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Antimicrobial-modified sulfite pulps prepared by in situ copolymerization

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Abstract

Grafting guanidine polymer (PHGH) onto cellulose fibers was conducted via in situ free-radical polymerization using ceric ammonium nitrate (CAN) as an initiator. The optimum reaction conditions were obtained, under which the grafting percentage and the grafting efficiency reached over 20% and 50%, respectively. Atomic force microscopy (AFM) images revealed that the grafted polymer tended to form grains with diameters ranging from 60 to 200 nm. AFM also enabled us to identify the location of the grafts on the surfaces of cellulose fibers by the measurements of the adhesion and attraction forces between a colloid probe and the samples. The cellulose fibers were rendered antimicrobial in the presence of 1.0% (wt) grafted polymer, and an excellent antimicrobial activity (over 99% inhibition) toward *Escherichia coli* was achieved. The AFM results also demonstrated that the antimicrobial mechanism of PHGH is to destroy the membrane of the cells.

Keywords: Cellulose fiber; In situ graft polymerization; Guanidine polymer; AFM; Antimicrobial

1. Introduction

Infection control is of utmost importance in various places, which require a high level of hygiene. Hospitals, pharmaceutical production units, food factories, etc., need to be rigorously disinfected in order to destroy pathogenic microbes (Woo, Mittelman, & Santerre, 2000). To address this purpose, the antimicrobial modification for various raw materials has been extensively developed and applied.

Cellulose is a naturally occurring polysaccharide and the most abundant renewable organic raw material in the world. Its derivatives have many important applications in fiber, paper and packaging industries. To endue this material with antimicrobial property is a current need of society, especially for the products used in the occasions that need a high degree of safety for the civilian population.

The properties of cellulose may be modified by changing the surface chemical structure of fibers. The graft copolymerization method has gained importance in modifying the chemical and physical properties of pure cellulose and was investigated in the last few decades. The graft copolymerization of varieties of monomers onto cellulose has been carried out by different techniques, such as irradiation with ultraviolet light, gamma rays, plasma ion beams, atom-transfer radical polymerization, and by ceric (IV) ion initiation methods. The ceric (IV) ion initiation offers great advantages of forming radicals at cellulose backbone through a single-electron-transfer process to promote grafting of monomers onto cellulose (Casinos, 1994; Guapta & Khandkar, 2002; Mcdowall, Gupta, & Stannett, 1984; Shukla, & Athalye, 1994).

Most of the common antibiotics are designed to affect the metabolism of bacteria. However, a growing number

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of bacterial species show resistance to these antibiotics and cause serious health problems. A promising possibility to overcome these difficulties is the development of a concept to kill bacteria based on physical interactions (Kügler, Bouloussa, & Rondelez, 2005). It has been proved that the chemicals with positively charged groups, such as quaternary ammonium (Thome, Holländer, Jaeger, Trick, & Oehr, 2003) or phosphonium (Kanazawa, Ikeda, & Endo, 1993; Popa et al., 2003), can kill bacteria upon contact. Polycations are the safe alternative to common disinfectants such as formaldehyde, ethylene oxide, chlorine or hypochlorite solutions, iodine, alcohols, phenols, or other compounds and have been widely applied to human life as nontoxic disinfectants or additives.

Guanidine polymer is a kind of water-soluble polycations and has a broad spectrum of activity against both Gram positive and Gram negative bacteria, and low mammalian toxicity. Its mode of action has been extensively studied (Broxton, Woodcock, & Gilbert, 1983; Broxton, Woodcock, Heatley, & Gilbert, 1984; Chawner & Gilbert, 1989). The lethal action of guanidine polymers to bacterial cells is suggested to be based on an irreversible loss of essential cellular components as a direct consequence of cytoplasmic membrane damage. The lethal sequence consists of a series of cytological and physiological changes, some of which are irreversible, which culminate in the death of the cell.

Guanidine polymer can be synthesized by condensation of a guanidine salt with a diamine. In case of the crosslinking reaction and the high melt viscosity, it is difficult to obtain the products with high molecular weight through melting condensation. Zhang, Jiang, and Chen (1999) investigated the melting polymerization of polyhexamethylene guanidine hydrochloride and polyhexamethylene biguanidine hydrochloride. Albert et al. (2003) studied the structure-activity relationships of oligoguanidines. The molecular weight of guanidine polymer they prepared ranged from 1000 to 3000, which are too low to be applied directly in the products of cellulose fibers due to the potential migration problems.

In this paper, we aimed at developing an approach of grafting guanidine polymers onto cellulose fibers via in situ polymerization, visualizing the effect of the grafting on the surface morphology of the fibers, and evaluating the antimicrobial effect after the modification. An important advantage of this novel approach is that the biocidal molecules are attached covalently to the substrates. It will eliminate the problems (e.g., leaching or migration) encountered by the conventional antimicrobial chemicals that are often simply blended or mixed with modified substrates. Ceric ammonium nitrate [$Ce(NH_4)_2(NO_3)_6$] was used as an initiator in the graft copolymerization to maximize the grafting occurring on fibre surfaces. Atomic force microscope (AFM) is the key tool for revealing the images of the modified fibers and identifying the location of the grafted polymers.

