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# Notes

## Preparation of Homogeneous Dispersions of Tunicate Cellulose Whiskers in Organic Solvents

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### Introduction

The incorporation of small amounts of high-stiffness, high-aspect ratio nanometer-sized fillers into polymers can lead to nanocomposites, which display a significant mechanical reinforcement when compared to the neat polymer.[1] Microcrystalline fibrous cellulose[2−4] is currently attracting significant interest in this context, mainly due to its intriguing mechanical properties and the abundance of cellulose in the biomass.[5] Tunicin, which can be isolated from sessile sea creatures known as tunicates, is one intensely studied type of biosynthesized, highly crystalline, cellulosic nanofiber.[5,6] Controlled hydrolysis of the tunicate mantles with strong acids affords almost defect-free monolithic cellulose crystals with a typical diameter of around 15 nm and a length of between 1 and 2 $\mu$m.[7] These fiber-like crystals—referred to as tunicate whiskers—display an elastic modulus of 120−150 GPa, as established by experimental as well as theoretical studies.[8] In addition to the extraordinary mechanical properties of single tunicate whiskers, tunicate dispersions in water, like other high-aspect ratio cellulose crystallites, show macroscopic anisoptropy.[6] Tunicate whiskers have been incorporated into a variety of polymer matrices to yield nanocomposites with improved mechanical properties.[5−7,9−16] The combination of aqueous polymer emulsions and whisker suspensions and subsequent drying has been the main process to transfer cellulose whiskers from an aqueous phase into an organic polymer phase. The combination of polymer solutions in water miscible cosolvents such as THF with aqueous whisker suspensions allowed the creation of nanocomposites with moderately polar hydrogen-bond accepting polymers including ethylene oxide-epichlorohydrin copolymers.[15] Freeze-drying and redispersion of tunicate whiskers as aggregates in toluene were used to integrate these fillers into atactic polypropylene, which served as a very good example of a solution processable hydrophobic polymer.[16] The strong aggregation of the tunicate filler observed, for example, in these polypropylene-cellulose nanocomposites, illustrates an important aspect of the nanocomposite approach: good dispersability of the whiskers in the polymer as well as in the processing solvent is a prerequisite to create polymer/whisker nanocomposites that display a significant mechanical reinforcement.[16] The dispersion of whiskers in aqueous solutions requires the presence of electrostatic repulsion between the individual rigid cellulose crystals. Commonly, this electrostatic repulsion is achieved by sulfuric acid hydrolysis, which introduces negatively charged sulfate groups on the whisker surface by way of esterification.[17] Stable suspensions of tunicate whiskers with negatively charged sulfate groups ($SO_4$−TW), the most commonly used variant, have only been obtained in water or, as most recently reported, *N*,*N*-dimethyl formamide (DMF).[18] It was recently demonstrated that dispersable whiskers can also be obtained by the TEMPO mediated oxidation of the amorphous cellulose fraction, resulting in highly carboxylated, negatively charged whiskers.[19] A variety of other solubilizing schemes has been explored to improve the dispersability in organic media, including the use of surfactants,[16,20] silylation,[21] grafting of PEO[22] or maleated polypropylene,[16] and acylation.[23] However, these surface modifications can also change the interactions among the whiskers and hence might significantly affect the macroscopic mechanical properties of the nanocomposite.

Cellulose whiskers without surface modification display strong interactions and have been reported as notoriously difficult to disperse in water. Araki et al. reported the preparation of stable cellulose microcrystal suspensions by HCl hydrolysis of bleached softwood kraft pulp.[24] This hydrolysis protocol was shown to yield microcrystalline cellulose that is virtually free of surface charges. Rheological experiments as well as electron microscopy images, however, seem to suggest that particles thus produced have a strong tendency to aggregate in water.[24] An independent recent study also seems to suggest strong whisker aggregation in a very similar system.[19] To the best of our knowledge, HCl hydrolysis of tunicate cellulose has not been reported to yield stable suspensions.

In the light of the previous discussion, we investigated the dispersability of tunicate whiskers with non-functionalized surface prepared by HCl hydrolysis (HCl−TW) and tunicate whiskers with negatively charged sulfate groups ($SO_4$−TW) prepared by sulfuric acid hydrolysis in a series of polar protic and aprotic organic solvents and elucidated the morphology of the whisker structures obtained upon deposition from these dispersions. We identified a variety of new organic solvents that allow the preparation of dispersions of cellulose whiskers and are well-suited for the creation of reinforced polymer nanocomposites without the need for elaborate surface modification, cosolvents, or surfactants.

