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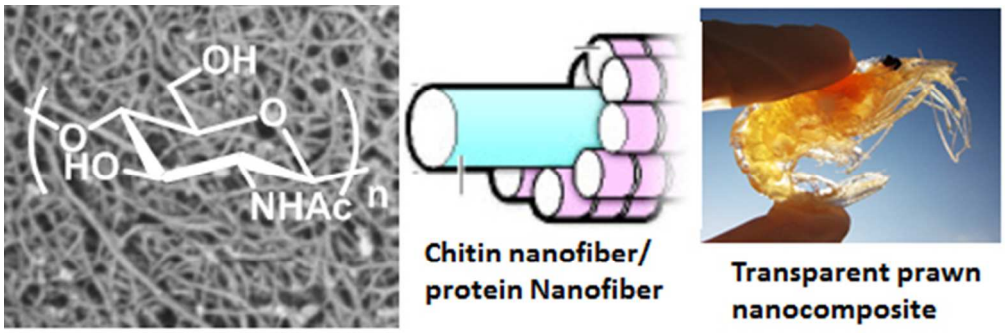

Graphical Abstract
182x62mm (72 x 72 DPI)

Journal Name

ARTICLE

Preparation of a protein-chitin nanofiber complex from crab shells and its application as a reinforcement filler or substrate for biomineralization

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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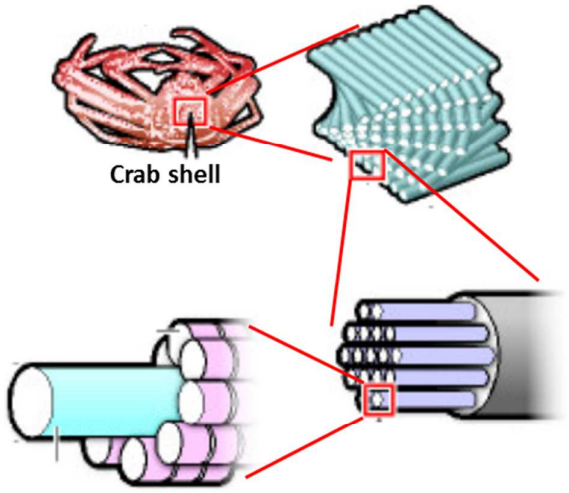
A protein-chitin nanofiber complex was successfully prepared from crab shells by a mechanical treatment after the removal of $CaCO_3$. One step in the conventional series of treatments—i.e., the removal of proteins—was omitted to bring down the production cost of the nanofibers. The obtained protein-chitin nanofibers had uniform width of approximately 20 nm and a high aspect ratio. These characteristics were similar to conventional chitin nanofibers. The optically transparent protein-chitin nanofiber composite was fabricated with acrylic resin. The nanofibers reinforced the acrylic resin film and thereby increased its mechanical properties. Proteins on the chitin nanofiber surface affected the biomineralization of $CaCO_3$ in the crab shell. Mineralization of $CaCO_3$ on the protein-chitn nanofiber was carried out by the gas-diffusion method. Protein molecules on the chitin NFs increased the chances for biomineralization to occur. The protein molecules stabilized the formation of vaterite and inhibited the transformation of vaterite to calcite.

Introduction

Chitin is a highly abundant carbohydrate polymer existing mainly in the exoskeletons of crabs. The crab shell has a strictly hierarchical organization (Fig. 1). The chitin molecules are aligned in an antiparallel manner to form α-chitin nanofibers (NFs).[1,2] The NFs are wrapped with a protein layer. The next



level consists of the cluster of a number of protein-chitin NFs. The clusters form a twisted plywood layer, which is gradually rotated about its normal axis. Crystalline calcium carbonate is embedded within the space of the helicoidally shaped structure.

