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Contact Antimicrobial Surface Obtained by Chemical Grafting of Microfibrillated Cellulose in Aqueous Solution Limiting Antibiotic Release

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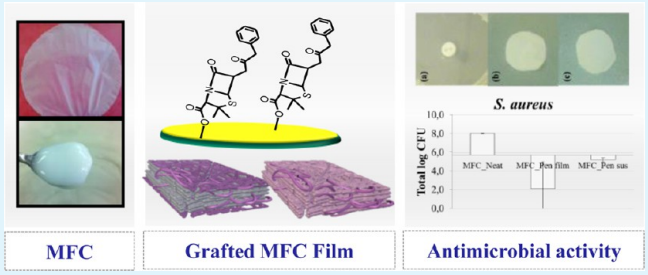
ABSTRACT: Contact active surfaces are an innovative tool for developing antibacterial products. Here, the microfibrillated cellulose (MFC) surface was modified with the β-lactam antibiotic benzyl penicillin in aqueous medium to prepare antimicrobial films. Penicillin was grafted on the MFC surface using a suspension of these nanofilaments or directly on films. Films prepared from the penicillin-modified MFC were characterized by Fourier transform infrared spectroscopy, contact angle measurements, elemental analysis, and X-ray photoelectron spectroscopy and tested for antibacterial activity against the Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. Penicillin-grafted MFC films exhibited successful killing effect on Gram-positive bacteria with 3.5-log reduction whereas bacteriostatic efficiency was found in penicillin-grafted MFC suspension. The zone of inhibition test and leaching dynamic assay demonstrated that penicillin was not diffused into the surrounding media, thus proving that the films were indeed contact active. Thus, penicillin can be chemically bound to the modified substrate surface to produce promising nonleaching antimicrobial systems.



KEYWORDS: *microfibrillated cellulose, benzyl penicillin, chemical surface modification, esterification, X-ray photoelectron spectroscopy, contact-active antimicrobial surfaces*

INTRODUCTION

Properties such as biodegradability, biobased, hydrophilic character, mechanical strength, and biocompatibility as well as the broad possibility for chemical modifications makes cellulose an important and fascinating polymer for many applications in fields ranging from engineering to medicine.[1] Researcher's team at ITT Rayonier Inc. developed a novel type of cellulose material called microfibrillated cellulose (MFC)[2,3] that is produced by the mechanical disintegration of the wood pulp. This mechanically produced nanocellulose is different from cellulose nanocrystals, obtained by acidic treatment, which are stiff and short.[4] MFC possesses several interesting properties, such as high expanded surface area, flexibility, high barrier, lightweight, and very high aspect ratios. The hydroxyl groups in its molecular structure can be employed for chemical modification of the surface (when using nonswelling conditions).[5] Such modification aims to increase the compatibility of MFC with other materials as well as develop a variety of value-added advanced materials.[6,7] MFC and functionalized MFC have been exploited for various innovative processes such as composite[8,9] rheology modifier,[3] fuel cells,[10] and emulsions.[11] In the past few years, in addition to these applications, new areas such as biomedical,[12] controlled drug delivery,[13] and antimicrobial surfaces[14] have been explored as well.

The need for functional materials and surfaces such as antimicrobials is increasing. In food packaging and for medical applications, noncontaminated materials are needed to improve the shelf life of food and to limit the spread of nosocomial diseases. In both cases, release and spread of active molecules should be avoided in order to maintain organoleptic food properties, limit resistance against bacteria, or both.[15]

In this study, antibiotic benzyl penicillin was chemically grafted on MFC in aqueous solution. Benzyl penicillin is the β-lactam antibiotic produced by the *Penicillium* species in the form of secondary metabolite.[16] It is a narrow spectrum antibiotic that is mainly active against Gram-positive bacteria. The penicillins attach to the enzyme receptors, called penicillin-binding proteins (PBPs), that are located in cytoplasmic membrane. By binding covalently with PBPs, penicillins interfere with the peptidoglycan production, resulting in osmotic instability and lysis of the bacteria. The mechanism of bacterial destruction might involve triggering of an autolytic process in Gram-positive organisms.[17] Utilization of β-lactam antibiotics have been, however, decreased due to the emergence of antibiotic resistant microorganisms. The continuous use of

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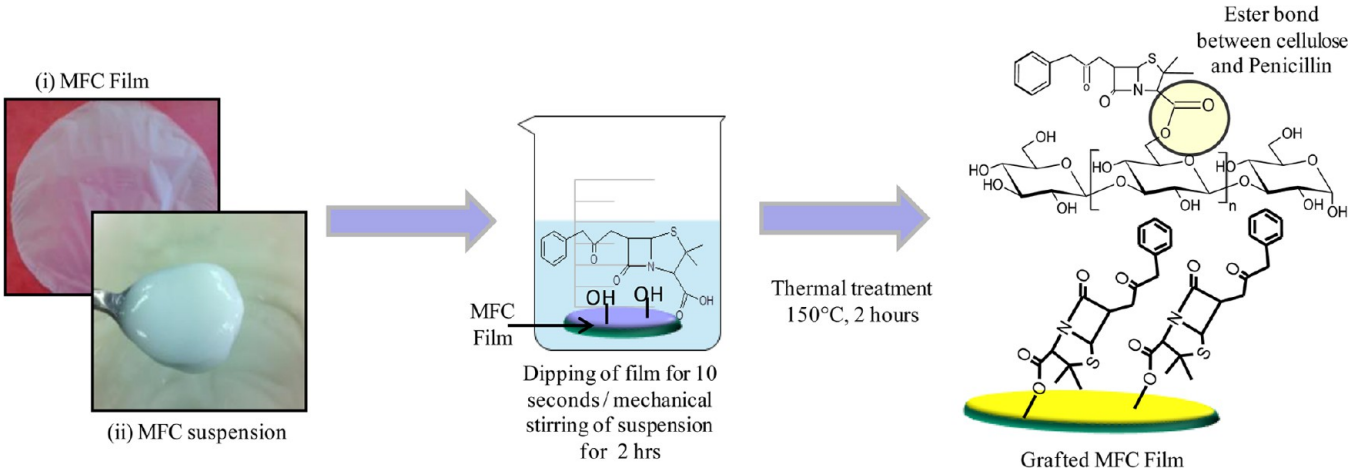
Scheme 1. Schematic Illustration for the Esterification of Microfibrillated Cellulose Using Benzyl Penicillin As an Active Agent to Prepare Contact Active Antimicrobial Surfaces



antibiotic induces a change in the gene expression of the microorganism, which leads to the production of new enzymes such as β lactamase and penicillinase. These enzymes hydrolyze the β-lactam ring, rendering the antibiotic ineffective.[18] Benzyl penicillin was selected among available antibiotics due to the presence of a carboxyl group and less steric hindrance that make it readily available for grafting. In addition, this antibiotic is widely used in hospitals and also have lower minimum inhibitory concentration which made it a suitable candidate for production of antimicrobial surfaces.[17] However, today some legislation tends to limit the use of this molecule family.[19] Therefore, our objective was to limit the use and the release of this molecule (penicillin) at the surface.

