

Article

## Hierarchically Self-Assembled Nanofiber Films from Amylose-Grafted Carboxymethyl Cellulose

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**Abstract:** In this paper, we report the formation of hierarchically self-assembled nanofiber films from amylose-grafted sodium carboxymethyl celluloses (NaCMCs) that were synthesized by a chemoenzymatic approach. First, maltooligosaccharide primer-grafted NaCMCs were prepared by a chemical reaction using two kinds of NaCMCs with different degrees of polymerization (DPs) from Avicel and cotton sources. Then, phosphorylase-catalyzed enzymatic polymerization of  $\alpha$ -D-glucose 1-phosphate from the nonreducing ends of the primer chains on the products was conducted to produce the prescribed amylose-grafted NaCMCs. The films were obtained by drying aqueous alkaline solutions of the amylose-grafted NaCMCs. The scanning electron microscopy (SEM) image of the film fabricated from the material with the higher DP from the cotton source showed a clear, self-assembled, highly condensed tangle of nanofibers. The SEM image of the material with the lower DP from the Avicel source, on the other hand, showed an unclear nanofiber morphology. These results indicate that the DPs of the main chains in the materials strongly affected the hierarchically self-assembled nanofiber formation. The SEM images of the films after washing out the alkali, furthermore, showed that the fibers partially merged with each other at the interfacial area owing to the double helix formation between the amylose-grafted chains. The mechanical properties of the films under tensile mode also depended on the self-assembled morphologies of the amylose-grafted NaCMCs from the different sources.

**Keywords:** self-assembled nanofiber; amylose; carboxymethyl cellulose; chemoenzymatic

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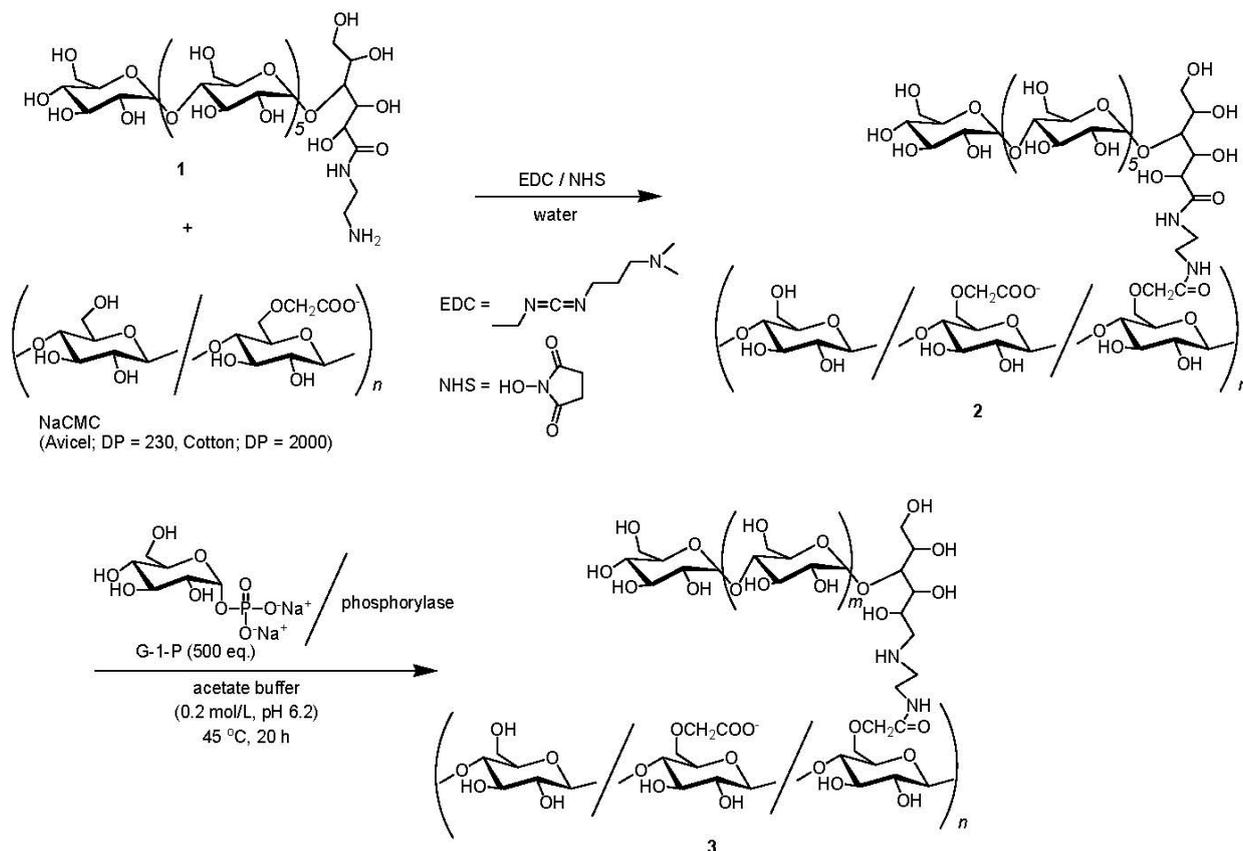
## 1. Introduction

