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Short communication

A simple method to tune the gross antibacterial activity of cellulosic biomaterials

Lambrini Adamopoulos [a], Jason Montegna [b], Greg Hampikian [c], Dimitris S. Argyropoulos [a], John Heitmann [a], Lucian A. Lucia [a,*]

[a] North Carolina State University, Department of Forest Biomaterials Science & Engineering, 3108 Biltmore Hall, Raleigh, NC 27695-8005, USA
[b] Georgia Institute of Technology, Institute of Paper Science & Technology, 500 Tenth Street, N.W. Atlanta, GA 30332-0620, USA
[c] Boise State University, Department of Biology, 1910 University Driver, Boise, ID 83725, USA

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Abstract

A very preliminary approach for grossly tuning the antibacterial activity of cellulosic fibers has been developed and its preliminary findings are described herein. The approach is universal for cellulosic-based substrates and first involves a physico-chemical adsorption phenomenon between fatty acid methyl esters (FAMEs) and cellulose. The cellulose biomaterials were in the form of disks 2 cm in diameter that were subjected to standard agar growth plates containing a gamut of gram positive and gram negative bacteria. Zones of inhibition were measured around the biomaterials which displayed a broad spectrum of antibacterial activity. This activity could be tuned simply by grossly changing the surface area of the cellulosic surface topology as indicated by the surface fibrillation of the microfibrils and hence the bioactive availability of the fatty acids. Thus, the potential application of these materials in the biomedical field appears promising.

Keywords: Antibacterial materials; Cellulose; Fatty acids

1. Introduction

Recently, a significant amount of both fundamental and applied research has been directed towards chemically and/or enzymatically engineering surfaces to withstand the proliferation of communicable pathogens (Appleton, Gosser, & Vogel, 1997; Engel et al., 2003; Fujishima, Chih, & Kubota, 2003; Hassinen, Durbin, & Bernhart, 1951; Kabara, Vrable, & Lie Ken Jie, 1977; Lee et al., 2004; Lin, Tiller, Lee, Lewis, & Klibanov, 2002; Marconi, Monopoli, Piozzi, Di Rosa, & Di Rosa, 2001; Melkonian et al., 2004; Mizunuma, Takayama, Fudanoki, & Abe, 1999; Neuberger, 1993; Ricke, 2003; Sarangapani, 1999; Seisen, Umezu, & Takiguchi, 1999; Tiller, Lee, Lewis, & Klibanov, 2002; Yano, Udagawa, & Sakai, 1996). In recent years, the real threat of modern bioterrorism coupled with the rapid evolution of new strains of bacteria and potentially harmful viruses, stresses the importance of developing materials to combat human pandemics,[1] However, the pragmatic fabrication of antimicrobial surfaces must address a number of fundamental issues. First, the materials must be very biocompatible, i.e., they must not be harmful to humans; second, they must maintain their antimicrobial activity under a variety of environmental conditions; third, they should be relatively facile to manufacture and implement, considering the economic constraints of scale-up. Thus far, very few antibacterial materials, meeting the above criteria, have been developed industrially. Fujishima et al. are among a few who successfully addressed these constraints by implementing specialized

* Corresponding author. Tel.: +1 919 515 7707; fax: +1 919 515 6302.
E-mail address: lucian.lucia@NCSU.edu (L.A. Lucia).

[1] http://www.ljworld.com/section/archive/story/197014: CDC chief warns of world virus outbreak. Paul Recer (AP).

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semiconductor materials that result in a broad spectrum bactericidal activity (Fujishima et al., 2003). In his work and other similar to it, the general approach has been to activate a surface either by inserting persistent bioactive materials (such as silver atoms) or by introducing a trigger such as light to stimulate an oxidative reaction that results in bacterial death. Typically, the surfaces that have been amenable to this type of modification have been metallic and porcelain in nature. "Soft" materials, such as biopolymers, however, have been almost virtually excluded.