Commercial chitin is generally prepared by treating crab shells with NaOH and HCl solutions to remove proteins and calcium carbonate, followed by drying in an oven.[3] The production of commercial chitin is quite expensive (3,000-5,000 JPY/Kg), and the major effluent purification treatments required to remove the abundant protein residue contribute the most to the costs. Indeed, the protein removal process accounts for approximately half of the production cost of commercial chitin. Moreover, the removal process with NaOH solution causes deacetylation and depolymerization of chitin molecules by alkaline hydrolysis.[4] Recently, we developed a method of isolating chitin NFs existing in crab shell.[5,6] Chitin NFs have a characteristic morphology,[7] high surface-to-volume ratio,[8] high mechanical strength,[9] and strong biological functions.[10-13] We expect that the addition of NFs will promote the use of chitin as a novel biomacromolecule. Chitin NFs from crab shells were prepared by a simple mechanical treatment after the removal of proteins and minerals. The chitin is easily disintegrated into NFs by mechanical treatment after removal of the embedding matrices. Protein layer on the NF surface are considered to play important roles in the biomineralization of calcium carbonate in crab shells.[14] In other words, proteins themselves basically do not provide as a solid support for the rigid exoskeleton of the crabs. Thus, it may be possible to skip the protein removal process during the preparation of chitin NFs. This simplification of the procedure would bring down the

Figure 1. Hierarchical structure of crab shell. Reprinted with permission from Asahi Shimbun (11/12/2014, Michi-wo-Hiraku).

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production cost of NFs. In this study, we report on the production of NFs from crab shells without the protein removal step. The obtained protein-chitin NF complexes are characterized in detail. Finally, we discuss potential applications of the characteristic NFs as a reinforcement filler or a substrate for the biomineralization of calcium carbonate.

Experimental

Materials

Raw crab shells of *Chionoecetes opilio* (snow crab) were the kindly provided as gift by Koyo Chemicals. The acrylic resin monomer, tricyclodecanedimethanoldimethacrylate (DCP), was kindly provided by Shin-Nakamura Chemical Co., and the other chemicals were purchased from Aldrich or Kanto Chemicals and used as received.

Preparation of protein nanofiber

The raw crab shells were treated with a large amount of 1M hydrochloric acid for 2 days at room temperature to remove the mineral salts, which consisted mainly of calcium carbonate.[3] After washing with distilled water, the sample was dispersed in water with a concentration of 0.7 wt%, and was roughly crushed by using a grinder (MKCA6-3; Masuko Sangyo Co., Ltd.). The rotation speed of the grinding stone was 1500 rpm. The grinded sample was passed through 30 cycles of a high-pressure water jet atomization treatment (Star Burst Mini, HJP-25001S; Sugino Machine).[15] The atomization system was equipped with a ball-collision chamber. The slurry was ejected from a narrow nozzle clearance with a diameter of 100 μm under high pressure of 200 MPa and collided with a ceramic ball with a diameter of 12.7 mm.

Preparation of acrylic resin reinforced with protein-chitin nanofibers

A 45 ml aliquot of protein-chitin NF dispersion with a 0.7 wt% chitin concentration was vacuum-filtered using a membrane filter. The obtained NF sheet was dried by hot pressing at 1 MPa and 100 ^{o}C for 30 min. The dried sheet was impregnated in a bi-functional acrylic resin monomer of DCP with 3 wt% photoinitiator, dimethyl 2,20-azobis(2-methylpropionate), under reduced pressure for 24 h.[16] The NF sheet impregnated with monomer was radically polymerized using UV curing equipment (Spot Cure SP-7; Ushio Inc.) for 8 min at 40 mW cm^{-1}. The composite film thus obtained had a length of 5 cm, width of 3 cm, thickness of 70 μm, weight of 128 mg and fiber content of 40 wt%.

Preparation of whole, transparent prawn and crab legs

Fresh prawns of *Litopenaeus vannamei* (White Leg Shrimp) and crab legs of *Chionoecetes opilio* (snow crab) were purchased from a local supermarket. They were treated in an excess amount of 1 M HCl for 2 days at room temperature to remove calcium carbonate. After being washed with an abundant amount of distilled water, the samples were refluxed in ethanol to remove pigment overnight. Finally, the samples were dipped in toluene for 2 days to obtain whole, transparent prawn and crab legs.

Mineralization of calcium carbonate on protein-chitin nanofibers

Mineralization was carried out by the gas-diffusion method.[17] Calcium dichloride dehydrate was dissolved in a protein-chitin NF dispersion (45 mL, 0.7 wt%) to 20 mM. The dispersion was placed in a desiccator. For mineralization, carbon dioxide gas was produced from ammonium carbonate powder at 40 ^{o}C. After 1 day, the sample was collected by filtration and washed thoroughly with water.