### Experimental Procedures

**Materials.** All reagents were purchased from Sigma-Aldrich and were used without further purification. Tunicates (*Styela clava*) were collected from floating docks at Snug Harbor (Jerusalem, RI). After gutting,[25] the incrustations on the tunicates' outer walls were removed by heating in aqueous potassium hydroxide (3 L, 5% w/w per 500 g of tunicate walls, 80 °C, 24 h), followed by mechanical agitation, scrubbing, and two more treatments with aqueous potassium hydroxide (3 L, 5% w/w, 80 °C, 24 h); this protocol represents a minor modification of the procedure reported by Yuan et al.[26] After washing

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the raw cellulose with water to pH 7, water (3 L), acetic acid (5 mL), and sodium hypochlorite solution (>4% chlorine, 10 mL) were added, and the temperature was raised to 60 °C. In 1 h intervals, additional portions of acetic acid (5 mL) and sodium hypochlorite solution (>4% chlorine, 10 mL) were added until the material's color changed from pinkish to pure white (usually two or three additions were required, depending on the particular batch of tunicates). Finally, the bleached de-proteinized walls were washed with deionized water and disintegrated with a Warring blender, yielding a fine cellulose pulp. Sulfate-functionalized tunicate whiskers ($SO_4$−TW) were prepared by sulfuric acid hydrolysis of cellulose pulp, according to the method described by Favier et al. with slight modifications.[9] To a cooled suspension of tunicate cellulose pulp in deionized water (600 mL, 0 °C), sulfuric acid (98%, 960 mL) was slowly added under vigorous mechanical stirring. Subsequently, the dispersion was heated to 60 °C and kept at that temperature for 90 min under continued stirring. The dispersion was cooled to 0 °C, filtered over a small-pore fritted glass filter, and washed with deionized water until neutrality was reached. Finally, $SO_4$−TW was re-dispersed in deionized water (1 L) by overnight sonication, and water was added to adjust the concentration to 8 mg/mL. Non-functionalized whiskers (HCl−TW) were prepared using concentrated hydrochloric acid hydrolysis of pulp, according to the method described by Paillet and Dufresne with slight modifications.[27] Concentrated hydrochloric acid (250 mL, 12 N) was added dropwise to a cooled suspension of tunicate cellulose pulp in deionized water (750 mL, 4 °C), resulting in a final HCl concentration of 3 N. The solution was refluxed for 1.5 h. The mixture was allowed to cool to room temperature and filtered over a small-pore fritted glass filter and washed with deionized water until neutrality was reached. Finally, HCl−TW was re-dispersed in deionized water (1 L) by overnight sonication, and water was added to adjust the concentration to 8 mg/mL.[28] The resulting aqueous dispersions ($SO_4$−TW and HCl−TW ~8 mg of dry cellulose per 1 mL of $H_2O$) were frozen in an acetone/dry ice cooled stainless steel container and subsequently lyophilized.

**Instrumentation.** Transmission electron micrographs (TEMs) were acquired using a JEOL 1200EX Transmission Electron Microscope. All samples were prepared on carbon-coated copper grids using a standard uranyl acetate negative staining method.[29] Whisker dimensions were determined using standard imaging software and are reported as average values ± standard error for about 10 whiskers. For ultrasonication of whisker dispersions, a Fisher FS60H ultrasonic bath was used. IR spectra were recorded using an ABB Bomen MB series FTIR spectrometer.

**Dispersability Test.** Lyophilized tunicate whiskers (2 mg) were transferred to a 5 mL glass vial, and the appropriate solvent (2 mL) was added. The concentration of 1 mg/mL was chosen since it represented the lower concentration limit above which the aqueous dispersions of tunicate whiskers prepared by sulfuric acid hydrolysis showed permanent birefringence due to macroscopic anisotropy (Figure 1).[6] The whisker solvent mixtures were subsequently sonicated at or slightly above room temperature for 6 h. At this point, the quality of the dispersion was assessed by inspection of the dispersion between two crossed polarizers. The presence of birefringence was used as the criterion for having good dispersability in that particular solvent.[6] Samples that were not well-dispersed at this stage were further sonicated as indicated and then reassessed. The quality of the final dispersions and the effect of prolonged sonication were evaluated by means transmission electron microscopy images.