Cellulose is the most abundant biological macromolecule, with a polysaccharide structure consisting of a chain of  $\beta$ -(1 $\rightarrow$ 4)-linked glucose residues [1,2], and is a very important renewable resource used in furniture, clothing, and medical products. Considerable efforts are also being devoted to developing new material applications of cellulose because of its biodegradable and eco-friendly properties. Self-assembled fibrillar nanostructures from cellulose, so-called nanofibers, are promising materials for practical applications in bio-related research fields such as tissue engineering [3–5]. Conventional approaches to the production of cellulose nanofibers are mainly top-down procedures that break down the starting bulk materials from natural cellulose resources [6–8].

In a previous study, we found that the self-assembly of amylose-grafted carboxymethyl cellulose sodium salt (NaCMC) forms nanofiber films upon drying its alkaline aqueous solution [9]. Carboxymethyl cellulose (CMC), an anionic water-soluble polysaccharide, is one of the most widely used cellulose derivatives, and its sodium salt (NaCMC) has a number of COONa groups that promote water solubility [10]. Our method for the formation of nanofibers from amylose-grafted NaCMCs is completely different from the aforementioned conventional top-down procedures because our method is a hierarchically self-assembling generative (bottom-up) route, in which fibrillar nanostructures are produced by regeneration from the solutions of the substrates.

Amylose-grafted NaCMC (**3**) was synthesized by a chemoenzymatic technique according to Scheme 1, which was combined of phosphorylase-catalyzed enzymatic polymerization with chemical reaction [11–19]. Because the enzymatic polymerization of  $\alpha$ -D-glucose 1-phosphate (G-1-P) is initiated at the nonreducing end of the maltooligosaccharide primer and produces amylose by the following propagation [20–27], the primer was first introduced on the NaCMC chain by the condensation of an amine-functionalized maltooligosaccharide (**1**) with carboxylates in NaCMC to give a maltooligosaccharide-grafted NaCMC. Then, the phosphorylase-catalyzed polymerization of G-1-P was conducted using the product to give the prescribed material, **3**. The introduction of amylose-graft chains contributed to the construction of a rigid NaCMC main chain, resulting in a nanofiber film upon drying the alkaline solution of the product. Furthermore, the long amylose-graft chains formed double helixes in the intermolecular NaCMC chains by washing out alkali from the film to produce a robust film with the merged nanofiber morphology.

In this paper, we describe the effect of the degree of polymerization (DP) of the NaCMC main chains on the formation behaviors of hierarchically self-assembled nanofiber films from **3**. For this purpose, the materials were synthesized by the aforementioned chemoenzymatic method using NaCMCs having similar degrees of carboxymethylation (DC). The NaCMCs were prepared from two kinds of cellulose with different DPs (microcrystalline cellulose (Avicel No. 2331), DP = *ca.* 230; cotton, DP = *ca.* 2500) [28,29].

