Antimicrobial/Antimold Polymer-Grafted Starches for Recycled Cellulose Fibers

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Abstract
In this work, an antimicrobial guanidine polymer (PHGH) was grafted onto starch as a carrier to form branched or grafted chains along the starch backbone. This grafting improved the antimicrobial properties and the adsorption of the starch on recycled cellulose fibers. Similar work was also conducted on bleached sulfite fibers for comparison. The results showed that the starch, grafted with 12 wt% PHGH, adsorbed more on recycled fibers than on sulfite fibers. By applying the antimicrobial-modified starch to recycled or sulfite pulps up to 20 mg/g, both antimicrobial and antimold performances of the papers were improved substantially. Additionally, the PHGH-modified starch increased the tensile index of papers, but decreased the tear index slightly. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were employed to investigate the morphologic changes of Escherichia coli bacteria and Chaetomium globosum fungi upon exposure to the PHGH-modified starch, thus demonstrating that the antimicrobial mechanism is based on the damage of bacterial membrane.

Keywords
Antimicrobial, antimold, guanidine polymer, cationic starch, recycled cellulose fibers

1. Introduction
Starch has been extensively used in papermaking as strength agent and retention aid [1]. The adsorption of unmodified starch on fibers is often driven by hydrogen bonding [2]. To increase the adsorption of starch on fibers, the conventional ap-
proach is to render the starch cationic by introducing quaternary ammonium groups [2, 3]. Most of such modifications improved the performance of starch in enhancing the paper strength [4, 5].

Several studies have been conducted to improve the antimicrobial performance of papers by using antimicrobial agents or organic compounds to create paper for food packaging industries [6, 7]. However, the application of conventional disinfecting agents on fibers affects the surface chemistry and eventually the bonding of fibers [8, 9]. It was reported that low molecular weight (MW) antimicrobial agents desorb easily from the fiber surface, or leach out during storage, thus impairing their antimicrobial performance [10]. In contrast, applying a high-MW antimicrobial polymer will not only eliminate the ‘leaching-out’ problem, but may also improve the strength properties of papers [9], which is desirable for paper industries to develop innovative products.

It is well known that the recycled fibers contain a substantial amount of impurities originating from the various sources of papers prior to recycling [11]. The strength of papers is reduced by recycling, mainly due to the hornification phenomenon [12]. Therefore, these impurities and the hornification phenomenon impair the antimicrobial and mechanical properties of papers, which diminish their practical application in some paper grades, e.g., food packaging. Due to the shortage of virgin fibers and environmental concerns, the application of recycled fibers is steadily increasing in paper industries, which increases the strong demands for the recycled fibers with improved quality [13–17].

In our previous work, the modification of starch with guanidine polymer (PHGH) was performed; and the optimal modification conditions for achieving the best antimicrobial performance were identified for the papers made from bleached sulfite fibers [18–20]. However, it is unclear whether PHGH-modified starch can achieve similar antimicrobial performance on various fibers and can increase the strength properties of papers made from different fibers. In the present work, the PHGH-modified starch was prepared based on the method established in our previous work, and applied to recycled fibers [19]. The effects of the PHGH-modified starch on the adsorption, antimicrobial and mechanical properties of the papers made from recycled fibers were systematically investigated. Moreover, the antimicrobial performance of such a polymer was evaluated, which has not been conducted previously. The success in antimold will expand the application of such antimicrobial polymers significantly. Similar work was also conducted using bleached sulfite fibers for comparison.

2. Materials and Methods

2.1. Materials

Recycled and bleached sulfite fibers were obtained from Cascades and Tembec, respectively. The bleached fibers were washed with distilled water three times prior to using. To conduct the research under paper mill conditions, we used recycled
pulp as received, to prevent any changes in the pulp properties prior to our analysis. Potato starches, glycerol diglycidyl ether (GDE), Poly(diallyldimethylammonium chloride) (Poly-DADMAC) with a MW of \((1–2) \times 10^5\), Lauria Bertani (LB) broth, LB agar and phosphate-buffered saline (PBS, pH 7.4) were all purchased from Sigma-Aldrich. Anionic polyvinyl sulfate (PVSK) with a MW of \((1–2) \times 10^5\) (97.7% esterified) was provided by Wako. Poly(hexamethylene guanidine hydrochloride) (PHGH) was synthesized according to the procedures described in our previous work [21].

