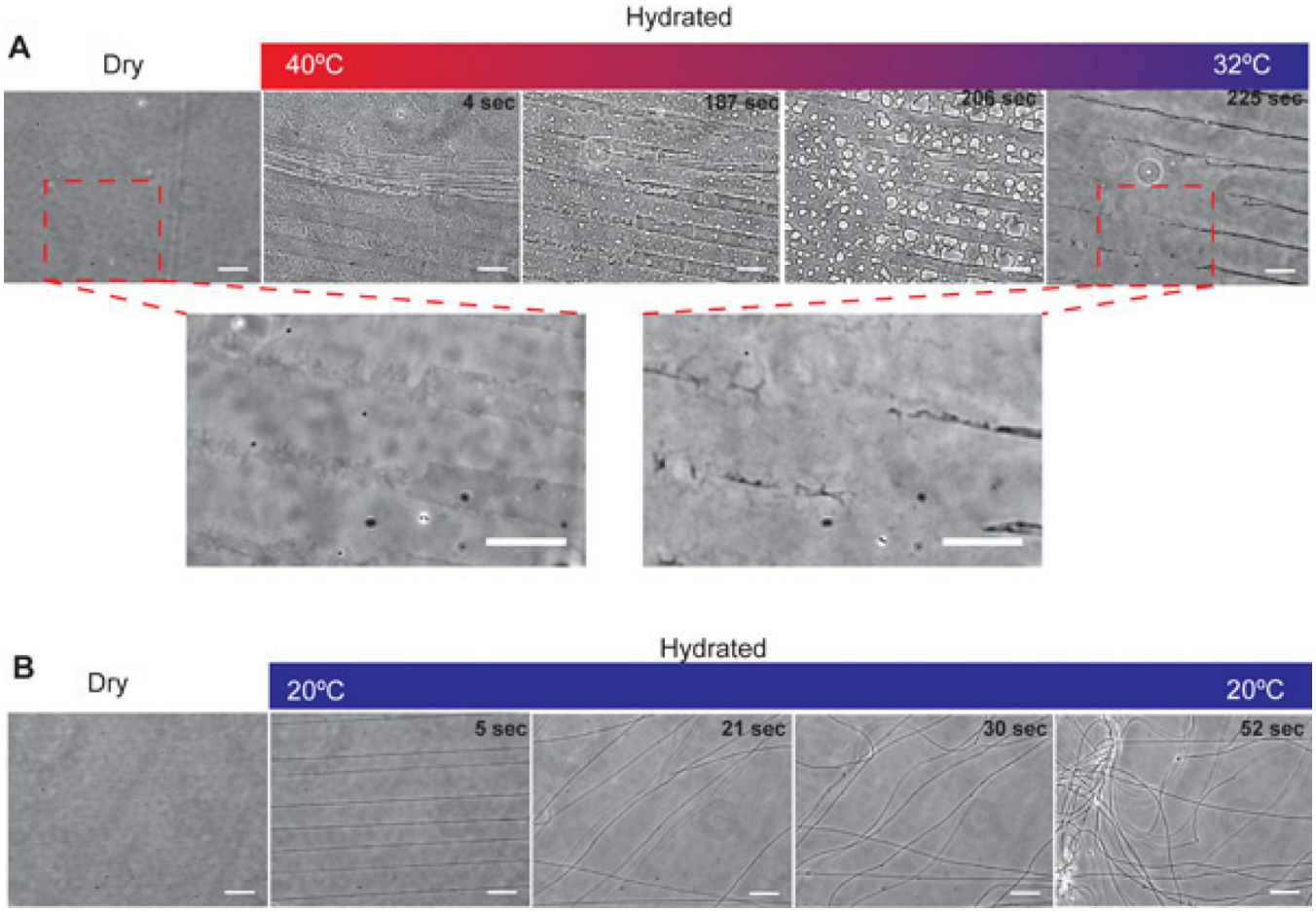

Figure 4. Examples of problems that may prevent proper SIA and protein release from the PIPAAm surface

(A) Poor microcontact printing of ECM proteins onto the PIPAAm results in fragmentation and other defects of a 20 μm wide FN line prevents the SIA of a continuous FN nanofiber and instead results in the formation many smaller FN fragments. **(B)** Rapidly releasing the FN nanofibers from the PIPAAm substrate may also affect the final fiber arrangement. In this case water at 20 °C, already below the LCST of PIPAAm, was added and triggered rapid dissolution causing the nanofibers to snap back, break (30 sec) and form random, unorganized configurations (52 sec). Scale bars are 50 μm.