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COMMUNICATION

Physical properties of TEMPO-oxidized bacterial cellulose nanofibers on the skin surface

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Abstract Water-dispersed bacterial cellulose nanofibers were prepared via an oxidation reaction using 2,2,6,6-tetramethyl-1-piperidine-*N*-oxy radical (TEMPO) as a catalyst. It was found that TEMPO-oxidized bacterial cellulose nanofibers (TOCNs) synthesized via sodium bromide-free methods are similar to those synthesized using sodium bromide. The TOCNs retained their unique structure in water as well as in emulsion. TOCNs adhere to the skin surface while maintaining nanofibrous structures, providing inherent functions of bacterial cellulose, such as high tensile strength, high water-holding capacity, and blockage of harmful substances. When gelatin gels as model skin were coated with TOCNs, the hardness representing the elasticity was increased by 20% compared to untreated gelatin gel because TOCNs could tightly hold the gelatin structure. When porcine skin was treated with TOCNs, carboxymethyl cellulose, and hydroxyethyl cellulose, the initial water contact angles were 26.5°, 76.5°, and 64.1°, respectively. The contact angle of TOCNs dramatically decreased over time as water penetrated the fibrous structure of the TOCN film. When observed by scanning electron microscopy and confocal microscopy, TOCNs on the skin surface provided physical gaps between particles and the skin, blocking the adsorption of particulate matter to the skin surface. On the contrary, the structure of water-soluble polymers was disrupted by an external environment, such as water, so that particulate matter directly attached to the skin surface. Characterization of TOCNs on the skin surface offered insight into the function of nanofibers on the skin, which is important for their applications with respect to the skin and biomedical research.

Keywords Bacterial cellulose · Surface modification · Physical property · Particulate matter filtration

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Introduction

The structure and function of the skin are vital because it acts as a crucial barrier to the external environment in the most superficial organs and as an epidermal barrier to water loss. Natural, synthetic, and organic polymers have been used as effective protective barriers on the skin. Especially, natural polymers,

such as cellulose, chitosan, and polysaccharides, are biocompatible and biodegradable, providing various functions, such as moisturization, elasticity, and protection of the skin surface.

Among these natural polymers, celluloses are not only the most abundant natural biopolymers on the Earth, but also representatives of microbial extracellular polymers. Conventional celluloses, such as natural cellulose, chemically modified cellulose derivatives, and bacterial cellulose (BC), have been used on the skin (Klemm et al. 2005). Natural cellulose crystals, which are tens of micrometers in size, are not soluble in water, and thus, their use is limited in the exfoliation of the dead skin cells. Cellulose derivatives could be obtained by chemical modification of alcoholic hydroxyl groups into other groups to become soluble in water. Carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC) are typical examples wherein the alcoholic group is substituted with a carboxyl group and hydroxyethyl group, respectively. These derivatives are polymers that cannot form structures, so they are only used as thickening agents or additives similar to other polymers. BC, which is synthesized by *Acetobacter xylinum*, belongs to the most promising class of biopolymers. It has unique properties, such as high water-holding capacity, high crystallinity, fine fibrous networks, and high tensile strength (Tahara et al. 1997; Naritomi et al. 1998; Lee et al. 2001; Svensson et al. 2005). Owing to the unique physical and mechanical properties of BC as well as its purity and uniformity, BC has various applications, such as high-quality audio membranes, electronic paper, biomedical materials as artificial skin and blood vessels, cosmetic materials as facial mask sheet, and in fuel cells (Fontana et al. 1990; Evans et al. 2003; Shah and Brown 2005; Czaja et al. 2006). However, despite the various advantages of BC nanofibers, their use on the skin has been limited in daily life because of their opaque form.