2.2. Preparation of PHGH-Modified Starch

Coupling PHGH onto starch to create branched antimicrobial polymers was carried out according to our previous work [19]. At first, 2.4 g GDE was added drop-wise within 20 min to 200 ml of starch aqueous solution (10%), while the pH was adjusted to 10 using 1 M NaOH. After 30 min of the reaction, 8 g PHGH was added to the solution. The reaction was stopped after 2 h of adding PHGH by neutralizing with HCl.

2.3. Analysis of PHGH-Modified Starch

The grafting percentage of the PHGH on starch was determined using a thermogravimetric analyzer (TGA) SDT Q600 (TA Instruments). The charge density of the PHGH-modified starch was determined using a Particle Charge Detector, Mütek PCD 03 (Herrsching) at neutral pH.

2.4. Adsorption of the PHGH-Modified Starch on Fibers

For the adsorption measurements, different amounts of the PHGH-modified starch solution were added to the fiber suspension. For all the measurements, the concentration of fiber suspension was fixed at 3% (1 g fiber in 33 g solution consisting water and polymer) at neutral pH in 125-ml Erlenmeyer flasks. The flask was kept shaking in a water bath shaker at 40°C and 150 rpm for 1 h. In our previous research, the adsorption of PHGH-modified starch was the highest at 40°C [20]. This temperature is also close to the operating temperature of papermaking. Control samples without pulps were prepared under the same conditions. Then, the pulp fibers were filtered and the supernatants were collected for the adsorption analysis using the PCD titrator. The amount of adsorbed polymer on fiber was then calculated as the difference between the added amount of polymer and the amount of polymer remaining in the solution. Moreover, the surface charge density of fibers was determined via a back titration method [8]. An equivalent to 0.1 g (o.d.) of different fibers was added to 50 ml poly-DADMAC (0.5 mM) and mixed for 2 h. After filtering, the supernatants were titrated using the PCD titrator. The concentration differences in poly-DADMAC solution in supernatants and their corresponding control samples led to quantify the surface charge density of fibers. Three repetitions were conducted to get an average value for each sample.
2.5. Preparations of Antimicrobial-Modified Fibers and Hand-Sheets

Fibers were dispersed in a 2-l three-neck glass flask at 3% consistency and stirred at 40°C for 1 h. Then, various amounts of PHGH-modified starch were added to the fiber suspensions. After mixing for 1 h, the fibers were washed twice with distilled water. Then, the hand-sheets with a grammage of 60 g/m² were made from each fiber suspension according to Technical Association of Pulp And Paper Industry (TAPPI) standard T 205, and the hand-sheets were kept overnight in a conditioning room in accordance with TAPPI T 402 prior to testing. The fines content of fibers were also determined using the Dynamic Drainage Jar (DDJ) according to TAPPI T 261.

2.6. Characterization of Antimicrobial Activities

The antimicrobial activities were tested against the Gram-negative bacteria *Escherichia coli* (*E. coli*, ATCC 11229). To investigate the antimicrobial properties of the PHGH-modified starch, the determination of minimum inhibition concentration (MIC) based on broth dilution, one of the popular antimicrobial techniques used for investigating the antimicrobial properties of polymers, was conducted in an attempt to identify the minimum concentration of modified starch required to deactivate all the bacteria in solutions [19]. Fresh cultured *E. coli* was diluted with LB broth to $10^6$ CFU/ml. Various dilutions of PHGH-modified starch were prepared by adding different amount of LB broth to have 8 ml polymer in solution. 2 ml of each polymer/broth solution was transferred into glass tubes and the remaining was kept as a control sample. Then, 0.2 ml *E. coli* ($10^6$ CFU/ml) was added to the polymer/broth solutions, and seeded tubes were incubated at 37°C for 18 h. The MIC was interpreted as the lowest concentration that could inhibit the visible growth of bacteria.

