Processing and Microfabrication of Self-Assembled Chitin Nanofibers and Composites

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Abstract

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Chitin (poly–(1,4)-N-acetyl-D-glucosamine) is the second most abundant natural polysaccharide after cellulose and is synthesized by a number of living organisms. Due to strong intermolecular hydrogen bonds, chitin is insoluble in water, diluted acid/base solutions and most organic solvents. The intractability and water insolubility of chitin impedes the use of water-based self-assembly generative route, which is common in the synthesis of other biogenic nanofibrils. We found that chitin dissolves in the solvent Hexafluoroisopropanol (HFIP) and self-assemble into 3nm nanofibers upon drying from solution, named chitin nanofiber ink. Chitin nanofiber ink provides great advantages to manufacture macro- and micro scale structures without disrupting the delicate nanoscale molecular self-assembly process. In this work first I use the nanofiber ink to fabricate macro scale nanofiber chitin films and study the structure-properties-processing relationships. Then I use the nanofiber ink to microfabricate nanofiber chitin substrates on supported platforms and free-standing films with micro-structures desired for tissue engineering. Finally I explored chitin silk composites nanofiber self-assembly.

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1. Introduction

Chitin, the world's second most abundant polysaccharide after cellulose, exists in wide range of living organisms. For example, the exoskeleton of crabs mainly is composed of chitin, protein and minerals to provide mechanical support to the body[1], or photonic crystals mainly composed of chitin, which emit iridescent colors and can be found in butterfly wings, or the cell walls of fungi, or insect cuticles [2]. Two centuries ago in 1811, a French professor of natural history, Henri Braconnot discovered chitin and named it "fungine" after the discovery of a "material particularly resistant to usual chemicals" by an English scientist A. Hachett in 1799[3]. Since then chitin never was let it go in scientists' dreams. Unlike some synthetic polymers which were invented, used, banned and disappeared in the past two centuries (such as polychlorinated biphenyl(PCBs), Chlorofluorocarbon(CFCs)), chitin has remained an under-utilized material since its discovery.[4]

The positive attributes of chitin are numerous. Every year it is estimated that about 10^{11} tons of chitin is produced by nature , yet only about 0.01% of the products are commercialized.[5, 6] In addition to its abundant renewable sources, chitin itself has a number of attractive properties. It is biocompatible, biodegradable, bio-absorbable, chemically and thermally stable, possesses antibacterial and wound healing abilities, and has low immunogenicity[7]. Accordingly, chitin has a very broad range of applications in different fields such as food industries, materials science, microbiology, agriculture, wastewater

treatment, drug delivery systems, tissue engineering, and biocompatible electronic devices. [8-10]

As a polysaccharide, the repeating structural unit in chitin is *N*-acetyl-D-glucosamine (~50-100%)(**Fig.1** m block) and D-glucosamine (~50-0%)(Fig. 1 n block) linked by β(1-4) glycosidic bonds, which form long linear chains. [2, 11]. The difference between these two blocks is the acetyl group in **m** block is replaced by amine group in **n** block. If the mole ratio between m and n is lower than 1:1, the polymer is typically referred to as chitosan, the important derivative of chitin.

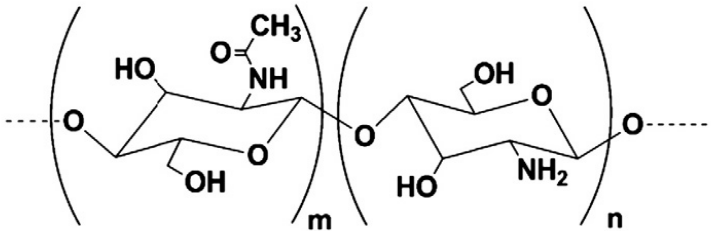

Figure 1. Chemical Structure of chitin / chitosan

There are two main types of chitin: α-chitin and β-chitin. α-chitin is by far the most abundant type, found in crab and shrimp shells as well as in insect cuticles and yeast cell walls.[12] β-chitin is much rarer: it is found in squid pens[12, 13] and in the tubes synthesized by pogonophoran and vestimetiferan worms.[14] The third allomorph γ-chitin has also been described, but it is just a variant of α family[15], therefore only α and β have been the focus of most research. The proposed crystal structure of α and β-chitin are presented in **Figure 2**.[16] In both crystal structures, a number of intra-sheet hydrogen bonds which are dominated by strong hydrogen bonds between CO and NH groups keep the chitin chains

organized in sheets. In α-chitin, inter-sheet hydrogen bonds that are associated with the hydroxymethyl groups of adjacent chains along the *b* parameter of the

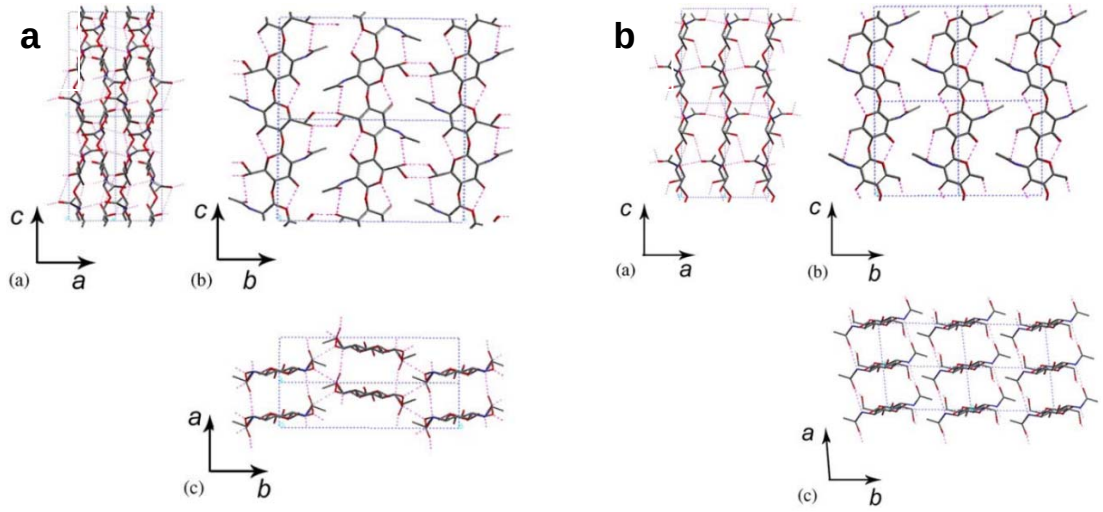

Figure 2. (a) structure of α-chitin *ac,bc* and *ab* projection respectively (b) structure of β-chitin *ac,bc* and *ab* projection respectively[16]

cell are also found, however such bonds are not found in β-chitin. Consequently the space arrangement in α-chitin is more densely packed than β-chitin. In addition there are two anti-parallel molecules per unit cell in α-chitin, whereas only one is present in β-chitin. This allows β-chitin to incorporates water molecules and other small molecules into its crystal lattice more easily than α-chitin, causing it to become hydrated and swollen easily in water.[17]

