Supplementary Material (ESI) for Soft Matter

Electronic Supplementary Information

A facile bottom-up route to self-assembled chitin nanofibers

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Chitin Nanofiber Fabrication:

Materials: Chitin squid extract (Industrial Research Ltd-New Zealand), Lithium chloride (Sigma Life Science), N,N-dimethylacetamide (Sigma Aldrich) and hexafluoro 2-propanol (Sigma Aldrich) are used as received. Silicon wafers (Universal wafers) or glass coverslips (Ted Pella, Inc) are carefully rinsed in ethanol and deionized water prior to use.

Preparation of Chitin Nanofibers/films: The chitin nanofibers/films are fabricated either directly from chitin/hexafluoro 2-propanol solution (Approach 1), or a 5.3 wt% lithium chloride/N, N-dimethylacetamide (DMAC) solution using deionized water as a precipitating solvent (Approach 2). The detailed fabrication procedures are listed below.

Chitin Nanofibers Preparation:

Approach 1 (3 nm nanofibers): Typically, 0.001-0.05 wt% chitin/HFIP solutions are prepared by dissolving chitin in HFIP while stirring, this procedure forms homogeneous solutions. 5 μL of the as-prepared solutions are then placed on top of freshly clean silicon wafers or glass coverslips. After evaporation of the solvent, the samples are washed with a copious amount of deionized water and air-dried under ambient conditions. Optical microscopy and AFM on several areas of the sample indicates that the fibers are not washed away by deionized water. Chitin is water insoluble, we thus assume that if there were any leftover monomer it would not be washed away either and it would stay on the surface. We thus estimate the yield of the nanofibers to be > 95 %.

Approach 2 (10 nm nanofibers): A 5.3 wt% LiCl/DMAC solution is prepared by dissolving 0.5 grams of LiC in 10 mL DMAC while stirring, this procedure forms homogeneous solutions. Complete dissolution in the solvent occurs in 3-5 days. A series of chitin solutions is then prepared by dissolving chitin in 5.0 wt% LiCl/DMAC. At a 0.5 wt% concentration, the chitin solution forms a robust gel structure. For nanofiber fabrication, 20 μL of as-prepared chitin/ (LiCl/DMAC) solution is dropped onto the freshly clean silicon wafers or glass slides, followed by addition of 500 μL of deionized water. After five seconds, another 200 μL of ethanol is added to facilitate the precipitation of chitin nanofibers onto the substrates. After approx. 1 min, the substrate is washed with copious amounts of water to remove the remaining LiCl salt. The substrates are then dried under constant N_2 gas flow at ambient temperature. We estimate the yield of nanofibers as Yield of nanofibers (%) = the net weight of final samples (silicon chip weighted before and after deposition)/ (the volume of chitin solution * the concentration of chitin solution). Three tests gave an average yield of 51 ± 9 % for this preparation. We do not observe any monomer on the sample.

Preparation of Chitin Films: 5μL 0.125 wt% chitin/HFIP solution is directly dropped onto freshly-made clean glass coverslips. After drying, the samples are washed with copious amounts of deionized water and air-dried at room temperature. The chitin films prepared in this fashion are composed of very dense randomly aggregated nanofiber networks. However,

the individual nanofiber features in the films are more difficult to discern than the structures produced from low concentrated Chitin/HFIP solution (Fig S3, Fig 2 a, 2b and 2c).
Sample Preparation for Fiber Characterization: The chitin nanofibers were prepared for various characterization techniques onto either a clean silicon wafer, glass slide, teflon plate, or TEM copper grids. The morphology and diameter the nanofibers were not affected by the collecting substrates indicating that fibers form before landing on the surface and surface interaction does not play a major role in the process. We used TEM copper grids and silicon wafer for TEM and AFM imaging, respectively. For XRD, the samples were directly deposited onto glass slides. For FTIR, a teflon plate or a glass slide was typically used The deposited chitin samples were then scraped off the teflon plates or glass slides via a regular razor.