The shaking flask method (quantitative test) was also used for evaluating the antimicrobial activities of the hand-sheets made from recycled or sulfite fibers treated with the antimicrobial polymer [19]. In this method, 0.10 g paper scraps and 5 ml bacterial culture ($10^6$ CFU/ml) were mixed and shaken at 200 rpm at 37°C for 1 h. After shaking, different dilutions, i.e., $10^{-1}$, $10^{-2}$, ..., $10^{-6}$, were made by adding 1 ml of a bacterial culture to 9 ml PBS. Then, 0.1 ml of this culture was seeded on an agar plate. The plates were put into an incubator at 37°C for over 24 h. The number of colonies was counted and 3 repetitions were made for each sample. The inhibition of cell growth was quantified based on (1):

$$\text{Growth inhibition of cell (\%)} = \left( 1 - \frac{B}{A} \right) \times 100,$$

where $A$ and $B$ are the number of the colonies detected from the control and treated samples, respectively.
2.7. Antimold Activity

The resistance of hand-sheets against fungal growth was determined by a series of antimold tests using Chaetomium globosum (C. globosum) according to TAPPI T 487. Random test specimens were prepared by cutting the 50 mm² of hand-sheets. Each specimen was inoculated with C. globosum and incubated for 2 weeks at 28°C. The resistance was determined by visual examination and the results were reported in the form of percentage of deactivating fungus on the surface of papers.

2.8. Atomic Force Microscope Analysis

To investigate the effect of PHGH-modified starch on E. coli cells, an Atomic Force Microscope (AFM, Nanoscope IIIa, Veeco Instruments) was employed. At first, fresh E. coli cells in LB broth were separated from the broth by centrifuging the bacterial suspension (10⁸ CFU/ml) at 5000 rpm for 1 min. Then, the cells were washed with the buffer solution of PBS twice and re-dispersed in distilled water. The bacterial prepared by this procedure was denoted as fresh E. coli. Treated E. coli was prepared by mixing the fresh E. coli with PHGH-modified starch solution at the concentration just above MIC, which was defined in the section for the characterization of antimicrobial activities. The mixture was shaken for 30 s. Fresh and treated E. coli solutions were dropped on a Silicon wafer (University wafer, South Boston) and air-dried. AFM images were obtained in tapping mode using a silica probe (NP-S20, Veeco Instruments) with settings of 512 pixels/line and 1 Hz scan rate.

2.9. Scanning Electron Microscopy

The images of unmodified and modified C. globosum with PHGH-modified starch, which were prepared for antimold test, were taken using a scanning electron microscope (SEM, JSM-6400, Jeol, Japan). At first, unmodified and modified C. globosum were fixed in 6% glutaraldehyde for 2 h. before washing with sodium cacodylate buffer. Then, they were fixed in 1% OsO₄ for 1 h, washed again with the same buffer solution, and dehydrated in ethanol. Finally, they were critical point dried and gold coated.

2.10. Characterization of Paper Properties

The brightness of hand-sheets was tested according to TAPPI T 452, by employing an optical tester (Technibrite Micro TB-1C). The tensile and tear strengths were measured according to TAPPI T 494, and T 414, respectively, using Lorentzen and Wettre (L&W) tensile and tear testers (Sweden).

3. Results and Discussion

3.1. Adsorption Behaviors of Antimicrobial-PHGH-Modified Starches

TGA analysis showed that the grafting percentage of PHGH on starch was approx. 12 wt%. It was observed in our previous work that by increasing the grafting
percentage of PHGH on starch, the charge density of PHGH-modified starch was increased [20]. The PHGH-modified starch with a charge density of 0.6 mequiv/g achieved the highest amount of adsorption on cellulose fibers, which was applied to the current study. Figure 1 shows the adsorption isotherms of the starch with pended PHGH chains on recycled and sulfite fibers. The driving force for such an adsorption is the electrostatic attraction between the cationic groups of PHGH-modified starch and anionic groups of fibers [5, 20]. Clearly, the antimicrobial polymer adsorbed more on recycled fibers than on sulfite fibers. The back titration analysis showed that the surface charge densities of recycled and sulfite fibers were $-220$ and $-20 \, \mu\text{equiv/g}$, respectively. Compared with sulfite fibers, the relatively high charge density of recycled fibers was claimed to be originated from anionic trashes and other impurities in recycled fibers [11]. The high specific surface areas of recycled fibers could be another reason. In other words, the impurities and anionic trashes increase the total surface areas available for polymer adsorption. The fines content of recycle and sulfite fibers were 21.2% and 7.4%, respectively, which suggests the higher available surface area of recycle fibers, thus leading to the higher adsorption of the antimicrobial polymers.