In the last five years, several research efforts have attempted to modify "soft" surfaces (cloth, gauze, clothing, paper, polymers, plastics, boxes, cartons, human tissue) by chemically manipulating carbohydrates and hydroxyl functionalities as anchor points for chemical derivatization. One of the prominent research efforts in this arena, spearheaded by Klibanov et al., demonstrated that *N*-alkylated poly(4-vinyl-pyrdine) groups created surfaces that killed wild-type and antibiotic-resistant bacteria (Lin et al., 2002; Tiller et al., 2002). Similarly, Cohen et al. Melkonian et al. (2004) fortuitously discovered that amine-derivatized carbohydrates (cotton, bulk cellulose, paper, wood, gauze) were surface active against a variety of both gram positive and negative bacterial strains. In their work, the carbohydrates were tosylated (coupling reaction with *para*-toluenesulfonic acid) and then reacted with a DABCO (1,4-diazabicyclo[2.2.2]octane) unit, possessing a lipophilic alkyl chain (Volpenhein, 1985), to remove the tosylate leaving group. The exposed long chained ammonium functionality on the surface of the materials killed a gamut of bacteria on simple contact, did not lose activity upon prolonged use, was not modified after its antibacterial activity, and was not removed upon washing. Indeed, the mechanism of activity was postulated to be purely physical in nature and to rely on the dynamic interaction of the surface with the cell wall of the bacteria. As a result, the bacteria cannot resort to any protective or immunological response mechanisms for survival. Such a technology would display a "detergent" character, i.e., it would involve the dissolution of the bacterial cell wall regardless of the strain (gram positive or gram negative). In effect, three general schemes for antimicrobial activity, illustrated in Fig. 1, can be elaborated.

The antimicrobial activity of a surface may be (1) external, (2) surface active, and (3) time-released. Each mode of action has its advantages and disadvantages. The external mode is represented by disinfectants as applied to surfaces that compromise the structural integrity of the microorganisms they contact. It is a blanket antimicrobial approach where sufficient quantities kill the microorganism and may also affect humans.

The surface-active mode can be represented by the selective transferal of antimicrobial surface agents into the microorganism until toxic accumulation occurs or membrane disruption occurs causing cellular leakage.

Finally, the time-released mode consists of discharging antimicrobials in response to an environmental trigger, such as a change in surface pH, pressure inductions, in which either of the latter conditions may be initiated by surface attachment of the antimicrobials, or a temperature change.

Our research focused on the surface-active mode of antimicrobial activity, using cellulosic substrates as platforms for physico-chemically accumulating fatty acids in the production of antimicrobial agents. Our work has determined that the fatty acid esters from soybean oil (principally linolenic acid) are able to resist the proliferation of various G+ and G− bacterial strains. This preliminary report thus presents the adsorption methodology, antibacterial activity, and how the bioactivity was grossly tuned through a change in the surface area of the biomaterials.