**Scheme 1.** Chemoenzymatic synthesis of amylose-grafted NaCMC (**3**).

## 2. Experimental Section

### 2.1. Materials

Microcrystalline cellulose from Merck (Avicel, No. 2331) and absorbent cotton from Kakui Co. Ltd. (Kagoshima, Japan) were used. Carboxymethylation of the cellulose was carried out by the reaction of cellulose with sodium chloroacetate according to the literature procedure [30]. The DC values were estimated by the titration method described in the literature [31]. Thermostable phosphorylase (*Aquifex aeolicus* VF5) was supplied by Ezaki Glico Co. Ltd., Osaka, Japan [23,32,33]. An amine-functionalized maltooligosaccharide (**1**) was prepared according to the literature procedure [16]. Other reagents and solvents were used as received.

### 2.2. Synthesis of Maltooligosaccharide-Grafted NaCMC (**2**)

To a solution of NaCMC (from Avicel, DC = 0.46, 0.020 g, 0.0101 mmol) in water (3.0 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (0.0387 g, 0.202 mmol) and *N*-hydroxysuccinimide (NHS) (0.0232 g, 0.202 mmol), and the mixture was stirred at room temperature for 1 h. Then, **1** (0.245 g, 0.202 mmol) was added to the solution and the mixture was further stirred at room temperature for 24 h. After the reaction solution was dialyzed in a dialysis bag (molecular cut off: 12,000–14,000) against water overnight, the obtained material was purified further by precipitation into methanol (300 mL). The precipitate was isolated by filtration, washed with dimethyl sulfoxide (DMSO) and methanol, and dried under reduced pressure to give

maltooligosaccharide-grafted NaCMC (**2**) (0.133 g);  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  3.00–4.44 (sugar protons of H2–H6,  $\text{NCH}_2\text{CH}_2\text{N}$ ), 4.44–4.68 (H1 of NaCMC), 5.17, 5.34 (H1 of maltooligosaccharide). The degree of substitution (DS) for the grafting was determined by the integrated ratio of the H1 signal of maltooligosaccharide to that of NaCMC to be 0.074. Maltooligosaccharide-grafted NaCMC from cotton was synthesized according to a similar procedure (DC = 0.43, DS = 0.070).

### 2.3. Synthesis of Amylose-Grafted NaCMC (**3**)

The aforementioned **2** (from Avicel, 0.0080 g, 0.0212 mmol) was dissolved in an aqueous sodium acetate buffer solution (0.2 mol/L, pH 6.2, 3.0 mL) and G-1-P disodium salt (0.486 g, 1.60 mmol) was added to the solution. After the pH value was adjusted to 6.2 by the addition of 0.2 mol/L aqueous acetic acid, thermostable phosphorylase (16 units) was added to this solution, which was then maintained at 45 °C for 20 h with stirring. After the resulting gelic mixture was immersed in water (100 mL) for 3 h, the gel was lyophilized to give amylose-grafted NaCMC (**3**, 0.107 g);  $^1\text{H}$  NMR (1 mol/L NaOD/ $\text{D}_2\text{O}$ )  $\delta$  3.00–4.44 (sugar protons of H2–H6,  $\text{NCH}_2\text{CH}_2\text{N}$ ), 4.44–4.68 (H1 of NaCMC), 5.13, 5.27 (H1 of amylose). Amylose-grafted NaCMC from cotton was synthesized according to a similar procedure.

### 2.4. Formation of Nanofiber Film from **3**

Amylose-grafted NaCMC **3** (0.040 g) was first dissolved in a 0.50 mol/L NaOH aqueous solution (1.5 mL) by stirring the mixture at room temperature. The solution was thinly cast onto a glass plate and dried under ambient conditions to give a film. The resulting film was immersed twice in water (10 mL for 10 min and 5 mL for 5 min) to remove the NaOH and dried under ambient conditions.