For this work, esterification was performed using a simple, convenient, and solvent free method at high temperature. The hydroxyl group of MFC forms ester bonds with carboxylic acid at the C3 position of the β-lactam ring of penicillin, as shown in Scheme 1. A large amount of data is available on cellulose and its fibers, but only a few studies dealing with the esterification of the MFCs surface can be found in the literature.[20,21] In a previous study, the researchers used a heterogeneous catalytic method in which the final product was used in nanocomposite applications to enhance interfacial adhesion with matrix like poly(lactic acid).[22] or poly(propylene carbonate).[23] In another study, researchers used gas-phase esterification on NFC films with trifluoroacetic acid anhydride (TFAA) and acetic acid (AcOH) at 2 ratios (1:2 and 2:1) to prepare hydrophobic nanofibers.[24] In yet another study, the authors modified the bacterial cellulose nanofibers on their surface with acetic acid, hexanoic acid, or dodecanoic acid using *p*-toluenesulfonyl chloride as a catalyst in pyridine under nitrogen medium[25] to prepare oil in water emulsion stabilized by grafted nanofibers.[26]

In the literature, all the studies on esterification were performed using different solvents with strategies not adaptable for penicillin. Moreover, their prime objective was to increase the hydrophobic character of nanocelluloses, in order to obtain a homogeneous distribution and enhanced interfacial compatibility in the nanocomposites.[27] Indeed, this research used an esterification process for the preparation of antimicrobial surfaces by using specifically an aqueous solution.

This study focuses on using a novel and simple esterification reaction to covalently link benzyl penicillin to the surface of cellulose microfibrils to provide surface-only antibacterial activity. To the best of our knowledge, this is the first attempt to graft antibiotic on the surface of microfibrillated cellulose.

MATERIALS AND METHODS

Materials. For the preparation of nanocellulose, high quality bleached softwood cellulose (Domsjo, Sweden) was produced from a controlled mixture of spruce and pine (60% and 40%, respectively). Microfibrillated cellulose was produced on a pilot scale (CTP, France). According to the protocol developed during the SUNPAP project, MFC was isolated from sulfite softwood-dissolving pulp (Cellulose Plus, Domsjö, Sweden) using refining followed by enzymatic pretreatments and then mechanical treatment in a homogenizer (Ariete NS3075, GEA Niro Soavi, Italy). Five passes (one at 1000 bar and four at 1450 bar) were successively performed through the homogenizer to produce a homogeneous and gel-like MFC suspension with 2% (weight/weight%) consistency.[28] Benzyl penicillin (Sigma-Aldrich), in the form of sodium salt, was converted into benzyl penicillin before the experiments. Ethanol and acetic acid (Chimie Plus) and deionized water were used for all the experiments.

Staphylococcus aureus ATCC 6538 and *Escherichia coli* ATCC 10536 were provided by DSMZ, Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures). Frozen bacteria were maintained (−80 °C) and transferred onto PCA (plate count agar) composed of 5 g/L tryptone, 2.5 g/L yeast extract, 1 g/L glucose, and 9 g/L neutralized bacteriological agar on a monthly basis. A suspension of *Bacillus subtilis* (10^7 spores/mL) was purchased from Humeau (France) and used as the test microorganism to evaluate antibacterial activity. Spores were revived by growing in the nutrient broth for 16 h. A nutrient agar adapted for the development of the spores was also purchased from Humeau (France). The agar powder was dissolved in the deionized water to reach a final concentration of 28 g/L and then boiled to obtain a clear nutrient agar solution. Petri dishes with a diameter of 90 mm were purchased from Humeau (France) and used for the antibacterial tests.

Methods. *Preparation of the Penicillin Grafted MFC Films.* First, 0.1 g/mL of sodium penicillin solution was prepared at pH 4 using 0.1 M acetic acid. An MFC film was prepared using a classic handsheet former. MFC was filtered on nitrocellulose membranes having a pore size of 65 μm at 600 mbar pressure for 2 min under vacuum. Drying was performed using pressure dryers (Karl Frank GMBH) at 80 °C for 15 min. After that, MFC film (~0.2 g) was immersed in the prepared solution for ~10 s at 25 °C. The film was cured at 150 °C under flowing air to covalently graft the penicillin onto the surface of MFC films. The cured MFC film was subjected to Soxhlet extraction with ethanol for at least 12 h before any characterization.

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Further, 0.1 g/mL of sodium penicillin solution was mixed with MFC at 0.5% concentration (w/w); the pH was reduced to 4 using 0.1 M acetic acid. The suspension was stirred for 2 h followed by filtration and thorough washing with distilled water, to remove the physically adsorbed penicillin. Film was casted with the penicillin treated MFC and was cured at 150 °C to covalently graft the penicillin onto the surface of MFC films. The cured MFC film was subjected to Soxhlet extraction with ethanol for at least 12 h before any characterization.

Quantitative Assessment of Antimicrobial Activity. The quantitative assessment of antibacterial activity against *E. coli* and *S. aureus* was based on the AATCC Test Method 100-1998 performed under static conditions. Unmodified and modified nanocellulose was dry sterilized at 70 °C for 24 h before antimicrobial assay. All solutions were autoclaved at 121 °C for 20 min at 15 psi. The cell suspensions prepared with 20 mL of nutrient broth were grown overnight at 37 °C, under horizontal shaking at 100 rpm. An aliquot of 200 μL of cell suspension was then placed onto at least 2 replicates of each sample (modified and unmodified surfaces). After 24 h of incubation at 37 °C, the bacteria were extracted using 50 mL of neutralizing solution: 3 g/L L-α-lecithin, 5 g/L sodium thiosulfate, 1 g/L L-histidine, 30 g/L Tween 80, and 10 mL/L of a buffer solution (KH_2PO_4 0.68 g/L) (pH 7.2 ± 0.2). The numbers of colony forming units (CFU) within the resulting suspensions are then enumerated using the pour plate count method. The bacteria log reduction, i.e., the antibacterial activity of the films, was calculated according to the following formula:

$$\log \text{reduction} = \log \text{CFU T24 untreated sample} - \log \text{CFU T24 treated sample} \quad (1)$$

All experiments were repeated twice ($n = 2$).

