Structural information of biopolymer nanofibrils by infrared nanospectroscopy

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A B S T R A C T

Biopolymer nanofibrils, such as chitin and silk nanofibrils, are abundant and critical structural components in nature and materials engineering. However, characterizing these nanofibrils at single fibrillar scale is still a challenging task. Herein, infrared nanospectroscopy (nanoIR), a technique that combines the advances of both atomic force microscopy and infrared spectroscopy, was applied to detect the chemical and structural information of single chitin and silk nanofibrils. The results of this study reveal that nanoIR is a powerful approach to identify the single biopolymer nanofibrils with spatial resolution reaching up to ~10 nm. In addition, for silk nanofibrils, the results support that nanoIR can be used to analyze the relative content of each secondary structure by deconvolution, because the content of each secondary structure is consistent with the results obtained from the deconvolution of the traditional infrared spectrum. However, the prerequisite for this semi-quantitative analysis is the use of high signal-to-noise ratio spectra, and characteristic peak positions of each secondary structure in nanoIR spectra were predetermined.

1. Introduction

Biopolymer nanofibrils are abundant and critical architectural components of materials found in nature [1]. These biopolymer nanofibrils comprise different biopolymer chains, i.e., cellulose, chitin, and silk fibroin. However, they are typically well-organized into sophisticated arrangements at higher structural levels, such as highly-oriented fibers, anisotropic lamellae, and 3D-helicoids [2–4]. These unique mesoarchitectures form central features in hierarchical structures of natural materials, endowing high mechanical performance and key functions of biological tissues. For example, the nanoconfinement and nanofibril orientation of spider dragline silk fiber (a fiber produced by the major amputal gland of spiders as a lifeline and used in the construction of the orb web outer rim and spokes) contributes unparalleled mechanical properties, exhibiting a unique combination of high tensile strength (a tensile strength in the range 1–2 GPa) and extensibility (50–60% strain at failure) [5]. These mechanical features enable a silk fiber to absorb a large amount of energy before breaking attributing toughness several times higher than that of steel and Kevlar fibers [5–7]. The anisotropic lamellae in cell walls allow trees to adjust mechanical properties of woods to adapt to wind and regulate tree growth [8]. The 3D-helicoids of chitin nanofibril (CN) layers in animal exoskeletons efficiently resist fracture through energy dissipation and prohibiting crack propagation [4].

In recent years, beyond these natural roles, applications of biopolymer nanofibrils are being explored extensively as a path towards design of novel materials with features that surpass those achievable with synthetic polymers [9–11]. These characteristics, combined with sustainability, availability, low-cost, and biocompatibility provide added incentives to explore biopolymer nanofibrils in the design of new materials. Accordingly, the insight into the structures of biopolymer nanofibrils offers the opportunity to elucidate structure–property–function relationships of natural materials and inspire new processing routes of these biopolymer nanofibrils. Defined regions in nanofibrils should be critically investigated in order to achieve the abovementioned characteristics. Biopolymers in nanofibrils show considerable complexity and variability and can be traced to diverse chemical components and growth environments, as
well as differences in processing conditions. However, experimentally examining the structural organization of biopolymers at the single nanofibril level remains a challenging task. The characteristic size of these nanofibrils (approximately 2–20 nm in diameter [12]) is much smaller than the spatial resolution of most advanced structural characterization techniques, such as synchrotron Fourier transform infrared (S-FTIR) microspectroscopy (diffraction limit, ~2.5–75 μm [13–15]), Raman spectroscopy (~2 μm [14]), and synchrotron coherent small-angle X-ray scattering (spot size, 25 × 10 μm²) [16].

Infrared nanospectroscopy (nanoIR), a hybrid technique that combines the spatial resolution of atomic force microscopy (AFM) with the chemical analysis capability of infrared spectroscopy, allows the characterization of single nanofibril possible [13,17,18]. This technique uses AFM tip to locally detect thermal expansion of a sample resulting from the local absorption of IR radiation. The AFM tip itself thus acts as the IR detector and can detect the thermal expansion with spatial resolution approaching the AFM tip radius (Fig. S1). Thus, the nanoIR technique can overcome the spatial resolution limits of S-FTIR microspectroscopy [13].