2,2,6,6-tetramethyl-1-piperidine-*N*-oxy radical (TEMPO) is a water-soluble, commercially available, and stable nitroxyl radical that can oxidize alcoholic hydroxyl groups of celluloses to aldehydes, ketones, and carboxyl groups under mild conditions. TEMPO-oxidized celluloses have been studied in various applications, such as paper, oil removal, gas barrier film, heavy metal elimination, and biomedical applications for cell delivery (Shah and Brown 2005; de Carvalho et al. 2016; Tarrés et al. 2016a, b).

For applications to the skin surface, TEMPO-oxidized bacterial cellulose nanofibers (TOCNs) could have numerous advantages over those from plant cellulose. In the case of plant cellulose, a pre-treatment process is required to generate nano-meter size fibers, and it is difficult to form a network covered on the skin because the fiber could be excised randomly or into a short length during this process. BC, on the contrary, has not only a relatively constant length of the fiber that could be networked to the skin, but also excellent inherent physical properties. However, there have only been a few studies on TEMPO-oxidized celluloses for skin applications (Fu et al. 2013; Kalia et al. 2014). We found that TEMPO-mediated BC, a transparent form dispersed in the aqueous phase, not only takes advantage of the inherent properties of BC, but also has complete control over the surface of the skin.

In this study, we investigated water-dispersed BC nanofibers obtained by oxidation via TEMPO in terms of physical properties, such as increase in elasticity and water adsorption, and we evaluated a filtrating particulate matter (PM) model on the skin. Characterization of TOCNs for the skin revealed that they have unique properties with potential applications in the skin and biomedical research.

Materials and methods

Materials

Sheets of BC were purchased from EZ Costec Co. Ltd. (Gyeonggi, Korea). TEMPO, sodium bromide, sodium hypochlorite, fluorescein isothiocyanate (FITC), nile red, poly(lactic acid) (PLA), polyvinyl alcohol (PVA), and gelatin from porcine skin were purchased from Sigma-Aldrich (St Louis, MO, USA). Ethanol was purchased from Daejung Chemical & Metal Co. Ltd. (Gyeonggi, Korea). All other reagents were purchased from Sigma-Aldrich in the highest grade commercially available.

TEMPO oxidation of BC

BC sheets (20 g wetting weight) were cut into small pieces and suspended in 500 mL distilled (DI) water containing 20 mg TEMPO. Subsequently, 15 mL NaOCl solution was added to the cellulose suspension

to initiate oxidation while maintaining the system at pH 10 with 0.5 M NaOH. The mixture was vigorously agitated overnight using a magnetic stirrer at 25 °C. The oxidation reaction was quenched by adding ethanol to the suspension. The products were washed with DI by centrifugation at 10,000×*g* several times. For the synthesis of TOCNs with NaBr, 0.5 g NaBr was added in the initial reaction and the remaining process was carried out in the same manner.

The carboxylate and aldehyde contents of samples were measured as described previously (Saito and Isogai 2004). The carboxylate content in the solid samples was determined using an electric conductivity titration method. Aliquots of each sample were further oxidized with $NaClO_2$ at pH 4–5, and the increase in carboxyl groups was regarded as the amount of aldehyde groups present in cellulose.

Scanning electron microscopy characterization

Oil in water (o/w) emulsions containing TOCNs comprised 0.03% TOCNs, 1% dimethicone, 3% pentaerythrityl tetraethylhexanoate, 1% hydrogenated polydecene, 7% dipropylene glycol, 7% glycerin, 0.4% methyl glucose sesquistearate, 3% cyclopentasiloxane, 4% xanthan gum, 12% carbomer, 1.5% sorbitol, 0.4% chelating agent, 2% trisodium EDTA, 2% pH adjusting agent, and 44.83% water. To investigate the surface characteristics, 0.05% TOCNs with and without NaBr during the synthesis, TOCNs from Bamboo, original BC, and o/w emulsions containing TOCNs were prepared by casting the solution on porcine skin (1 × 1 cm) and drying at room temperature overnight. These samples were observed using a field-emission-type scanning electron microscopy (SEM; Hitachi S-4000) after platinum sputtering at 20 mA for 120 s.