3.1.1. Effect of PHGH-Modified Starch on the Morphology of \textit{E. coli}

Figure 2 shows the AFM images of untreated or treated \textit{E. coli} with the PHGH-modified starch at the minimum inhibition concentration. Figure 2A–C shows the section images, topography and height, respectively. For fresh \textit{E. coli}, the surface membrane was structured and integrated, and there were no indentations and grooves on cell surface. Also, there were no leaked residues around it. From height and section images, \textit{E. coli} cells showed elliptical with middle high and
end low shape with a height difference of around 200 nm. After treatment with the PHGH-modified starch, the E. coli cell collapsed and the cell membrane was completely destroyed. Significant losses of intracellular components from the bacterial cells caused the membrane to collapse, leading to a reduction in height to 100 nm. This is consistent with our previous findings [19, 21]. The cell membrane of E. coli is composed of proteins and phospholipids. The bacterial membrane is stabilized by sodium and potassium ions and phospholipids in the cell. The PHGH-modified starch replaces the metal ions, binds with acidic phospholipids and induces a phospholipides phase separation, thus damaging the cell membranes [20, 22, 23].

3.2. Antimicrobial Performance of the Recycled Fibers Treated with PHGH-Modified Starch

The antimicrobial testing showed that the MICs of the unmodified and PHGH-modified starches were more than 2000 ppm and about 15.6 ppm, respectively. Such results suggested that the starch pended with PHGH chains significantly increased its antimicrobial performance. Figure 3 shows the growth inhibition of bacteria (%), conducted by the shaking method, versus the adsorption dosage of PHGH-modified starch. As can be seen, by increasing the adsorption of the PHGH-modified starch up to 25 mg/g (approx. 0.3 wt% PHGH, based on fibers), the complete growth inhibition (i.e., 100%) was almost reached. Upon applying PHGH-modified starch up to 25 mg/g, the growth inhibition of the recycled fibers treated with PHGH-modified starch appeared to be slightly lower than that of the treated sulfite fibers. The results are mainly due to the higher impurities in recycled fibers than in sulfite fibers, as described earlier. Overall, the excellent antimicrobial performance of cellulose fibers was achieved by adding the antimicrobial polymer at a very low
dose (20 mg/g of PHGH-modified starch on fibers, whereas the amount of PHGH grafted onto the starch was about 12 wt%). This modification allows the branched PHGH polymers using starch as a core or carrier to be a very promising polymer for rendering various paper products antimicrobial.

### 3.3. Effect of PHGH-Modified Starch on the Morphology of C. globosum

Figure 4 shows the morphologies of unmodified and modified fungi with the PHGH-modified starch observed by SEM. Clearly, the entire body of *C. globosum* can be observed without any further distortion for the untreated sample, while only the spores of *C. globosum* can be found on the surface of the treated sample. Therefore, it is inferred from Fig. 4 that the PHGH-modified starch successfully inhibited the growth of *C. globosum* [24].

### 3.4. Antimold Performance of the Recycled Fibers Treated with PHGH-Modified Starch

Figure 5 shows the percentage of antimold activities for the PHGH-modified fibers versus the amount of PHGH-modified starch applied. As seen, by increasing the adsorption of the PHGH-modified starch up to 25 mg/g (approx. 0.3 wt% PHGH, based on fibers), the growth of fungi was completely stopped, regardless of the type of fibers. The improvement in antimold properties of papers are due to the fact that the adsorbed PHGH-modified starch on fibers did not allow *C. globosum* to grow, as seen in Fig. 4.
3.5. **Effect of PHGH-Modified Starch on Paper Properties**