Chitin Nanofiber Characterization:

Tapping mode (TM) AFM is performed on a Veeco Multimode V with a Nanoscope IV controller using Veecoprobes Sb-doped Si cantilevers (ρ = 0.01-0.025 Ω-cm, k = 40 N/m, ν ~ 300 kHz). The average nanofiber diameter is estimated by measuring the height profile of 50 different individual nanofibers in a 10 × 10 μm or 2 × 2 μm AFM height image (see Supp. Info.). Bright-field TEM images are collected on a FEI Tecnai G2 F20 S/TEM operated at an accelerating voltage of 200 kV after staining the samples with uranyl-acetate.
For FTIR, dried chitin samples precipitated from the two above solutions are ground into powder, mixed with KBr (sample/KBr 1:20 w/w), and compressed into KBr pellets. FTIR spectra are then obtained with a Bruker vector 33 FTIR spectrophotometer. The original chitin sample is also grounded into powder and compressed into pellets for FTIR analysis.
XRD spectra are acquired at room temperature with a wide-angle X-ray diffractometer (2θ = 5–50^{o}) (Bruker D8 Focus) with Cu Kα radiation, operated at 40 kV and 40 mA.

Detailed Procedures for TEM Sample Preparation:

For TEM analysis, 1 μL 0.01% chitin//HFIP solution is directly deposited on carbon film-supported 400 mesh copper grids (Ted Pella, Inc.) Excess solution is quickly wicked away with a piece of filter paper and the sample dried. To produce nanofibers from chitin/ (LiCl/DMAC) solution, the TEM samples for were prepared by placing a 2 μL 0.05% chitin/ (LiCl/DMAC) solution onto the carbon film-supported copper grid; the sample is then carefully washed with small amounts of water, blotted with a piece of filter paper, and air dried. Once dried, both samples are negatively stained by washing with 2 wt% uranyl acetate. A thin layer of uranyl acetate solution remains after blotting and the samples are dried under ambient conditions.

Stability of Chitin Nanofibers:

The stability of chitin nanofibers in PBS solution was assessed by comparing the AFM images of nanofibers before/after immersion into 1 mL 1× PBS solution for 1 week. The stability of chitin nanofibes in dry state was also examined by comparing morphologies of fibers before /after the as-prepared fibers get exposed to air under ambient conditions for 3 months. The morphology and size of nanofibers in both cases remain unchanged, suggesting that the nanofibers are stable in PBS solution for at least one week and for 3 months in ambient conditions.

Cell Culture & Cytotoxicity:

Cell Culture: Chitin nanofibers (3 nm, 10 nm) and films deposited on clean glass coverslips are sterilized with ethanol for 1 hour and rinsed with phosphate buffered saline (PBS, Invitrogen) prior to cell culture. The nanofibers are stable in PBS solution and do not show significant morphology changes after incubation. Schwann cells (SCs, ATTC, RT4-D6P2T, Manassas, VA) are maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Invitrogen), and 1% penicillin-streptomycin (Invitrogen). Each chitin sample is cultured with SCs at a density of 12,500 cells/well in 24-well culture plates for five days, with media changes every two days.

Schwann Cell Proliferation: The alamarBlue (Invitrogen) colorimetric assay is used to analyze the cell number and proliferation of the SCs on the chitin samples. After one day, the samples are transferred to a fresh well-plate to ensure that only cells on the samples are analyzed. After 1, 3 and 5 days, the samples are washed twice with PBS and incubated with 10 vol% alamarBlue in DMEM with 10% FBS. 300 μL of the alamarBlue solution is transferred to an opaque 96 well plate for fluorescent measurement (ex. 560 nm, em. 590 nm). The fluorescent intensity of the alamarBlue is converted to cell number using a standard curve.

Schwann Cell Morphology Analysis: SC attachment to the chitin samples is imaged with Scanning electron microscopy (SEM). Samples are removed from culture after three days, rinsed with PBS and fixed with Karnovsky's fixative overnight. After fixing, samples are briefly rinsed with DI water and dehydrated with sequential rinses of 50, 75, 95 and 100% ethanol for 15 min each. Samples are dried using a Denton DCP-1 critical point dryer with CO_2. Samples are sputter-coated with Au/Pd for 30 seconds at 18 mA and are imaged with a JEOL 7000-SEM with a probe current of 90 μA and a 5KV accelerating voltage. The degree of cell morphology difference between chitin film and chitin nanofiber-based substrates is quantified using Photoshop. Specifically, three sets of chitin samples cultured with SCs were imaged with SEM at a magnification of 1000x. The cell surface area (μm^2) was measured with Photoshop and averaged to the number of cells within each field of view (11, 450 μm^2). The calculated surface area per cell for the chitin film, chitin fiber (3 nm) and chitin fiber (10 nm) are 191.0 μm^2, 284.5 μm^2, 262.5 μm^2, respectively.