The non-solubility of chitin in almost all common solvents has been an important factor blocking in its appropriate utilization. [11] Due to its rigid crystalline structure based on the strong bonds between acetamido group, hydroxyl group and carbonyl group, it is insoluble in water, diluted acid/base solutions and almost all common solvents. It can dissolve in highly concentrated acids as well as hexafluoroisopropanol (HFIP) or hexafluoroacetone and recently it was found to

dissolve in calcium solvents in mild conditions. [18] Chitin is also found dissolve in concentrated H_2SO_4 or HCl, LiSCN/H_2O, chloroethanol/ H_2SO_4, trichloroacetic acid, urea/NaOH and ionic liquids. However all these solvents have their own limitations such as toxicity, corrosiveness, high costs and environmental concerns. [19]

In nature, chitin is found as semi-crystalline nanofibers with hydrophobic interaction between glucosamine rings and hydrogen bonding along the linear chains. [11] In the exoskeleton of the lobster *Homarus americanus* it shows a pronounced hierarchical structure of the material and strong crystallographic texture of the α-chitin protein network(**Fig.3**).[1] Inspired by Nature, fiber

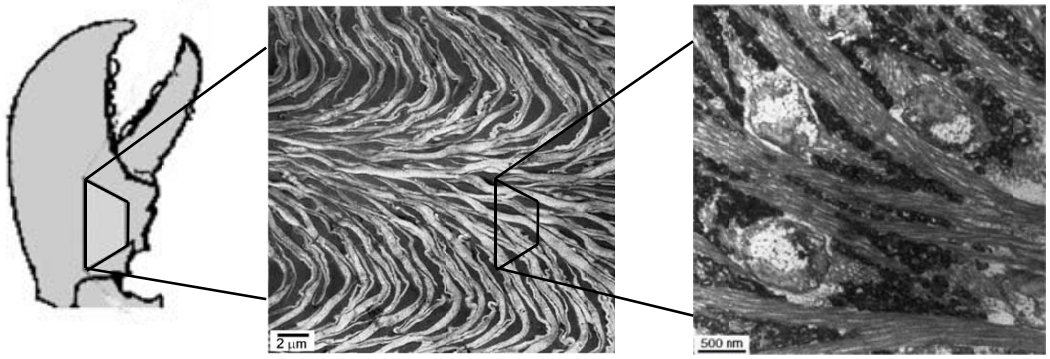

Figure 3. TEM images of hierarchical structure of the exoskeleton the lobster *Homarus americanus*[1]

formation has been long interest for the researchers. Various methods have been tried, such as acid hydrolysis, tempo-mediated oxidation, ultrasonication, electrospinning, mechanical treatment and gelation. [20] Most of the methods require harsh processing conditions (highly acidic or basic), which results in the depolymerization and deacetylation of chitin. Furthermore, such methods can only produce large diameter nanofibers; smaller nanofibers, as present in nature, are difficult to produce. However in 2009, we developed a novel method to

produce chitin nanofibers *in vitro* in mild conditions by dissolving β-chitin in either HFIP or LiCl/N,N dimethylacetamide(DMAC). [21] For β-chitin/HFIP, simply drying solution on substrates in ambient conditions led to micro-long nanofibers of α-chitin with a small diameter (2.8±0.7nm) (**Fig.4**).This size of nanofibers are similar to biogenic nanofibrils produced by living organisms; for example the exoskeleton of the lobster *Homarus americanus* mentioned above. For chitin/LiCl/DMAC, drying the solution is not practical. Instead fibers were precipitated out upon the addition of ample amounts of water about 10-25 times of the original volume. α-chitin fibers formed in this way have a larger diameter (10.2±2.9nm) with a similar length as those prepared from HFIP.

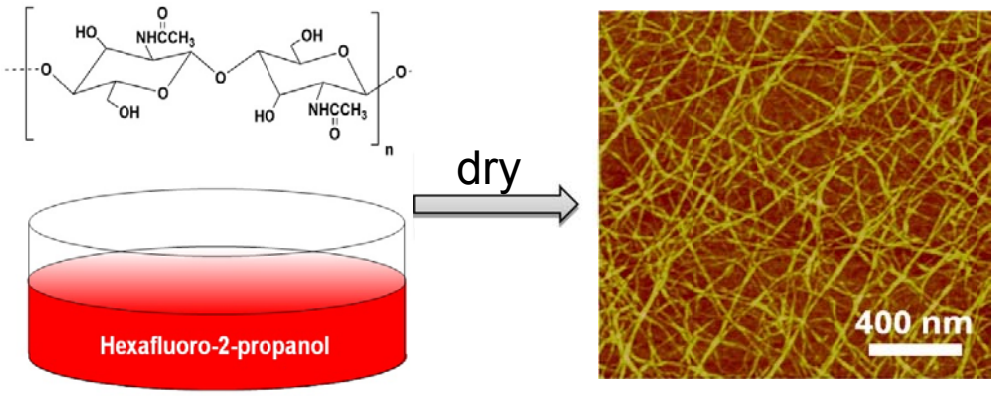

Figure 4. self-assembly of 3nm chitin nanofibers from the chitin/HFIP solution upon drying in ambient conditions

The underlying mechanism of the self-assembly process is driven by the intramolecular hydrogen bonding of the acetyl groups.[22] Chitosan are not distinct chemical entities from chitin, but chitosan (low degree of acetylation) does not self-assemble into nanofibers from solution because it lacks chitin's acetylamide groups. In chitin/HFIP, the self-assembly process is initiated by solvent evaporation. The resulting self-assembled nanofibers are composed of a single

self-assembled filament without any surface corrugation. However, in chitin (LiCl/DMAC) prepared fibers, intermediate (*ca*. 6nm) and small (*ca*. 3nm) nanofibers adhere to the large nanofibers at some sites, which indicates the fiber self-assembly process for the two methods might have proceeded along different routes.

XRD (X-ray diffraction) measurements show that the nanofibers produced by both methods are made of highly crystalline α-chitin (**Fig.5**). The transition of β-chitin to α-chitin is probably due the higher thermodynamic stability at α-chitin with respect to β-chitin; that is because of the higher number of hydrogen bonds present in α-chitin. [2, 23] In addition deacetylation and depolymerization are not observed in the nanofibers, which are significant improvements to common limitations of current chitin nanofiber production methods.