Fluorescence image characterization for PM filtration of TOCNs

The optically sectioned fluorescence images of the porcine skin were acquired with a home-built confocal laser scanning microscope (CLSM) operating with three fluorescence channels at 30 frames per second. The details of the microscopy were described previously (Song et al. 2015). In short, the home-built CLSM is based on confocal detection utilizing two diode-pumped solid-state (DPSS) lasers and two photomultiplier tubes (PMT) with 100-μm pinholes. Two DPSS lasers with wavelengths of 488 and 561 nm were employed to excite fluorescein isothiocyanate (FITC) and Nile red, respectively. A water immersion objective lens from Olympus with a numerical aperture of 0.6 and a magnification of 40 × was used. The working distance of the objective lens was 170 μm and the field of view was set to 400 μm by 400 μm.

The axial resolution of the CLSM can be defined as the axial position at which the detected intensity drops to half of the value on the focal plane. The measured axial resolution was approximately 2 μm, i.e., the CLSM can optically section the image planes every 2 μm. The analog signals from the PMTs were fed into the frame grabber along with the video synchronization signals. The digitally converted signals from the 10-bit frame grabber were used to acquire the snapshot images and movie streams with a visual basic-based custom-written software.

For PM filtration of TOCNs, three 100 μL samples (DI, 0.1% polysaccharide, and TOCNs) were coated on 1 × 1 cm porcine skin, which expressed FITC. PLA particles as a model PM were synthesized by the following protocol. PVA (150 mg) was dissolved in 100 mL purified water. PLA (200 mg) and Nile red (1 mg) were dissolved in 5 mL dichloromethane and the mixture was dispersed in the prepared PVA solution at 1300 rpm overnight. The resultant mixture was excessively washed with purified water 3 times and stored at 4 °C until use. A solution of PLA particles (1–5 μm), which expressed Nile red, was dropped onto the sample-coated porcine skin. The prepared porcine skin was imaged using confocal microscopy.

Contact angle measurements

The surface wettability of various cellulose structures was evaluated by contact angle measurements using a Contact Angle System OCA (Dataphysics), combined with a high-speed camera. Water droplets were deposited directly at the surfaces of dried cellulose solution covering porcine skin, and the water contact angles were measured. Three measurements were performed per sample and averaged. The volume of the water droplet was 10 μL, and the tip used was a precision stainless steel tip (Gauge 32, EFD).

Hardness measurement

To determine the effect of TOCNs on elasticity, hardness testing of the cellulose nanofibers deposited on gelatin gel was carried out using a texture analyzer (TA.XT plus, Stable Micro Systems, UK). Four milliliters of 5% gelatin gel was prepared in 6-well plates, and 1 mL of TOCNs was cast and dried. All samples were placed in a concentric cylinder with a diameter of 5 mm and placed under a cylindrical probe (P/5). The setting of the texture analyzer was kept at a pre-test speed of 1 mm/s, test speed of 0.4 mm/s, hold time of 10 s, and post-test speed of 1 mm/s with 5 g trigger force. The initial significant peak force from the resulting curve was considered as the initial fracture force, and the absolute peak force was considered as the hardness of the samples. All hardness measurements were performed 5 times.

Results and discussion

Characterization of TEMPO-oxidized bacterial celluloses

Water-dispersed cellulose nanofibers were obtained by oxidizing BC with TEMPO. Primary hydroxyl groups at the C6 carbon of BC were converted to carboxylate groups by the oxidation reaction. In agreement with previous studies (Saito et al. 2006, 2007), TOCNs showed a good dispersion capability even after allowing the suspension to stand for at least 1 month at room temperature (see supporting information, Fig. S1). Conventional oxidation methods for synthesizing dispersed cellulose nanofibers use TEMPO/sodium bromide/sodium hypochlorite. However, the remaining bromide chemicals could generate toxicity because of handling and cause safety concerns. Oxidation methods of α-D-glucopyranoside, corn starch, and sucrose without sodium bromide have been reported (Bragd et al. 2000; Lemoine et al. 2000).