Assessment of Antimicrobial Releasing Activity. This protocol was based on the standard AFNOR EN 1104 procedure. It consists of placing the samples to be tested, onto preinoculated agar (with *Bacillus subtilis*) followed by incubation for 3 days at 30 °C. The leaching ability of modified MFC was concluded, by the presence of zone of inhibiton. All experiments were repeated thrice ($n = 3$).

In total, 100 mg of each dried sample was taken in a closed Erlenmeyer flask and 10 mL of 1/500 nutrient broth was added to each unmodified and modified MFC and placed on the shaker for 24 h at 30 °C. After 24 h, the samples were filtered using glass filter paper to remove fibers. Bacteria (10^4 CFU/mL) were added to the filtrate and the samples were incubated in an orbital shaker overnight at 30 °C. The numbers of colony forming units within the filtrate were calculated using the pour plate method. All experiments were repeated twice ($n = 2$). Characterizations of MFC were performed by the following methods.

Infrared Spectroscopy (FTIR). Infrared spectra were recorded for neat and modified MFC in ATR mode, using a PerkinElmer Spectrum 65. All spectra were recorded between 4000 and 600 cm^{-1}, with a resolution of 4 cm^{-1} and 16 scans. For each sample, at least 5 spectra were obtained, and FTIR spectra shown in the figures are representative of the samples.

Elemental Analysis (E.A.). Elemental analysis was carried out by the "Service Central d'Analyse (Vernaison France)" of the "Central National de la Recherche Scientifique" (CNRS). Carbon, hydrogen, nitrogen, and sulfur contents were measured for unmodified and modified MFC. For each sample, duplicates were performed and the values were averaged. The overall degree of substitution was calculated on the basis of the carbon content of the neat and the grafted MFC, using the following eq 2:

$$\text{DS} = \frac{(\text{Mx in cellulose} - x\%(\text{mass of cellulose}))}{(\text{mass of grafted molecule} \times x\% - \text{Mx in grafted molecule})} \quad (2)$$

where Mx is mass of carbon in cellulose (72.07), mass of anhydrous cellulose (162.14), molecular mass of penicillin (334.39), and mass of carbon in penicillin (156.13). The DS corresponds to the number of grafted hydroxyl function molecules per anhydroglucose unit within the bulk of material.

Contact Angle Measurement. Contact angle measurements were performed by depositing 5 μL of water droplets on the surface of the MFC and angles obtained were recorded using an OCA dataphysics system, equipped with a CCD camera. The contact angle acquisition was done by static sessile method during the first 60 s after deposition. All the measurements were performed on dried film and at least 4 times for each sample. Average values are presented in this study.

Thermogravimetric Analyses (TGA). A PerkinElmer simultaneous thermal analyzer (STA 6000) was used. About 30 mg of sample was placed in a pan and tested at a heating rate of 10 °C/min from ambient temperature to 900 °C. All experiments were performed at least twice.

X-ray Photoelectron Spectroscopy (XPS). X-ray photoelectron spectroscopy was carried out using the PHI Quantera SXM. Samples were placed in an ultrahigh-vacuum chamber (10^{-8} mbar), with collection of electrons by a hemispherical analyzer at an angle of 90°. Signal decomposition was determined using Spectrum NT, and the overall spectrum was shifted to ensure that the C−C/C−H contribution to the C_{1s} signal occurred at 285.0 keV. Comparison of the elementary surface composition was performed using eq 3:

$$\frac{O}{C} = \left(\frac{I_1}{I_2}\right) \times \left(\frac{S_1}{S_2}\right) \quad (3)$$

where I_i is the intensity of signal i (carbon, oxygen, or nitrogen) and S_i (S_C = 0.00170, S_O = 0.00477, and S_N = 0.00299) denotes the atomic sensitivity factor whose values were calculated from eq 4:

$$T_1 = \frac{1}{(E_i^{\text{kin}})^{0.7}} \quad (4)$$

with T_i, λ_i, and σ_i being the transmission energy, the electron inelastic mean free path, and the photoionization cross section for the X-ray source, respectively. T_i depends on the atomic kinetic energy E_i^{kin} (eV) with E_C^{kin} = 966.6 eV, E_O^{kin} = 722.6 eV, and E_N^{kin} = 851.6 eV. The Penn algorithm was used to calculate the electron inelastic mean free path λ (λ_C = 2.63 nm, λ_O = 2.11 nm, and λ_N = 2.39 nm) and the values were taken from Scofield[29] (σ_C = 1, σ_O = 2.85, and σ_N = 1.77). XPS data could be used to determinate the DS of the surface (DSS), taking into account the first surface layers. For the calculation of DSS, several methods can be considered, but the most common one is based on that reported by Goussé et al.,[30] who defined the DSS (based on the amount of nitrogen) as shown in eq 5:

$$\text{DSS} = \frac{M_{\text{AGU}} \times x}{(100 \times M_C) - (M_{\text{group\_grafted}} \times x)} \quad (5)$$

where M_{AGU} is the molar weight of one anhydroglucose unit (162.14 g mol^{-1}); M_C, the molar weight of the carbon atom (12 g mol^{-1}); $M_{\text{group\_grafted}}$, the molecular mass of the grafted moieties (334.39 g mol^{-1}); and x, the mass concentration of carbon. XPS was performed on the dried film for unmodified and modified fibers.

Atomic Force Microscopy (AFM). Neat MFC and grafted MFC were imaged using an atomic force microscope (AFM; Nanoscope III, Veeco, Canada). Films were glued to the steel plates and dried overnight under room conditions in order to have adhesion between the plate and the film. Each sample was characterized in tapping mode with a silicon cantilever (OTESPA, Bruker) at different locations. Images were subjected to the first order polynomial flattening in order to reduce the effects of bowing and tilt.

