Exploring the Fibrous Nature of single-stranded DNA-Collagen Complexes: Nanostructural Observations and Physicochemical Insights

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Supplementary Information

Materials & Methods

As per our previously published protocols[1-4], NACC complexation occurs at room temperature, simply by pipetting the two reagents together at the desired ratios. Bovine collagen type I was supplied by Advanced Biomatrix (PureCol EZ Gel, 300 kDa, diluted to a final working concentration of 1.5 mg/mL). We define dilution as adding deionized water *with ssDNA* (for NACCs) or *without ssDNA* (as the collagen-only control). ssDNA oligonucleotides were ordered from IDT Technologies and resuspended in ultrapure deionized water to a final concentration of 10 μM.

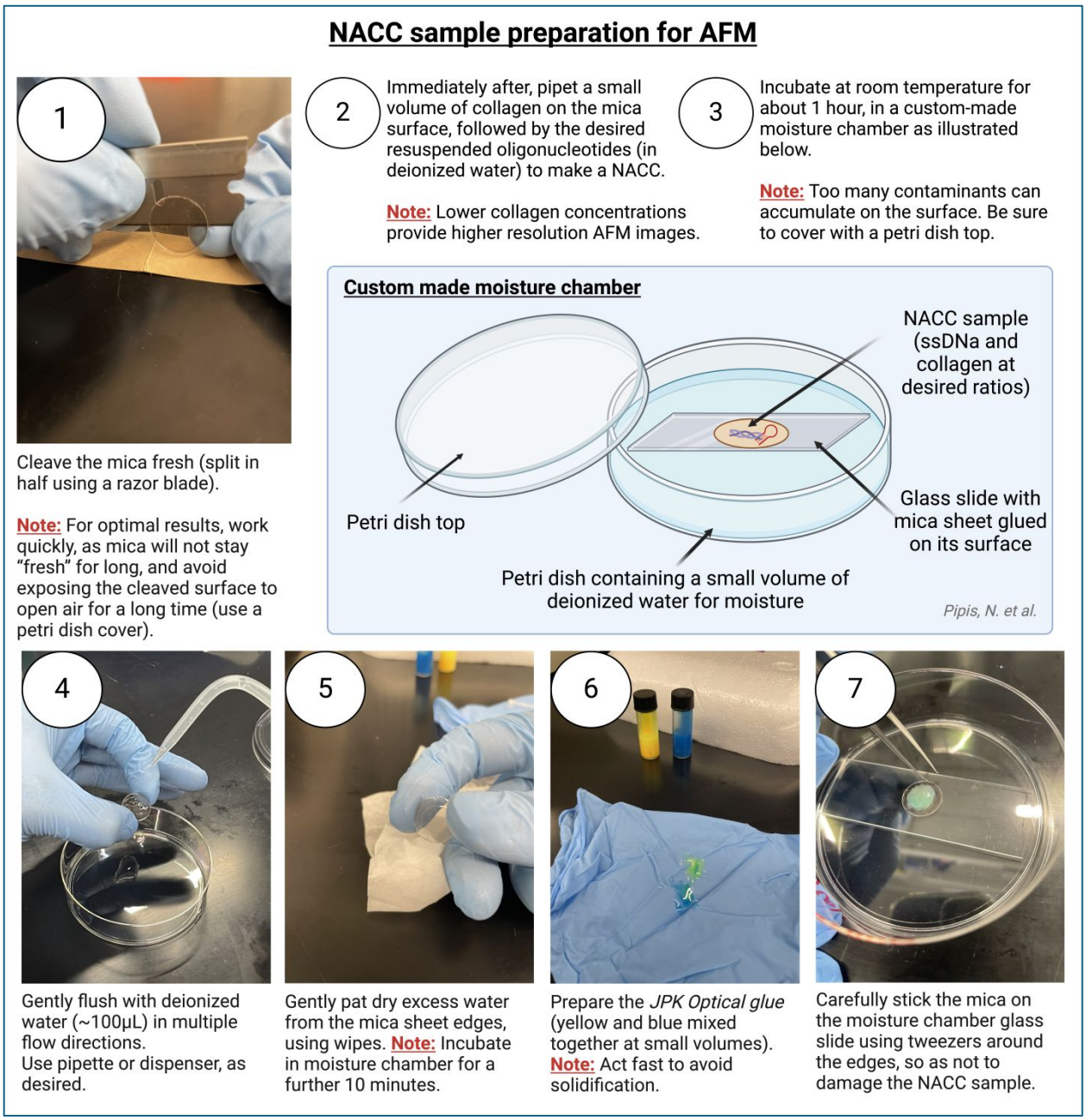
80-nucleotide random sequence: 5' – AAT ATC TCG CGC GAT AGC GAT CGA CTA GCT GAG CTA TGC TAG CAA CTG ACA TAC TGA GCT AGC CTG AAC GTG ACT GAA CG – 3', Mw = 24,681 g/mol

