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Fabricating Fibrinogen Microfibers with Aligned Nanostructure, as Biodegradable Threads for Tissue Engineering

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Biocompatible and biodegradable polymer fibers from several microns to few submicron sizes are appealing for various biomedical applications, such as tissue engineering, wound dressings, and drug delivery of sensitive biomolecules.[1,2] Especially, the natural biopolymers received much attentions for the past two decades due to their non-immunogenicity, biodegradability, and biocompatibility, where they could easily bind with variety of cells through specific biochemical interactions. Furthermore, the polymer carriers with aligned nanostructures with pores could be more useful for sustained release of active biomolecules, as well as, providing orientations for specific cell growth. In addition, porous surface in fiber could also maintain controlled bioavailability and drug delivery capability, facilitating cell growth, proliferation, accommodation, and transportation of nutrients and waste metabolites.

For diverse biomedical applications, the natural protein-based nanofibers could provide many advantages over the synthetic polymers for their natural intercellular or biochemical interactions in subsequent scaffold remodeling, like degradation and formation of extracellular matrix (ECM), or epidermis. However, the available common methods for the polymer fabrications of microfibers or nanofibers relied on electrospinning, phase separation, and self-assembly, limited the use of natural proteins. For example, the electrospinning would not be suitable partially for aqueous soluble proteins due to less spinnability. The self-assembly method would involve many complex laboratory procedures with selective synthetic polymers, like diblock copolymers, triblock copolymers, triblocks from peptide-amphiphile, and dendrimers. Furthermore, the nanofibers made from above procedures appeared to have relatively weak mechanical strength, making difficulties in handling *in vitro* and *in vivo* for the post modifications of fiber.[4,5] Hence, fibrinogen (Fbg) nanofiber could be one of the promising materials in tissue engineering, due to their nanometer size (50-500 nm) and natural interactions with native ECM. Functionally, Fbg nanofiber could provide a passage for cells to communicate between neighboring cells, as strong anchor for cell adhesion and their survival, by modulating the biochemical interactions and activating the intracellular signals. In future, Fbg nanofiber may even completely regurgitate the structural and biological properties of tissues or organs.[2]

Previously, we observed unaligned inter connected nanostructure networks within Fbg microspheres.[6] Based on the previous results, Fbg microfibers with aligned nanostructures towards one direction were fabricated for the first time by a simple gel-solvent extraction method, utilizing micron size silicon rubber tube with mechanical strength and without high power voltage and high pressure.

To compare the alignment of nanostructure formations, Fbg solutions were prepared in two different concentrations,

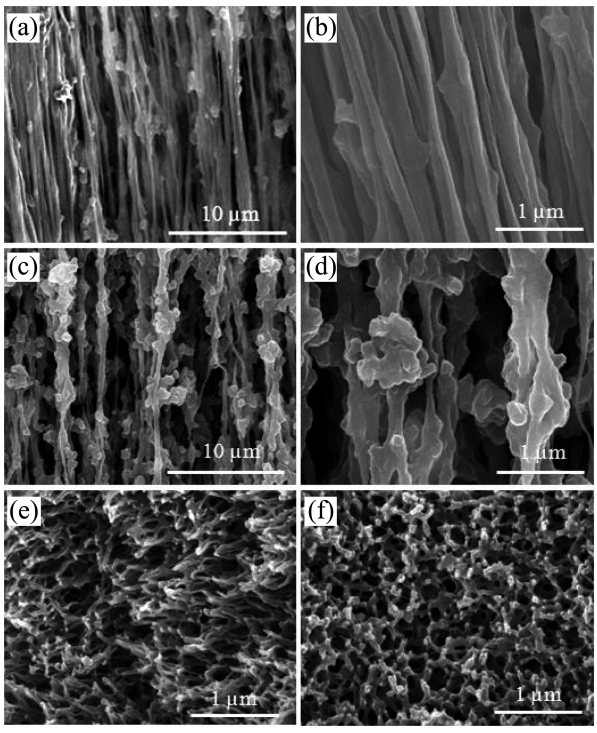

Figure 1. SEM images of Fbg microfibers made from two different concentrations, 10 wt % and 15 wt % concentrations. Well aligned nanostructures of Fbg microfiber could be observed from Fbg concentration of 15 wt % (a, b), and highly interporous with aggregated structure could be seen from Fbg concentration of 10 wt % (c, d). Figure (b) and (d) represented the expanded images of Fbg microfibers (a), (c), respectively. The cross-sectioned SEM image also clearly confirmed the higher degree of nanostructured alignment from Fbg microfiber made from 15 wt % concentration (e) than 10 wt % (f), which revealed higher degree of interporous structure.