### 2.5. Measurements

$^1\text{H}$  NMR spectra were recorded on a JEOL ECX-400 spectrometer. Scanning electron microscopy (SEM) images were obtained using an Hitachi SU-70 electron microscope. X-ray diffraction (XRD) measurements were conducted using PANalytical X'Pert Pro MPD with Ni-filtered Cu  $K\alpha$  radiation ( $\lambda = 0.15418$  nm). The stress–strain curves under tensile mode were measured using a tensile tester (Little Senster LSC-1/30, Tokyo Testing Machine).

## 3. Results and Discussion

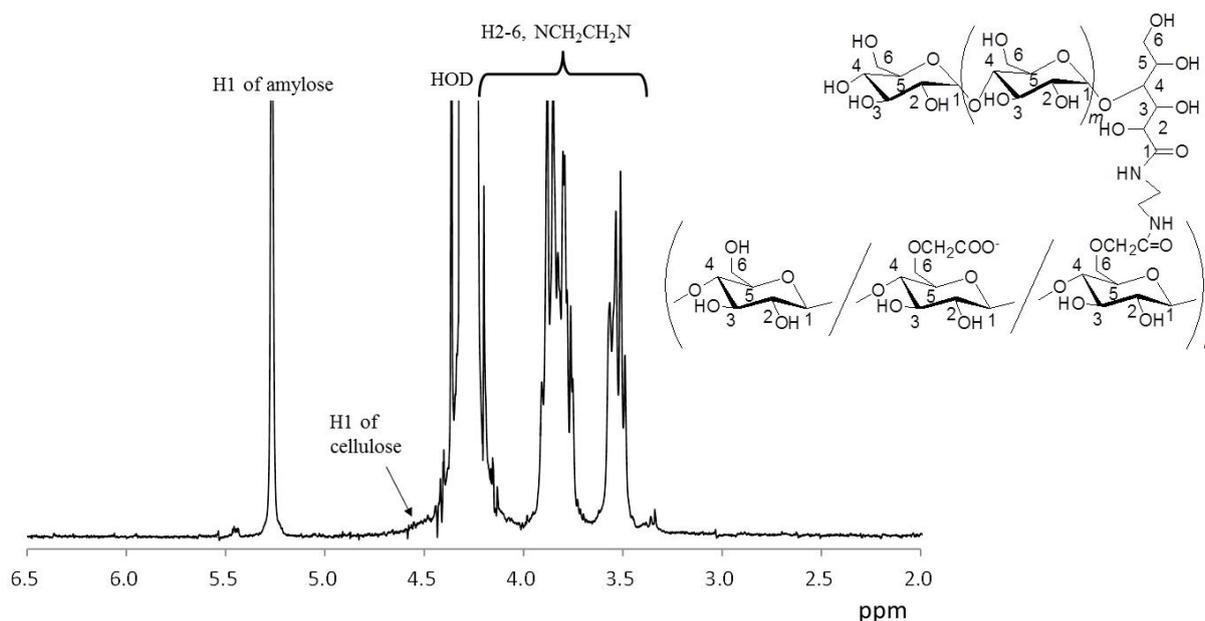
### 3.1. Chemoenzymatic Synthesis of Amylose-Grafted NaCMC **3**

For the chemoenzymatic synthesis of the amylose-grafted NaCMCs (**3**), in this study, two kinds of NaCMCs with similar DC values and different DP values were prepared by the carboxymethylation of Avicel and cotton [30]. The NaCMC from Avicel had DC and DP of 0.46 and 230, respectively, and the NaCMC from cotton had DC and DP of 0.43 and 2500, respectively [28,29]. The introduction of maltooligosaccharides onto the NaCMC chains was performed by the condensation of **1** with carboxylates in the NaCMCs using the EDC/NHS condensing agent in water to give **2** (Scheme 1). The ratios of the introduced maltooligosaccharide chains to the repeating units (functionality) in the

products from the two kinds of NaCMCs were adjusted to have similar values (DS = 0.074 from Avicel and DS = 0.070 from cotton) by appropriate reaction conditions, which were determined by the integrated ratios of the H1 signal of the maltooligosaccharides to that of the NaCMCs in the  $^1\text{H}$  NMR spectra in  $\text{D}_2\text{O}$ .