Scanning Electron Microscopy-Electron Dispersive X-ray Spectroscopy. Chemical characterization in the SEM was performed using the nondestructive energy dispersive X-ray analysis (EDX) (Jeol JSM 6400) with a silicon drift detector. The electron beam stimulates the atoms in the sample with uniform energy, and they instantaneously emit X-rays of specific energy for each element, which provides information about the elemental composition of the sample. SEM-EDX was conducted to investigate the sulfur and nitrogen distribution in the neat and grafted films. A carbon conductive coating was done on the samples to allow the use of high voltage (15 kV) for X-ray spectra and X-ray mapping. An accelerating voltage of 10 kV was used during the 600 s of the measurement.

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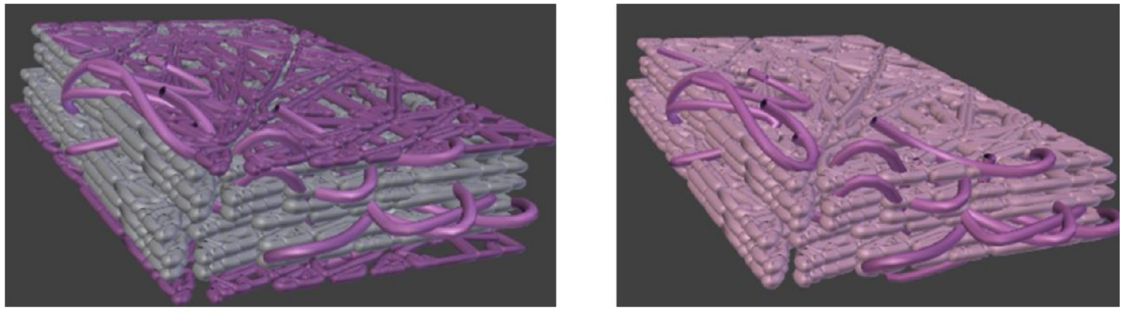

Figure 1. Pictorial representation for the two strategies for modification: (a) on the surface of MFC film and (b) grafting on the fiber surface.

Table 1. Penicillin Weight, Contact Angle, And Thermogravimetric Analysis for Neat and Grafted MFC

			penicillin mass		contact angle			thermogravimetry
	grammage (g/m^2)	thickness (μm)	before extraction	after extraction	before curing at 150 °C	after curing at 150 °C	after Soxhlet extraction	after Soxhlet extraction
MFC_Neat	69.1	50 ± 1					52° ± 4	300
MFC_Pen film	66.8	44 ± 2	8.7 ± 2	5.9 ± 2	40.2 ± 2	32.7 ± 1	89° ± 10	246
MFC_Pen sus	31.4	30 ± 2	ND	ND	45.8 ± 9	61.0 ± 9	93° ± 10	278

RESULTS AND DISCUSSION

The main objective of this study was to obtain nonleaching antimicrobial films having higher quantities than the minimum inhibitory concentration. Therefore, grafting an active molecule on the high surface area of MFC was our solution for this. Figure 1 describes the two ways of performing the grafting of penicillin on the surface of MFC. To the best of our knowledge, only a few authors have followed the same strategy before but they have mainly worked with silanes and bacterial cellulose[6,14a,12b]. Bacterial cellulose is highly pure cellulose but it is difficult to produce on the industrial scale and, in addition, the films obtained are barely redispersible for further conversions (like coating). In this study, the active molecule selected was penicillin because of its high activity toward a wide range of bacteria and, most important, possible esterification in water.

MFC Grafting with Penicillin. During the esterification process, the carboxyl group of benzyl penicillin reacts with the hydroxyl group on the surface of MFC, resulting in the formation of antimicrobial MFC. MFC film was dipped into the penicillin solution to absorb the active molecule. In MFC suspension, first penicillin was adsorbed onto the MFC fibers before formation of the film. When the modified samples were heated, the covalent bonding between MFC and benzyl penicillin may occur.

Table 1 depicts the increase in the weight of the MFC film after grafting. Before Soxhlet extraction, 8.7% of penicillin uptake was observed in the dried film. After Soxhlet washing with ethanol for 12 h, the average weight of the penicillin grafted on the MFC film was reduced to 5.9%. This decrease in the weight corresponds to the removal of all the physically adsorbed and nongrafted penicillin onto the MFC film surface. Besides, the thermal stability of neat and modified MFC was assessed by thermogravimetry (Table 1). The neat MFC exhibited the typical thermal degradation profile of cellulosic substrates, with a maximum decomposition rate around 370 °C. Degradation temperature of MFC_Neat, MFC_Pen Film, and MFC_Pen sus started at 300, 246, and 278 °C respectively. Reduction in degradation temperature was the consequence of the modification of MFC. Indeed, the initial degradation temperature is lower when penicillin was grafted on the film then in MFC suspension.

It is well-known that MFC is hydrophilic. This is depicted by the contact angle measurement in Table 1. The esterification process reduces the water wettability considerably and renders the film surfaces hydrophobic[31,28]. Water contact angle was examined before and after the Soxhlet extraction. Contact angle of MFC_Neat, MFC_Pen Film, and MFC_Pen sus was 52 ± 4°, 89 ± 10°, and 93 ± 10°, respectively. The apparent increase of the penicillin-modified samples is clear evidence of their surface modification. Furthermore, before Soxhlet extraction, contact angles of grafted MFC (32.7 ± 1° for MFC_Pen film and 61.0 ± 9° for MFC_Pen sus) were lower than those of the neat MFC (52 ± 4°) due to the presence of free penicillin (confirmed by the penicillin mass difference before and after extraction), while after Soxhlet washing, all the ungrafted penicillin was removed leaving only the grafted MFC with higher contact angle.

Qualitative Analysis of Grafted MFC Films. FTIR spectra was analyzed to compare microfibrillated cellulose before and after grafting (Figure 2). Unmodified cellulose nanofibers display several characteristics bands of cellulose macromolecules at 3 350 cm^{-1}, 1 110 cm^{-1} (and also used for the normalization of all spectra), 2 868 and 2 970 cm^{-1} for hydroxyl groups (OH), C−O of secondary alcohol, C−H from −CH_2−, respectively.[6] In MFC_PEN Film, a new band appeared between 1700 and 1750 cm^{-1} with a profound peak at 1735 cm^{-1} corresponding to a weak carbonyl ester bond.[32] No peak was detected on MFC_PEN sus, which might be due to the penicillin present, which could be too low to be detected with MFC.