2. Results and discussion

2.1. NanoIR spectrum of single α-TOCN

In this study, nanoIR was used to analyze and image biopolymer nanofibrils, including chitin and silk nanofibrils. First, α-chitin nanofibril (α-TOCN) was characterized, attributing to its critical roles in modulating the optical and mechanical properties of natural materials together with broad availability for material applications [1]. α-TOCN, a cellulose analog comprising a long-chain polymer of a (1, 4)-β-N-acetylglucosamine [2], is the most abundant crystalline polymorph of chitin found in animal exoskeletons.

Fig. 1A presents the characteristic morphology of α-TOCN produced by 2,2,6,6-tetramethylpiperidine 1-oxyl radical-mediated oxidation. These nanofibrils show needle-like shapes with the lengths of approximately 200–500 nm. Fig. 1B displays both the longitudinal (green line) and the transversal height traces (purple line), indicating the characteristic height profile (with a diameter of ~6 nm) and smooth nanofibril surfaces. Before nanoIR characterization, FTIR spectrum of α-TOCN films was recorded. Fig. 1C shows a series of specific absorption bands in the 1800–1000 cm⁻¹ region. For example, two absorption peaks at 1655 and 1624 cm⁻¹ are observed in the amide I region (1700–1600 cm⁻¹, C–O stretching [19]). The most accepted explanation for the two peaks
is the presence of two types of amide bonds. Half of the carbonyl groups are bonded through hydrogen bonds to the amino group inside the same chain (C=O…H–N) (the insert image in Fig. 1C, highlighted by purple circles), which is responsible for the vibration mode at 1655 cm\(^{-1}\). The rest of the carbonyl groups form the same bond with the –OH group from the neighboring chain [19] (the insert image in Fig. 1C, highlighted by green circles). This additional bond decreases the intensity of the amide I band at 1624 cm\(^{-1}\). In addition, the sodium carboxylate groups present in the TOCN (if they were not converted to free carboxyl groups by acid treatment) may also contribute the amide I bands, as the C=O stretching band of the carboxylate group also shows adsorption at the amide I region. However, as for the carboxyl groups due to the oxidation of the chitin, previous study has provided a method to protonate the carboxylates to shift them out from under the amide regions [20]. Due to neutralizing the pulp, the carboxyl groups should be all carboxylates, and thus this carboxylate band significantly overlaps with the amide bands.

In addition to the amide I band, the spectrum shows two other amide bands, including amide II at 1560 cm\(^{-1}\) (the C–N stretching and the N–H in-plane bending) and amide III at 1317 cm\(^{-1}\) (C–N stretching and N–H in-plane bending) [19-21]. The assignments of all the characteristic peaks in \(\alpha\)-TOCN are listed in Table 1.

Fig. 1F shows the nanoIR spectrum of single \(\alpha\)-TOCN which the AFM image and nanoIR image collected by the nanoIR system shown in Fig. 1D and E, exhibiting good resolution for both amide I and II bands in the region 1800–1500 cm\(^{-1}\), making them suitable for qualitative analysis. As in conventional FTIR spectra, both the amide I and II bands in the nanoIR spectrum are suitable for in situ analysis and imaging chemical structures of single \(\alpha\)-TOCNs. As an example, Fig. 1E displays the nanoIR image of single nanofibrils mapped by the infrared amplitude of 1566 cm\(^{-1}\). Compared to the AFM image shown in Fig. 1D, the \(\alpha\)-TOCN region showed significant chemical contrast (the yellow parts in Fig. 1E), providing much higher infrared amplitude than the substrates (the black area in Fig. 1E). More remarkably, the nanoIR image (the white frame region in Fig. 1D and E) shows the small nanoparticles, indicating that nanoIR imaging can clearly distinguish single \(\alpha\)-TOCNs with a resolution of \(\sim 10\) nm, which is approximately 1000 times higher than the resolution obtained by S-FTIR microscopy [14,15].