2. Experimental

2.1. Materials

The bleached sulfite pulps were supplied by Fraser Papers East Operation, Edmundston, New Brunswick, Canada. The pulp was purified with distilled water for three times. Ceric ammonium nitrate (CAN, 99.99%) and glycidyl methacrylate (GMA) were purchased from Sigma–Aldrich and used as received. Polyhexamethylene guanidine hydrochloride (PHGH) was prepared by condensation polymerization of hexamethylene diamine and guanidine hydrochloride.

2.2. In situ copolymerization experiments

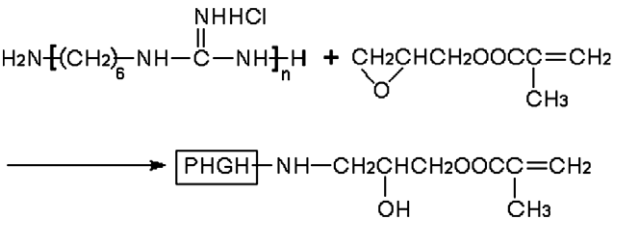
First, the unsaturated double bonds were introduced to PHGH by reacting with GMA (Scheme 1). The molar ratio of amino and epoxy groups is about 0.75–1.5. The reaction was carried at room temperature in aqueous solution for 6 h.

Then a certain amount of sulfite pulp and distilled deionized (DD) water were added into a 250 ml erlenmeyer flask, then stirred and purged by passing nitrogen for 20 min. After that, GMA modified PHGH and CAN were added into the flask. 1 N HNO_3 and 1 N NaOH were used to adjust the pH value of the pulp suspension. The reaction (Scheme 2) was carried out by placing the flask in a water bath at 30–70 °C for 30–180 min. After the desired time had elapsed, the reaction was stopped by adding 0.5% hydroquinone solution. Because the homopolymer of PHGH is water soluble, they can be removed by washing the treated fibers thoroughly with DD water for 3 times. The grafting percentage (GP) and grafting efficiency (GE) were determined as follows:

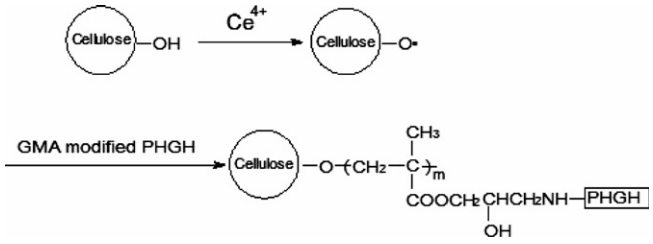
$$\mathrm{GP} = \frac{W_g - W_o}{W_o} \times 100, \quad \mathrm{GE} = \frac{W_g - W_o}{W_p} \times 100$$

Where W_o, W_g and W_p represent the weights of the original fibers, grafted fibers and the added polymer, respectively.

In this work, all the data of GP and GE were obtained without extraction. In fact, three samples were selected and extracted with DD water in a Soxhlet apparatus for 24 h. The maximum weight loss was less than 5%. Herein we simply ignored the effect of extraction, and discussed the results under the same treating conditions.



Scheme 1. Reaction between PHGH and GMA.



Scheme 2. In situ copolymerization of PHGH onto cellulose.

2.3. Molecular structure and element analysis

Attenuated total reflectance–Fourier transform infrared (ATR–FTIR) spectra were obtained with a NEXUS 470 spectrophotometer [Nicolet Thermo Instruments (Canada) Inc.], using a ZnSe reflection element. Elements of the grafted fibers were analyzed by energy dispersive X-ray spectroscopy on a JEOL 2011 scanning transmission electron microscopy.

2.4. Charge density

Charge density of modified fibers was determined via back titration using a Particle Charge Detector MÜtek PCD 03 (Herrsching, Germany). About 0.1 g of pulp suspension (10% fiber consistency) was added into 40 ml anionic polyvinyl sulfate (PVSK) solution (concentration = 0.5 mM). The sample bottle was immersed in a water bath shaker (Innova 3100, New Brunswick Scientific) shaken (150 rpm) at 40 °C for 24 h. Then the suspension was filtrated with a 150 mesh polytetrafluoroethylene screen. The filtrate was diluted to 50 ml (pH ranges from 7.5 to 8.0). Ten milliliter of this solution was added into the measure cell and titrated with standard cationic polyelectrolyte [poly (diallyldimethylammonium chloride) (poly-DADMAC)] (concentration = 1 mM). Three repeats were conducted to obtain an average value for each sample.

2.5. AFM analysis

The atomic force microscope (AFM) is not only a powerful analytical tool to characterize the surface structure with high-resolution images, but also a promising tool for the investigation of interaction and adhesion between two surfaces. Discussion on the different imaging modes and applications of AFM has been detailed in a number of books and review articles (Bottomley, 1998; Ikai, 1996; Takano et al., 1999; Vansteenkiste Davies, Roberts, Tendler & Williams, 1998).