The morphological characteristics of unmodified and modified MFC were investigated using atomic force microcopy. Figure 2 shows that the fibrous nanostructure remained unchanged after the modification on the films. Indeed, the

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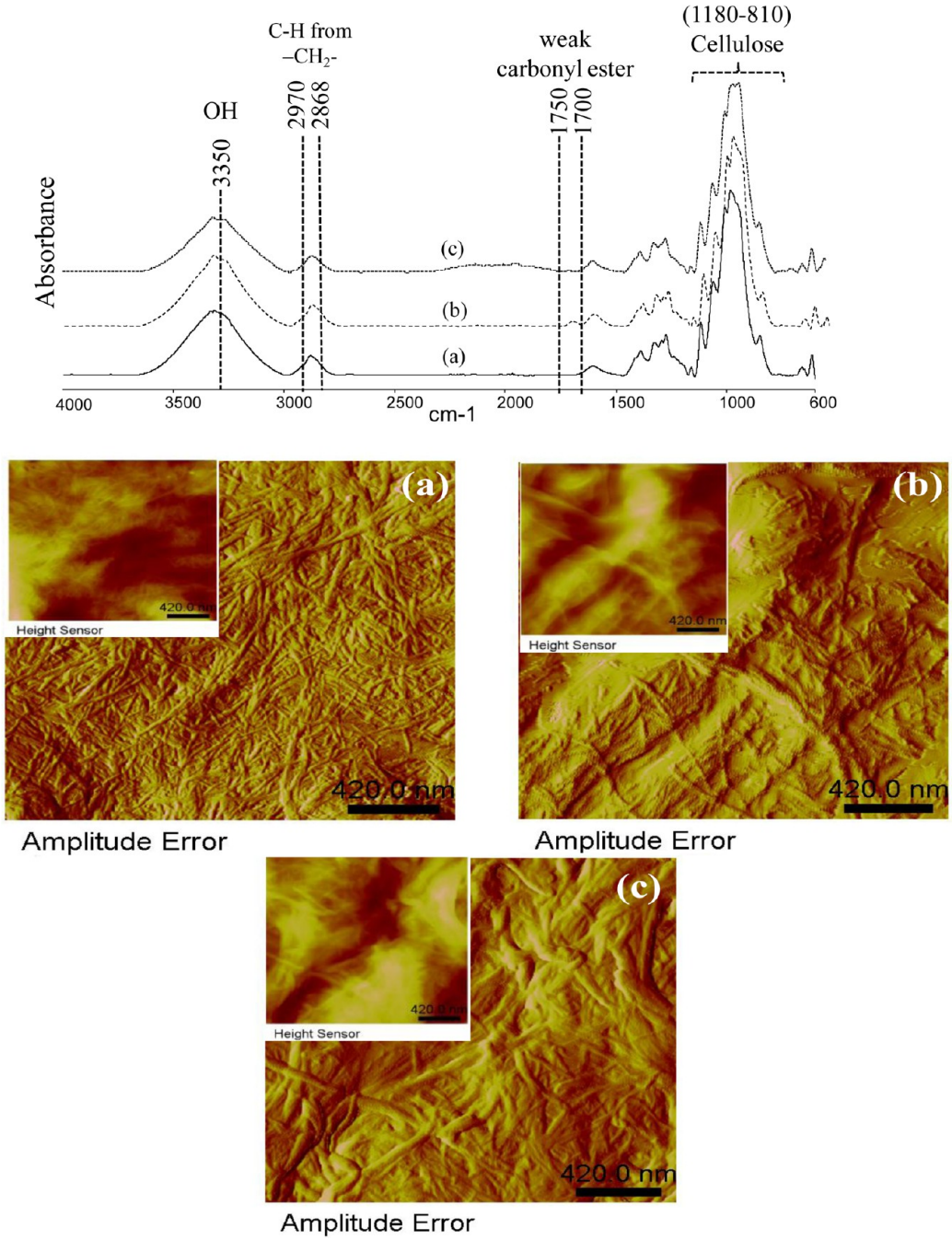

Figure 2. Qualitative analysis of neat MFC (a), MFC_Pen film (b), and MFC_Pen sus (c) through FTIR spectra and AFM pictures.

chemical modification here was heterogeneous, since cellulose was not dissolved by water, it is expected that only surface modification of the microfibers would occur. It is important to mention that the backbone structure of cellulose and the morphology of the microfibers is preserved and so it is likely that the molecular weight of cellulose was conserved, hence, its physical properties (including mechanical properties).

Quantitative Analysis of Grafting Efficiency. In order to ensure the distribution of penicillin throughout the MFC film, grafted samples were analyzed by SEM-EDX. Therefore, cross sections were prepared and the elemental distribution was visualized by energy dispersive method to analyze the diffusion. Since, nitrogen (green dots) and sulfur (blue dots) were introduced by penicillin, it is assumed that the distribution of element corresponds to diffusion of benzyl penicillin in MFC film (Figure 3).

For penicillin-grafted MFC, sulfur and nitrogen atoms are uniformly distributed within the MFC, which contradicts the hypothesis that penicillin will only modify the surface of the film when grafted directly on MFC film. However, the spectrum with EDX showed a significant concentration of sulfur present in penicillin grafted throughout the film in MFC_PEN film, although at the same time, no peak was seen with penicillin grafted on suspension (MFC_PEN sus). Infact, it is difficult to have a precise inference with the maps as the bands obtained are from elements but from the background noises as well. Overall, SEM-EDX micrographs confirmed the presence of penicillin on the MFC films. The results with SEM