**Preparation of Whisker Films.** Films used for FTIR characterization were prepared by casting of water or formic acid whisker dispersions (~10 mg/mL) onto PTFE sheets. After drying overnight in a well-ventilated hood, the films were released from the PTFE substrate and dried for 12 h at 50 °C and 12 mmHg.

## Results and Discussion

**$SO_4$−TW.** Sulfate-functionalized tunicate whiskers ($SO_4$−TW) were prepared by sulfuric acid hydrolysis of cellulose pulp derived from tunicates (*Styela clava*) harvested off the New England coast according to well-established procedures.[9,23] These high-aspect ratio nanoparticles formed colloidal suspensions in water, which at a concentration of 1 mg/mL or higher displayed the permanent birefringence that reveals macroscopic anisotropy (Figure 1).[6] This birefringence is usually taken as an indication for good whisker dispersion,[18a] but we also confirmed the formation of dispersions of individualized whiskers by transmission electron microscopy (TEM, Figure 2a). The TEM images show that the whiskers are well-individualized, but as in earlier studies,[30] completely isolated microfibrils could rarely be observed, even after extensive dilution, in which case domains with a significant whisker concentration were observed on the carbon-coated copper grids. While the aqueous $SO_4$−TW dispersions can be directly used for the preparation of polymer nanocomposites,[9,11] freeze-dried whiskers are often used as the starting material for the preparation of dispersions in organic solvents.[16,31]

**Figure 1.** Photographs of (A) dispersions of as-prepared $SO_4$-TW in water and (B) freeze-dried $SO_4$-TW re-dispersed in water, viewed through crossed polarizers. Concentrations are from left to right: 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 mg/mL.

As was shown by others, freeze-dried $SO_4$−TW can be readily re-dispersed in water and, as recently reported, also in DMF[18] (Figures 1 and 3), although we found that extensive ultrasonication is required to achieve full dispersion in this solvent (Table 1). This led us to explore a number of other polar solvents as potential organic dispersants for $SO_4$−TW; the results of this dispersability study, which for a first screening relied on the criterion of birefringence,[6] are summarized in Table 1 and Figure 3. In view of the rather encouraging results with DMF, our selection of other aprotic solvents included dimethyl sulfoxide (DMSO)[32] and *N*-methyl pyrrolidine (NMP). Gratifyingly, both these solvents were found to disperse $SO_4$−TW well; the dispersion process appears to proceed more quickly in NMP than in DMF or DMSO. We also extended our investigation to protic solvents other than water and selected formic acid and *m*-cresol, which are well-known for their tendency to break hydrogen bonds.[33] Indeed, both solvents were found to rapidly and completely re-disperse freeze-dried $SO_4$-TWs. In addition to the birefringence-based dispersability tests, we confirmed the whisker individualization by means of TEM. The TEM micrographs (Figure 2b,c) of redispersed, lyophilized $SO_4$−TW show no evidence of large-scale aggregation but reveal a level of dispersion that is identical to that of as-prepared (not freeze-dried) $SO_4$−TW after deposition from water (Figure 2a). A detailed inspection of the TEMs (Figure 2) suggests a slightly lower aspect ratio for the re-dispersed $SO_4$−TW. For each of