Carboxyl and aldehyde contents of original BC and TOCNs with and without NaBr are summarized in Table S1. The carboxyl and aldehyde contents, determined by conductivity titration method, of the TOCNs without NaBr were 1.08 ± 0.12 and 0.09 ± 0.04 mmol/g, and those of TOCNs with NaBr were 1.11 ± 0.17 and 0.06 ± 0.02 mmol/g, respectively. The carboxyl and aldehyde groups of both the TOCNs were increased compared to those of the original BC, but those of the two samples were found to follow a similar trend. The SEM images of both the TOCNs reveal that TOCNs from BC regardless of the use of NaBr during synthesis have similar diameter and were able to cover the surface uniformly (Fig. S2). Consequently, there were no remarkable differences in carboxyl contents and structure of TOCNs synthesized with/without NaBr.

Structure of TOCNs on model skin

The surface of TOCNs was observed by SEM. Figure 1 illustrates the process of preparing surface-modified cellulose nanofibers. After oxidation, the highly interlaced fibers of pure BC were swollen and dispersed in DI water because of electrostatic repulsion between the negatively charged fiber surfaces. It was confirmed that synthetic cellulose nanofibers in TOCN solutions covering the porcine skin (2×2 cm) maintained the original structure of nanofibers. In addition, o/w emulsion (oil:water = 1:9) containing TOCNs also exhibited nanofibrous structures. Furthermore, the average diameter of the BC nanofibers was approximately 100 nm and decreased to 30–50 nm after oxidation. TEMPO-mediated oxidation could have occurred regioselectively on the surface of cellulose fibrils because this reaction proceeded from regions that were accessible to the chemical solution (Saito et al. 2007). This reaction introduces carboxyl groups to the surface of nanofibers, resulting in weakening of inter-fiber hydrogen bonds between fibrils which bundle them into fibers. In other words, the repulsion due to ionic charge on the surface of the nanofibers leads to reduction of the ability of the fibers to come into close contact, thereby forming and stabilizing smaller diameters (Spaic et al. 2014). Therefore, oxidized BC was able to maintain its original fibrous structure with a slightly decreased fiber diameter.

To confirm the formation of structures of TOCNs from plant cellulose and BC on the skin surface, structures of both the TOCNs at the same concentration were evaluated based on the SEM images (Fig. S2). Unlike the structure formation of the TOCNs from BC on the skin surface described above, the results of SEM images revealed that TOCNs from plant cellulose could not cover the skin uniformly, but