To determine the morphology of the samples, AFM measurements were performed using a Nanoscope IIIa from Veeco Instruments Inc., Santa Barbara, CA. The images were scanned in Multimode mode in air using commercial silicon tapping probe (NP-S20, Veeco Instruments) with a resonance frequency of about 273 kHz. For each sample images were scanned on at least ten different fibers. Usually five different areas of each fibre were investigated. Only representative images are shown.

Although AFM has the ability to obtain high-resolution morphological images, it has apparently not been used to localize grafts on cellulose fibers (Gustafsson, Ciovica, & Peltonen, 2003; Maciel & Christopher, 2002; Niemi & Paulapuro, 2002). In this work, we tried to identify the grafts by measuring the interaction forces or deflection distance (DD) curves between the probe and samples. The measurements were performed in picoforce mode using a contact probe with spring constant 0.32 N/m. To eliminate the effect of the geometry relevant for the interaction zone, "colloid probe technique" (Ducker, Senden, & Pashley, 1991, 1992) was employed. Here, we used a spherical borosilicate particle ($\Phi = 5\mu$) probe, supplied by Novascan Technologies Inc. (2501 North Loop Drive, Ams, LA 50010, USA), to detect the interaction between the probe and samples. The experiments were carried out at room temperature in air and the scan size was 500×500 nm. Within each experiment, the force-distance or deflection distance (DD) curves were acquired on the surface at five different spots and on each spot 20 times at a constant tip velocity of 0.5 μm/s, from which (i.e., 100 readings) the average adhesion and attraction force was determined as follows:

$$F_{\text{attraction}} = k_{\text{tip}}\Delta Z_{\text{jump-in}}, \quad F_{\text{adhesion}} = k_{\text{tip}}\Delta z_{\text{jump-off}}$$

where k_{tip} is the spring constant of the probe, $\Delta z_{\text{jump-in}}$ and $\Delta z_{\text{jump-off}}$ are, respectively, the distance of jump-in or jump-off.

Both borosilicate and cellulose are negative charged materials, and grafted polymer is a cationic polymer. So the attraction and adhesion forces between negative charged tip and cellulose fibers and the tip and grafted polymers are supposed to be obviously different. With the help of AFM, we can determine which area of the sample is the grafted polymer and which area is cellulose.

2.6. Antimicrobial test

The antimicrobial tests were carried out at the Research and Productivity Council (RPC) (Fredericton, New Brunswick, Canada). A shake-flask method was used to quantify the antibacterial activity of cellulose fibers and the modified fibers against *Escherichia coli* (ATCC #25922). Five milliliter of the diluted cell solution was added to a triangle flask (200 ml) and mixed with 70 ml phosphate-buffered saline (PBS, pH 7.2–7.4) and 1.0 g modified or unmodified fibers. The final cell concentration was 1.5×10^6 cells/ml. After the cultivation was shaken (150 rpm) at 37°C for 1 h, 0.1 ml of the cell solution is taken from three different parts in the flask and seeded on three agar plates and incubated at 37 °C for 48 h. The number of the colonies was counted by measuring the colonies formed and compensating with the degree of cell dilution. The inhibition of the cell growth was calculated using the following equation:

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

Where A and B are the number of the colonies detected from the control sample and the wood fiber samples, respectively. Three repeats were conducted to get an average value for each sample.

3. Results and discussion

3.1. In situ copolymerization

The copolymerization was confirmed by the ATR–FTIR spectra and the results from energy dispersive X-ray spectroscopy. Fig. 1 shows the IR spectra of cellulose fibers and grafted fibers. It was confirmed the introduction of PHGH from the adsorption peak at 1633 cm^{-1} due to imino groups and at 1727 cm^{-1} due to carboxyl groups. Fig. 2 shows the energy dispersive X-ray spectroscopy of grafted fibers. The peaks of elements N, and Cl are attributed to the grafted PHGH. It should be noted that the height of peaks is not proportional to the actual constituent owing to the feature of the technique (i.e., weak in quantitative analysis). On the other hand, the peak location of nitrogen is quite near to those of carbon and oxygen, thus creating the overlapped peaks with nitrogen to some extent.

To optimize the reaction time and temperature for better grafting polymerization, the effects of time and temperature on the grafting yields were investigated at 5 different temperatures: 30, 40, 50, 60 and 70 °C, reaction time ranging from 10 to 180 min. The results are shown in Fig. 3. Clearly, both reaction time and temperature are of the vital factors in determining the extent of graft. In general, grafting yield can be increased by increasing temperature. The curves of grafting efficiency have the similar trends to those of grafting percentage. The increase of reaction temperature may lead to multiple effects, such as larger swelling of fibers, increasing the diffusion of PHGH, facilitating redox initiator system, enhancing the chain propagation but likely increasing the rate of termination and homopolymerization in bulk phase as well. For the current systems, the grafting percentage and efficiency increase with the increase of reaction time at first, then decrease. This outcome may be attributable to the degrafting after a certain time. The maximum 14.26% of grafting percentage and 35.64% of grafting efficiency were observed at 70 °C and 30 min reaction time.