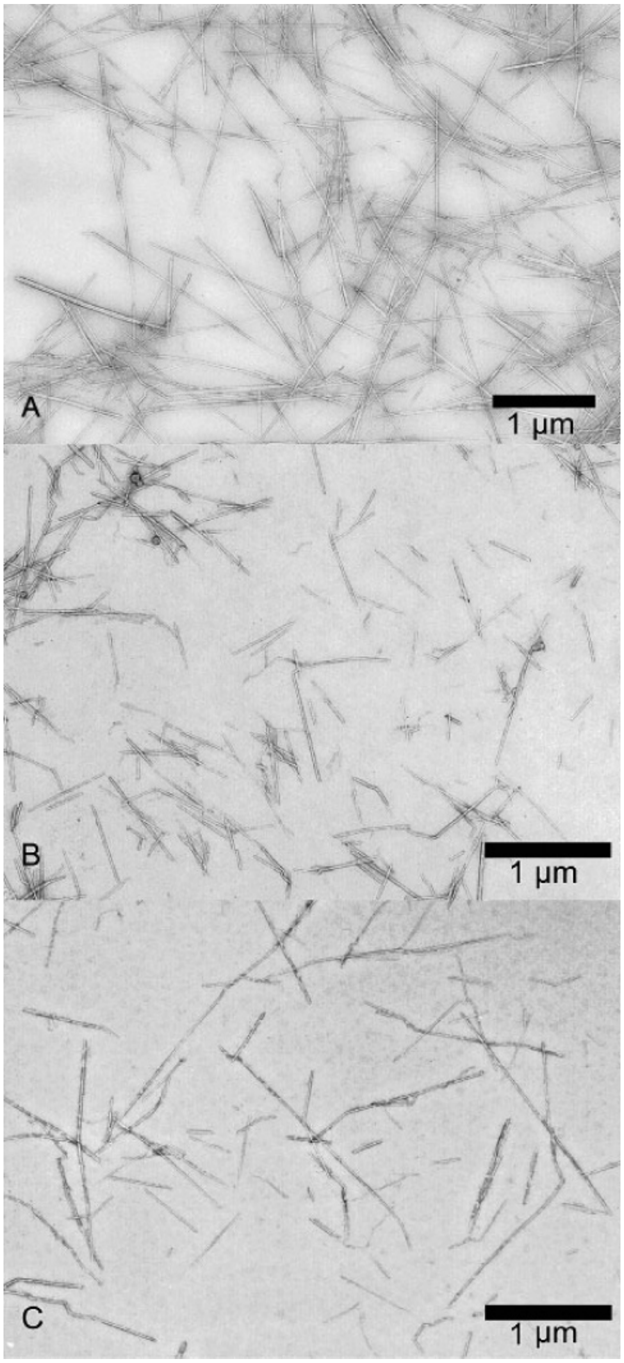

**Figure 2.** Transmission electron micrographs of (A) as-prepared $SO_4$-TW deposited from water; (B) freeze-dried $SO_4$-TW deposited from *N*-methyl pyrrolidone; and (C) freeze-dried $SO_4$-TW deposited from formic acid.

the three systems (as prepared, lyophilized, and processed from NMP and lyophilized and processed from formic acid), the diameter was established from the TEMs to be 20.0 ± 3.0 nm. The length of the whiskers varied between 2.20 ± 0.20 $\mu$m (as-prepared), 0.63 ± 0.05 $\mu$m (lyophilized and processed from NMP), and 1.61 ± 0.16 $\mu$m (lyophilized and processed from formic acid). This finding is related to the effect of prolonged sonication, which is consistent with previously reported data for cellulose whiskers prepared from cotton[34] and highlights the importance of rapid and smooth re-dispersion, which can, as our data show, be achieved by processing from formic acid.

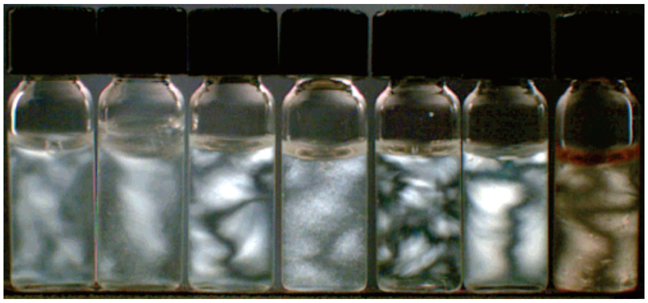

**Figure 3.** Photographs of dispersions of $SO_4$-TW viewed through cross polarizers at 5.0 mg/mL. From left to right: as-prepared in water, freeze-dried, and re-dispersed in water, DMF, DMSO, *N*-methyl pyrrolidone, formic acid, and *m*-cresol.