2. Materials and methods

2.1. Generation of the cellulose-fatty acid disks

Four hundred and fifty grams of soybean oil (used as is from Natural Oils International Inc.) was dissolved into 500 mL of ACS grade methanol in a three-necked flask. NaOH (0.5 g) was added to this solution. The mixture was then heated to 70 °C for 30 min. (with stirring), and allowed to cool. The upper layer of the two layers that appeared had an off-yellow color and contained the ester, as determined by TLC (90:9:1 petroleum ether:diethyl ether:acetic acid). The latter was extracted and dissolved in an alkaline solution (0.5 g KOH) of 500 mL methanol. It was then refluxed for 2 h producing the fatty acid soap as indicated again by TLC. This was dried overnight in a vacuum oven, and a dark yellow oil was produced. In the next step, a mass ratio of approximately 10:1 soap:cellulose disks was used. This dictated that 100 g of the oily soap based on a starting mass of 10 g of 2 cm diameter cellulose disks be utilized, all of which was dissolved in 500 mL methanol. A catalytic amount of potassium carbonate was added (100 mg, 1 mmol), after which the solution was heated and the methanol was driven off. At 100 °C, a vacuum was applied to the reaction and it was heated for 2 h at 130 °C. No purification was done on the disk samples given the dilution factor of methanol, i.e., only adsorbed products remained in the disks while the starting materials remained in solution. To determine the presence of the FAMEs and quantitate their abundance, quantitative FT-IR microspectroscopy was done. Spectra of the cellulose disks were collected by reflectance mode Fourier transform infrared microspectroscopy. Spectra were recorded at wavenumbers 4000–600 cm^{-1} by a Continuum IR scope coupled to a ThermoNicolet Nexus 670 FT-IR and using OMNIC software (ThermoNicolet, Madison WI). For each spectrum, 120 interferograms were averaged using collection parameters set at a resolution of 8 cm^{-1}, 0.6329 scan speed, and Happ-Genzel apodization. Spectra were collected on two different samples and compared to a control disk of underivatized cellulose. Fig. 2 illustrates a typical FT-IR spectrum of the cellulose-fatty acid disk.

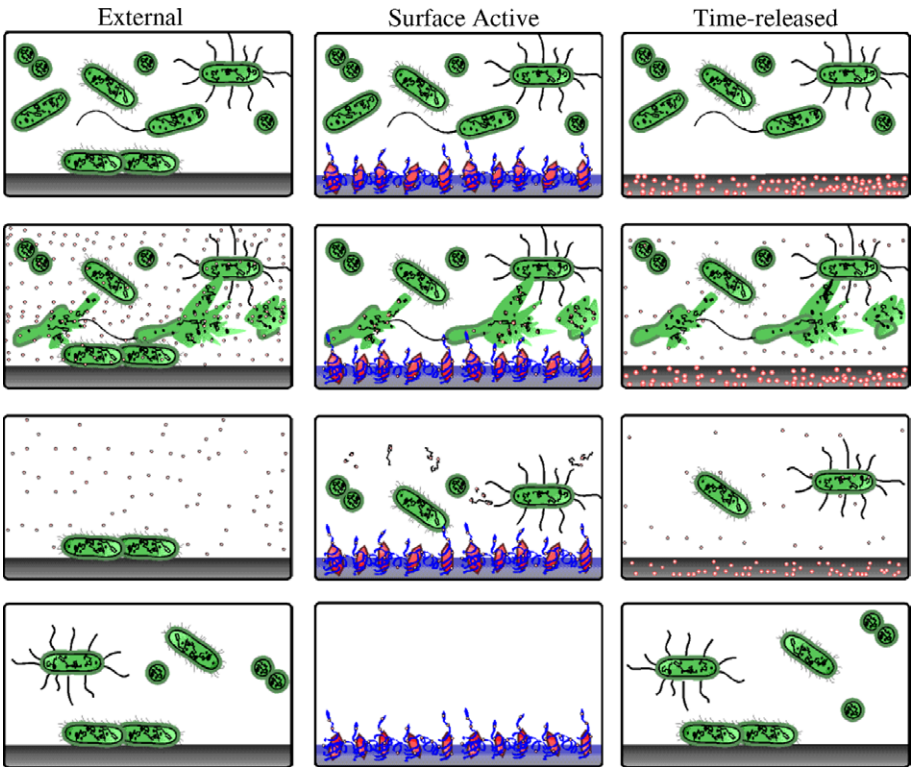

Fig. 1. The three general modes of antimicrobial surface-mediated activity.

It was determined that there was up to 40% coverage of the available cellulosic hydroxyls by the fatty acids. This was calculated by a simple mathematical difference of the hydroxyl peak integration between the sample impregnated with the fatty acid versus the control sample, i.e., the sample hydroxyl peaks would be associated with the acid functionalities via hydrogen bonding and attenate those hydroxyl signals. It was determined that >95% of the fatty acids could be removed by robust Soxhlet extraction conditions (14 h reflux with ACS grade hexane) to give the original control IR spectrum.