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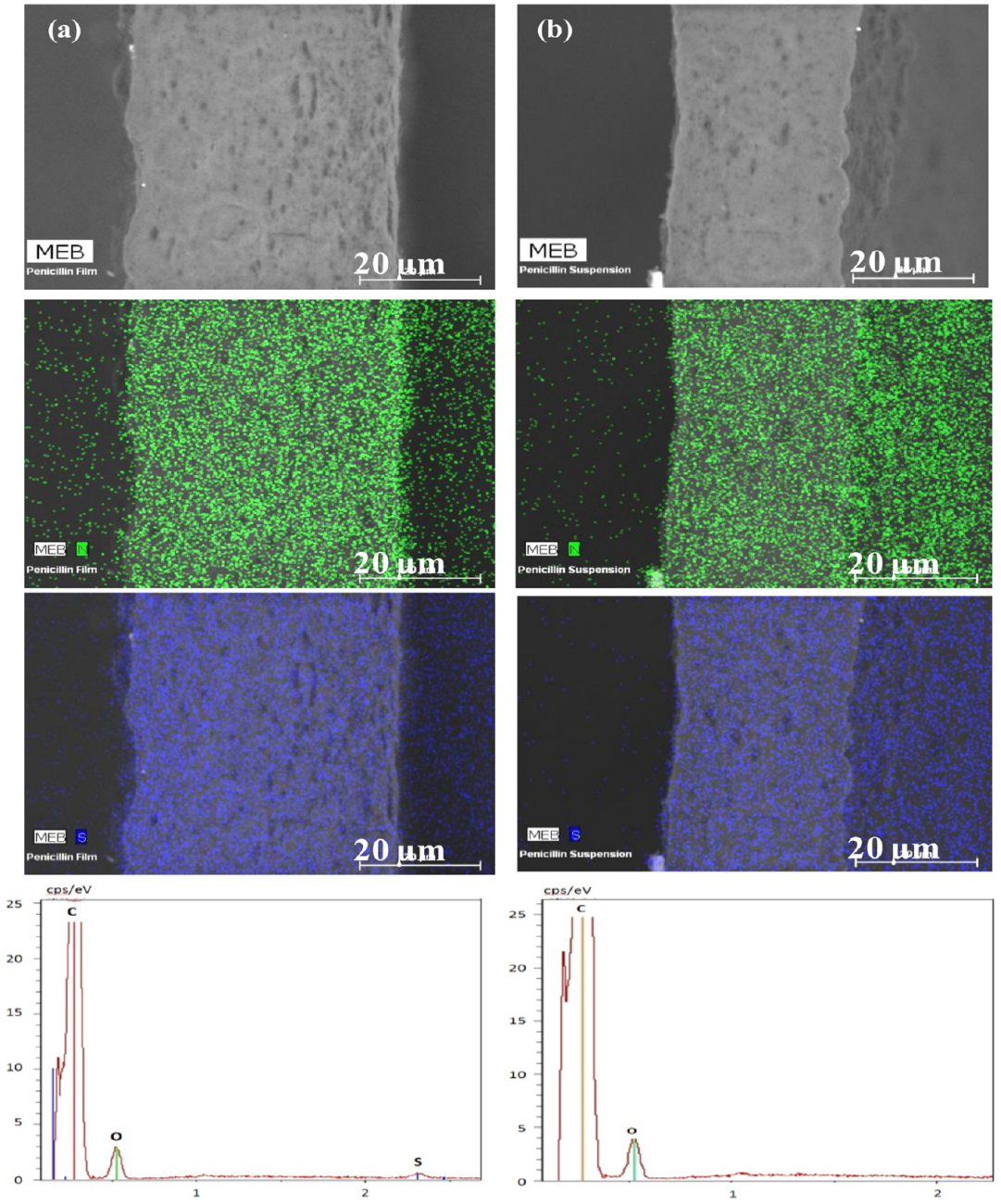

Figure 3. Nitrogen (green dots) and sulfur atomic (purple dots) distribution cartography in grafted MFC (a) MFC_Pen film and (b) MFC_Pen sus.

Table 2. Carbon, Hydrogen, Sulfur, and Nitrogen Concentrations for Neat and Grafted MFC as Obtained by Elemental Analyses[a]

	experimental values				normalized values	
	% C	% H	% S	% N	% C	DS
MFC_Neat	40.4	6.5	<0.1	<0.1	44.4	
MFC_PEN film	41.2	6.1	0.3	0.3	45.3	0.04
MFC_PEN sus	41.6	6.4	<0.2	<0.1	44.9	0.02

[a]Standard deviation up to 0.1 for all elements.

EDX were further confirmed by elemental analysis with quantification of each element.

Elemental analysis for neat and grafted MFC were shown in Table 2. With the grafting of penicillin on MFC, there is high content of carbon in grafted MFC compared to the neat MFC which apparently verify the grafting of MFC. An increase in nitrogen and sulfur content in grafted MFC is in agreement with the introduction of a β-lactam ring on the surface of nanocellulose, which is significantly higher for penicillin-grafted directly onto MFC films; this confirms the results obtained from SEM-EDX.

The degree of substitution obtained was 0.04 and 0.02 for penicillin-grafted MFC film and MFC suspension, respectively.

Next, the XPS analysis was explored in addition with elemental analysis, which provided insight into the chemical composition of the surface of the MFC films, before and after esterification. The XPS data for each element and deconvolution of C 1s peak for neat and grafted MFC are summarized in Table 3. The main peaks are detected at 285 and 533 eV, corresponding to C and O atoms, respectively. After grafting

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Table 3. Atomic Concentration from XPS Survey Scans of Neat and Grafted Samples As Well As C 1s Deconvoluted Peaks[a]

	experimental values				decomposition of C 1s				
	% C	% O	% N	O/C	C1	C2	C3	C4	C1/C3
MFC_Neat	57.4	42.6	0	0.74	16.1	60.2	19.9	3.9	0.8
MFC_PEN film	60.1	39.1	0.8	0.65	31	51.8	15.0	2.3	2.0
MFC_PEN sus	59.5	40	0.5	0.67	25.3	56.3	16	2.3	1.6

[a]Standard deviation up to 0.1.