The amylose-grafted NaCMCs were synthesized by the phosphorylase-catalyzed polymerization of G-1-P from the nonreducing ends of the maltooligosaccharide (primer) graft chains on **2** in the G-1-P/primer feed ratio of 500 (Scheme 1). As the polymerization progressed, the gelation of the reaction mixtures took place. The gelic products were immersed in water and the resulting gels were lyophilized to give **3**. The isolated products were insoluble in water but soluble in aqueous alkaline solution. Thus, the structures of the products were characterized by the  $^1\text{H}$  NMR spectra measured in 1 mol/L NaOD/ $\text{D}_2\text{O}$  (Figure 1, from the cotton source), which showed an obvious increase in the integrated ratios of the H1 signal of amylose to the H1 signal of NaCMC as compared with that in the  $^1\text{H}$  NMR spectra of **2**. The average DPs of the amylose-graft chains in **3** from Avicel and cotton were calculated on the basis of the elemental analysis data, and the functionalities of the maltooligosaccharide chain (the DS values) in **2** were found to be 187 and 218 for Avicel and cotton sources, respectively.

**Figure 1.**  $^1\text{H}$  NMR spectrum of amylose-grafted NaCMC (**3**) from cotton (NaOD/ $\text{D}_2\text{O}$ ).

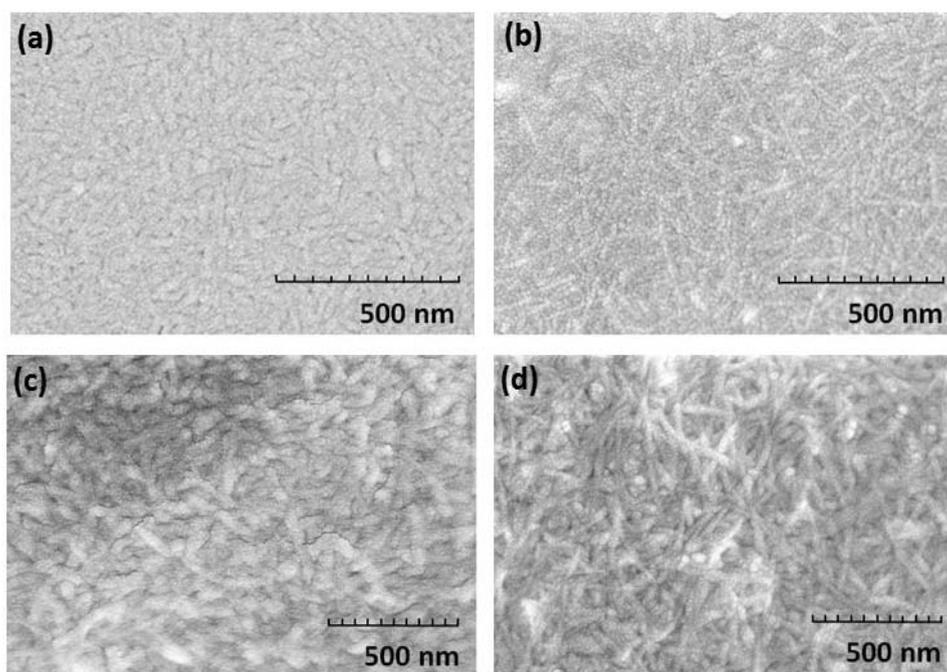