This random sequence, 80-nt long ssDNA, was selected because it falls within the typical aptamer oligonucleotide length and the "fiber-forming region" of NACCs (~15–90 nt long). Additionally, NACC complexation is independent of ssDNA sequence (GC% content). Similarly, this ssDNA-to-collagen molar ratio used was based on our previous publications[1-4] since it enables NACC fibrillogenesis.

Atomic Force Microscopy (AFM)

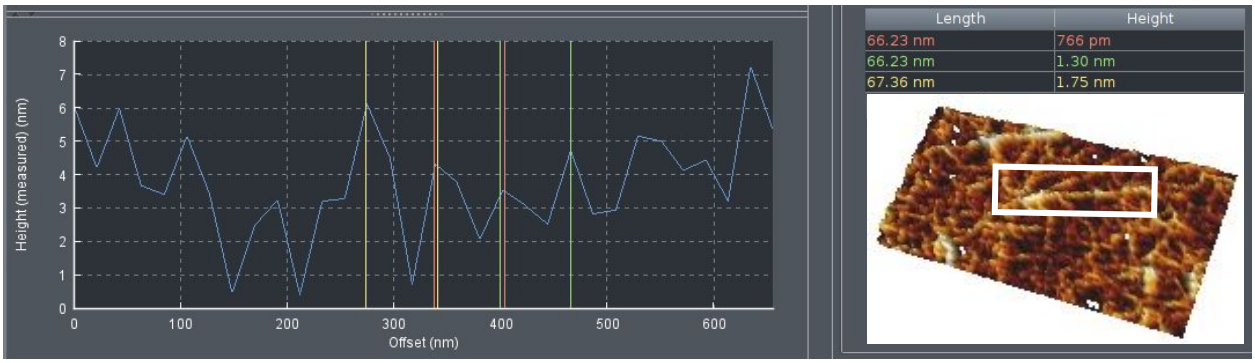
Protocol for sample preparation: Muscovite Mica Sheets (V-1 Quality, Electron Microscopy Sciences) were cleaved fresh using a razor blade. Immediately after, on one surface, we deposited 70μL of collagen alone, followed by 30μL water (as the collagen-only control, devoid of ssDNA). On the other mica surface, we deposited 70μL collagen with 30μL of the 80-nucleotide long ssDNA oligonucleotide in order to form the NACC. The final collagen concentration was 1.5

mg/mL on each mica sheet. The sheets were then incubated at room temperature for at least 1 hour in a custom-made moisture chamber to minimize evaporation. Samples were then flushed twice with 100µL of deionized water in multiple flow directions. Excess water was gently pat dried from the edges of the mica sheet using Kimwipes, and the samples were incubated in the moisture chamber for a further 10 minutes, half-covered with a petri dish. Samples were then fixed onto a glass slide using the *JPK Bio-Compatible Glue* for subsequent AFM imaging.



Supplementary Figure 1 A schematic diagram of our protocol for preparing NACC (or collagen-only) samples for AFM.

Data collection and analysis: The Bruker NanoWizard 4 system with ScanAsyst-Fluid+ probes (triangular pyramid tip) was used to obtain high-resolution images and data of the samples in Quantitative Imaging contact mode. The AFM images were analyzed through Bruker's JPK Data Processing software (Version 7.0.184). For height profiles (measuring the D-band periodicities), the "Cross Section" button was selected, followed by a mouse drag along the fiber of interest. Three-dimensional images were generated using the "3D View" feature, to illustrate results clearer. Mechanical characterization (stiffness measurement) was performed by deflection calibration of the tip on a freshly cleaved mica sheet (without any loaded sample), followed by a linear fit on the "Vertical Deflection Vs Height" curve. The "Spring Constant" was calibrated (Sensitivity: 15.65 nm/V, Spring Constant: 0.5 N/m), data was "Smoothed" (Gaussian method), and "Baseline Offset" was subtracted (5.837 nN). The "Contact Point" was automatically calculated (7.288 µm). Mica sheets with samples were then loaded, and data was acquired after identifying areas of interest. The "Fit Parameters" for elasticity (Young's modulus) were set as "Hertz/ Sneddon" (model type) and "Triangular Pyramid" (tip shape). Then, image data was acquired in a similar method as described above for the height measurements (by dragging the mouse along fibers of interest). Height and stiffness raw data were exported and visualized in GraphPad Prism version 10.1.2.