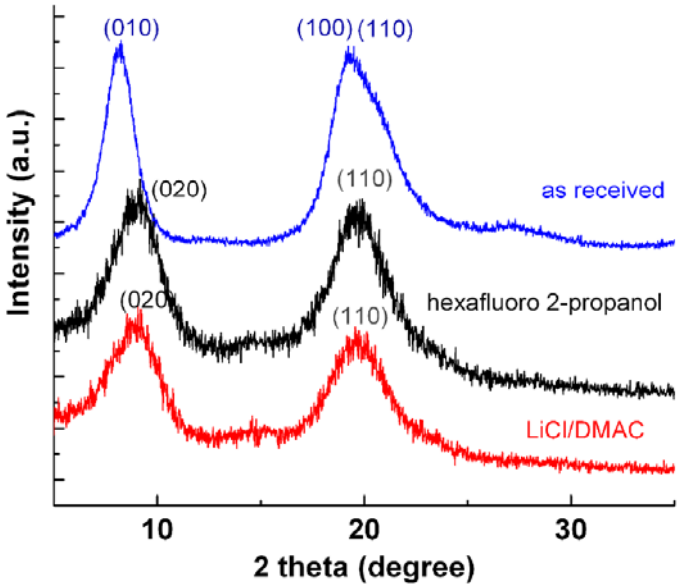

Figure 5 XRD spectra of chitin samples: as received chitin sample (blue), chitin nanofibers prepared from the chitin/HFIP solution (black), and chitin nanofibers from the chitin/(LiCl/DMAC) solution (red). The presence of peaks at 9.2°, 19.5°, indexed as (020) and (110) reflections, indicates a α-chitin structure for the nanofibers samples. The peak at 8.1°, indicative of (010) reflection in the XRD spectra of the as-received chitin samples, indicates mainly β-chitin structure for the as-received sample.

Subsequently chitin nanofiber ink is created. Chitin nanofiber ink is the homogeneous solution of β-chitin dissolved in HFIP that self-assembles into 3nm α-chitin nanofibers as mentioned above. The ability of controlling the organization of molecular building blocks at the nanometer level is of utmost importance, not only from the viewpoint of scientific curiosity, but also for the development of next-generation organic devices with electrical, optical, chemical, or biological functions. [24] This self-assembled chitin nanofiber solution offers great potential in the manufacturing of nanoarchitectures (nanostructures and nanopatterns) over large areas by using top-down fabrication methods. [25] Top-down fabrication (microfabrication technique) has been used to produce ordered micro- and nanoscale structures over the past two decades. It rose from the semiconductor and microelectronics industries where it was initially used to fabricate integrated circuits of microprocessors at micrometer length scales. [26] Combining of these microfabrication techniques with the advantages of self-assembled nanofiber solution provides a great future for applications in biology.

In this report, first, I will discuss my development of two novel macro-scale chitin film fabrication processes with the chitin nanofiber ink. Dried chitin materials from its solution suffer brittleness and dimensional distortion as hydrogen bonds between adjacent chitin chains form due to coalescence of chitin chains on contact with moisture.[27] In order to study chitin bulk properties, it is very important to produce a flexible chitin film with minimum distortion and uniformity. I studied the relationships among the nanofiber structures, mechanical properties and the chemical processing of the prepared chitin films. Second, I will discuss

my development of top-down microfabrication methods to make micropatterned chitin substrates using the developed chitin film fabrication process and replica molding technique for tissue engineering. Engineered tissues require enhanced organization of cells and the extracellular matrix (ECM) for proper function. The cells reorganize according to the interaction with the ECM based on the topography and mechanical properties of the ECM. [28] To promote cellular organization, substrates with controlled micro- and nano patterns have been developed as supports for cell growth, and to induce cellular elongation and orientation visa contact guidance in engineered tissues. [29]. Finally I will discuss my study of the nanofiber formation of chitin/silk composites in HFIP. Natural structural materials based on chitin nanofibers in a silk matrix are made up of a biomineralized organic phase with exceptional fracture toughness. [30] Mixing self-assembled chitin nanofiber ink with the natural protein fiber silk and studying the final micro- and nano-structure provides the fundamentals for the development of chitin-silk or similar biocomposites.

2. Research

2.1 Chitin Processing-Structure -The Mechanical Properties

2.1.1 Chitin processing and films fabrication

In order to characterize the mechanical properties of chitin films, uniform and homogeneous films with minimum flaws and impurities are required. According to ISO and ASTM standards on determination of tensile properties of plastics, the method of testing, test specimens, devise for measuring the width and thickness

of test specimens, the apparatus to perform the test, the condition of test, the number of tests all shall satisfy the standard regulations and restrictions. The test specimens shall be free of twist and shall have mutually perpendicular pairs of parallel surface, and that the surfaces and edges must be free from scratches, pits, sink marks and flash. [31, 32]

It is challenging to make chitin films that can be used for the mechanical properties characterization test using chitin nanofiber ink. In the processing, chitin films drying from solution at room atmosphere easily shrink, twist, become uneven and are not transparent. Previously simple drop casting methods were used to make chitin films.[21] Briefly chitin nanofiber ink was poured into Polydimethylsiloxane(PDMS) or glass mold and left dry at room temperature with or without parafilm cover to control the solvent evaporation speed. The films usually turned out unpleasant (**Fig.6**). The films made by this method have no consistency from sample to sample. Also it requires large amount of solutions in order to find a good part in the center of films, which is time consuming.

Figure 6. β-chitin films made by simple drop cast method

To solve this problem, I developed two new routes to make chitin films, Vacuum Drying (VD) and Vacuum-assisted Filtration (VF) (Fig.7). For VD, chitin nanofiber

ink was poured into a mold and left in a vacuum desiccator that was connected to the house vacuum. By this method HFIP evaporation was expedited and at the same time environmental influences were minimized. The drying time was reduced to approximately 2-3 days depending on the amount of solution and the designed thickness of final films. For VF, chitin nanofiber ink was supported on a paper filter coated with a very thin layer of PDMS while drying. The