10 wt % and 15 wt %. The field SEM images of Fbg microfibers (Figure 1) revealed the highly aligned nanostructures from Fbg microfiber made from 15 wt % concentration (a, b), and highly porous nanostructures were observed fiber made from 10 wt % (c, d). The bulk morphology was observed by optical microscope (images not shown). The concentrations of the Fbg in solution seemed to have profound effects on the aligned structure and overall size, where diameters of Fbg microfiber was observed at approximately 175-200 μm for fiber made from 15 wt % and 125-150 μm for 10 wt % Fbg solution.

In addition, the rate of solvent extraction seemed to depend on the tube dimension. If no chemical reaction occurred between aqueous and organic phases during the solvent extraction process, the extraction would be governed by mass transfer at the interface of aqueous and organic phases. Next, the high density phase would lead to solvent extraction process with spinodal decomposition. Furthermore, the kinetic rate of spinodal decomposition would govern the surface chemistry and the pores of the polymer fiber, since the solvent extraction would be based on tube's inner diameter and the coaxial parameters, such as viscosity, channel dimension, and mass transfer from the secondary flow.[7,8]

In the case of the tubular gel-solvent extraction method, the initial fluctuation occurred at the interface between aqueous and organic phase, which could induce the surface-directed spinodal decomposition and capillary force, resulting fiber formation. On the other hand, our hypothesis was that the pore formation could be due to the interplay between polymer-particle's interfacial tension and wall-surface wetting, and the relative dynamics of diffusion based transport. Because highly concentrated polymer particles could be formulated with highly aligned structure, whereas polymer particles in lower concentrations could increase the pores. The experimental results also indicated that the Fbg microfiber made from concentration of 15 wt % showed highly aligned nanostructures with leaner configuration, because of its relatively higher concentration. On the other hand, the Fbg microfiber at lesser concentration of 10 wt % displayed increased pores. These differences in Fbg microfiber with concentration of 10 wt % could be due to the higher volume ratio of solvent in aqueous extraction, resulting numerous lumps with more interconnected networked structures. Above results were confirmed by their respective cross sectioned images from SEM in Figure 1(e) and (f).

When the rate of extraction was studied as a function of channel dimension, the faster rate was observed with the coiled shape tube than the straight tube. For example, the 15 wt % Fbg-gel took approximately 20-25 min to flow through 1 meter length tube in a straight tube, whereas the coiled shape tube took only 8-10 min. Faster flow with the coiled shape tube could be from the higher laminar flow and enhanced generation of tangential secondary flow. Based on above results, the centrifugal force moved sharply forward to the walls surface, especially, at higher linear velocity. Simultaneously, the aqueous fluid with lessor velocity would

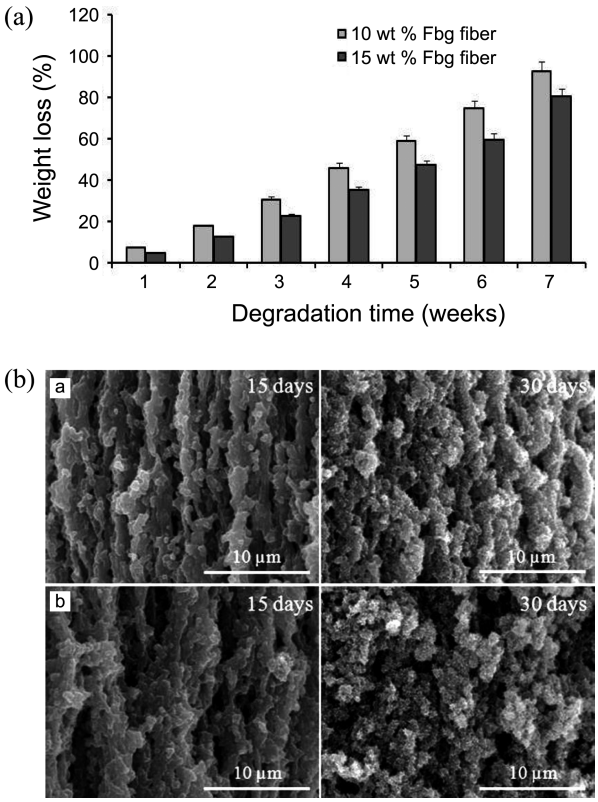

Figure 2. (a) The weight loss of Fbg microfibers fabricated from two different concentrations, 10 and 15 wt %, during degradation in the fibrinolytic degradation medium. (b) The SEM image of Fbg microfibers, after cross-linked with 5 mM EDC and tPA induction for fibrinolytic degradation at 15 and 30 days, Fbg microfiber made from 15 wt % concentration (a) and 10 wt % concentration (b).