**Table 1.** Overview of the Dispersability of Lyophilized Tunicate Whiskers in Polar Solvents

| solvent | $SO_4$-TW | HCl-TW |
|---|---|---|
| Water | ++[a] | − |
| *N*,*N*-dimethyl formamide | + | − |
| dimethyl sulfoxide | + | − |
| *N*-methyl pyrrolidone | ++ | − |
| formic acid | ++ | ++ |
| *m*-cresol | ++ | ++ |

[a] Dispersability was examined at a concentration of 1 mg/mL using birefringence as an indicator. ++ = Dispersed after 6 h or less of sonication. + = Dispersed after 24 h (72 h in the case of *N*,*N*-dimethyl formamide) of sonication at ca. 60 °C. − = Did not disperse.

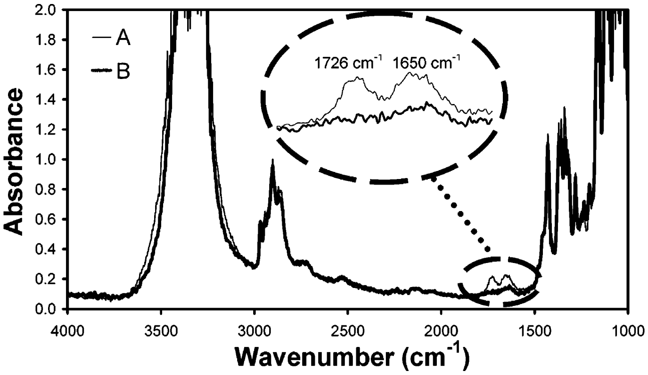

**Figure 4.** FTIR spectra of $SO_4$-TW films cast from (A) formic acid and (B) water.

One might be concerned that the use of formic acid might lead to esterification of the cellulose hydroxyl moieties on the surface of the whiskers, which, as discussed earlier, would be important from a polymer nanocomposite point of view and might also cause the obliteration of the whiskers. Therefore, dispersions of $SO_4$−TW were cast from formic acid and water (for reference purposes), and the resulting thin films were characterized by FTIR spectroscopy (Figure 4). The spectra of the films produced from aqueous and from formic acid dispersion are virtually identical, with the exception of two peaks of very low intensity at 1650 and 1726 cm$^{-1}$. The signal around 1650 cm$^{-1}$ is attributed to the symmetric deformation vibration of adsorbed water molecules on the cellulose whiskers,[35] which proved to be difficult to fully eliminate as most of the samples quickly re-adsorb water from the environment. The peak at 1726 cm$^{-1}$ is attributed to the C=O stretch vibration and is indeed indicative of formate ester moieties. However, the low intensity of this (typically very strong) C=O stretch vibration suggests that only small amounts of the latter are formed.[23] However, the stability of these dispersions is limited to a few days because of this chemical reaction. Similarly, dispersion of TW in *m*-cresol is limited in stability by the oxidative stability of *m*-cresol itself. To the best of our knowledge, this is the first demonstration of dispersion of $SO_4$−TW in solvents other than