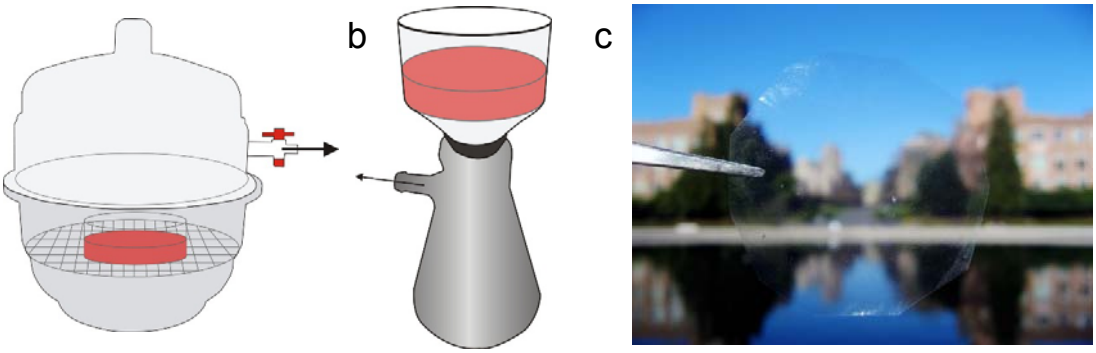

Figure 7. (a) Vacuum drying method (VD) (b) Vacuum assisted filtration method (VF) (c) Chitin films made by VF taken in the campus of university of Washington

paper filter was rinsed with acetone before the deposition of thin PDMS layer to reduce the amount of impurities from paper filter that possibly diffuse into the chitin solution while drying. The PDMS layer served the dual purpose of providing a smooth surface for the formation of the film and controlling the solvent evaporation rate. Then the PDMS coated filter paper was placed in a Buchner funnel connected to the house vacuum and covered with a glass cover in order to minimize evaporation from the top. The chitin films dried in approximately 1 day. The films made by these two methods are transparent, uniform and homogeneous, which completely satisfy the standards that ISO or ASTM regulated for mechanical tests purpose. (Fig.7 c)

2.1.2 Chitin nanofiber structure and mechanical properties characterization

Using the methods I developed, I made chitin films from different concentration of chitin nanofiber ink, and then I studied the microstructure-mechanical property-processing relationship of the chitin nanofiber network. For both processing methods, chitin nanofibers were self-assembled in the films (**Fig.8**).

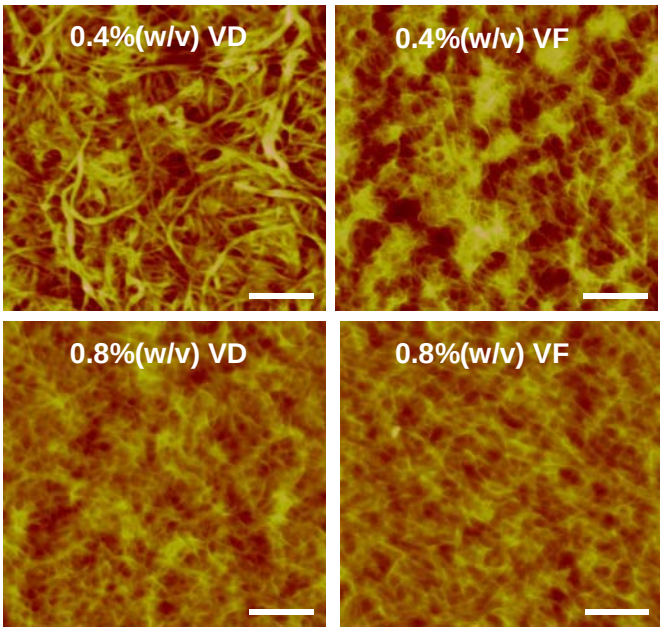

Figure 8. AFM images for 0.4% (w/v) chitin nanofber ink prepared by VD and VF (scale bar 200nm)

The observation of self-assembled nanofiber of 3nm diameter was consistent with what was previously reported for drop-casting, in which nanofibers always form as long as the drying time was slow enough to produce a solution of critical concentration. However VD films of 0.4%(w/v) concentration appeared to have nanofibers of larger diameters fibers than 3nm.[21] The size of these larger fibers was similar in appearance to the fibers from LiCl/DMAC precipitation in water previously reported. [21] It is possible that with the faster drying times associated with the VD films, the fibers may assemble into thicker bundles rather than

creating a uniform smaller network. For both methods, the initial surface morphology inspection suggests that the films from the solution with higher concentration (0.8%w/v) are denser than the films made from the solution with lower concentration (0.4%w/v). And later the density measurement proved such conjecture (not shown here).

In order to study whether the different methods affect the crystalline structure of the chitin nanofibers, we used XRD **(Fig.9 a)**. In XRD, α-chitin and β-chitin are distinguished by the different position of the diffraction peak corresponding to the

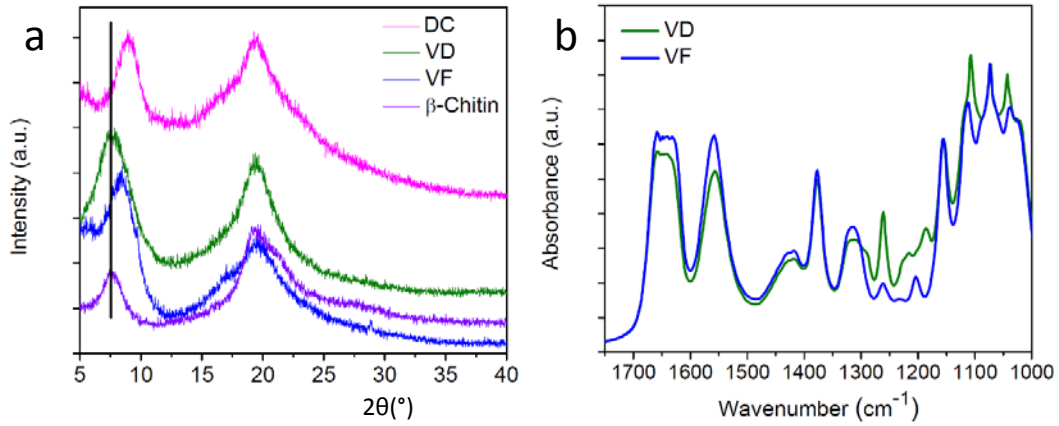

Figure 9 (a) XRD of chitin films prepared by VD and VF in comparison with the as received chitin powder and DC. (b) FTIR of chitin films prepared by VD and VF.

(020) inter-sheet distance. For β-chitin, this peak is at 7° while for α-chitin this peak is shifted to higher angles of 9°-9.5° corresponding to smaller (020) inter-sheet distance due to the increased degree of hydrogen bonding between the chitin molecules. We have seen that drop casting chitin nanofiber ink resulted α-chitin films because α-chitin is thermodynamically more stable. For films prepared by VF the (020) peak is at 8° and films made by VD the (020) peak is at 7.5°. Both of the peaks located in between α-chitin and β-chitin peaks, which means both films went through β to α transition during the drying process;

however due to the drying time and drying condition difference, the transition is not completed. Fourier Transform Infrared spectroscopy (FTIR) (Fig9 b) confirmed the insights from XRD. The presence of α-chitin is confirmed by the amid I (C=O stretching) split peaks at 1657 cm^{-1} and 1620 cm^{-1} .[33] These peaks are attributed to the hydrogen bonding of the C=O group of chitin to the –NH group of an adjacent chitin chain (1657 cm^{-1}) and with an –OH group on the same chitin chain (1620 cm^{-1}). The peak at 1635 cm^{-1} is generally associated with C=O binding only to –OH in β-chitin. In the α-chitin nanofibers it may correspond to the C=O groups of the six chitin molecules on the surface of the nanofibers that do not fully participate in inter molecular hydrogen bonding or to some presence of β-chitin in our samples.