flow outward from the coil axis. Overall, the higher concentrations of Fbg had more significant impact on microfiber, forming aligned nanostructures and its relatively larger diameter. And, the coiled flow system significantly speed up the rate of extraction.

The basic criteria of the developing degradable scaffolds would be depended on their applications, including the pre-configuration of microfiber for the short or long termed degradations and surface roughness in adhesion and proliferation of cells and tissues. To achieve long term degradation, both fibers were cross-linked with concentration of 5 mM EDC for 12 h. And to confirm the *in vitro* degradation, cross-linked fibers were incubated with addition of plasminogen and tPA for 7 weeks. Every 7 days the degradation ratio was monitored by measuring fibers' weight loss. After 7 weeks of fibrinolytic degradation, weight of Fbg microfibers (made from 15 wt % concentration) decreased to 19.8% from its initial weight, whereas Fbg microfibers from 10 wt % concentration decreased further to 7.4% from its initial weight, as shown in Figure 2(a). Plasminogen could be activated by tPA to serine protease, plasmin, which could degrade Fbg by cleaving Arginine or Lysine residues at their carboxyl terminal peptide bond. The fibrinolytic degradation also measured qualitatively by SEM at different time intervals

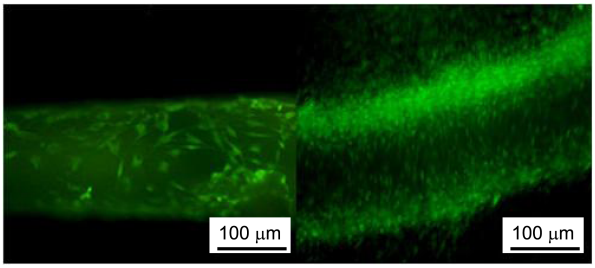

Figure 3. The fluorescence images revealed the C2C12 cell attachments on Fbg microfiber after uptaking Calcein AM fluoroflore: cells anchored tightly to Fbg microfiber and grew well.

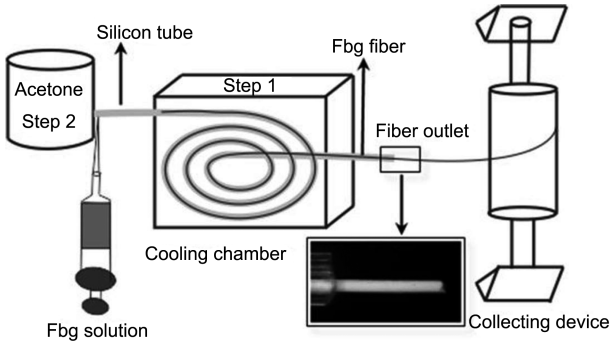

Figure 4. Schematic diagram of the fabrication process for Fbg microfiber (inlet: optical micrograph of fiber from silicon tube outlet).

of 15 and 30 days in Figure 2(b). However, data from the weight loss experiments and SEM images indicated that the highly porous fiber degraded faster than the lessor porous fiber. In another word, less porous fiber, Fbg microfiber made from 15 wt % concentration could have contained more cross-link forms the high surface to volume ratio, consuming exposed Lysine residues on the surface of the Fbg microfiber. Hence, plasmin could have taken longer time to degrade lessor porous and leaner Fbg microfiber.

Since the surface roughness would also be important for cells interaction and proliferation, values for the surface roughness were calculated using three-dimensional morphology atomic microscopic image. The surface roughness values came out to be 42.643 nm for Fbg microfibers made from 15 wt % and 78.531 nm for 10 wt %, respectively. The higher roughness value could be due to the high volume ratio of aqueous extraction.