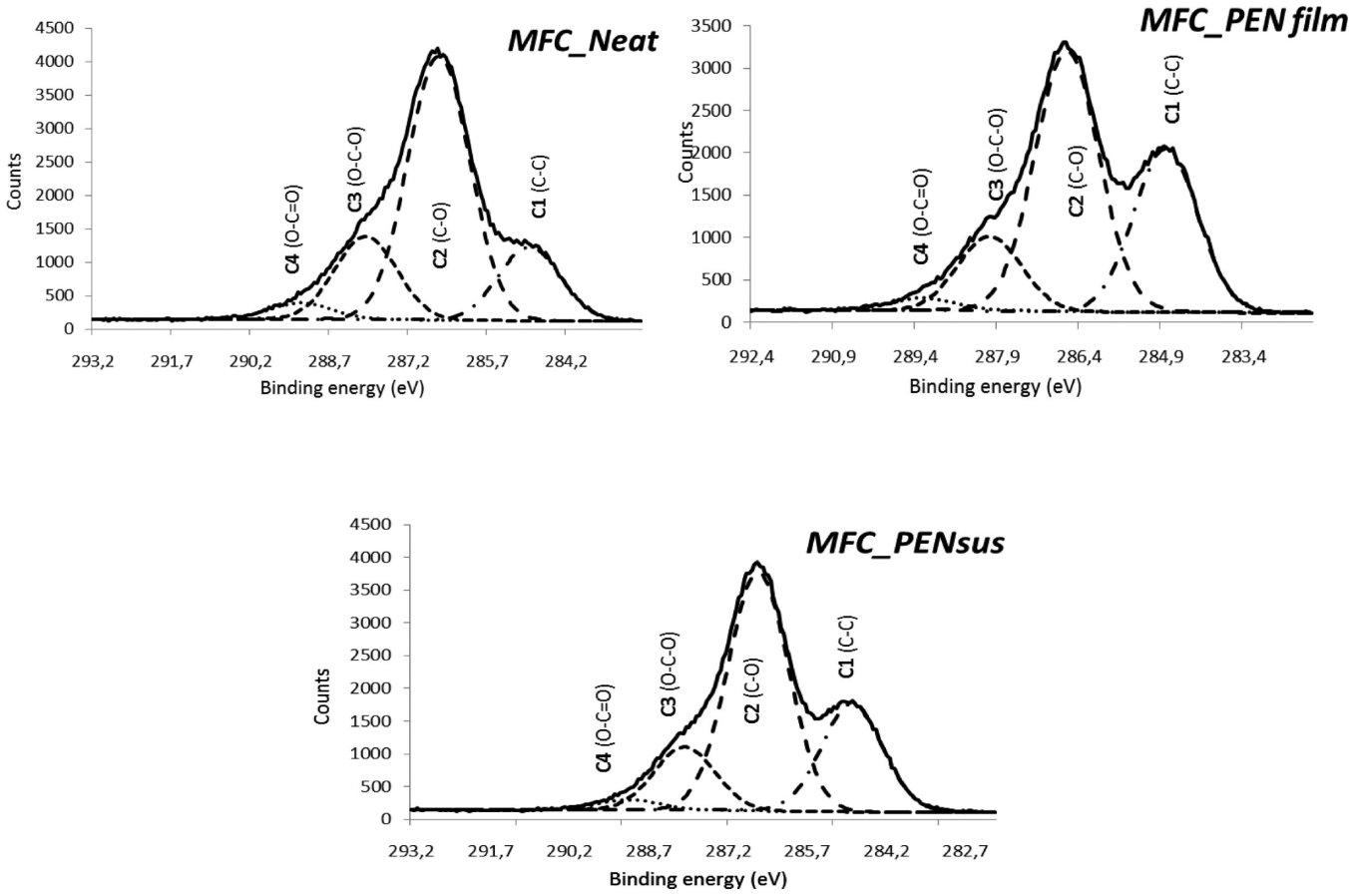

Figure 4. High-resolution XPS spectra of the C 1s peak in neat and grafted MFC.

with penicillin, in addition to the classic peaks, a new peak is observed at 400 eV attributed to N atoms. According to XPS data obtained, carbon and nitrogen content is higher for the samples having penicillin. This is in agreement with data obtained by elemental analysis of the samples. Theoretically, the surface O/C ratio for pure cellulose is 0.83. Despite this fact, experimental values obtained, i.e., 0.74 is different from the theoretical values. The probable reason for this difference is due to the presence of carbon rich contaminants in the surface of nanofibers.

The high-resolution C 1s XPS spectra of the same samples were also obtained. The usual carbon peaks were detected: C1 of carbon bonded to another carbon and/or hydrogen only; C2 of carbon bonded to one oxygen, C3 of carbon bonded to two atoms (acetyl or carbonyl); C4 of carbon bonded with three groups, that is, oxygen atoms, carboxyl, or carbonyl groups.

The deconvolution of C 1s signal is presented in Figure 4, in order to quantify the grafting and to corroborate the occurrence of surface grafting. This deconvolution reveals four peaks, which are, respectively, attributed to C1 (C−H), C2 (C−O), C3 (O−C−O and/or C═O), and C4 (O−C═O), with a binding energy of 285.0, 286.6, 287.8, and 289.2 eV, as summarized in Table 3. This table shows that the intensity of C1 (C−C/C−H) increases from 16% to 31% and 25%, respectively, for the neat, penicillin grafted on film, and penicillin grafted on MFC suspension. Furthermore, the ratio C1/C3 indicates the number of aliphatic carbons per glucose unit. The C1/C3 ratio change from 0.8 for neat NFC to 2.0 and 1.6 for penicillin grafted on film and penicillin grafted on MFC suspension provide clear evidence of increase in carbon per glucose unit, respectively.

The relative increase in the C1 peak indicates the substantial amount of aliphatic carbon in the grafted MFC films, which solely denotes the presence of penicillin. The C 1s is comparatively higher in penicillin-grated on films than in suspension, which proves that grafted MFC is present on the surface of MFC_Pen film and in bulk in MFC_Pen sus.

The degree of substitution was obtained by elemental analysis, which corresponds to the bulk analysis of all the films, whereas the degree of surface substitution was calculated by XPS, which characterizes the surface of the films whose thickness is up to 3 nm. DSS was calculated using the surface

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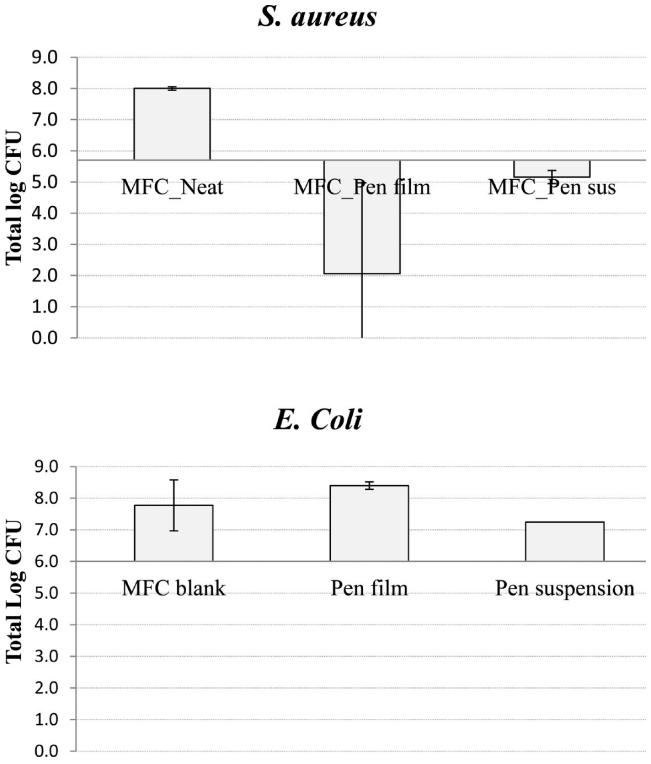

Figure 5. Antibacterial activity assessment of neat and grafted MFC with *S. aureus* and *E. coli* using the ATCC method.

nitrogen mass concentration obtained from the areas of the N 1s peak at 399 eV in the XPS spectra. The degree of substitution (shown in Table 3) is 0.04 and 0.02 for MFC_Pen film and MFC_Pen sus, respectively, while the degree of substitution of surface DSS is 0.11 and 0.08 for MFC_Pen film and MFC_Pen sus, respectively. Eventually, higher DSS value confirms that all the grafting took place on the surface of the cellulose.