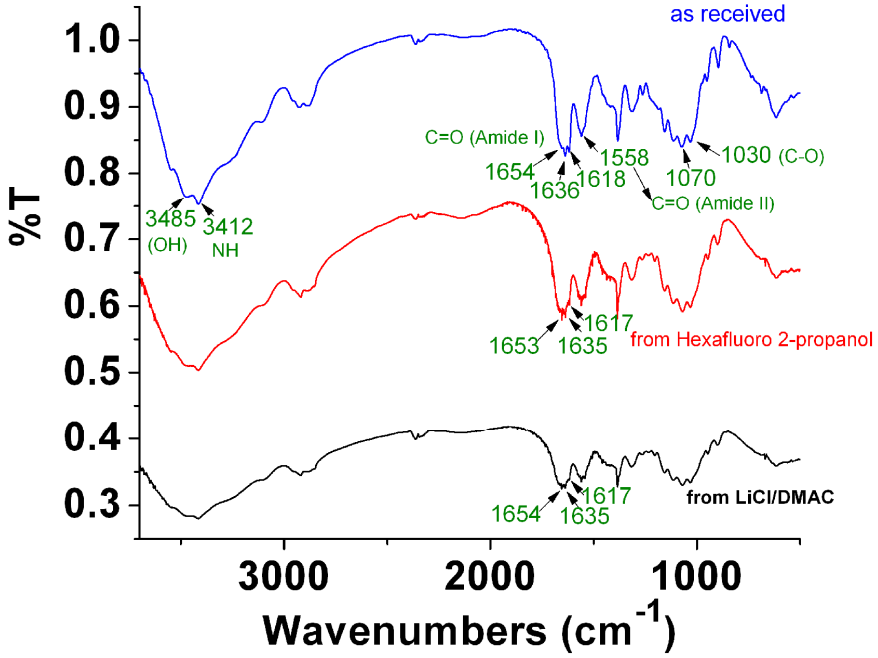

Fig. S1 FTIR spectra of chitin samples: (a) as received chitin sample, (b) chitin nanofibers prepared from the chitin/ HFIP solution, and (c) chitin nanofibers from the chitin/(LiCl/DMAC) solution. The split peaks at 1654 cm^{-1}, 1618 cm^{-1} (signature of α-chitin) and 1635cm^{-1} (signature of β-chitin) are present in the spectra of all the samples,[1] implying that a combination of α- and β-chitin structures exist in these samples. However, the main structure is α-chitin in the nanofibers samples, as indicated by the strong split peaks at 1654 cm^{-1}, and results from XRD analysis (Fig SI2). In addition, the β-chitin signature 1636 cm^{-1} peak becoming weaker in both nanofiber samples (compared with the as-received sample), suggest that β-chitin to α-chitin transformation take place during self-assembly process. This result is consistent with previous reports that pure β-chitin has not been possible to obtain either from solution or through in vitro synthesis.[1] The degree of N-acetylation can also be evaluated from FTIR using the ratio A_{1560}/ A_{1030} following previous literature procedures.[2] Values of 0.770 (96.8% N-acetylation), 0.749 (95.0% N-acetylation), and 0.755 (95.4% N-acetylation) were obtained for the as received sample, 3nm nanofibers and 10 nm nanofibers respectively. This indicates that negligible, if any, deacetylation occurs during the nanofiber fabrication processes.