To investigate the macro-scale mechanical properties of these chitin films, I performed tensile testing. The stress strain curves show relatively consistent data for films fabricated by VF for the two concentrations 0.4%w/v and 0.8%w/v solution (**Fig. 10**), however the same is not true for VD. For the films fabricated by VD and VF from 0.4%w/v, the elastic moduli are 1.3GPa and 1.9GPa and from 0.8%w/v the elastic moduli are 1.8GPa and 2.0GPa respectively. Generally, the results are consistent with the denser film having higher elastic modules. Previously we measured the elastic modulus of the drop cast films to be 2.3GPa. [34] DC films are approximately as dense as the VF films. The higher elastic modulus for the DC films may be attributed to a higher α-chitin content in the DC films. The tensile strength (σ_{ts}) are 54.6MPa (VD) and 129.3MPa (VF) for 0.4%w/v solution and 111.3 MPa (VD) and 123.13MPa (VF) respectively. The

lower tensile strength for VD compared with VF can be attributed to a lower film quality and existence of flaws and notches according to Weibull statistics. [35]

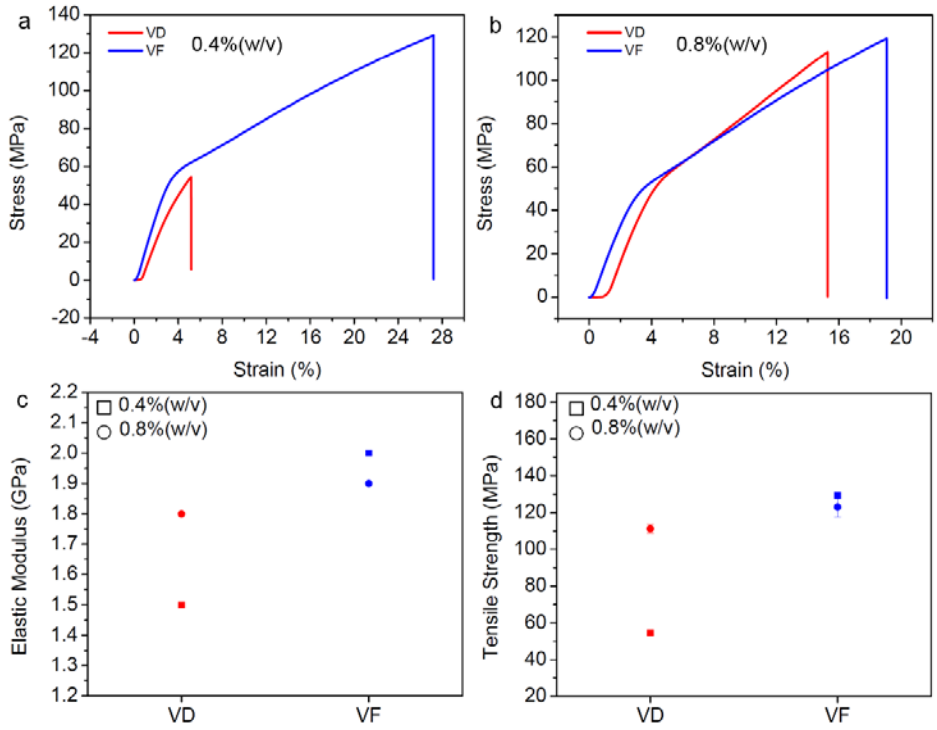

Figure 10. Tensile properties of chitin films prepared by two methods, vacuum drying(VD) and vacuum assisted filtration(VF). (a,b) Stress-strain curves of films prepared by the two methods for 0.4%(w/v) and 0.8%(w/v) solution. (c) Elastic modulus as a function of fabrication method and solution concentration (d) Tensile strength versus fabrication methods and solution concentrations.

In conclusion, I have developed two new reproducible and consistent fabrication techniques to make uniform and homogeneous chitin films that comply with the requirements ISO and ASTM regulated for mechanical properties tests. With these two methods, chitin nanofiber film density, network structure and mechanical properties can be tuned and manipulated easily by changing the processing parameters. Such ability provides this material a promising future for specific applications.

2.2 Micro-fabrication of self-assembled chitin nanofibers substrates for Tissue Engineering

In vivo, cells are capable of sensing and responding to a plethora of signals, consisting of biochemical and biophysical cues provided by the extracellular matrix (ECM), which acts as a cellular scaffold. [36, 37] The ECM delivers signals to cells using two types of physical cues: one is topographies [38, 39] and the other is mechanical stiffness. [40, 41] Various substrates with unique physical properties mimicking the ECM over a broad range of length scales with nano- to micrometers structures are fabricated to control the cellular behavior. [42-44] The substrates are usually on a support platform or on ultrathin biodegradable substrates that are implanted in the host tissue. The ultrathin substrates need to be thin and flexible for conformal contact to the tissue of choice and robust for the ease of handling. [45]Robustness of the substrate is particularly important in myocardial tissue repair. [46] To fabricate robust biodegradable substrates, structural biopolymers such as collagen, chitin and chitosan are particularly appealing for their biocompatibility and mechanical strength. [47, 48] Specifically, the 3-D assembly of nanofibrous structures with chitin and its deacetylated derivative chitosan are known to mimic the natural ECM and promote cell attachment and spreading ability. [49] While chitin nanofibers already exist in nature, chitosan nanofibers are typically produced by electrospinning. [50, 51] Electrospinning is difficult to couple with micro- and nanofabrication to yield micropatterned substrates. However chitosan nanofibers have found broader use than chitin due to the intractability and insolubility of chitin in common organic

solvents. With the explored mechanical properties of chitin films and the methods developed in section 2.1, I developed methods to fabricate micro-patterned chitin substrates on supported sturdy platforms as well as ultra-thin, mechanically robust, yet flexible free standing chitin films with chitin nanofiber ink. These fabricated substrates then were employed for tissue engineering to culture NIH-3T3 fibroblast cells. This work was done in the collaboration with Prof. Khademhosseini's lab at Harvard University.