Characterizations

The infrared spectra of the samples were recorded with an FT-IR spectrophotometer (Spectrum 65; Perkin-Elmer Japan Co., Ltd.) equipped with an ATR attachment (diamond/ZnSe crystal) with 4 scans at a resolution of 4 cm^{-1}. For field emission scanning electron microscopic (FE-SEM) observation, the prepared NF slurry was diluted with EtOH and dried in an oven to obtain a cast film. The film was coated with an approximately 2 nm layer of Pt by an ion sputter coater and observed by FE-SEM (JSM-6700F; JEOL, Ltd.) operating at 2.0 kV. X-ray diffraction profiles of the NFs were obtained with Ni-filtered CuK_{α} from an X-ray generator (Ultima IV; Rigaku) operating at 40 kV and 40 mA. The light transmittances of acrylic resin reinforced with protein-chitin NFs were measured using a UV-Vis spectrophotometer (V550; JASCO, Tokyo, Japan). Mechanical properties were tested using a universal testing instrument (AG-X; Shimadzu) for samples 30 mm long and 10 mm wide at a crosshead speed of 1 mm min^{-1} with a gauge length of 10 mm. At least three and five specimens were tested for neat resins and their nanocomposites, respectively.

Results and discussion

Preparation of protein-chitin nanofibers

Red crab shells were used as a starting material. The contents of chitin, protein, and calcium carbonate in the original shells were 25 %, 12 %, and 52 % as w/w, respectively. These compositions were estimated by means of a ninhydrin-hydrindantin protein test and gravimetric analysis.[18] Calcium carbonate was removed from the crab shells by conventional HCl treatment. This method is known to remove most of the calcium carbonate from crab shells.[3] After removal of calcium carbonate, the yield of residual product was approximately 45 %. After the acid treatment, the compositions of chitin and protein were increased to 69 % and 24 %, respectively. Then, the shells were passed through a high-pressure water atomization system. After 30 cycles of treatments, the sample was dispersed homogeneously in water at a concentration of 0.7 wt%; the water was a light yellow color. An SEM image of the dispersion revealed that the sample was successfully disintegrated into NFs (Fig. 2a). The sample had a homogeneous NF network and a high aspect ratio with a width of approximately 20 nm. The morphology of the NFs was very similar to that of the conventional neat chitin NFs (Fig. 2b).[5] This indicates that 24 % protein part exists on the chitin nanofiber surface to form a original protein-chitin NF complex as shown in Figure 1.

Figure 3 shows the FT-IR spectra of (a) crab shells and (b) newly prepared NFs obtained through the acid and mechanical treatments of crab shells. After treatments, the transmittance bands at approximately 870 cm^{-1} and 1450 cm^{-1} derived from calcium carbonate have completely disappeared.[19] Moreover, an OH stretching band at around 3450 cm^{-1}, an NH stretching band at 3240 cm^{-1}, amide I bands at 1640 cm^{-1} and 1620 cm^{-1} and an amide II band at 1550 cm^{-1} of the NFs are observed. These strong peaks in the carbonyl region are especially characteristic of α-chitin.[20] Thus, the hydrochloric acid treatment was sufficient to remove the calcium carbonate and produce protein-chitin NFs.