2.2. Antimicrobial testing

The antimicrobial testing was done in accord with accepted biological practices to test antibacterial efficacy. A series of sterile Petri plates (100 mm × 15 mm) containing a tryptic soy agar with 5% sheeps blood nutrient was used for growing bacterial lawns. Selected bacterial cultures were used to swab the plates after which 3 × 2 cm cellulose-ester disks were symmetrically arrayed on the plate. This was repeated with a number of bacterial cultures, all of which were incubated overnight at 37 °C to encourage the growth

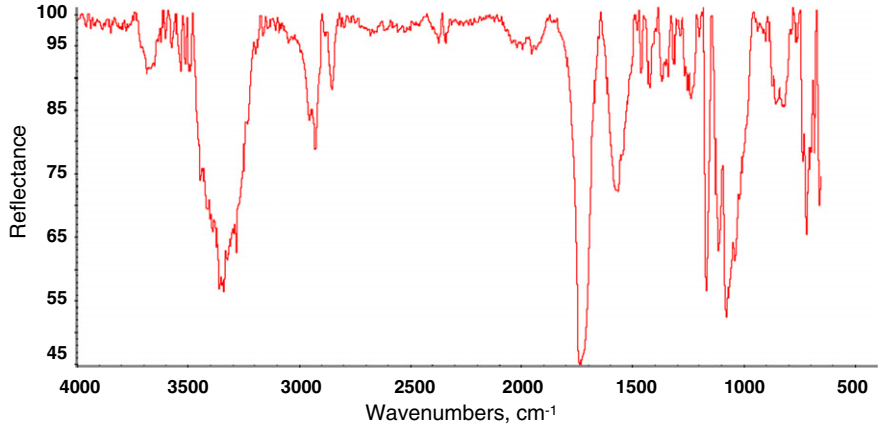

Fig. 2. The FT-IR spectrum of the cellulose-fatty acid disk is shown which illustrates the presence of the signature FAME ester linkage (1735 cm^{-1}).

of the bacterial lawn. The zones of inhibition were determined by measuring the bacteria-free region from the edge of the disk to the edge of the lawn. Selected duplicates were run to ensure reproducibility which was within 15%.

3. Results and discussion

3.1. Cellulose antibacterial properties

We determined that the FAMEs were physico-chemically trapped within the cellulose disks since they required significant Soxhlet extraction time (14 h) to fully remove them. We explored the activity of these cellulose materials against the bacterial samples shown in Table 1.

The cellulosic disks were tested for their antibacterial properties after two distinct treatments: they were either washed or not washed with water. This was done to change the available surface area of the external fibrils to determine if it was a factor in the antibacterial response.

After applying the disks to various agar media where isotropic bacterial lawns were allowed to grow, we found an array of responses. Shown in Fig. 3 is a typical setup for the experiment that includes three sample disks (cellulose ester water rinsed, non-rinsed, and the control cellulose disk). A very good antibacterial response is observed in both test cases. The test disks display a fairly good "zone of inhibition," or region in which the bacterial species refuses to propagate. The intensity of this inhibitory effect is quantified by measuring the distance (in mm) from the edge of the disk to the outer annulus of bacterial growth. We obtained data such as shown in Fig. 3 for all of the bacterial cultures indicated in Table 1.