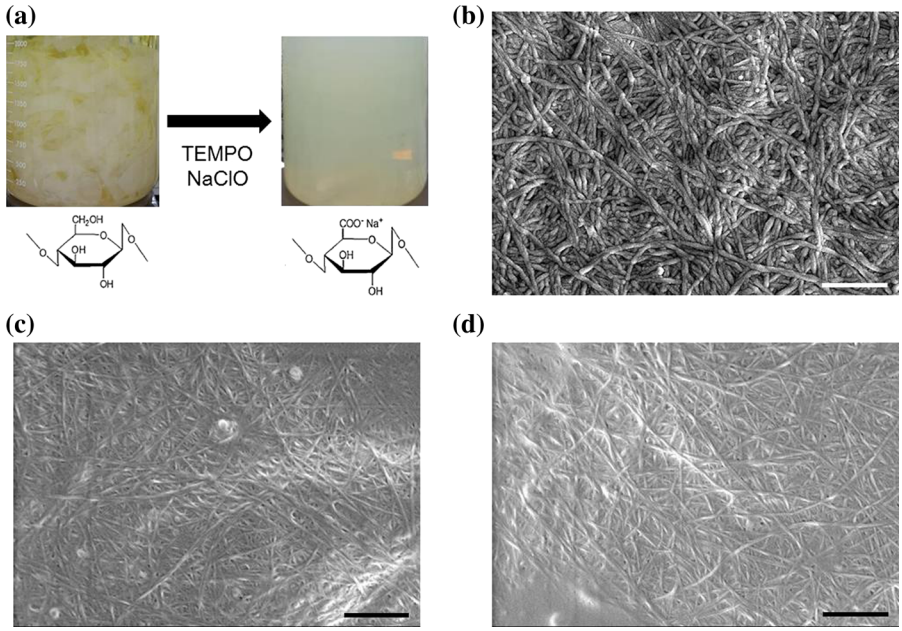

Fig. 1 **a** Schematic diagram representing the TEMPO oxidation of BC. SEM images of **b** BC sheet and TOCNs in **c** water and **d** o/w emulsion on porcine skin. Scale bar = 1 μm

it only covered several parts in the form of spots. It means that the network of TOCNs from plant cellulose consisting of short length fibers could lack the ability to cover the overall surface.

Elasticity measurement of TOCNs

Elasticity measurements were performed to determine the efficacy of TOCNs. Gelatin, a proteinaceous product derived from collagen, was selected as model skin because it is known to function as a scaffold in the skin and biomedical applications (Choi et al. 1999; Perng et al. 2007) and has been studied for its mechanical characteristics (Rahman and Al-mahrouqi 2009). Gelatin gel (4 mL, 5% w/w) was prepared in a 6-well plate and TOCN solution (1 mL, 0.03% w/w) was added to the gelatin gel for 1 h. The remaining solution was removed, and the dried samples were measured using a texture analyzer for measuring hardness.

The hardness curve generated by the texture analyzer was determined by plotting force as a function of time. Hardness was defined as the maximum force required to compress the probe on a sample. The specimen in the all-round properties analyzer was pressed down on the gelatin gel at a constant rate to measure the pressure. The press pressure increased the strength of the surface structures as a result of increased elasticity. As shown in Fig. 2, the hardness of the TOCN-coated gelatin gel increased by 20% compared to that of gelatin gel without TOCNs. Original BC, present in a sheet form, which cannot be dispersed in the aqueous solution, is not suitable to apply on the gelatin gel. Alternatively, the original BC was chopped using a universal grinder to measure the hardness. The hardness of chopped BC was 18% higher than that of the untreated sample, which was similar to that of TOCNs at the same concentration. The increased resistance of the gelatin gel to external force means that the nanofiber structure of TOCNs firmly holds the gelatin structure.

The relative hardness, which is defined as the ratio of hardness of TOCNs coated on gelatin gel to that of gelatin gel without TOCNs, was obtained at each concentration of TOCNs ranging from 0 to 0.15%. As shown in Fig. S3, the relative hardness showed a steep increase of up to 0.03%, which then increased steadily, suggesting that at least 0.03% TOCNs (33.3 μg/cm^2) were required for the cellulose network structure to cover most of the surface to increase elasticity.

Water-absorbing capacity of TOCNs

It was known that as compared to other celluloses, BC has high water-absorbing ability (Fontana et al. 1990; Fu et al. 2013). A water droplet was placed on porcine

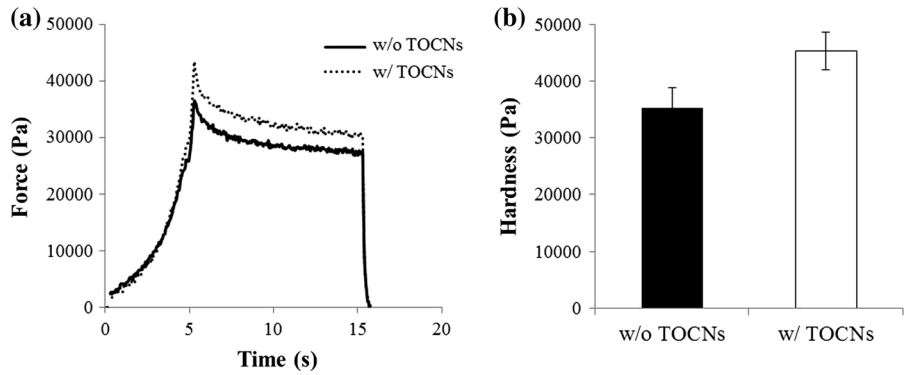

Fig. 2 Hardness measurement of TOCNs deposited on the gelatin gel and gelatin gel without TOCNs. **a** Force as a function of time. **b** Maximum force (hardness)