To fabricate micro-patterned supported chitin substrate and free standing chitin films, I used micro-patterned PDMS master mold with 3.16um spacing (G1) and 12um spacing (G2) microstructures. G1 is smaller than the average cell diameter and G2 is lightly larger than the average cell diameter. The patterned PDMS master mold was simply replica molded from the commercialized glass gratings. The process to make the patterned PDMS master mold was the standard soft lithography method. [52]

The processes of fabricating micro-patterned supported chitin substrates and free standing chitin films are described below (**Fig 11)**. For supported chitin substrates (Fig.11 a), chitin nanofiber ink was poured on top of PDMS master mold and immediately a clean glass slide was placed on top of the solution. By the natural gravity, the glass slide would press down on the solution evenly, resulting in uniform and thin chitin films on glass slide upon drying. This simple strategy afforded the attachment of chitin nanofiber substrates to an arbitrary support without requiring additional adhesives, which may be toxic to the cells.

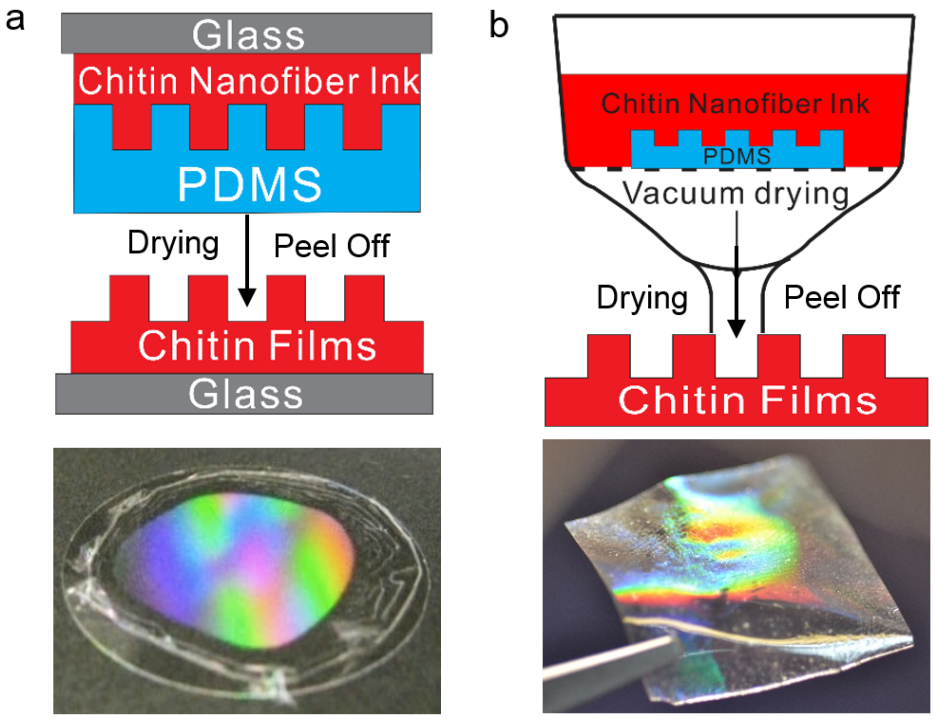

Figure 11. (a) supported chitin substrates fabrication process and samples (b) free standing chitin substrates fabrication process and samples

For flexible free standing chitin films, the vacuum assisted filtration method was used. The methods remain consistent with those in section 2.1 about this method except a thin micro-patterned PDMS master mold was placed on top of the paper filter. Once the ink was dry, the patterned chitin films were peeled off directly from the PDMS mold with a pair of lab tweezers. By this method uniform, transparent and robust, at the same time patterned free standing chitin films were produced. The thickness and size of the films and the drying time can be easily adjusted by changing the amount of solution, the size of the Buchner funnel and the thickness of PDMS layer or vacuum pressure.

Both supported and free standing substrates were easy to handle and stable in cell culture media for extended periods of time, which was proved by the later work in the lab. The micro-fabricated substrates prepared in this fashion

exhibited the desired microstructure, which is inferred from the diffraction patterns in the optical images (Fig.11).AFM micrographs of patterned supported chitin substrates and free standing chitin films were investigated (**Fig.12**).

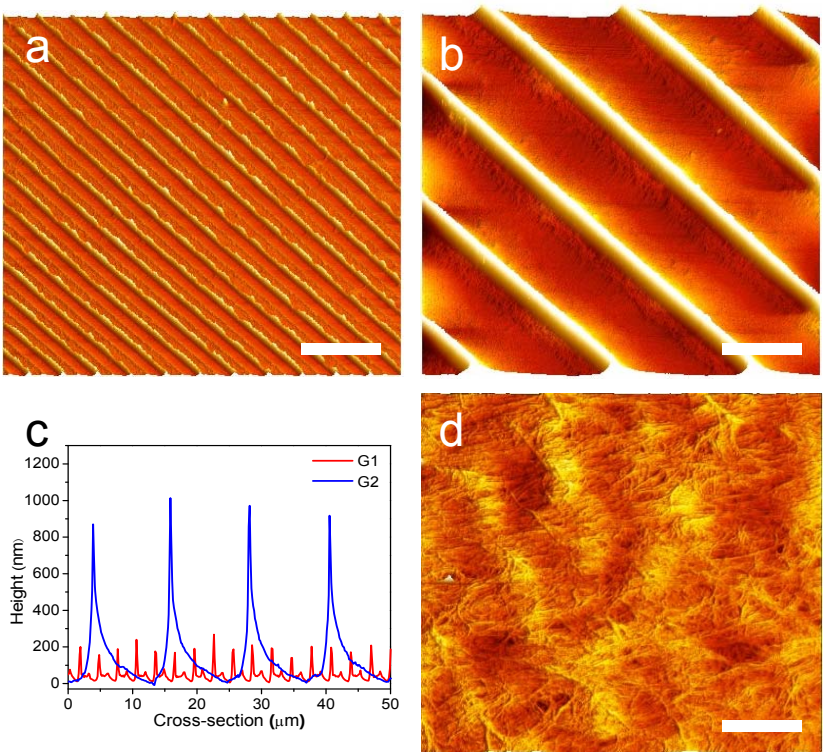

Figure 12. AFM imaging of the supported chitin substrates. (a,b) AFM height images of chitin substrate G1 and G2 respectively(scale bar 10um). (c) Cross-sectional height profiles of (a) and (b). (d) Magnified view of (b) showing the chitin nanofiber morphology (scale bar 200nm)

Micro-patterned substrates G1 and G2 closely replicated the spacing and height of the original gratings (Fig.12 c). Both G1 and G2 substrates had a saw tooth cross section that derived from the cross section of the diffraction grating. The features in G1 were on average 193nm tall, while the features on the G2 substrates were 943nm tall. For both substrates, uniform features were found in extended areas of up to several square centimeters only limited by the size of the original master and mold. With appropriate masters and molds, features of arbitrary shape and size ranging from a few nanometers to hundreds of microns

could be achievable with the chitin nanofiber solution, expanding these kinds of substrates available for future studies. As previously reported, the random morphology of chitin nanofibers was maintained intact in the both processing methods (Fig.12 d).