Supplementary Figure 2 Representative collagen-only measured height indicating D-band periodicities of the boxed region (screenshot from the JPK Data Processing Software).

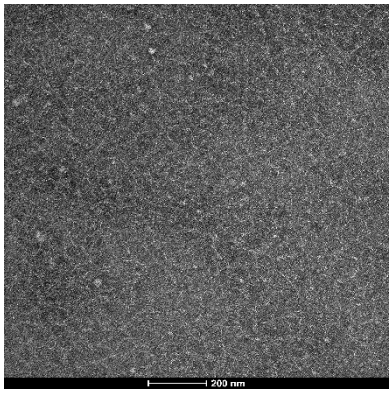
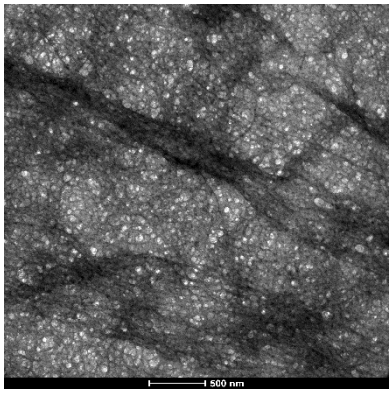
Phase Contrast, TEM, and Cryo-Electron Microscopy

- Phase contrast images were taken using the Keyence BZ-X800 and a PlanFluor 20 × 0.45/8.80–7.50 mm lens.

- For TEM, the Talos L120C G2 was used. Samples were stained with 1% uranyl acetate for subsequent negative stain imaging. The images were acquired on a CETA camera (Thermo Fisher Scientific). On top of performing TEM on bovine collagen samples, this was repeated on rat-tail collagen as well, with similar observations as shown in *Supplementary Figure 3*.

- For cryo-EM, after mixing bovine collagen (0.75 mg/mL) and ssDNA (10µM), three microliter aliquots of suspension were immediately applied to a glow discharged, quantifoil 2/2 grid with 2 nm carbon substrate (Quantifoil Micro Tools GmbH) and vitrified using a VitrobotTM Mark IV (FEI Co.) operated at 4°C and with ~90% humidity in the control chamber. Collagen-only fibers (0.75 mg/mL) were also plunge-frozen for comparison. The vitrified samples were stored under liquid nitrogen and transferred into a Gatan cryo-holder (Model 626/70) for imaging.

The samples were examined using a 4k × 4k CCD camera (Gatan, Inc.) on a Tecnai (FEI Co.) G2 F20-TWIN Transmission Electron Microscope operated at a voltage of 200 kV using low dose conditions (~20 e/Å2). Images were recorded with a defocus of approximately –2µm to improve contrast.

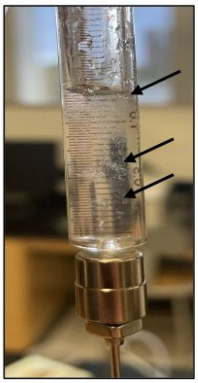
(a) TEM – Rat-tail collagen only (1.5 mg/mL)	(b) TEM – Rat-tail NACCs (1.5 mg/mL)
	

Supplementary Figure 3 Representative TEM image panel of rat-tail type 1 collagen, 1.5 mg/mL (Note: scalebars vary) ***(a)*** *Rat-tail collagen only.* Only individual fibrils are distinguishable ***(b)*** *Rat-tail NACCs*. A clear induction of sheet-like fibril formation is demonstrated upon addition of ssDNA. ssDNA induces the formation of collagenous fibrous sheets, leading to the emergence of a network that was previously non-existent in the absence of ssDNA.