### 3.2. Formation and Characterization of Self-Assembled Nanofiber Films from **3**

We previously reported that amylose-grafted NaCMCs (**3**) with DPs of 140–214 and an amylose-graft chain functionality of 35.4 synthesized from commercially available NaCMC (DP = ca. 1200 and DC = 0.7) formed self-assembled nanofiber films after drying their aqueous alkaline solutions. In the present study, we evaluated the effect of the DPs of the NaCMC main chains in **3** on the formation behavior of the self-assembled nanofiber films using two materials synthesized as aforementioned. Solutions of the materials in 0.50 mol/L NaOH aq. were cast onto a glass plate and dried under ambient conditions to give the product films. It was confirmed from the SEM image that

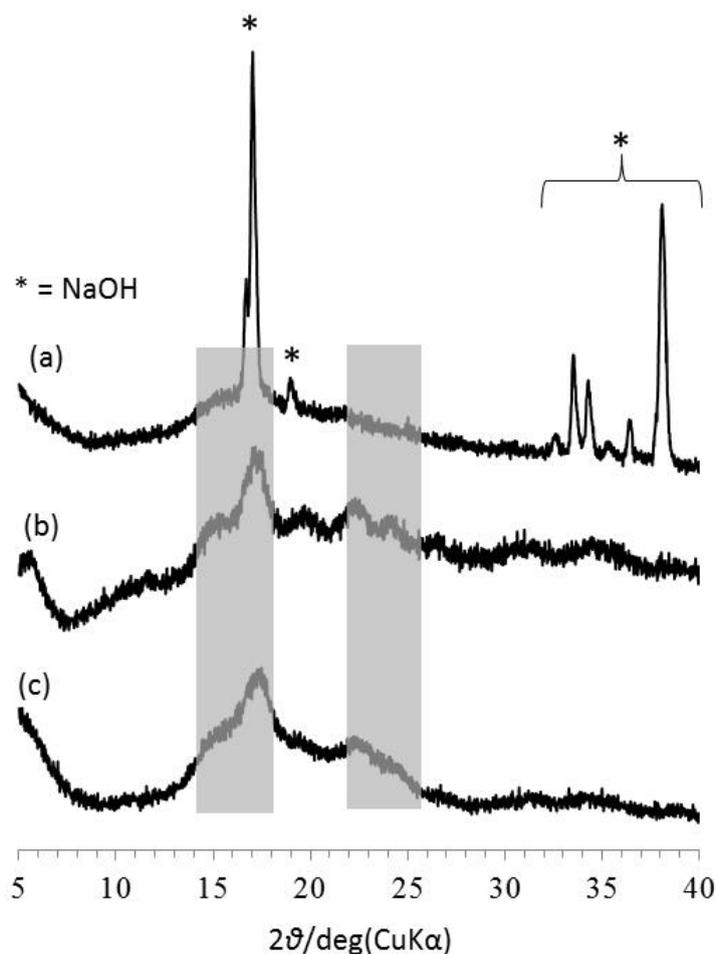
the film of **3** from the cotton source was constructed from nanofibers arranged in a highly condensed tangle (Figure 2b). On the other hand, such a clear nanofiber morphology was not seen in the SEM image of the film fabricated from the Avicel source, although it showed some broad fibrillar entanglement (Figure 2a). The SEM images of the films after washing out the alkali showed that the fibrils partially merged with each other at interfacial areas with the remaining fibrillar morphologies (Figures 2c,d). The SEM results suggest that the longer NaCMC chain in **3** is favorable for producing the regularly controlled self-assembly needed to construct the clear nanofiber morphology.

**Figure 2.** SEM images of films prepared from alkaline solutions of **3** from Avicel and cotton ((a,b), respectively) and respective films after washing out alkali ((c,d), respectively).