The effect of initiator concentration on grafting percentage and efficiency is presented in Fig. 4. In the case of grafting initiated by a chemical initiator, the extent of grafting increases with the increase of initiator concentration up

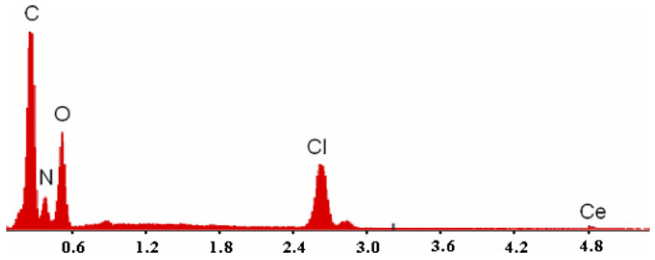


Fig. 2. Energy dispersive X-ray spectroscopy of grafted cellulose fibers.

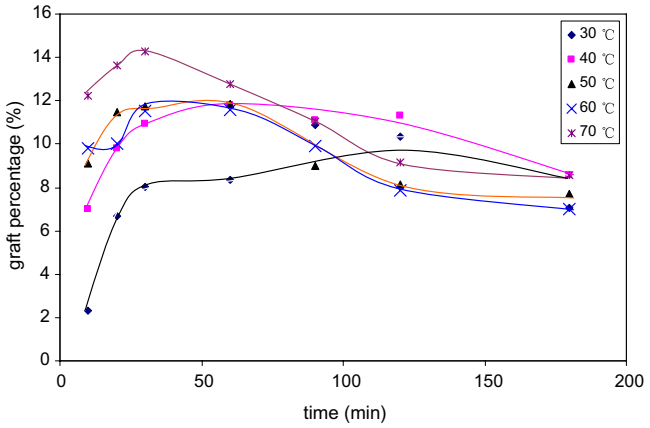


Fig. 3. Effect of temperature and time on the graft percentage of cellulose fibers.

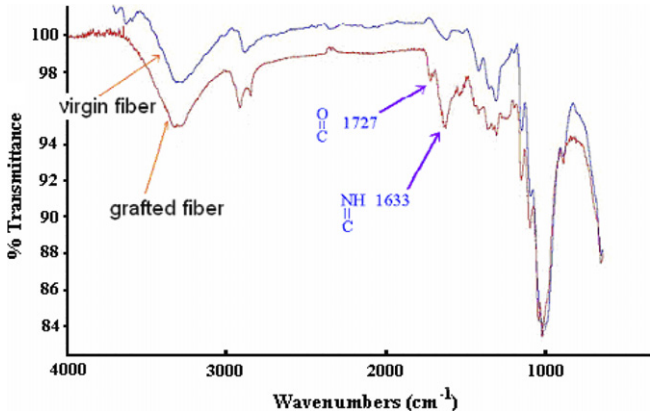


Fig. 1. Infrared spectra of virgin and grafted cellulose fibers.

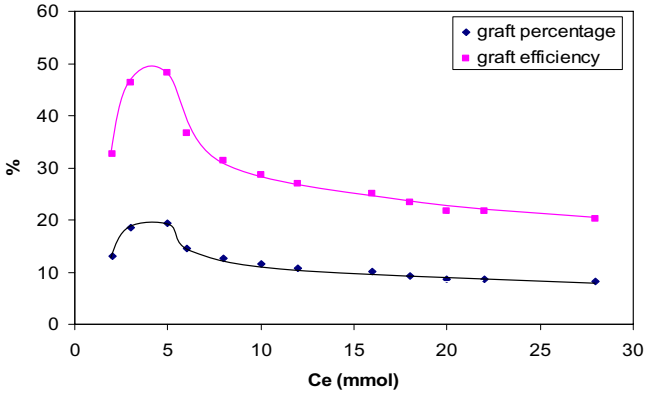


Fig. 4. Effect of CAN concentration on the graft percentage and efficiency of cellulose fibers.

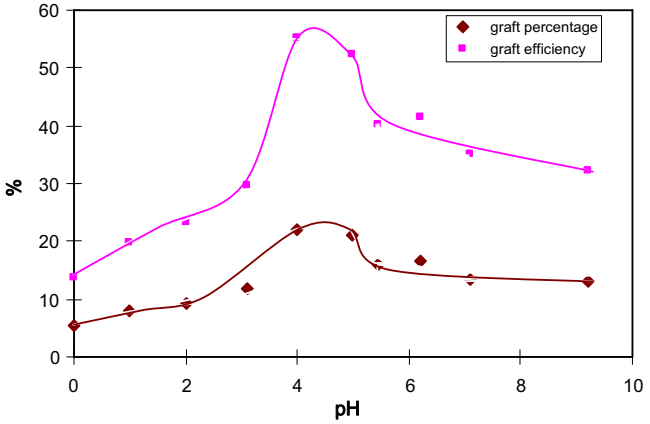


Fig. 5. Effect of pH on the graft percentage and efficiency of cellulose fibers.