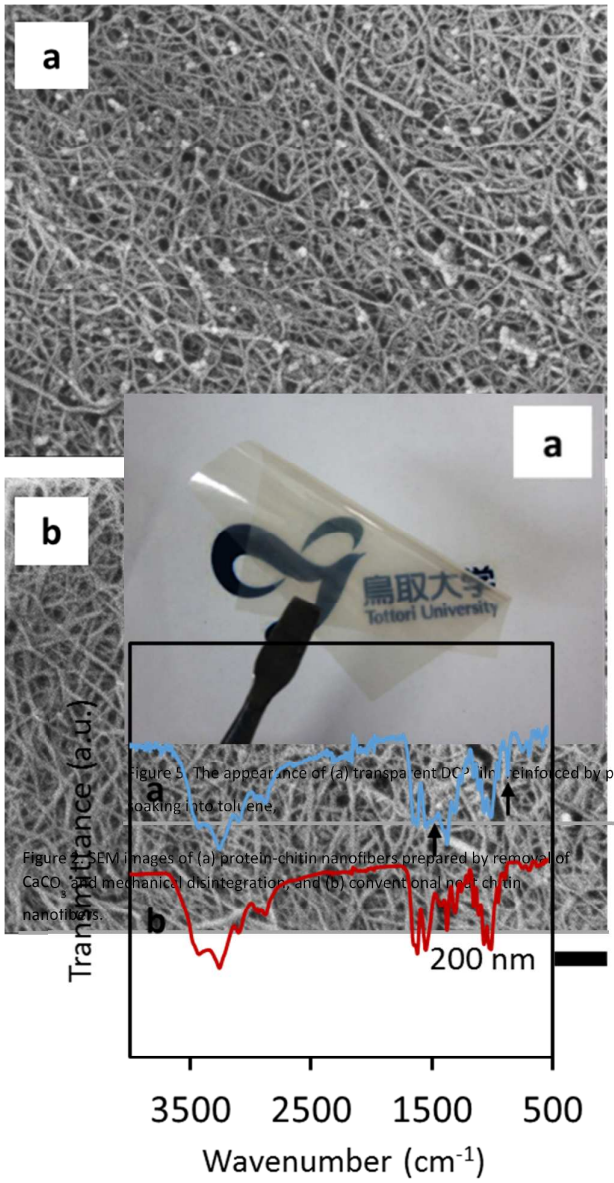

Figure 3. SEM images of (a) protein-chitin nanofibers prepared by removal of $CaCO_3$ and mechanical disintegration, and (b) conventional neat chitin nanofibers.

Figure 4 shows the X-ray diffraction profiles of (a) crab shells and (b) protein-chitin NFs. The strong diffraction peak at 29°, which is a typical crystalline pattern for calcium carbonate (calcite),[19] was completely absent from that of the NFs after acid treatment, indicating the mineral component was completely removed from the protein-chitin NFs. Moreover, the four diffraction peaks of chitin NFs observed at 8.7, 18.6, 22.3, and 25.3°, which corresponded to the 020, 110, 120, and 130 planes, respectively, are typical crystal patterns of α-chitin.[21] This result also indicated that protein-chitin NFs were successfully prepared from the crab shells.

Preparation of acrylic resin film reinforced with protein-chitin nanofibers

In a previous study, we prepared an acrylic resin composite reinforced with chitin NFs.[16] Due to the nano-size structure and excellent mechanical properties of chitin NFs, the composite was highly transparent, and had a high Young's modulus and a high tensile strength. Here, we prepared an acrylic resin (DCP) composite film reinforced with protein-chitin NFs. The nanocomposite film became transparent after complexation with acrylic resin, despite the high fiber content of 40 % (Fig. 5a).