skin covered with TOCNs and water-soluble cellulose from wood, such as CMC and HEC, and changes in the water contact angle with time were measured. As shown in Fig. 3, the initial water contact angle of TOCNs was 26.5°. The water contact angle changed dramatically with time as a result of partial penetration of water into the film. However, the initial contact angles of CMC and HEC films were 76.5° and 64.1°, respectively, when measured under the same conditions. These results suggest that the high carboxylate content of TOCNs could contribute to their excellent water-absorbing ability, resulting in low resistance to water in addition to their unique properties.

The contact angles of the three types of cellulose were measured to investigate the treatment method of BC, such as before/after TEMPO oxidation and with/without NaBr during synthesis (Fig. S4). The initial contact angles and the change in the contact angle of TOCNs with time and with/without NaBr showed a similar trend. The initial contact angle of original BC membrane was lower than that of TOCNs, but it was found to be similar within 1 min. This result indicates that the TOCNs retain the inherent properties of the original BC even after oxidation.

PM filtration of TOCNs

Suspended PM, classified as either PM10 or PM2.5, is one of the most serious sources of air pollution, and especially, is harmful to humans because its small size could penetrate the human skin as well as the bronchi and lungs (Li et al. 2016). Nanofibers for air filtration to block PM could provide a dramatic increase in filtration efficiency with a relatively small decrease in permeability (Wang et al. 2015). We hypothesized that the structural formation of dispersed nanofibers on the skin could block PM. To confirm this hypothesis, we observed SEM and confocal images of model PM on porcine skin covered with different materials. Solutions of PLA particles of 1–5 μm as model PM were dropped onto dried porcine skin samples. Based on the results of the SEM analysis, model PM particles were

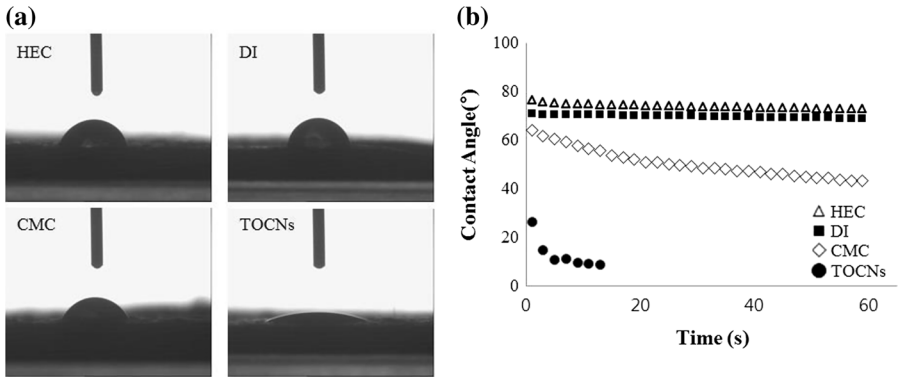

Fig. 3 Contact angle measurement of different types of celluloses covering the porcine skin. **a** Microscopic images of water droplet on various celluloses covering the porcine skin at the initial stage. **b** The change in the contact angle with time