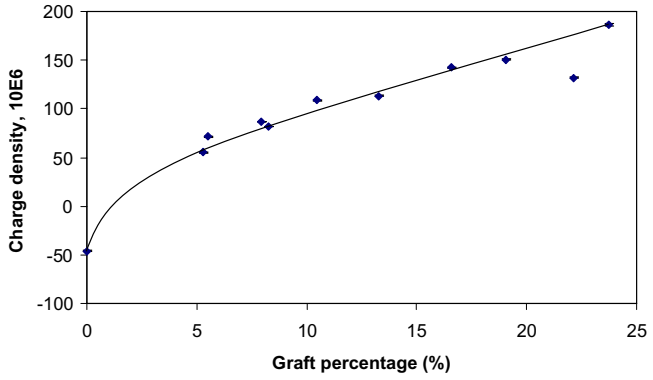


Fig. 6. Charge density of cellulose fibers.

to a certain limit, beyond which grafting will decrease. At a low concentration, the increase of grafting percentage may be due to catalyst exhaustion or an increase in grafting rate. At a high concentration, the decrease of grafting percentage could be due to the decrease in the rate of polymerization. The increasing ceric ions will lead to an increase in cellulose radical termination of growing grafted chains and homopolymerization. As shown in Fig. 4, the highest percentage and efficiency were obtained using the CAN concentration at around 5 mmol. The percentage and efficiency of grafting attained 19.31% and 48.21%, respectively.

The process of graft copolymerization is strongly dependent on the pH of the medium. The effect of pH on the percentage and efficiency of graft is presented in Fig. 5. The use of acids in the grafting reaction improved the graft by causing inter and intracrystalline swelling of cellulose fibers, thus enhancing the macromonomer accessibility. Acid also acted as a catalyst and enhanced the oxidizing capacity of the initiator. At a higher concentration, however, acid might act as an inhibitor for free radicals, thus decreasing the grafting rate. In the most of literatures of graft copolymerization on cellulose fibers using CAN as the initiator, the optimum of pH is around 2. But in this work, the optimum is about 5. Cellulose is often negative-charged. The higher pH, the more negative-charged the cellulose fibers will be. Acrylate and acrylic acid are the most common monomers studied for grafting on the cellulose backbones. They bear the negative-charged ions or groups; and therefore, the low pH assists to decrease the negative-charge density and improves the monomer accessibility. In the current systems, however, cationic macromonomers were employed for grafting. As a result, it is easier for the macromonomer to approach the fibers at a higher pH via electrostatic association. That is the reason why the optimum pH found in this study is higher than that of other reports.

From what we discussed above, the optimum conditions of graft copolymerization obtained are as follows: temperature, 70 °C; pH 5; reaction time, 0.5–1 h; the concentration of initiator, 5 mmol. The grafting percentage and efficiency could reach over 20% and 50%, respectively.

The results in Fig. 6 show the relationship between the charge density and the grafting percentage of the modified fibers. The charge density of virgin cellulose is -45.89×10^{-6} eq/g. After being grafted with cationic polymers, the charge reversal on cellulose fibers occurred, i.e., from negative to positive-charged. The increase in charge follows the same trend of the grafting percentage. The results provide the direct evidence that surface property of fibers has been changed by in situ copolymerization; and also prove that the method we employed to determine the grafting percentage is reliable.

3.2. AFM analysis

AFM was used to image samples in a tapping mode, as shown in Figs. 7–9. AFM revealed a pronounced difference between the virgin and grafted cellulose fibers. It can be clearly seen the fibrillar structure of the virgin fiber surface. The random orientation of the cellulose microfibrils (CMFs) indicates they belong to the primary layer of the fiber. The diameter of the CMFs ranges from 12 to 56 nm. After graft copolymerization, the surface of fibers appears to be covered with the granules. The size of these granules varies between 60 and 200 nm. Beside the granules, linear oriented part can be seen in Fig. 8. The similar structure could not be found in the virgin sulfite fibers even though nearly one hundred of sample points were tested by AFM. To confirm the relationship between this structure and the graft reaction, another experiment was conducted. Fibers were treated at the same condition as the grafted samples without PHGH in the reaction system, only the cellulose fibers and initiator. The linear oriented part can also be readily found, as shown in Fig. 9. Part of out-layers is likely destroyed during graft copolymerization, having the inner layer exposed. This is similar to the effect created by high shear force in mechanical pulping process.