To evaluate the use of chitin substrates for tissue engineering, NIH-3T3 fibroblast cells were cultured on G1 and G2 and control chitin substrates. Cell attachment, alignment and proliferation were measured. On the chitin micropatterned substrates, G1 and G2 (**Fig.13**a and b) cells with a spindle-like morphology aligned their cytoskeletal structure along the major axis of the micropatterned features. In contrast, the cells grown on the control chitin substrates did not have any preferred orientation. Cell nuclei alignment on G1 and G2 micropatterns after 5 days of cell culture was different from control as well. In G1 and G2, a larger proportion of the cells aligned within the 0-10° preferred angle range however in control such arrangement was not observed (Fig. f). In addition, on G1 and G2, cell elongation increased with cell alignment. This increase indicated that the substrate topography affected the cell morphology along with cellular alignment (Fig. 13h). Cell attachment and proliferation were also evaluated with the direct cell counting method at days 1 and 5 of culture. NIH-3T3 fibroblast cells proliferated at day 5 of culture compared to day 1, indicating that the chitin substrates were non-cytotoxic. In addition there was no significant difference in cell proliferation on different micropatterned samples (G1 and G2) compared to the control substrate (Fig. i).

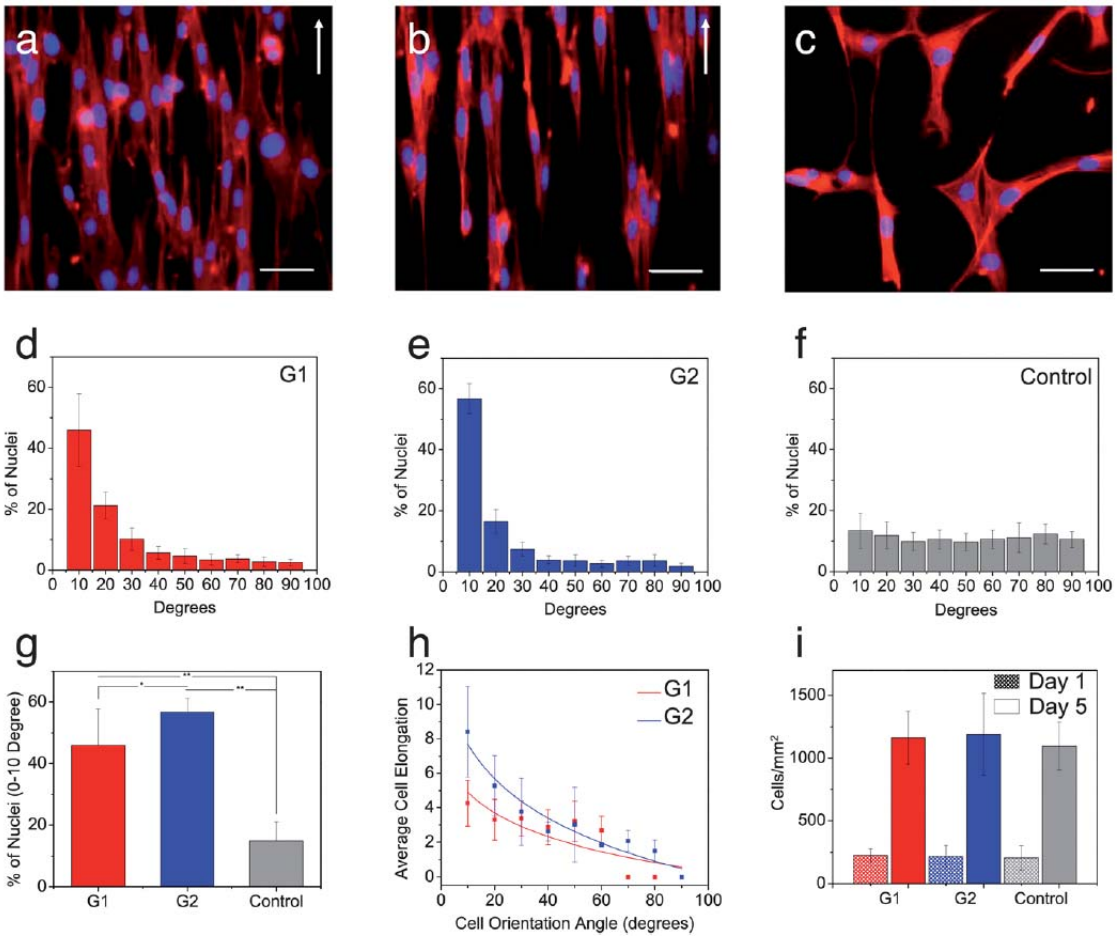

Figure 13 Fluorescence images of the actin cytoskeleton of the cells on (a) G1, (b), G2 substrates and (c) control sample after 5 days of culture (Scale bar 50 μm). The white arrow shows the longitudinal direction of the patterns. (d-f) Distribution of cells nuclei alignment angles on the patterned and control samples, (g) Percentage of cells that have orientation angles within 0-10 degree angle, (h) Cellular elongation function of cell orientation angle within the patterned substrates (i) Proliferation of the cells on the patterned and control substrates

In conclusion, simple replica molding method and vacuum-assisted filtration method were successfully used for micro-fabricating supported chitin substrates and free-standing chitin films. The AFM micrographs showed high pattern fidelity to the original masters. In addition the seeded NIH-3T3 cells on the substrates aligned along the major micropattern axis. Overall these two fabrication methods were low cost, easy to use, high throughput and did not require expensive clean room equipment and the prepared chitin substrates were transparent, robust,

ultra-thin which could make a very promising candidate to form 3D functional tissue and mimic the complex hierarchical structure of the ECM.

2.3 Chitin silk composites processing and its nanofiber formation

In crustaceans, the material of exoskeleton is chiefly designed to resist mechanical loads, which is a multilayered composite tissue consisting mainly of chitin which is associated with various proteins. Its structure is similar to other natural structural materials, such as bone, tooth, wood, arthropod cuticle, which have in common a bio-mineralized organic phase with exceptional fracture toughness, but couple with low weight. Inspired by these natural composites, chitin silk bio-composite was developed.

Previously chitosan and silk have been mixed to produce large diameter electrospun nanofibers for tissue engineering scaffolds. [53] The chitosan-silk mixtures have poor mechanical properties possibly resulting from the inability of chitosan to crystallize into nanofibers and form an ordered composite structure with silk. [54] Chitosan does not form nanofibers because it lacks chitin's acetylamide groups that contribute to the hydrogen bonding during the chitin nanofiber self-assembly process, as is mentioned above.