Typical mechanical and physical properties of the papers made from the recycled or sulfite fibers treated with PHGH-modified starch are listed in Table 1. In terms of the results shown in Fig. 1, by adding 12.5 mg/g of PHGH-modified starches on fibers, which was applied for the physical testing of papers, almost all of the polymers adsorbed on fibers, regardless of recycled or sulfite pulps. The tensile
Table 1.
Properties of papers made from the sulfite and recycle pulps treated with PHGH-modified starch

<table>
<thead>
<tr>
<th>Amount added (mg/g)</th>
<th>Tensile index (Nm/g) SF</th>
<th>Tear index (Nm²/kg) SF</th>
<th>Apparent density (kg/m³) SF</th>
<th>Brightness (% ISO) SF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SF RF</td>
<td>SF RF</td>
<td>SF RF</td>
<td>SF RF</td>
</tr>
<tr>
<td>0</td>
<td>9.1 ± 1.2 27.7 ± 0.9</td>
<td>11.9 ± 0.4 8.8 ± 0.5</td>
<td>510.1 ± 3.2 475.0 ± 6.3</td>
<td>88.2 ± 0.2 40.7 ± 0.1</td>
</tr>
<tr>
<td>12.5</td>
<td>11.3 ± 0.8 33.8 ± 0.1.1</td>
<td>11.2 ± 0.3 8.5 ± 0.7</td>
<td>522.2 ± 2.6 487.7 ± 4.2</td>
<td>84.9 ± 0.4 38.9 ± 0.2</td>
</tr>
</tbody>
</table>

SF, sulfite fiber; RF, recycle fiber.

The tensile and tear indices of papers were affected by the PHGH-modified starch, whereas the tear index was decreased to some extent (Table 1). The changes reflect the improvement in fiber bonding, and are in agreement with previous work [20, 25]. The changes in the tensile and tear indices of papers upon polymer adsorption were comprehensively demonstrated in the literature [9, 26]. There are two main factors influencing the tear index of papers: fiber breakage and bonding breakage. The decrease in the tear index of papers, while the tensile index is increased, has been attributed to the increase in the fiber breakage, which requires less force than the bonding breakage (pulling-out of fibers) in a tear test [9, 25]. Additionally, the apparent density of the papers was increased. The variations in the apparent density were more pronounced in the papers made from the recycled fibers than in those from the sulfite fibers. These results might be due to the retention of more impurities and fines in the papers made from the recycled fibers. However, the brightness of papers was reduced by applying PHGH-modified starch, at a high dosage in particular, regardless of the type of pulps.

To further elucidate how the tensile and tear indices of papers were affected by the PHGH-modified starch, the effect of polymer was investigated in a broad range of dosages (up to 25 mg/g). The variations in tensile and tear indices (%) versus the various amounts of the PHGH-modified starch are shown in Fig. 6. Clearly, an approx. 40% increase in tensile index and 15% decrease in tear index were achieved for the sulfite fibers containing approx. 25 mg/g of the PHGH-modified starch. In one study, the addition of 20 mg/g of cationic starch caused 11.4% increase in the tensile index of papers [1, 27]. In the current system, the enhancement in the tensile strength induced by the PHGH-modified starch appeared to be more pronounced. However, the tear index was reduced by 10%, which is similar to the findings in the literature [1, 27]. It is also of interest to notice that the variations in the tensile and tear indices for the recycled fibers were less significant than those for the sulfite fibers.

4. Conclusions

PHGH-modified starch adsorbed more on recycled fibers than on sulfite fibers. The modification of starch pended with guanidine-based polymers (PHGH) increased its
antimicrobial and antimold activities significantly. With the addition of the starch containing 12 wt% PHGH at the dosage as low as 20 mg/g on fibers, the excellent antimicrobial activities were achieved, regardless of the type of fibers. The AFM images demonstrated that the cell membrane of *E. coli* bacteria was collapsed upon exposed to the PHGH-modified starch at the concentration higher than MIC (15.6 ppm). The SEM images also showed that the polymer inhibited the growth of *C. globosum*. Moreover, upon applying 25 mg/g of PHGH-modified starch on fibers, the tensile index of papers was increased, whereas the tear index was decreased. Therefore, the starch pended with PHGH chains not only renders the recycled fibers antimicrobial and antimold, but also potentially increases the fiber bonding.

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**References**