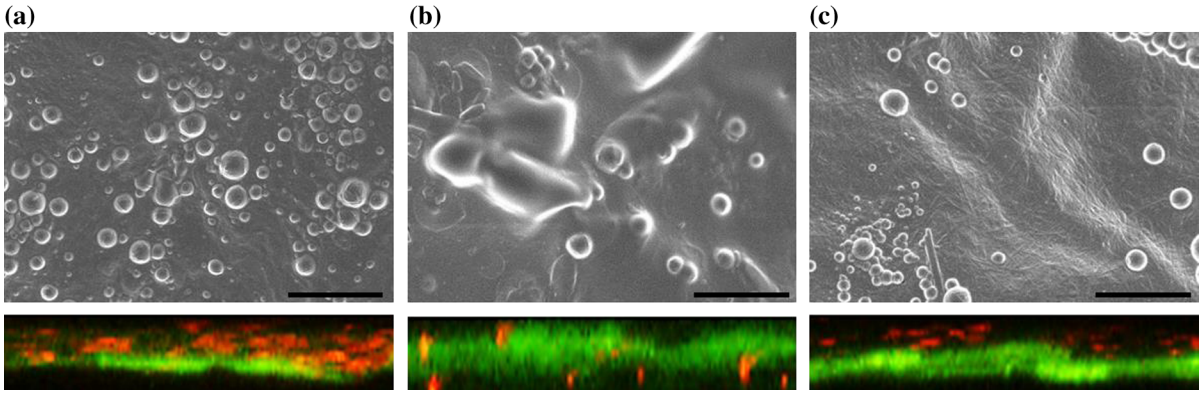

Fig. 4 SEM (top) and confocal (bottom) images of model PM on porcine skin covered with different materials. **a** Non-treated, **b** polysaccharide, and **c** TOCNs. In the confocal study, two DPSS lasers with wavelengths of 488 and 561 nm were employed to excite fluorescein isothiocyanate (FITC) and Nile red, respectively. Scale bar = 5 μm

directly adsorbed on non-treated porcine skin (Fig. 4a). In the case of polysaccharides, xanthan gum as a water-soluble polymer, the particles in the solution penetrated the polysaccharide film because its structure was disrupted or dissolved in the aqueous solution. As a consequence, some particles were buried under the polysaccharide film and the others were attached to the skin surface directly (Fig. 4b). However, TOCNs, which were not soluble but were dispersed in water, could block the particles and prevent their attachment to the skin surface (Fig. 4c).

To confirm the effect of TOCNs as a barrier between PM and the skin, en face confocal florescence image stacks of the control, polysaccharides, and TOCNs on the porcine skin were taken using a Z-axis translation stage at every 2 μm in the direction of depth from the surface. Four spots were imaged in each sample. To assess the imaging depth for different samples, we compared the typical B-scan images (2D cross-sectional view orthogonal to the en face image), which were reconstructed from acquired en face image stacks with a rendering software (Image J, National Institutes of Health, USA).

Cross-sectional images of control groups, which were non-treated porcine skin and polysaccharide-coated porcine skin, confirmed that model PM with diameters of 1–5 μm (red) attached directly to the porcine skin (green). However, cross-sectional images of TOCNs-coated samples showed that PM was separated from the porcine skin. This means that barriers with nanofibrous structures of less than 2 μm in thickness can block PM from penetrating the skin.

Conclusions

TOCNs, transparent and dispersed in aqueous solution, were investigated on the skin surface. Our study is significant as it is the first attempt at applying nanofibrous structures to confirm physical properties, such as water-absorbing capacity, elasticity, and ability to block PM on the skin surface. Although, as compared to plant cellulose, BC has advantageous features for the skin tissue materials, conventional uses of BC nanofibers for the skin have been difficult to expand because of the spatial limitations of the opaque sheet form. However, the nanofibrous structures of TOCNs have unique properties and could be controlled freely in solution form on the skin regardless of the dimension. Moreover, their unique structure is retained in water as well as in emulsion.

TOCNs have a great potential not only to apply the functions of elasticity and moisture on the skin, but also to prevent the direct adhesion of PM on the skin as a nanofiber filter. At the same time, they provide a cornerstone for the development of fiber-cosmetics.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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