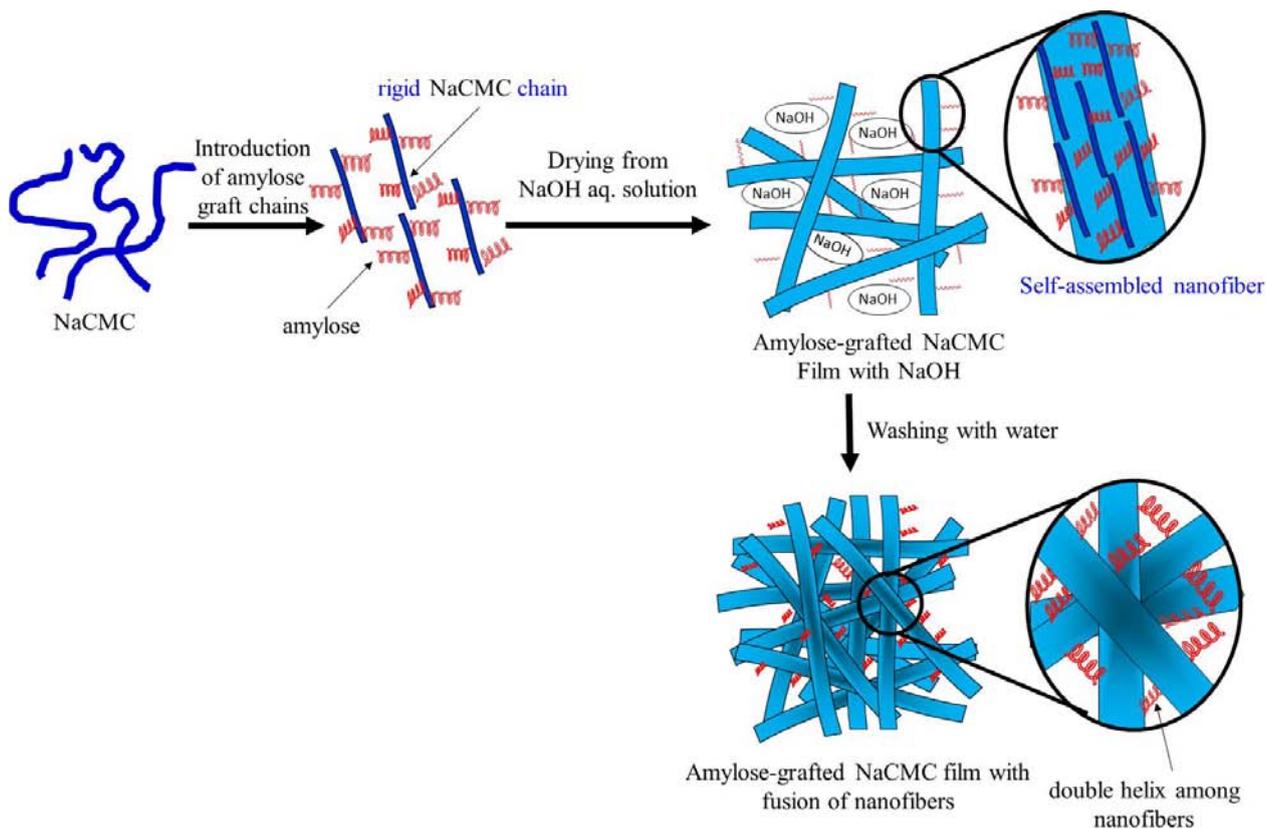
XRD measurements of the films were conducted to evaluate the hierarchically self-assembled structure of **3**. The XRD profile of the film of **3** from cotton before washing out the alkali slightly show diffraction peaks due to amylose helix at around 17 and 23° [34] besides NaOH crystalline peaks (Figure 3a), indicating that the amylose graft chains only partially formed double helix conformation in the film. Because the aqueous alkaline solution is a good solvent for amylose, the formation of an amylose double helix is mostly prevented during the drying process of the solution. After washing out the alkali from the film, the XRD profile exhibited diffraction peaks obviously due to the amylose helix (Figures 3b,c), indicated with shadows), suggesting the progress of double helix formation during the washing process.