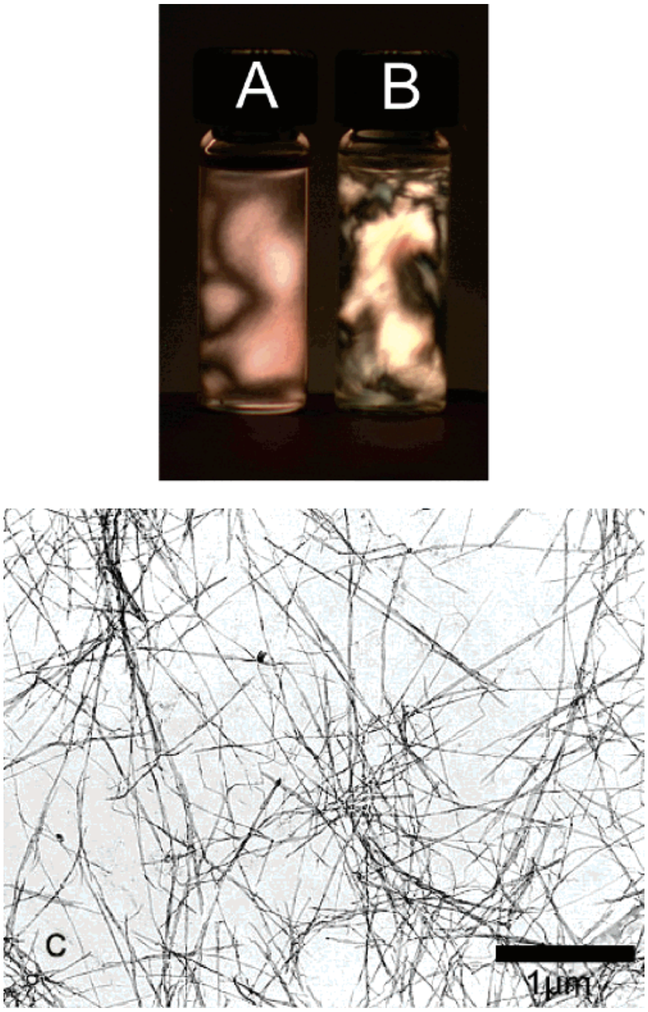

**Figure 5.** Photographs of dispersions of freeze-dried HCl-TW redispersed in (A) *m*-cresol and (B) formic acid at 5.0 mg/mL (viewed through crossed polarizers). Transmission electron micrograph of HCl-TW after deposition from formic acid (C).

water, DMF, or DMSO. These results expand the possibilities for nanocomposite preparation using $SO_4$−TW.

**HCl−TW.** Combining the protocols of Yuan et al.[23] and Paillet et al.,[27] we also prepared tunicate whiskers without ionic surface groups by hydrolysis of the cellulose pulp with HCl rather than $H_2SO_4$ and subsequent lyophilization. Conducting the same dispersability experiments as were carried out with $SO_4$−TW (Table 1), we found that neither $H_2O$ nor any of the non-protic solvents (DMF, DMSO, and NMP) used in our study was capable of homogeneously dispersing the HCl−TW. This result is not surprising since it is well-known that the surfaces of $SO_4$−TW feature a significant number of negatively charged sulfate ester moieties, which limit the formation of hydrogen bonds between individual whiskers and stabilize (aqueous) dispersions by electrostatic repulsion.[6] The fact that DMF, DMSO, and NMP are capable of dispersing $SO_4$−TW (vide supra) but not HCl−TW highlights the importance of surface charges, which play an important role in assisting these solvents to break inter-whisker hydrogen bonds. We discovered that the situation is quite different in the acidic protic solvents used here. Indeed, formic acid and *m*-cresol not only disperse $SO_4$−TW well, but also HCl−TW, as is evident from the stable birefringence of the dispersions thus produced (Figure 5a,b). The ability of these solvents to disperse both types of whiskers more readily than water is consistent with the well-known tendency for these solvents to break hydrogen bonds. TEM micrographs of HCl−TW deposited from formic acid (Figure 5c) support that the microfibrils were well-individualized, nearly identical to as-prepared $SO_4$−TW after deposition from water (Figure 2a) and $SO_4$−TW after deposition from formic acid (Figure 2c). Judging from the TEM micrographs of HCl−TW, it seems that this type of whisker has the tendency to aggregate during the drying process required for TEM sample preparation (i.e., in the absence of the dispersant), which is almost certainly a consequence of the lack of surface charges. The fact that only a few fully individualized HCl-TWs can be seen in the TEMs made it difficult to establish their length, but their diameter of 19.9 ± 0.1 nm could be accurately established and was found to be virtually identical to that of $SO_4$−TW.

## Conclusion

The presence of negatively charged sulfate ester moieties on the tunicate whisker surface is the determining factor in the dispersability in polar aprotic solvents. Without surface charges, unmodified tunicate whiskers (e.g., HCl−TW) do not disperse in polar aprotic solvents. Protic solvents such as formic acid and *m*-cresol were shown to effectively disrupt the hydrogen bonds in aggregated whiskers, dispersing even the non-charged HCl−TW. To the best of our knowledge, this is the first time birefringent dispersions of unmodified tunicate whiskers were successfully produced. Tunicate whisker dispersions in organic solvents are valuable intermediates for the preparation of polymer/tunicate whisker nanocomposites. The preparation of such composites was previously stifled, due to the lack of a common solvent/dispersant for the polymer and the whiskers. The absence of any surface modification promises to allow the formation of a percolating hydrogen-bonded whisker network, which is often claimed to be responsible for the good mechanical properties of tunicate whisker nanocomposites.[9c] Further research exploring the scope and limitations of organic solvent dispersed tunicate whiskers in the preparation of polymer nanocomposites is currently in progress.

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