Of great interest is that there are few granules on the linear oriented area. To identify the component in each

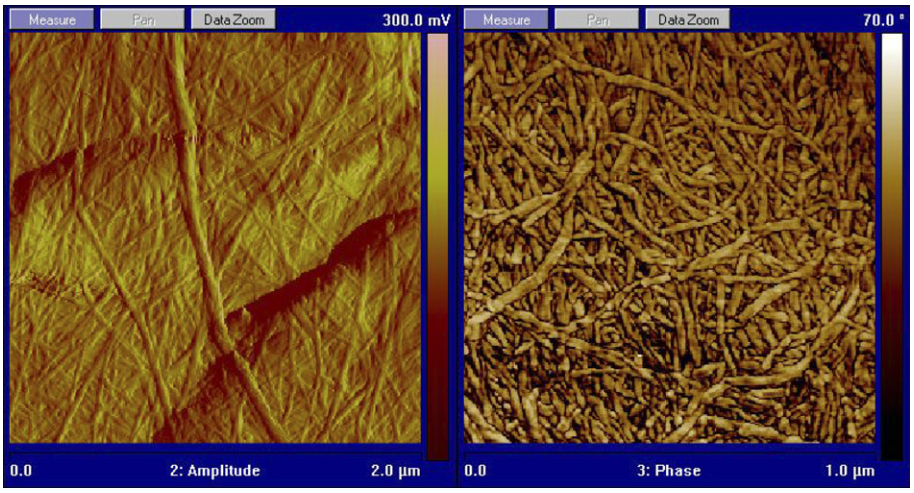


Fig. 7. AFM images of virgin fiber.

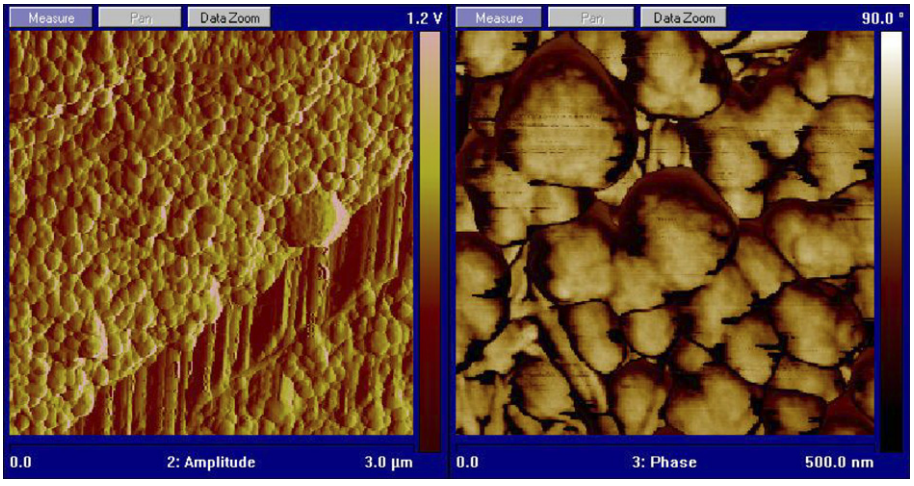


Fig. 8. AFM images of grafted fiber.

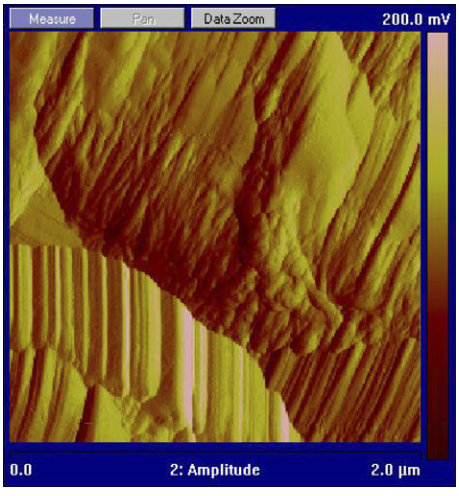


Fig. 9. AFM images of control sample. (Fibers were treated at the same condition as the grafted samples, but there was no GMA modified PHGH in the reaction system, just the cellulose fibers and initiator.)

region, AFM was used to localize the grafts in the cellulose fibers by measuring the adhesion and attraction forces between a colloidal probe and the fiber samples. The results are listed in Table 1.

Theoretically, the attraction and adhesion forces between the materials with opposite electrostatic charges are much stronger than those between the materials with same kind of electrostatic charges. Both of the glass probe and cellulose fibers are negative charged material, while grafted polymer is positive-charged. Typical AFM force curves are shown in Figs. 10 and 11. From Table 1, it can be found that the forces of virgin fiber and oriented area of grafted fiber are almost the same, which are much weaker than those of granular area of grafted fiber. The attraction and adhesion forces of the later were increased by about 15 and 4 times, respectively. The more profound difference occurs in the attraction forces, which are usually due to the electrostatic attraction and surface tension forces. There is also a great difference of the standard deviations of the force between the granular area and the virgin

Table 1
The adhesion and attraction force between the colloid probe and cellulose fibers

Sample	Adhesion force		Attraction force	
	Average (nN)	S* (nN)	Average (nN)	S* (nN)
Virgin fiber	81.9	9.4	3.5	0.3
Orient area of grafted fiber	80.2	10.3	3.5	0.3
Granular area of grafted fiber	320.6	153.5	53.3	26.3

* S represents the standard deviation.