Antibacterial effects were detected around the non-rinsed disks for all of the bacterial species studied. Interestingly, the gram positive bacteria (the first three strains in Table 1) were the most affected. Since all of the incubations were performed on the same media (blood agar), this difference may be due to dramatic dissimilarities in the cell wall materials of the two types of bacteria. Gram positive (G+) cell walls are principally composed (90%) of peptidoglycan, a macromolecule composed of amino acids and sugar,

Table 1
Shown are the zones of inhibition for the various bacterial species used in this study as a function of rinsed and non-rinsed samples

Bacteria strain (Gram Stain)	Zone of inhibition (mm)[a]		
	Non-rinsed	Rinsed	Control
Bacillus megaterium (G+)	20	10	0
Bacillus subtilus (G+)	15	10	0
Staphylococcus aureus (G+)	14	11	0
Salmonella cholerus (G−)	10	8	0
Seratia marcescius red (G−)	9	0	0
Enterobacter (G−)	9	0	0
Escherichia coli (G−)	9	8	0

P. aeruginosa and *P. vulgaris* did not grow a bacterial lawn.
[a] For purposes of comparison, a zone of 40 mm is considered to be the maximum level of inhibition.

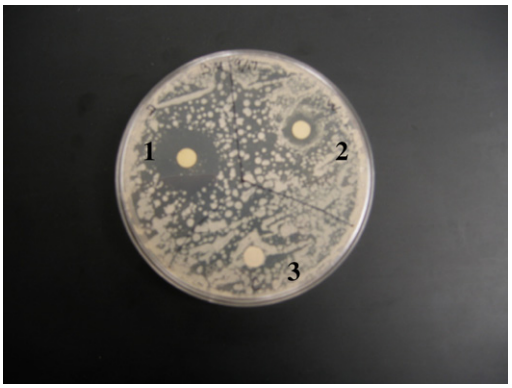

Fig. 3. Shown above is a bacterial lawn growth consisting of colonies of *Bacillus megaterium* as a function of varying disk treatments: 1 – non-rinsed cellulose-ester disk, 2 – rinsed cellulose-ester disk, and 3 – control.

which has the ability to form more than 20 layers in the cell wall. In addition, the Bacillus species have the ability to form endospores, which are highly resistant to a wide range of biocidal agents. The gram negative bacteria, however, are low in peptidoglycan content, but high in lipid content. This lipopolysaccharide component contains Lipid A (endotoxin), primarily responsible for pathogenic effects such as fever and shock. A number of these bacteria are very virulent accounting for a variety of human maladies, including gonorrhea, meningitis, UTIs, and food poisoning. In this study, the action of the cellulose disks may be predicated upon a mechanism in which the diffusible fatty acids interact with the bacterial cell wall and cause its disruption. This should be more pronounced in the G− bacteria due to topological considerations in which lipid–lipid chemical interactions should increase membrane fluidity. Our data shows that there is no remarkable difference in sensitivity of the various gram types to the biomaterials which may point to the general detergent nature of the antimicrobial mechanism. In fact, Raychowdhury provided evidence that enhanced membrane fluidity may be a potential mechanism for fatty acid-induced bacterial cell wall disruption based on the presence of bacterial cell contents in the bulk (Raychowdhury, Goswami, & Chakrabarti, 1985). Our data do not differ appreciably from the work done by Cohen et al. who were able to develop quaternized cellulosic biomaterials which displayed similar antibacterial responses (Engel et al., 2003; Melkonian et al., 2004). As a followup to the mechanism for their activity, we attempted to explore the surface topology of the cellulose disks to determine if there were any physical considerations that influenced bacterial response.

We obtained a series of standard SEM micrographs of disks that were rinsed with a number of solvents and show several micrographs in Fig. 4 for comparison. As observed in the micrographs, clear superficial architectural patterns are observed. In the control disk that was not subjected to wash, microfibrillar arrays extending from the secondary wall of the softwood fibers can be observed. These microfibrils are generated prior to matting the fibers by a mechanical refining process to ensure sufficient surface area is available