To study the fiber formation, we mixed squid pen β-chitin and *B.Mori* cocoon silk co-dissolved in HFIP and stirred by magnetic bar for a period of time to form homogenous chitin silk/HFIP solution. The mixed chitin/silk solution is similar to chitin nanofiber ink. It can be processed using methods described in the previous

sections. Specifically in this topic, I studied the chitin silk nanofiber formation using AFM (**Fig.14**).

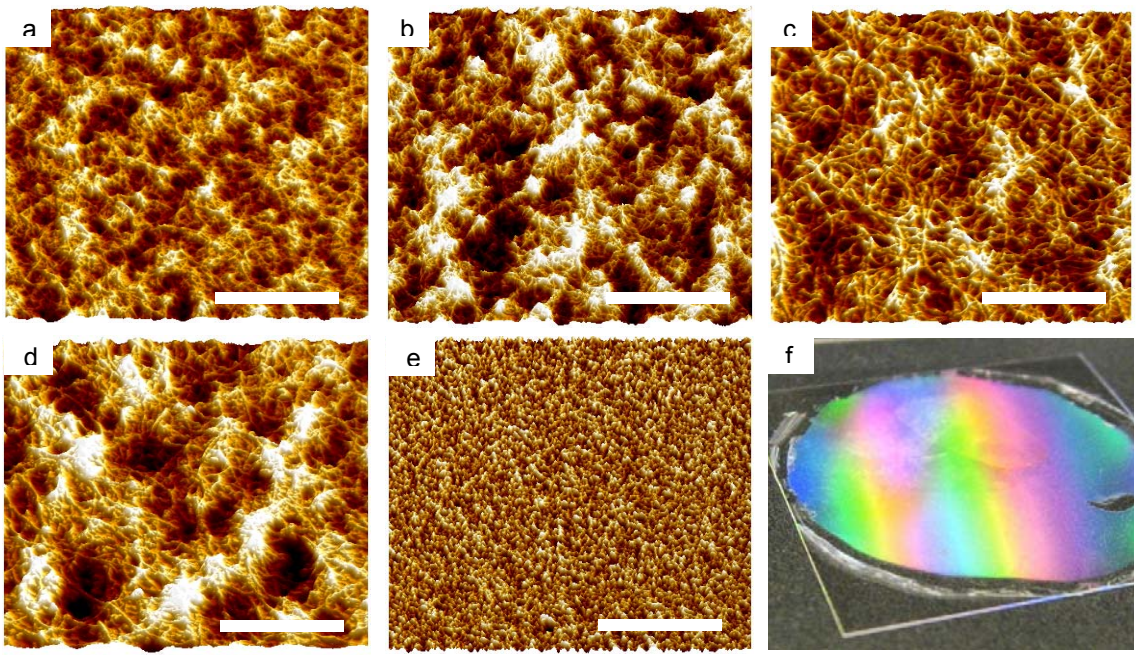

Figure 14. (a-e) Topographic AFM images obtained from films of chitin, CS31, CS11, CS13 (CSXY,where X:Y=chitin:silk weight ratio) and silk respectively. Scale bars represent 1um. (f) Optical microscopy image of transparent chitin nanofiber-silk hybrid formed into a diffraction grating form solution-based replica molding.

The pure chitin film is made of highly entangled ultrafine chitin nanofibers (Fig.14 a). The AFM results show the chitin-silk bio-composite are made of the ultrafine chitin nanofibers embedded in the silk fibroin matrix. The chitin nanofibers in the co-assembled composite have the same entangled structure as the chitin nanofibers self-assembled from a chitin only-HFIP solution. With this property the chitin nanofiber content of the biocomposite is easily tunable by varying the solution chitin/silk weight ratio in CS31, CS11, and CS13(CSXY=chitin:silk weight ratio). This is a desirable feature that affords a simple strategy to fine-tune the biocomposite properties. In contrast, the surface structure of the silk dried from the silk/HFIP solution is smooth and does not contain nanofibers (Fig.14 e),

confirming that addition of chitin is essential to create the biocomposite nanostructure. The self-assembly of chitin nanofibers in the silk matrix was robust and occurred for a chitin/silk ratio as low as 1:30. As a proof of concept, chitin/silk membrane on supported platform with diffraction gratings (fig.13 f) was produced to demonstrate the compatibility of chitin/silk solution to the microfabrication method discussed earlier.

3. Conclusion

I have developed two new reproducible and consistent fabrication techniques, vacuum drying and vacuum-assisted filtration to make uniform and homogeneous chitin films. The processing parameters of these two methods can be easily adjusted to control chitin nanofiber film density, network structure and mechanical properties. The new simplified processing methods along with the good mechanical properties of these chitin films shows promise for future applications. With such ability and knowledge, later I fabricated supported chitin substrates and free standing chitin films with delicate micro-structures on both for the purpose of biological cell culture. The AFM micrographs showed high fidelity from the original masters. The seeded NIH-3T3 cells on the substrates aligned along the major micropattern axis. Finally nanofiber formation of chitin silk composites was studied. AFM results showed robust self-assembled chitin nanofibers embedded in the silk fibroin matrix were formed up to chitin silk weight ratio of 1:30.

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5. Publications

1.P. Hassanzadeh, **W. Sun**, J. de Silva, G.L.W. Cross, M. Rolandi, "Self-Assembled Chitin Nanofiber Mechanical Properties: Individual Fibers and Networks", Journal of Materials Chemistry B, 2014,2: 2461-2466. Selected as Cover Article 17/2014.

2.P. Hassanzadeh, M. Kharaziha, M. Nikkhah, S. Shin, J. Jin, S. He, **W. Sun**, C. Zhong, M.R. Dokmeci, A. Khademhosseini, and M. Rolandi, "Chitin Nanofiber Micropatterned Flexible Substrates for Tissue Engineering", Journal of Materials Chemistry B, 2013**,** 1: 4217-4224. Selected as Cover Article 34/2013.

3.J. Jin, P. Hassanzadeh, G. Perotto, **W. Sun**, M.A. Brenckle, D. Kaplan, F.G. Omenetto, and M. Rolandi, "A Biomimetic Composite from Solution Self-Assembly of Chitin Nanofibers in a Silk Fibroin Matrix", Advanced Materials, 2013, 25: 4482-4487. Selected as Cover Article (Back) 32/2013.

4. E.Josberger, Y.X Deng, **W. Sun**, R. Kautz and M.Rolandi, "Two-Terminal Protonic Devices with Synaptic-Like Short-Term Depression and Device Memory", Advanced Materials,2014: p.n/a-n/a.

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