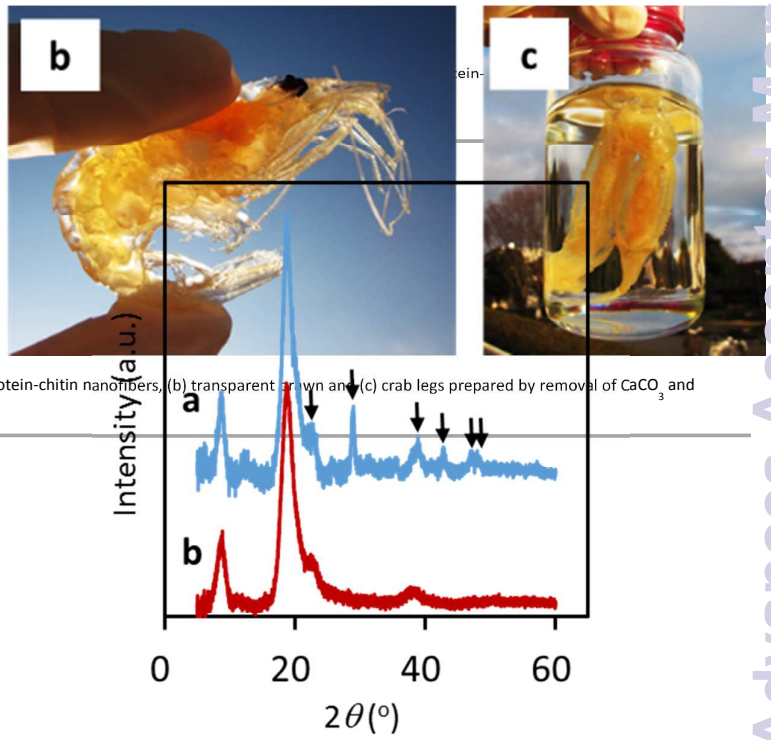

Figure 5. The appearance of (a) transparent DCP film reinforced by protein-chitin nanofibers, (b) transparent brown and (c) crab legs prepared by removal of $CaCO_3$ and soaking into toluene.

Figure 4. X-ray diffraction profiles of (a) dried crab shell and (b) protein-chitin nanofibers. Arrows: $CaCO_3$.

Figure 6 shows the regular light transmittance spectra of the neat DCP resin (refractive index: 1.5), and the nanocomposites reinforced with chitin NFs and protein-chitin NFs. The regular light transmittance of the DCP composite film with protein-chitin NFs was 78.5 % at 600 nm. This value was sufficiently high, although protein and pigment remained in the NFs and these components absorbed visible light. The high transparency was obviously due to the size effect, as well as the conventional neat chitin NF/DCP composite film. Since the approximately 20 nm width of the protein-chitin NF was much

thinner than the wavelength of visible light (400-800 nm),[22] the composite film was free from light scattering at the interface between protein-chitin NF and DCP.

Since chitin NFs have an antiparallel extended crystal structure, they have a high mechanical strength and Young's modulus.[23] Therefore, protein-chitin NFs may also be available as a reinforcing element to improve the mechanical properties of composite materials. Table 1 shows the mechanical properties of a DCP film and composite reinforced with conventional chitin NFs and the newly prepared protein-chitin NFs. The neat DCP resin was extremely brittle due to its high crosslink density. The Young's modulus, fracture strength and strain were 2.1 GPa, 10.6 MPa and 0.5 %, respectively (Table 1). On the other hand,

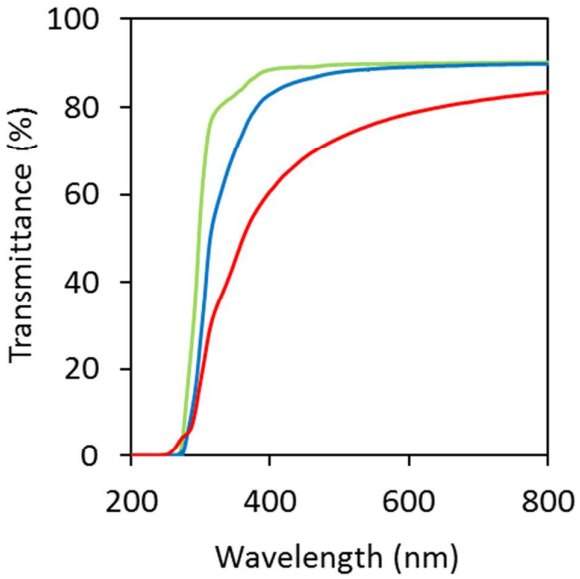

Table 1 Mechanical properties of neat DCP and the composites reinforced with protein-chitin nanofibers and chitin nanofibers.

	Young's modulus (GPa)	Fracture strength (MPa)	Strain (%)
Neat DCP	2.1	10.6	0.5
DCP/protein-chitin NF	2.3	52.1	4.6
DCP/chitin NF	2.5	51.4	5.2

when using protein-chitin NFs, these mechanical properties were significantly increased by 2.3 GPa, 52.1 MPa, and 4.6 %, respectively. These enhanced properties of the protein-chitin NFs were comparable to those of conventional chitin NFs. Thus, the results support the idea that protein-chitin NFs function as a reinforcing element as effectively as neat chitin NFs.