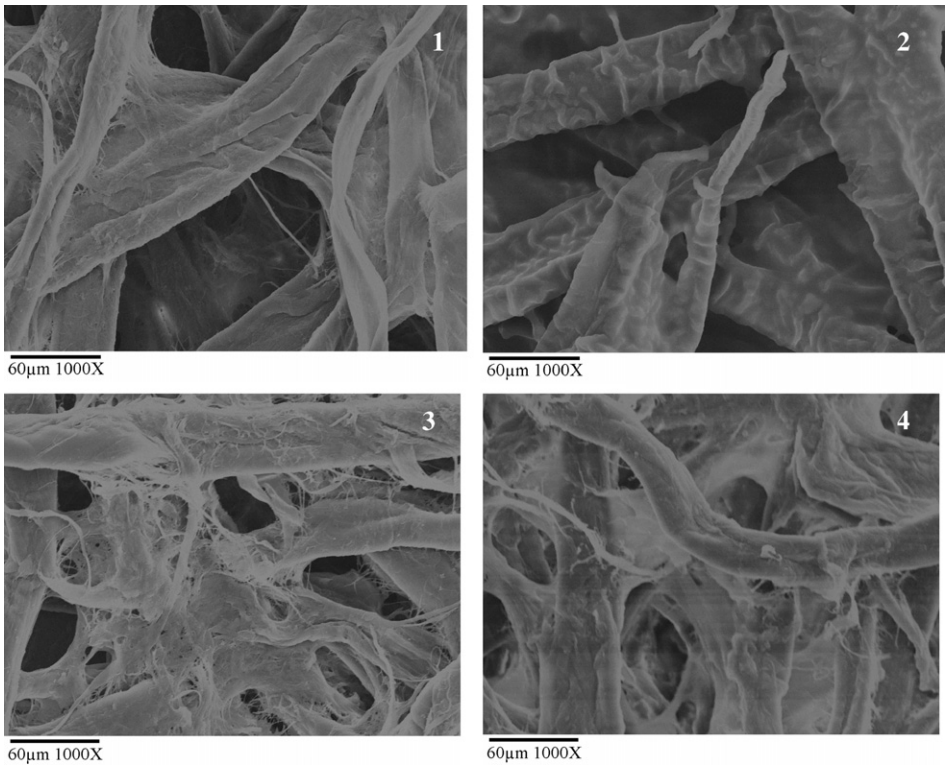

Fig. 4. Shown above are the SEM micrographs of the cellulose-ester disks as a function of (1) control cellulose disk, (2) water wash, (3) hexane wash, and (4) ethanol wash.

for increased bonding. Interestingly, the water rinse used to mechanically disperse residual fatty acids causes a type of microfibril coalescence as observed in the second micrograph of Fig. 4. This surface topological anomaly is not observed in ordinary paper fibers. Quite the opposite effect is apparent – fibers tend to swell quite efficiently. It is likely due to a hydrophobic interaction between the water and the adsorbed fatty acids causing them to aggregate as a result of the hydrophobic interaction. This was verified by introducing a non-polar solvent, hexane, to the fibers. Notice the extreme extent of fibrillation in micrograph 3 of Fig. 4. After exposure to a non-polar solvent, typical non-treated fibers would display the type of topological features shown in micrograph 2. In fact, when we used ethanol, a solvent having a polarity between water and hexane, we saw that fibrillation did occur, but not to the extent observed in micrograph 3. We also suspect that the extent of dissolution of the fatty acids chains in the ethanol is not as pronounced as it is in the hexane. These final results were reproducible and also point to the ability to control the degree of surface area availability of the adsorbed fatty acids on the fibers. It was found that the fatty acids required a very intense hexane extraction to remove them from the fibers since they were well entrained in the pores.

4. Conclusions

In summary, cellulose disks are a grossly tunable medium to strongly retain fatty acids for the express purpose of demonstrating antimicrobial behavior. It was found that these substrates displayed antimicrobial properties against various G+ and G− bacterial species. In general, the effects do not appear to show a bias between the two gram type bacteria. SEM analysis of these substrates suggests that by increasing the surface fibrillation and hence area, more pronounced antibacterial effects may be obtained, again likely owing to increased availability of diffusible fatty acids.

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