Isothermal Titration Calorimetry (ITC)

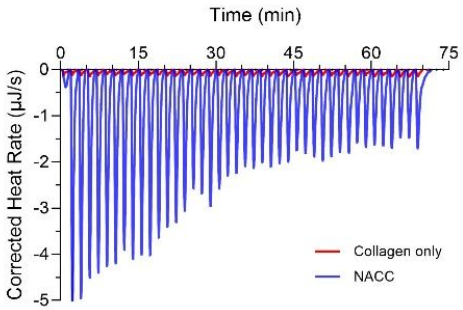
Experimental Procedure: ITC experiments were performed with a TA Instruments NanoITC. The ssDNA oligonucleotide solution in the buret syringe (10 µM) was titrated over aqueous type I collagen (950 µL, 1.5 mg/mL) in the sample cell at 25 ± 0.1 °C. Rat-tail collagen (Corning, 300 kDa) was used for this experiment due to the innate formulation of EZgel PureCol to solidify within ~30 minutes of being at room temperature, thereby unsuitable for this experiment. 4.76 µL ssDNA injections were carried out at 100s intervals, and the constant stirring speed in the sample cell was set to 250 rpm. The reference cell contained deionized water (free from ssDNA). Data was analyzed using the NanoAnalyze software version 3.12.5 and visualized using GraphPad Prism version 10.1.2.

(a) NACCs

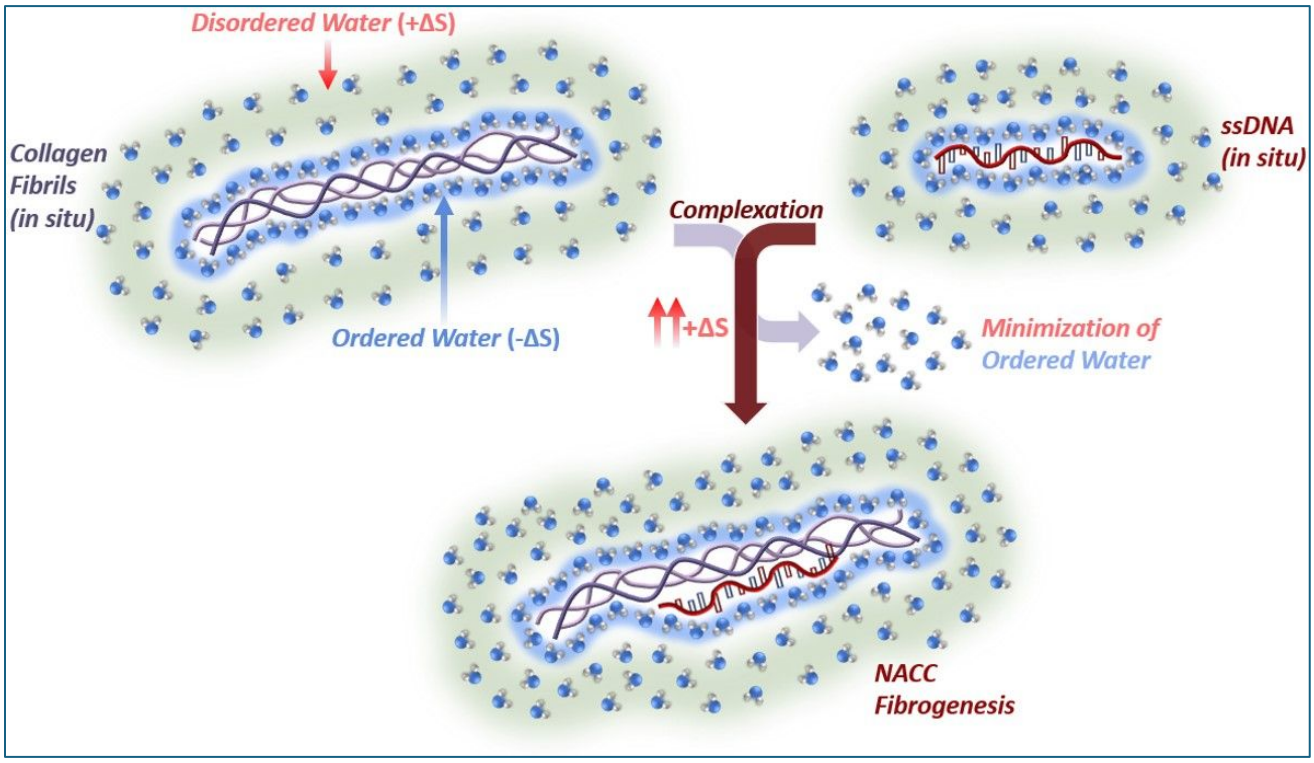


Independent	Kd (M)	6.866E-5	±6.700E-5
	n	2.352	±0.187
	ΔH (kJ/mol)	-2.082	±0.515
	ΔS (J/mol·K)	72.72	
	Confidence Level:		**95%**