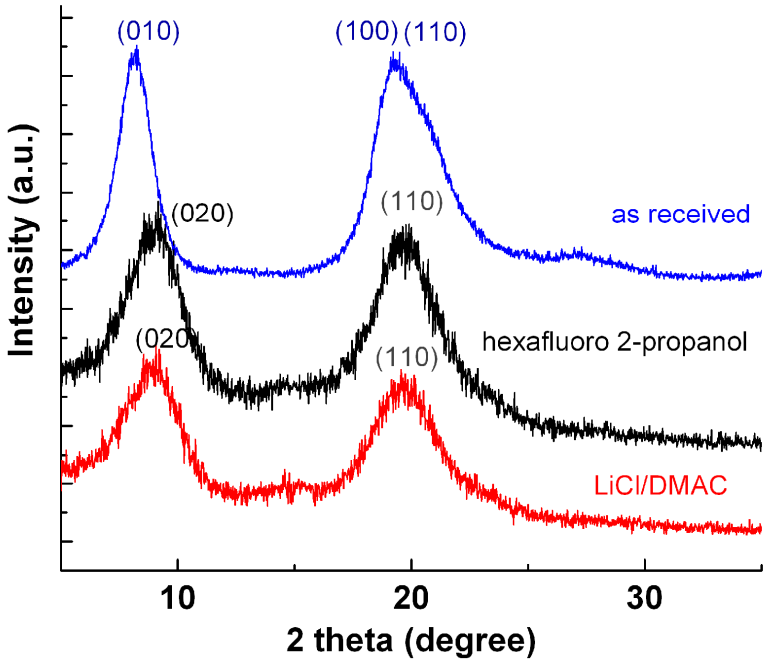

Fig. S2 XRD spectra of chitin samples: as received chitin sample (blue), chitin nanofibers prepared from the chitin/ HFIP solution (black), and chitin nanofibers from the chitin/ (LiCl/DMAC) solution (red). The presence of peaks at 9.2°, 19.5°, indexed as (020) and (110) reflections, indicates a α-chitin 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. This indicates that a transformation the β-chitin structure into α-chitin occurs during the nanofiber self-assembly process. Crystallinity measurements of the as-received sample (0.71), 3nm nanofibers (.68) and 10 nm nanofibers (0.72) confirm nanofiber crystallinity is conserved in both methods. Crystallinity was measured by taking the ratio of the sum of the area under the crystalline diffraction peaks to the total area under the curve from 5° to 30° accordingly to published procedures.[3]

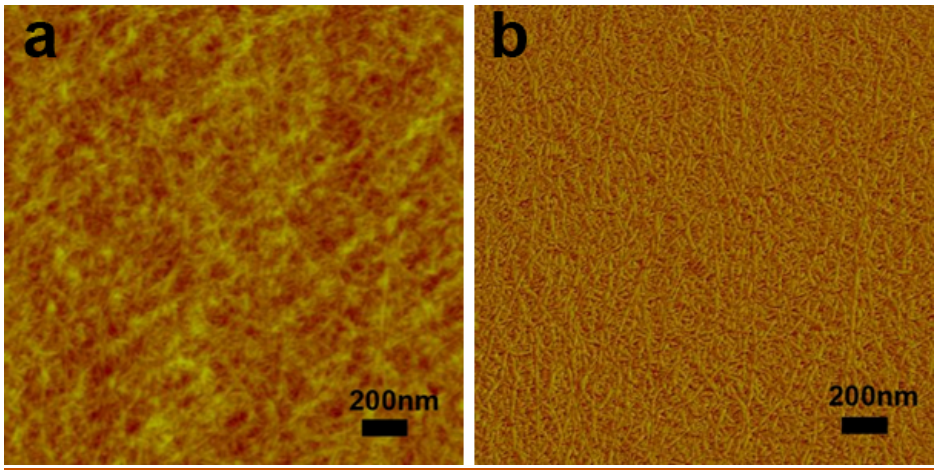

Fig. S3 Chitin films produced from a 5 µL 0.125 wt% chitin/HFIP solution. (a) AFM height image, (b) AFM phase image. The film is composed of a dense and random aggregated nanofibers network.

Fiber Width (Diameter) Determination

Fiber diameter is estimated from fiber height to avoid tip-convolution effects. The average chitin nanofiber diameter is estimated by measuring the height of 50 different individual nanofibers based on the AFM topography image.

A typical measurement of fiber height is shown Figure S4.

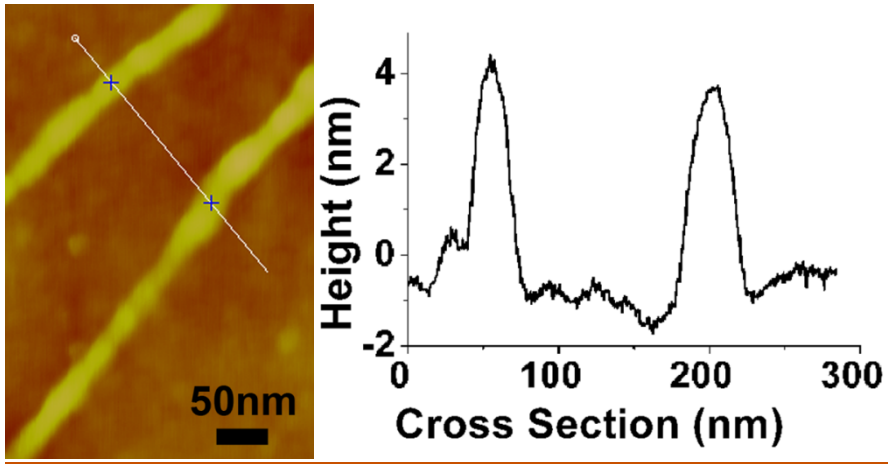

Fig. S4 AFM height image of single nanofibers prepared from Chitin/HFIP solution and corresponding cross-sectional height profile.