However, the nanoIR and FTIR spectra showed difference in the 1500–1000 cm\(^{-1}\) region (Table 1). Several peaks, such as amide III (1300–1200 cm\(^{-1}\), 1157, 1115, and 1075 cm\(^{-1}\), were not observed. The primary reason for this difference comes from two aspects. First, the infrared intensity of the globar source of traditional FTIR spectroscopy is much higher than that of nanoIR technique. The spot size of traditional FTIR spectroscopy is around 2 mm in diameter, and thus it can collect the accumulated vibration information of the test sample within such a size region. NanoIR technique, instead, only collects vibration information of samples within around 10 nm. Therefore, the sample signal intensity collected by traditional FTIR spectroscopy is much higher than that of nanoIR technique. The signal intensity of the sample affects both the signal-to-noise ratio and spectral resolution. Poor signal-to-noise ratio often leads to the indistinguishability of two adjacent absorptions since the smoothing processing is necessary for nanoIR spectrum. This processing usually couples two adjacent absorption peaks into a broad one when the signal-to-noise ratio of the spectrum is poor. In addition, the signal of the fingerprint region below 1300 cm\(^{-1}\) in IR spectra is ascribed to minor vibration information of samples, so these fingerprint peaks cannot be detected accurately when the signal intensity is not strong enough. The spectral resolution of nanoIR can be improved obviously by testing a high-density stacked sample, enhancing the sample signal intensity significantly. For example, when the \(\alpha\)-TOCN membrane was used for testing, the resolution of the spectrum significantly improved (as shown in Fig. 1G and H), matching well with the spectrum collected from traditional FTIR spectroscopy. However, this approach compromises the spatial resolution of nanoIR since it is impossible to obtain the structure information of a single nanofibril in these tests, but the accumulated signal of multiple nanofibrils. From Fig. 1H, it can be found that the intensities of the peaks at 1157, 1075, and 1028 cm\(^{-1}\) are even higher than that of the peaks in the 1720-1580 cm\(^{-1}\) region, which may be because these wavenumber regions are collected by different lasers. In the nanoIR system, four laser sources are integrated, the wavenumber regions they detect are 1910–1650 cm\(^{-1}\), 1650-1347 cm\(^{-1}\), 1347-985 cm\(^{-1}\), and 985-750 cm\(^{-1}\), respectively. Accordingly, it is difficult to quantify the intensity changes using a standard internal band (like at 1030 cm\(^{-1}\)) [20].

In addition, the driving force of nanofibrillation is the presence of anionic carboxyl groups. Therefore, it can be reasonably inferred that TOCNs have a higher carboxyl content than the unfibrillated chitin bundles. In other words, TOCNs and unfibrillated chitin bundles should have differential amide absorption. The nanoIR spectra of TOCNs and unfibrillated chitin bundles were thus collected, as shown in Fig. S2. Both samples show the carbonyl peaks at 1655 cm\(^{-1}\) and 1624 cm\(^{-1}\), but their absorption intensities are different. In unfibrillated chitin bundle (Figs. S2A and S2B), the intensity of 1655 cm\(^{-1}\) was significantly lower than 1625 cm\(^{-1}\). In contrast, after nanofibrillation (Figs. S2C and S2D), the strength of 1625 cm\(^{-1}\) was almost equal to (or even higher than) that of 1655 cm\(^{-1}\).