Finally, the biocompatibility of the cross-liked Fbg microfibers was evaluated with mouse muscle fibroblast cells (C2C12). The fluorescence light microscopic images revealed the intact attachment of cells on microfibers, showing good biocompatibility. C2C12 cells seemed to anchor well on the Fbg microfibers for cell attachment and migration (Figure 3). The aligned nanostructures within Fbg microfiber would be used as a scaffold for directing cells to adhere and proliferate in vascular tissue engineering, wound dressings and supports. In addition, Fbg microfiber with aligned nanostructures could be used reservoirs for controlled short or long term releases of sensitive biomolecules, such as, DNA, peptides, growth factors and antibiotics.

Experimental Procedures

Fabrication of Fbg Microfiber. Bovine Fbg, fraction Type I, was purchased from Sigma-Aldrich Inc. (MW 340 KDa, St. Louis, US). Fbg microfiber was prepared by the gel-solvent extraction method using silicon rubber tube (Sigma-Aldrich Inc., St. Louis, US). The schematic setup of Fbg microfiber production was explained in Figure 4, which consisted of two steps, gellation prior to desolvation. Briefly, Fbg solution was prepared with phosphate buffered saline (PBS, Sigma Inc. St. Louis, US). In step 1, Fbg solution was injected from one end to other end of the coiled shape silicon tube (ID - 0.76 mm) by single injection without break and cooled to 0-4 °C in an ice bath for 30 min without shaking. In the second step, the gel tube (injected end) would interface at room temperature with previously cooled acetone to 4 °C (Sigma-Aldrich Inc., St. Louis, US). Once the complete solvent extraction was finished, the white color fiber rolled out from the opposite-end of the tube. Moreover, until the fiber was completely extracted from the tube, injected-end was interfaced with acetone for better lubricant effect. Finally, rolled Fbg microfiber (uncrossed-linked) was dried under vacuum for 2 days.

Cross-linking of Fibrinogen Microfibers. Fbg microfiber was cross-linked using 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC, Sigma-Aldrich Inc., St. Louis, US), as a cross-linking agent. Approximately, 300 mg of uncross-linked Fbg microfiber were suspended in 25 mL of acetone and water mixture (4:1 ratio), containing EDC (5 mM). The cross-linking reaction was carried out for 12 h at 4 ^{o}C with mild shaking. Then, the cross-linked fibers were washed with distilled water, followed by moisture removal with 50% and 100% acetone.

Morphological Characterization of Fbg Microfiber. The morphology of the Fbg microfibers was characterized by both optical microscope (Nikon Eclipse TE 2000-U, Nikon Inc., JP) and scanning electron microscope (SEM, EF-SEM, Hitachi S-4700 Type II, Hitachi Inc. JP). The optical microscopic image was taken to observe the bulk morphology, and SEM images were analyzed to characterize the outer morphology, ultrafine nanostructures, and porous structure of the microfibers. For the convenience of conforming cells attachment, the growth of fibroblast cells on Fbg microfibers were also examined with fluorescence microscope (Nikon Eclipse TE 2000-U, Nikon Inc., JP).

Degradation of the Fbg Microfiber. To study, *in vitro* biodegradation of Fbg microfibers, fibrinolysis was monitored with SEM after addition of plasminogen (Haematologic Technologies, Inc. Essex Junction, VT, US) and tissue-type plasminogen activator (tPA, Haematologic Technologies, Inc. Essex Junction, VT, US). The cross-linked microfibers (60 mg) were suspended with mixtures containing Tris buffered saline (TBS, 25 μL), $CaCl_2$ (10 μL of 50 mM), lys-plasminogen (2.5 μL of 1 mM) and tPA (20 μL of 20 μg/mL), and TBS was added to reach the final volume of 100 μL. The

mixture was incubated at 37 °C with mild shaking for 7 weeks. The degradation medium was changed each week with freshly prepared one.

Binding of Fibroblast Cells on Fbg Microfiber. The cytocompatability of the cross-linked Fbg microfibers was assessed by using mouse fibroblast cells. Cross-linked microfibers was sterilized with addition of absolute ethanol and was prewetted with GIBCO® Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Inc. Boston, US) for 1 h prior to seeding the cells. A cell density of 2×10^4 cells was seeded on 24 well plate with 1 mL of DMEM containing FBS (10%) and incubated overnight at normal growth condition for the cell attachment. Then, the medium was replaced with an equal amount of DMEM with FBS (1%), and the incubation continued again for 3 days. Afterwards, 0.2 μM of Calcein AM (Sigma-Aldrich Inc., MO, Kansas) was placed in each well and again incubated for 20-30 min. The viability of the fibroblast in presence of microfibers was observed using fluorescence microscope.

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