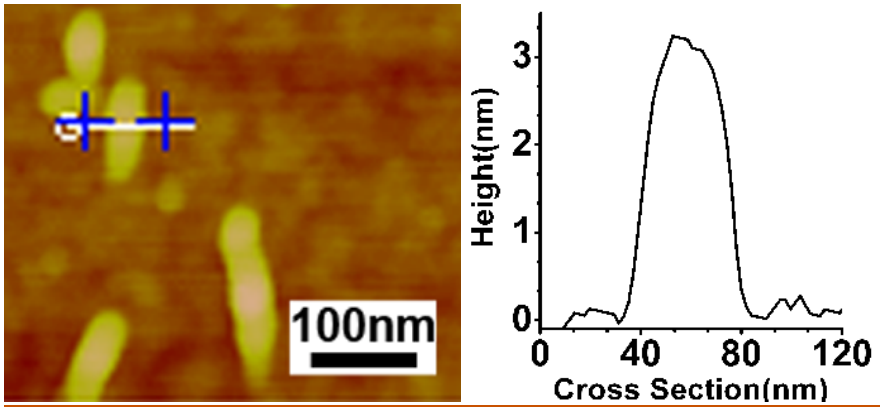

Fig. S5 Magnified AFM height image of Figure 2d, and corresponding cross-sectional height profile showing short nanofiber stub structures with a typical height of 3nm prepared from a 0.01 wt% chitin/(LiCl/DMAC) solution.

Kinetics study of nanofiber formation in Chitin/HFIP system:

To address the effects of kinetics in the chitin/HFIP system, we compared the nanostructures produced at controlled rate of solvent evaporation from solutions of the same concentrations. In particular, we studied the kinetics effect at the critical concentration of 0.002 wt% that is the lowest to produce micron long nanofiber at normal evaporation rate. The rates of solvent evaporation were controlled in three cases: 1) the solution was blow dried with N_2 gas (t= 3 sec.); 2) the solution was evaporated in air (t= 6 sec); 3) the solution was evaporated in a sealed petri dish (t=11 seconds). The morphologies of the resulting nanofibers are imaged using AFM (Fig S6).

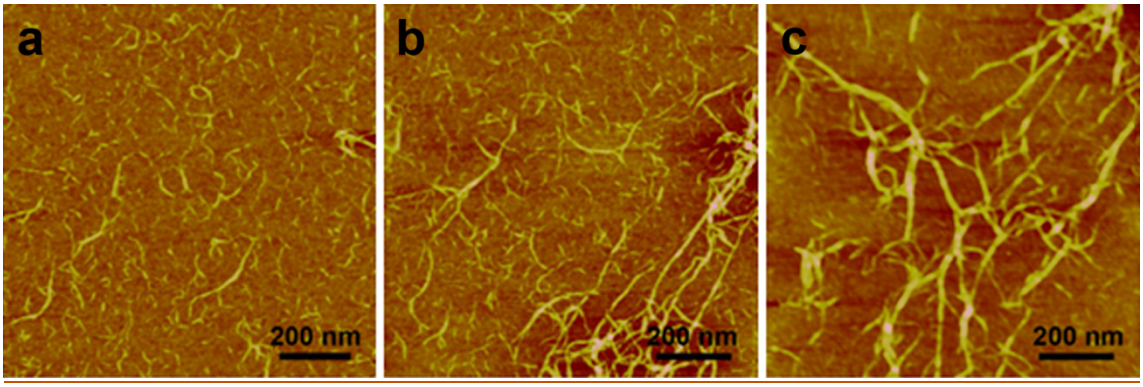

Fig. S6 AFM height images of chitin nanostructures deposited onto silicon wafers from 5 µL 0.002 wt% Chitin/HFIP solutions at different solvent evaporation rate: a) the solution was blow dried with N_2 gas (t= 3 sec.); b) the solution was evaporated in air (t= 6 sec); c) the solution was evaporated in a sealed petri dish (t=11 seconds).

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3 J. D. Goodrich, W. T. Winter, *Biomacromolecules* 2007, **8**, 252.