### Table 1

<table>
<thead>
<tr>
<th>Wavenumbers (cm(^{-1}))</th>
<th>Assignments</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1655</td>
<td>(\text{C} = \text{O} (\text{amide I}))</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1630</td>
<td>(\text{C} = \text{O} (\text{amide I}))</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1560</td>
<td>(\nu_{\text{C-N}} (\text{C-N-H}) + \delta_{\text{N-H}} (\text{amide II}))</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1422</td>
<td>(\delta_{\text{C-O}})</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1376</td>
<td>(\delta_{\text{C-H}} + \delta_{\text{C-H}})</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1312</td>
<td>(\nu_{\text{C-N}} + \delta_{\text{N-H}} (\text{amide III}))</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1255</td>
<td>(\delta_{\text{N-H}})</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1160</td>
<td>(\nu_{\text{C-O}, \alpha} (\text{ring}))</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1113</td>
<td>(\nu_{\text{C-O}})</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1070</td>
<td>(\nu_{\text{C-O}})</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1030</td>
<td>(\nu_{\text{C-O}})</td>
<td>[19-21]</td>
</tr>
<tr>
<td>1692</td>
<td>(\nu_{\text{C-O}} (\text{amide II}); \beta)-turn</td>
<td>[22-24]</td>
</tr>
<tr>
<td>1652</td>
<td>(\nu_{\text{C-O}} (\text{amide I}); \text{random coil and/or } \alpha)-helix</td>
<td>[22-24]</td>
</tr>
<tr>
<td>1624</td>
<td>(\nu_{\text{C-O}} (\text{amide I}); \beta)-sheet</td>
<td>[22-24]</td>
</tr>
<tr>
<td>1540</td>
<td>(\nu_{\text{C-N}} (\text{C-N-H}) + \delta_{\text{N-H}} (\text{amide II}); \text{random coil and/or } \alpha)-helix</td>
<td>[22-24]</td>
</tr>
<tr>
<td>1530</td>
<td>(\nu_{\text{C-N}} (\text{C-N-H}) + \delta_{\text{N-H}} (\text{amide II}); \beta)-sheet</td>
<td>[22-24]</td>
</tr>
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* Abbreviations: \(\nu\) = stretching, \(\delta\) = bending, \(\alpha\) = asymmetric stretching.  

#### 2.2. NanoIR spectrum of single silk nanofibril

In addition to \(\alpha\)-TOCN, a protein nanofibril, i.e., silk nanofibril (SNF), was also characterized by nanoIR. For protein samples, FTIR based techniques are not only sensitive to the molecular vibration but also useful for quantitatively evaluating the conformation of proteins, such as \(\beta\)-sheet, random coil, helix, and \(\beta\)-turns [25,26]. Fig. 2A presents the typical morphologies of the SNFs used for measurements. SNFs were produced from silk fibroin aqueous solution by heat-induced self-assembly [26]. Briefly, a 0.1 wt% silk fibroin aqueous solution was incubated at 60 °C for 7 days. These SNFs feature beaded-like structures with contour lengths up to several micrometers. The transverse height profile of SNFs (Fig. 2B) reveals a height of ca. 4-6 nm, while the longitudinal profile indicates random height fluctuations with roughness in the height of 2 nm.

The detailed infrared absorption of SNF was first monitored by conventional FTIR spectroscopy in the transmission mode (transmission mode spectroscopy for short) (Fig. 2C) and the full spectrum showed in Fig. S3. In this case, FTIR spectra of the SNF films assembled by vacuum filtration were collected in the wavenumbers between 1800 and 1400 cm\(^{-1}\). This region includes the amide I (1700–1600 cm\(^{-1}\)) and amide II
Fig. 2. AFM, FTIR, and nanoIR spectra of SNFs. (A) AFM image of SNFs. (B) Longitudinal (purple curve) and transverse (green curve) height profiles as indicated by the corresponding traces in Fig. 2A. (C) FTIR spectrum of SNF film obtained by transmission mode testing. (D) The deconvolution results of amide I band in Fig. 2C. Solid curve, original spectrum; dashed curve, deconvoluted peaks, Circles, simulated spectrum from summed peaks. (E) AFM image of SNFs collected by nanoIR system. (F) nanoIR spectra of single nanofibrils and substrate. nanoIR spectra of single SNFs were collected from the black cross-shaped points shown in Fig. 2E, while the nanoIR spectra of substrates were collected from red cross-shaped points. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample</th>
<th>(\beta)-sheet</th>
<th>random coil and/or helix</th>
<th>(\beta)-turn</th>
</tr>
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<tr>
<td>FTIR</td>
<td>SNF film</td>
<td>42 ± 1</td>
<td>54 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>NanoIR</td>
<td>single SNF</td>
<td>41 ± 1</td>
<td>53 ± 4</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td>SNF bundle</td>
<td>40 ± 2</td>
<td>56 ± 3</td>
<td>4 ± 2</td>
</tr>
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</table>