**Figure 3.** XRD profiles of films of **3** from cotton (a) before and (b) after washing out the alkali and (c) amylose.



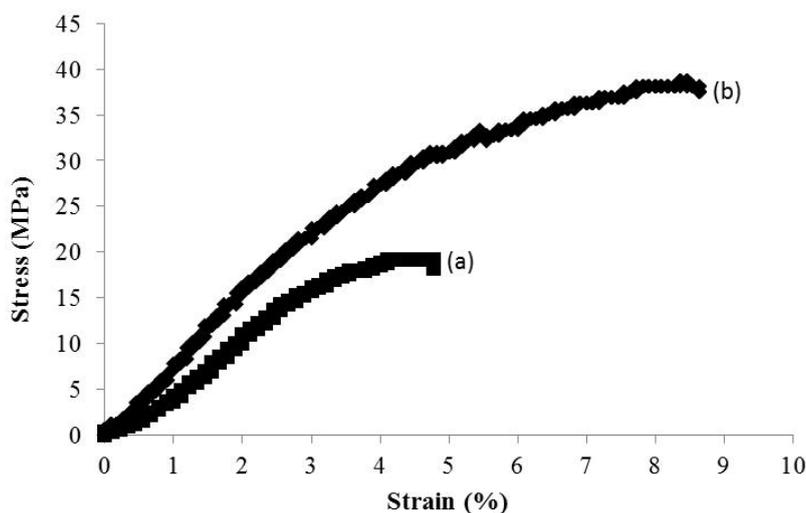
On the basis of the above results, the self-assembling process of **3** is proposed to lead to the formation of the nanofiber film (Figure 4). As already reported in our previous paper [9], the introduction of saccharide-graft chains on NaCMC prevented the construction of a random-coil conformation, resulting in the rigid nature of the NaCMC chain. While drying the aqueous alkaline solution of **3**, some of these rigid materials regularly assembled to induce nanofibrillation with the slight double helix formation from amylose graft chains on NaCMCs, but they did not construct large aggregates because the double helix formation of the most of the amylose chains was prevented due to the alkaline conditions. By washing out alkali from the film, the double helix was able to form on the nanofibers, leading to merging on the surface of the fibers. The average DP value of the NaCMC main chain of **3** from the cotton source (ca. 2500) was much larger than that of the amylose-graft chain (218), resulting in nanofibrillation with a high aspect ratio. On the other hand, because the two DP values of the main and graft chains in **3** from the Avicel source were comparable (230 and 187), the self-assembled nanofibers were not clearly formed.

**Figure 4.** Proposed self-assembling process of **3** under alkaline conditions leading to nanofiber film.



Finally, the mechanical properties of the films of **3** from the Avicel and cotton sources after washing out alkali were evaluated by tensile testing (Figure 5). The stress–strain curve of the film from the cotton source showed larger fracture stress and strain values than the film from the Avicel source. This result indicates that the DP of the main chain in **3** strongly affects the mechanical properties of the present nanofiber film.

**Figure 5.** Stress–strain curves of films of **3** from (a) Avicel and (b) cotton after washing out alkali.



## 4. Conclusions

This paper reports the formation of hierarchically self-assembled nanofiber films from amylose-grafted NaCMC films (3) with different DPs. The materials were synthesized by the chemoenzymatic method using NaCMCs from different sources, Avicel and cotton. The film with the clear nanofiber morphology was formed from the material with the higher DP (cotton) upon drying its aqueous alkaline solution, whereas the clear nanofiber morphology was not obtained in the film from the material with the lower DP (Avicel). By washing out the alkali from the films, the fibers merged at their interfacial areas. The obvious formation of the hierarchically self-assembled nanofibers in the film strengthened the mechanical properties under tensile mode.

## Acknowledgments

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## Conflicts of Interest

The authors declare no conflict of interest.

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