Antimicrobial Activity Assessment. The ability of penicillin-grafted MFC to inhibit the growth or even kill the bacteria was assessed for *S. aureus* and *E. coli*. The control experiment was run using ungrafted MFC (MFC_Neat). The 2 log CFU increase of MFC_Neat after 24 h of incubation for both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria shows that the experiment was done with good nutrient conditions for bacterial growth. As illustrated in Figure 5, for *S. aureus*, penicillin grafted on MFC film exhibit a strong antimicrobial killing effect, accounting for a decrease of 3.5 log CFU after 24 h of incubation, while penicillin-grafted MFC in suspension displayed only 1 log CFU decrease. This lower killing effect might be due to the presence of lower amount of penicillin (confirmed by previous analysis).

Besides, benzyl penicillin is not effective against *E. coli*. For penicillin-grafted MFC film, there was an increase of the bacterial population after 24 h of incubation and the bacterial growth for samples of penicillin grafted in MFC suspension are in the same range as that of the reference sample. This could be explained because penicillin is active against the cell wall of bacteria and Gram-negative bacteria have an outer layer made up of lipopolysaccharide that hinders the access of penicillin to the cell wall.[33]

AFNOR EN 1104 test was carried out with unmodified and modified MFC films against *B. subtilis* to check for the leaching effect of the samples. The leaching of an antimicrobial compounds into agar creates an inhibition zone around the samples under investigation (Figure 6a). In this study, no zone of inhibition was formed around the tested films even after 3 days of incubation indicating that penicillin does not leach out from the MFC film.

Analysis of the zone of inhibition gives qualitative information about the leaching of penicillin. Therefore, an

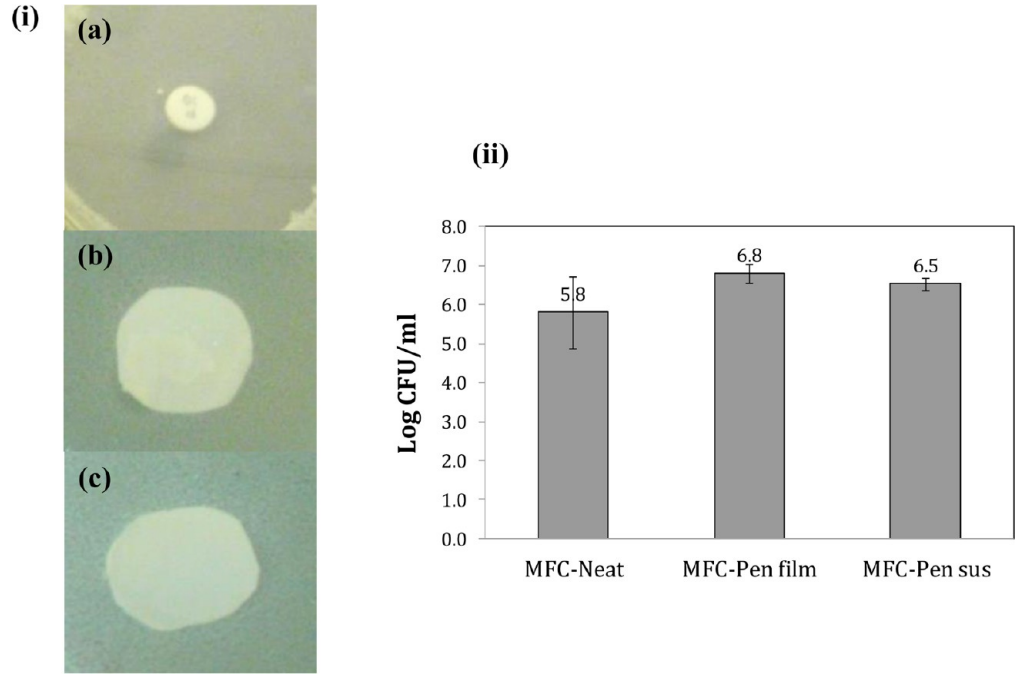

Figure 6. (i) Qualitative AFNOR EN 1104 test for (a) penicillin disk, (b) neat MFC, and (c) modified film; (ii) quantitative leaching assays for neat and grafted MFC against *Bacillus subtilis*.

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experiment was designed according to the previous method reported in the literature.[34] Figure 6 indicates that there is no significant difference in the growth of bacteria with unmodified and modified MFC. It was thereby concluded that no antibacterial activity was demonstrated by the filtrate. Altogether, the modified MFC films accomplish the requirements for a contact-active antibacterial material.

CONCLUSION

In this study, nonleaching antibacterial films were prepared through modification of the surface of microfibrillated cellulose with the antibiotic benzyl penicillin. This is the first work which focuses on the antibiotic grafting with esterification reaction on MFC in aqueous medium. Benzyl penicillin was efficiently covalently bonded on the surface of MFC which was confirmed by elemental analysis and XPS technique. Grafting quantity was higher when modification was done directly on film compared to when done on MFC suspension. Even with very low DS (0.04 for MFC_Pen Film and 0.02 for MFC_Pen Sus), these antimicrobial surfaces are very effective against Gram-positive bacteria. Moreover, when benzyl penicillin was grafted on MFC films, complete killing was observed with S. aureus (Gram-positive) but in contrast it did not demonstrate any activity against *E. coli* (Gram-negative), which was consequence of high resistance to antimicrobials. These contact active antimicrobial films can be used in various applications such as food packaging, medicine band aids, wound dressings, sterilized papers, and sterile syringe packaging.

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Notes

The authors declare no competing financial interest.

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