The relative content of different secondary structures can be obtained by the deconvolution of amide I band. The intensity ratio of amide I and II bands can afford molecular orientation of silk fibroin, because the C=O (amide I) and C-N stretching (amide II) in amide bond are perpendicular to each other. The assignments of the peaks in the amide I band agreed with the literature data. For instance, the amide I band of random coil/silk fibroin obtained by the conventional FTIR spectra shows specific absorption at 1690, 1660, and 1629 cm\(^{-1}\), which are the characteristic amide I bands of the \(\beta\)-sheet-rich structure. In contrast, the amide I band of random coil/another protein shows a broad peak at 1650 cm\(^{-1}\), and this observation is in agreement with the spectrum obtained by the transmission mode spectroscopy.

A series of nanoIR spectra of single SNFs were collected (Fig. 2E and Fig. S5). Using the same deconvolution methods with five wavenumbers deviation as given in Fig. 2D, the relative content of each conformation of SNFs was calculated. The deconvolution process and result for the nanoIR data shown in Fig. S6 indicated 41 ± 1% of \(\beta\)-sheet, 53 ± 2% of random coil/helix, and 6 ± 1% of \(\beta\)-turn. This relative conformational content obtained by the deconvolution is in line with that obtained by the deconvolution of the transmission-mode spectra, revealing that the nanoIR spectra can be used to evaluate the relative content of secondary structure of the proteins. However, it is worth noting that the deconvolution processing of infrared spectrum needs to follow strict rules. For example, the spectra used for deconvolution need to be collected at well-controlled conditions, such as vacuuming and complete water removal. Only the second derivative spectrum obtained under these conditions can truly reflect the conformation of the protein. Otherwise, the interference of environmental water vapor will cause significant distortion of the second derivative spectrum. These well-controlled test conditions are still difficult to achieve with nanoIR technology; thus, its second derivative spectra can no longer be used as a criterion for the determination of peak positions of each secondary structure. Here, the deconvolution of nanoIR spectrum was performed according to the peak position determined by the transmission spectrum collected from well-controlled conditions (Fig. S7A). Therefore, in principle, this operation is only suitable when the conformation-wavenumber relationships have been strictly verified. For an unknown protein, or a protein with unclear conformation assignments, the accuracy of the deconvolution method needs to be further
investigated.