(b) ITC comparison



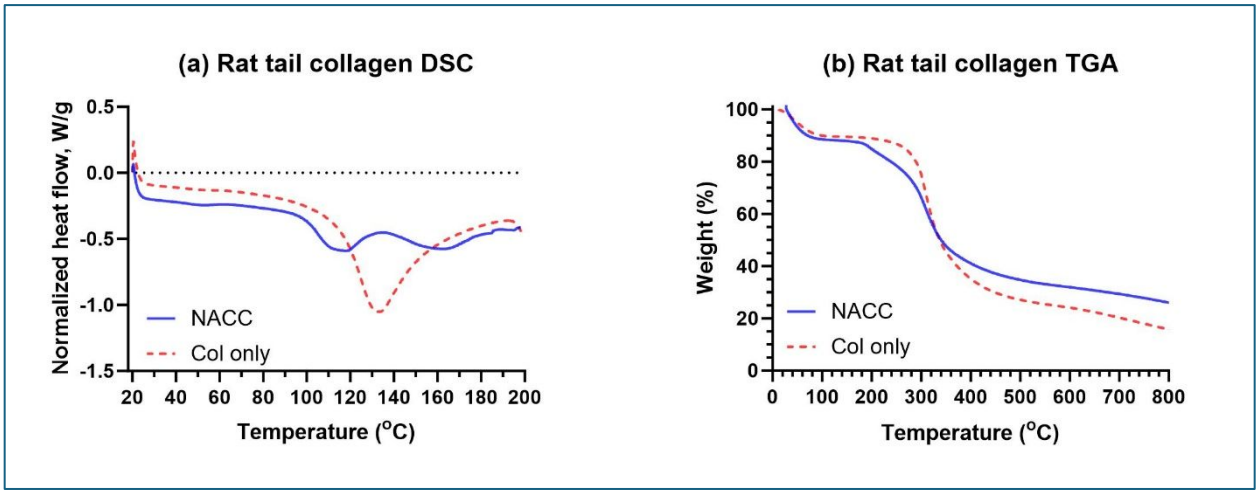
Supplementary Figure 4 (a) Arrows on the left picture indicate NACC fibrous complexes after completion of the ITC experiment. Additional metrics not mentioned in the main text include a Dissociation Constant, *Kd,* of 6.866E-5±6.700E-5 and Stoichiometry, *n*, of 2.352±0.187 ***(b)*** Comparison of ITC enthalpograms for addition of ssDNA to collagen (i.e., NACC) versus addition of deionized water, devoid of ssDNA (i.e., collagen dilution). A constant generation of lower heat values is evident for collagen only, in contrast to NACC formation which involves collagen aggregation and fibrillogenesis.



Supplementary Figure 5 A suggested mechanism for NACC self-assembly and fibrogenesis. We believe the phenomenon of complexation underpins the thermodynamically favorable supramolecular interaction between collagen and ssDNA.

Differential Scanning Calorimetry (DSC) & Thermogravimetric Analysis (TGA)

Experimental Procedure: Collagen-only and NACC samples were lyophilized for 24 hours. For DSC, measurements were carried out on a TA DSC2500 instrument. Around 5 mg of the sample were placed in aluminum containers. Scans were in the 20–200 °C range, using a temperature ramp of 10 °C/min. For TGA, samples were analyzed using a TA TGA5500 instrument, with approximately 5 mg samples placed in standard platinum pans and mass loss change monitored in the 50-800 °C range.



Supplementary Figure 6 To confirm and verify our findings with collagen derived from other species, we repeated the ***(a)*** DSC (exotherm up) and ***(b)*** TGA experiments with rat-tail collagen (Corning), observing similar trends with the bovine collagen as shown in the main text.

References

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