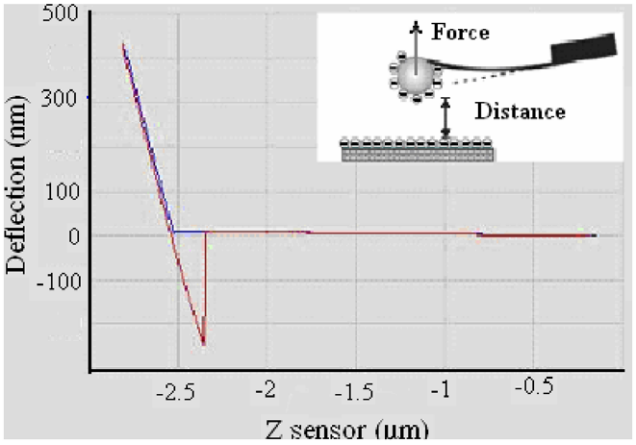


Fig. 10. Typical AFM force curve of virgin fiber or orient area.

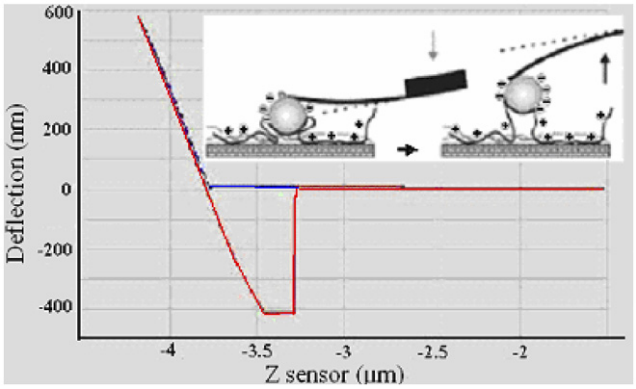


Fig. 11. Typical AFM force curve of grafted fiber.

Table 2
Antimicrobial activity of modified fibers against *E. coli*

Sample	Concentration of PHGH (wt%) (based on fibers)	Growth inhibition of cells compared to sample 1 after 48 h incubation (%)
Control*	0	
1	0	−95.97
2	0.5	88.85
3	1.0	99.86
4	2.0	99.99
5	4.0	100

* There were no cellulose fibers in the control sample. The control sample is normally used to determine how fast the bacteria are diluted out since they are growing at optimal conditions.

fibers or the oriented area of grafted fibers. Apparently, the uniformity of the granular area is much less than the others. The results imply that the grains are the grafted polymer, and the linear oriented area is composed of crystal cellulose microfibrils.

From the results of AFM, both images and force analysis, the morphology changes were obvious after graft copolymerization, and the location of grafted component could also be identified. The grafted polymer appears to form grains whose diameters range from 60 to 200 nm. The graft copolymerization more likely took place in amorphous areas (interphase of the CMFs) instead of crystal areas. That is why there are few granules on the

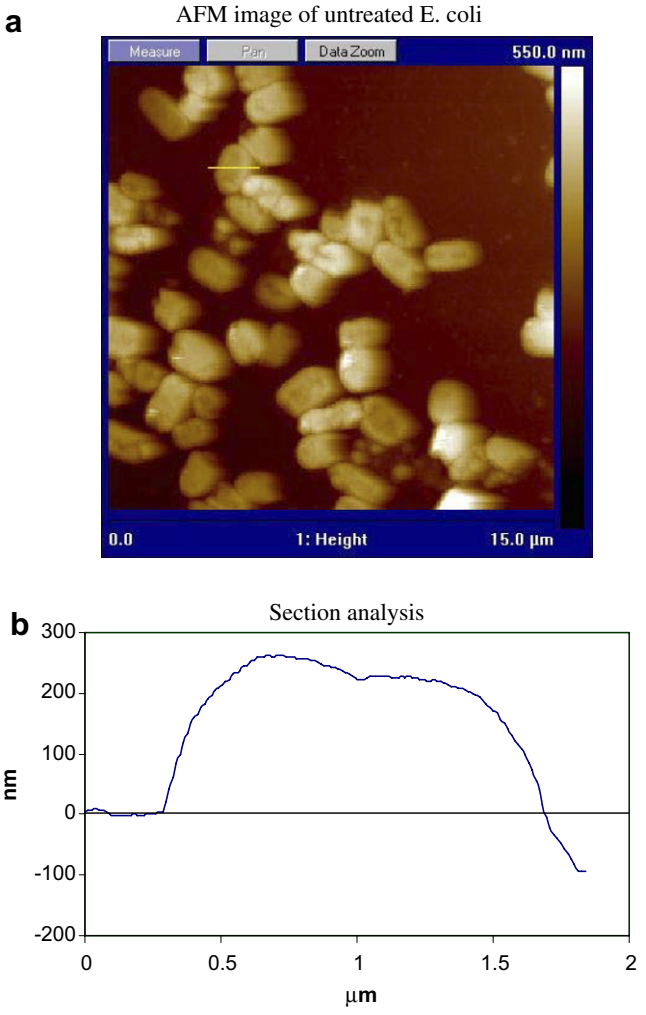


Fig. 12. AFM images of untreated *E. coli* (ATCC 11229).

linear oriented area. AFM provides the direct evidences of this view.