2.3. Visualization of SNFs via nanoIR imaging

The nanoscale resolution, together with the conformational sensitivity, enables nanoIR to visualize the interfacial structures between biopolymer nanofibrils and other functional nanomaterials. For example, the assembly of SNF on the graphene surface [27] monitored by nanoIR indicates that SNF almost only grows on the graphene surface, and the assembled SNF is a typical antiparallel β-sheet structure, because its amide I and II bands are almost consistent with the spectra of the single silk fiber detected by S-FTIR. However, significant distortion of amide I bands was detected in their associated single spectra. This situation mainly arises because of the elimination of the gold surface enhancement effect. The pristine SNF samples (obtained by heat-induced self-assembly) were prepared on the gold nanoparticles layer coated silicon substrate, in which gold can significantly amplify the infrared signals by enhancing local electromagnetic fields [13, 28]. However, for the SNF/graphene oxide hybrid system, as shown in Fig. 3A and Figs. S8-10, the SNFs grow directly on the surface of reduced-graphene oxide, eliminating the surface enhancement of gold nanoparticles, which results in the poor signal-to-noise ratio of the spectrum. However, the amide I and amide II bands can still be detected (Fig. 3B and C). To verify this inference, the nanoIR spectrum of the sample with a high SNF stacking area was collected (Fig. 3D). The resulting nanoIR spectra (Fig. 3E) indeed showed similar amide I absorption as that obtained by the conventional FTIR spectra, where the strong β-sheet peak was observed at ~1634 cm⁻¹, together with the amorphous phase at ~1667 cm⁻¹. These amide I bands were further deconvoluted using the same criteria as shown in Fig. 2D. These approaches showed considerable difference in the relative content of each secondary structure, for example, the relative contents of β-sheet, random coil/helix, and β-turn structures span in the ranges 38–42%, 53–59%, and 2–6%, respectively (Table 2). Again, these results agree well with the results obtained by transmission-mode characterization, further verifying the reliability of nanoIR in the semi-quantitative analysis. Another factor that affects the absorption of amide I band is the moisture in the environment and/or in the test sample, although most of the moisture has been removed during the sample preparation process and the testing process. For example, all the samples used for the measurements were thoroughly dried in the dryer for more than 12 h, and the nanoIR system was equipped with a N₂ purge accessory to remove the moisture in the sample chamber.

2.4. Polarized nanoIR spectra of single SNF

As mentioned, in principle, the molecular orientation of silk fibroin can be obtained by the intensity ratio between amide I and II bands, as the C=O and C–N stretching are perpendicular to each other. However, no prior experimental data can be used to evaluate the effectiveness of this method for determining the orientation in single biopolymer nanofibrils. Therefore, first, polarized synchrotron FTIR (S-FTIR) spectra of single Bombyx mori silk fiber were collected with the plane of polarization either parallel (the black curve in Fig. 4A) or perpendicular (the red curve in Fig. 4A) to the long axis of the fiber. Our previous finding revealed that silk fibroin has same orientational organization in single silk nanofibril and silk fibers [14, 15]. These two spectra revealed the infrared dichroism of the silk fibroin in silk fibers. For example, the β-turn peaks at 1700 cm⁻¹ showed significant dichroism parallel to the long axis of the fibers, while β-sheet peaks at 1630 cm⁻¹ showed perpendicular dichroism. In contrast, the peaks at 1550 and 1445 cm⁻¹ also showed significant parallel dichroism, but were not sensitive to the conformational change.

For polarized nanoIR measurements, first, the spectra of single SNF in parallel (p-pol) and vertical (v-pol) directions [29] (Fig. 4B) were acquired. Herein, the IR direction of the p-pol is paralleled to the x-y plane of the incident, while the IR direction of v-pol is perpendicular to the x-y plane of the incidence. For compassion, the p-pol and v-pol polarized nanoIR spectra of SNF bundles (Fig. 4C) and the SNF (Fig. 4D–F) with the anisotropic arrangement in the x-y plane, as well as
the silk protein-membrane with random molecular orientation (Fig. S11) were measured. Of note, the signal will be weaker but not necessarily indicate a change in the structure/chemistry when probed the edge or thinner fibril. For both SNF and SNF bundles, the intensity of the amide II band is significantly higher than the amide I band in the p-pol mode and this amide I/amide II ratio was reversed in v-pol mode. In contrast, for silk protein-membrane, the amide I bands were always higher in intensity than the amide II band intensity in both p-pol and v-pol mode. These results confirmed that the silk proteins in SNFs were anisotropic in the plane (p-pol) and out of the plane (v-pol) and were irrelevant to the arrangement of SNFs in the x-y plane.