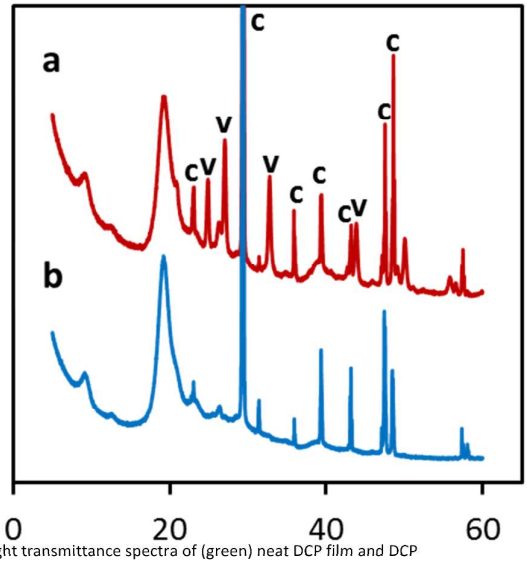

Figure 6. Regular light transmittance spectra of (green) neat DCP film and DCP film reinforced by (blue) chitin nanofibers, and (red) protein-chitin nanofibers.

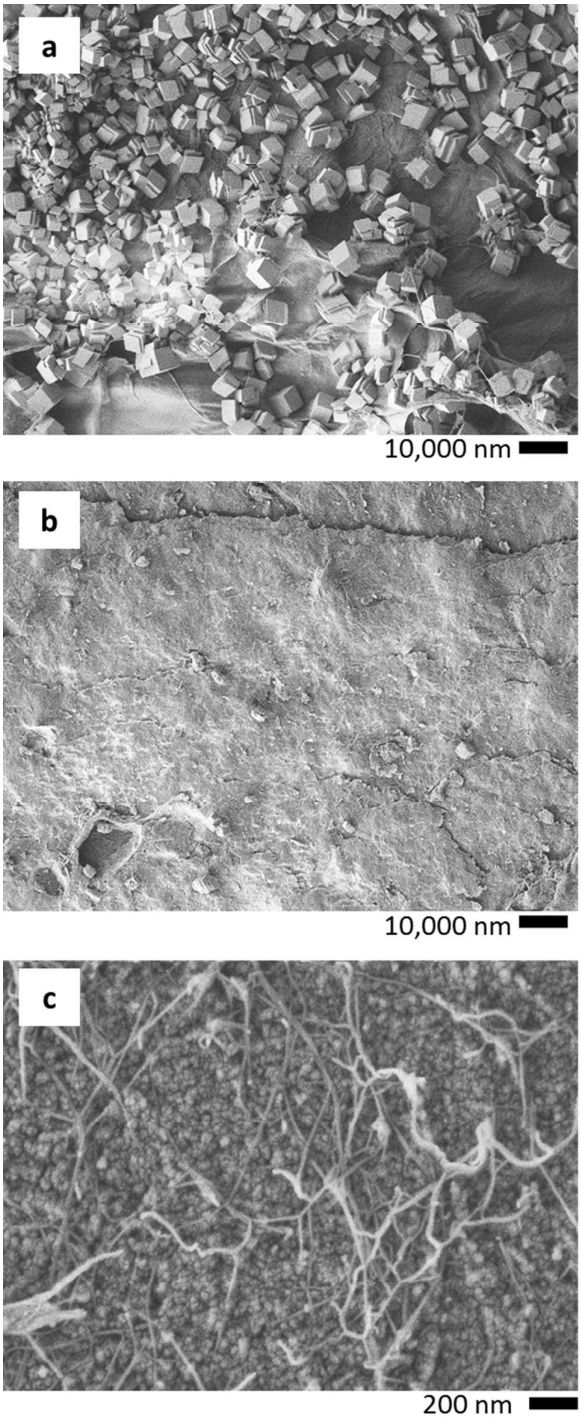

Figure 7. X-ray diffraction profiles of $CaCO_3$ crystals deposited on (a) protein-chitin nanofibers and (b) conventional chitin nanofibers. c = calcite, v = vaterite.

Shams et al. fabricated optically transparent crab shells. Since crab shells are composed of chitin NFs, they become transparent after the removal of proteins, calcium carbonate, and pigment composition, and subsequent complexation with acrylic resin.[24] Inspired by the transparent crab shells, we prepared whole, transparent prawn and crab legs (Fig. 5b and 5c). After the removal of calcium carbonate, the whole prawns and crab legs were immersed in toluene. Interestingly, after 2 days, the whole prawn and crab legs became transparent without changing their original structures. This implies that prawns and crab shells consist of protein-chitin NFs, and their nanostructures were preserved after the removal of calcium carbonate. Therefore, these shells became transparent after immersion in toluene (refractive index: 1.496), and the internal tissues were clearly visible. In the future, this feature may be applicable for establishing transparent biological specimens for anatomy instruction.