3.3. Antimicrobial test

The antimicrobial activity of the modified cellulose fibers containing various amount of PHGH against *E. coli* is shown in Table 2.

As can be seen from Table 2, the growth inhibition of sample 1 is −95.97%, which means the colony number of sample 1 is almost twice as much as that of control sample. This suggests the cellulose fibers might act as nutrient for the bacteria. In the presence of 1.0% (wt) grafted polymer in fibers, an excellent antimicrobial activity (over 99% inhibition) has been achieved. In the current test, the modified fibers were mixed with the cultivation only for 1 h, which indicates that the effective inhibition has been rapidly reached.

AFM was also used to investigate the antimicrobial mechanism, as shown in Figs. 12 and 13. After treated with 0.5% PHGH solution, the membrane of the cell was not intact and there were some clefts generated. For some cells, only part or fragments of membrane left. The membrane damage caused the leakage of low molecular weight cytoplasmic components and lead to the collapse of the cells. It is convinced by the section analysis. The height dropped from over 260 nm to less than 50 nm. These results clearly demonstrated that the antimicrobial mechanism of PHGH is to destroy the membrane of the cells.

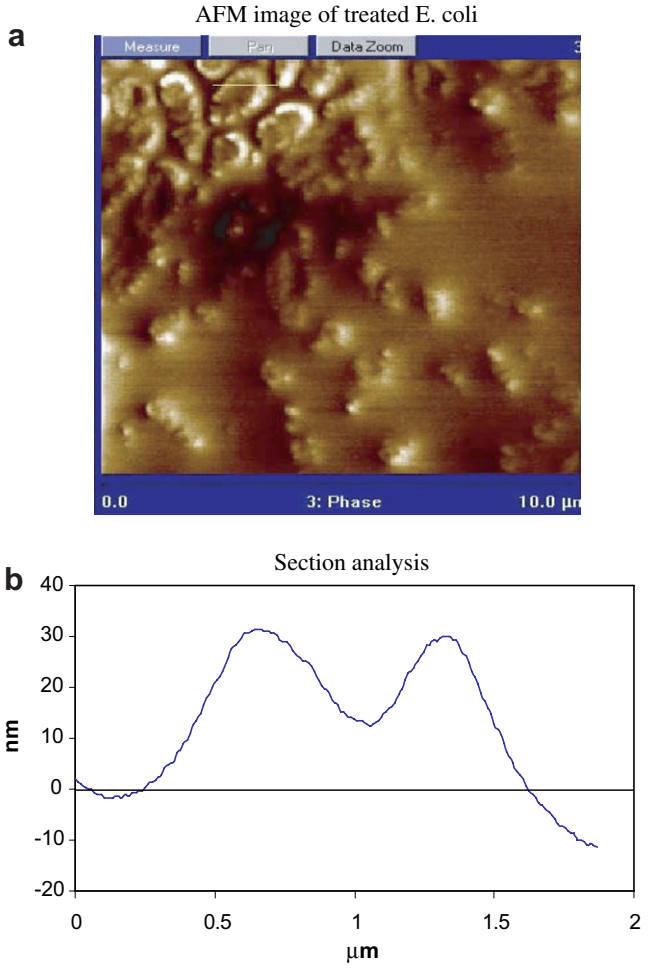


Fig. 13. AFM images of 0.5% PHGH solution treated *E. coli* (ATCC11229).

4. Conclusions

In situ graft copolymerization of GMA modified PHGH onto cellulose fibers (bleached sulfite softwood) was successfully conducted using CAN as an initiator. The optimum conditions of the reaction were obtained by a series of experiments. The grafting percentage and efficiency could reach over 20% and 50%, respectively.

The presence of grafted polymer on the grafted fibers was demonstrated by ATR–FTIR and energy dispersive X-ray spectroscopy. AFM images with high resolution revealed that the grafts appear to form the grains with diameters ranging from 60 to 200 nm. Part of out-layers of fibers was destroyed during graft copolymerization and the inner layer exposed. AFM was also successfully employed to localize the grafts in the cellulose fibers by measuring the adhesion and attraction forces between a colloid probe and the samples.

Cellulose fibers grafted with PHGH exhibited high antimicrobial activity against *E. coli*. In the presence of 1.0% (wt) grafted PHGH in fibers, the growth inhibition reached over 99%. The AFM results also demonstrate that the antimicrobial mechanism of PHGH is to destroy the membrane of the cells.

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