3. Conclusion

In conclusion, nanoIR was successfully used to disclose the chemical structure of single chitin and silk nanofibrils. The results of this study reveal that nanoIR offered a unique approach to identify the chemical composition of individual biopolymer nanofibrils with spatial resolution up to ~10 nm. For example, nearly identical peaks were observed in the nanoIR spectra obtained by FTIR for both single silk and chitin nano-fibrils. In addition, the experimental results of this study support the suitability of nanoIR technique for semi-quantitative analysis of secondary structures in single SNFs. However, further investigation is required to test its applicability to all protein systems in the near future. In summary, this analysis method provides a useful criterion to utilize nanoIR to investigate the structural details of single biopolymer nano-fibrils, with potentially significant interest in analytical chemistry, nanomaterials, biomaterials, and nanotechnologies.

4. Materials and methods

4.1. Preparation of TOCN

TEMPO-mediated oxidation was carried out with the following steps. First, 1 g chitin powder was suspended in 100 mL water containing 0.016 g TEMPO (0.1 mmol, Sigma-Aldrich, USA) and 0.1 g sodium bromide (1 mmol, Sigma-Aldrich, USA). The oxidation reaction was then started by adding the NaClO solution (10 mmol of NaClO per gram of chitin) at room temperature. During the reaction, the pH of the slurry needed to be maintained at 10, and this was achieved through the continuous addition of 0.5 M NaOH using a pH-Stat titration system. Once the alkali consumption was complete, the oxidation reaction was quenched by adding ethanol (~1 mL). Thereafter, the mixture was adjusted to neutral pH through the addition of 0.5 M HCl, followed by centrifuging at 12000 rpm for 5 min to remove the supernatant. The centrifugation step was repeated several times to remove soluble impurities thoroughly. A water-insoluble TEMPO-mediated oxidized chitin pulp was obtained through this process. The wet pulp was suspended in water at 0.1–0.2 wt% and treated by ultrasonic homogenization at 19.5 kHz with 300 W output power (7 mm in the probe tip diameter, US-300T, Nihonseiki, Japan) for 30 min to yield CNF aqueous solution. The carboxyl content of TEMPO-chitin was ~0.7–0.8 mmol/g.

4.2. Preparation of SNF

Bombyx mori (B. mori) silkworm cocoon silk fibers (purchased from Zhejiang Babei Co., China) were degummed by boiling in 0.5% (w/w) NaHCO₃ solution for 30 min twice. Then, the degummed silk fibers were washed with distilled water and allowed to air dry at room temperature. Further, a 10% (w/v) degummed silk fiber in aqueous solution 9.3 mol L⁻¹ LiBr (Macklin, 99.5%) solution was prepared by heating to 60 °C for 1 h. The solution was first cooled to room temperature and then dialyzed with deionized water at room temperature to yield silk fibroin solution with a protein concentration of ~5 wt%. To preform fibrillation, the concentration of silk fibroin solution was adjusted to 0.1–0.2 wt% and then incubated this diluted solution without perturbation at 60 °C for 7 days. When SNFs were formed, the solution changed from transparent to milky white, and exhibited obvious birefringence under polarized light. NaHCO₃ (Aladdin, AR ≥99.8%, MW: 84.01), LiBr (Macklin, 99.5%, MW:86.85), TEMPO (Adamas, AR ≥98%), NaOH (HUSHI, AR ≥96%), HCl (HUSHI, 36.0–38.0%).

Fig. 4. NanoIR images of SNFs. (A) S-FTIR spectra of single silk fibers. Black curve, single silk fiber measured with the infrared beam parallel to the fiber axis; red curve, single silk fiber measured with the infrared beam perpendicular to the fiber axis. (B) Schematic of the polarized nanoIR measurements. (C) Spectra of SNF bundles in parallel (p-pol) and vertical (v-pol) directions. (D) AFM image of the SNF mapped by nanoIR. (E) NanoIR spectra of the SNF in p-pol mode. (F) NanoIR spectra of the SNF in v-pol mode. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
4.3. SNF/graphene oxide composites

To produce SNF/graphene oxide composites, 0.1 wt% SF aqueous solution and graphene oxide/H$_2$O dispersion (4 mg mL$^{-1}$, monolayer content larger than 95%, Sigma-Aldrich, USA) were mixed at a mass ratio of 4:1. To that mixture (5 mL), 10 μL hydrazine monohydrazide was added to adjust the pH at 10.3 and incubated at 70 °C for 6 h.