Mineralization of calcium carbonate on protein-chitin nanofibers

Living organisms can prepare inorganic/organic composites *via* biomineralization. By studying the processes of biomineralization, materials scientists may be able to design novel composite materials. The acidic proteins are considered to play elemental roles in biomineralization. Several acid proteins with different amino acid sequences have been isolated from calcified tissues, such as teeth,[25] bones,[26] shells, and the exoskeletons of crustaceans.[14] Therefore, it would be of interest to study the $CaCO_3$ mineralization properties of the protein-chitin NFs prepared from crab shells. Mineralization of $CaCO_3$ was carried out by the gas-diffusion method using the gas-phase ammonium carbonate method, in a protein-chitin NF water dispersion.[17] The X-ray diffraction patterns of the mineralized protein-chitin NFs (Fig. 7a) as well as the mineralized neat chitin NFs (Fig. 7b) were obtained. The X-ray diffraction pattern of the mineralized neat chitin NFs showed the formation of the thermodynamically most stable calcite from the strongest (104) peak at $2\theta = 29.2^{o}$. In contrast, in the protein-chitin NFs, vaterite formation was also detected at mainly $2\theta = 24.9^{o}$, 27.1^{o}, 32.8^{o}, which corresponded to *hkl* 110, 112, and 114, respectively, in addition to calcite formation.[1]

The SEM images of the $CaCO_3$ crystalized chitin and protein chitin NFs are shown in Fig. 8. In the presence of the neat chitin NFs, many fine calcite crystals of rhombohedral shape and a size of several micrometers were formed (Fig. 8a). On the other hand, in the presence of the protein-chitin NFs, the characteristic cubic calcite crystals were not observed throughout the extensive area, while some patternless objects of several micrometers in size were observed (Fig. 8b).[19] Furthermore, at higher magnification, a large number of $CaCO_3$ grains were embedded within the space of protein-chitin NF network (Fig. 8c). The observed spherical structure with a bumpy surface is characteristic of vaterite. It is known that Ca^{2+} and CO_3^{2-} ions initially combine to form amorphous $CaCO_3$, and then the unstable amorphous $CaCO_3$ is immediately transformed to spherical vaterite.[27] Finally, the vaterite is rearranged to thermally stable calcite by dissolution and recrystallization. These results indicate that protein molecules on the chitin NFs increased the chances for biomineralization to occur, thereby providing a large number of fine crystals. Moreover, the protein molecules stabilized the formation of vaterite and inhibited the transformation of vaterite to calcite.

Figure 8. SEM images of $CaCO_3$ crystalized with (a) chitin nanofibers and (b,c) protein-chitin nanofibers.

Conclusion

Protein-chitin NFs were prepared from crab shells by the removal of calcium carbonate followed by mechanical treatment. Skipping the protein removal process will bring down the production cost of NFs. The protein-chitin NFs would also be available as reinforcement filler to increase the mechanical properties of acrylic resin as effectively as conventional neat chitin NFs without sacrificing the transparency and flexibility of the resin. Protein molecules on the surface of the NFs can accelerate the biomineralization of calcium carbonate crystals, resulting in a hybrid nanocomposite. Using these methods, novel bionanofibers could be inexpensively prepared through an eco-friendly process and have a very high surface-volume ratio, efficient mechanical strength, and characteristic surface properties. When we consider efficient utilization of protein layer, we can enhance the application of the nanofibers from crab shell waste. However, we should mind that shellfish protein sometimes causes an allergic reaction. Some people with shellfish allergy may have a reaction from handling protein-chitin NFs.

Acknowledgement

This research was partially supported by Grant-in-aids for Scientific Research (Young Scientists (A), No.26708026), Japan Society for the Promotion of Science, and by the research grant for Exploratory Research on Sustainable Humanosphere Science from Research Institute for Sustainable Humanosphere (RISH), Kyoto University.

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