4.4. AFM measurements

α-TOCN or SNF aqueous solution was diluted to $\approx 0.002$% (w/v) with distilled water. Then, a 10 μL aliquot of the resulting solution was added onto a mica wafer for 60 s for coating, followed by purging with nitrogen gas. The topographical structures of the SNFs were recorded using a Dimension ICON AFM fast scanning system (Bruker, Germany) in the tapping mode, where an aluminum reflective-coated silicon cantilever with a tip radius of 2 nm was used ($k = 40$ N/m).

4.5. NanoIR measurements

α-TOCN or SNF aqueous solution was diluted to $\approx 0.002$% (w/v) with distilled water. Then, a 10 μL aliquot of the resulting solution was dropped onto the silicon substrate, which had been coated with a thin layer of gold nanoparticles, followed by purging with nitrogen gas. The gold coating on the surface can amplify the infrared signals on membrane samples as thin as ~5 nm or single self-assembled monolayers because of the enhanced local electromagnetic fields [13,29]. NanoIR measurements were carried out using a nanoIR2-FS system (Anasys Instruments, Santa Barbara, CA) equipped with one pulsed QCL laser ranged from 1000 to 1800 cm$^{-1}$. AFM topography was scanned first in the tapping mode, and then a serial of locations was selected for AFM-IR spectrum measurement. (Cantilever: PR-EX-TnIR-A-10, Anasys Instruments, Santa Barbara, CA, spring constant: 1–7 nN/nm, radius of curvature: 20–30 nm). The laser sources we used is a combination of four lasers, which are used for detecting the wavenumber regions of 1000–1800 cm$^{-1}$, 1347–985 cm$^{-1}$, and 985–750 cm$^{-1}$, receptivity. The laser pulsed rate was optimized to match the cantilever’s resonance frequency to maximize the IR signal. All nanoIR spectra were recorded at 1 cm$^{-1}$ spacing, averaged four spectra, and smoothed using Savitzky–Golay (2–8 points) and Smooth (2–5 points) filter. The signal-to-noise ratio of spectra is approximately 2. Step discontinuity filter was used to process the shape gap of spectra. Notably, each spectrum shown in this study was from a single experiment, but the data obtained from the spectra (e.g., β-sheet content, etc.) are the average of more than four separate deconvolutions from different samples. In the deconvolution process, the wavelength range 1590–1710 cm$^{-1}$ was chosen to calibration baseline. The broad absorption at 1730 cm$^{-1}$ was excluded in the deconvolution process, because this band does not come from the absorption signal of the sample and is probably caused by the scattering or diffraction of the IR light due to the irregular shape of the sample. As shown in Figs. 1H and 3E, the absorption at ~1730 cm$^{-1}$ disappeared in both the nanoIR spectra of the α-TOCN membrane and SNF membrane. Further, the deconvolution of amide I bands was performed according to the peak position determined by the transmission spectrum collected from well-controlled conditions. After the local IR spectrum was collected, the wavelength is fixed at specific wavenumbers (the peaks positions in amide I and amide II for example), and then tapping AFM-IR image was collected at these wavenumbers to get the distribution of representative peaks of SNF. In order to eliminate the effect of the moisture in the environment and/or in the test sample in amide I band, all the samples used for measurements were thoroughly dried in the dryer for more than 12 h, and a N$_2$ purge accessory was used in the nanoIR system to remove the moisture in the sample chamber.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.polymer.2021.123534.

References

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