

NOVEL CHITOSAN DERIVATIVES AS FILMS WITH AN ANTIMICROBIAL EFFECT

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Abstract

*This paper presents the results of chitosan acylation with linoleic (LA) and dilinoleic acid (DLA). The chemical structures of the new derivatives with different degrees of substitution of LA and DLA were assessed by Fourier transform infrared spectroscopy. Polymer films were prepared by solution casting and drying. The resistance of films to bacterial degradation was tested according ISO 846 standard: "Plastic-Evaluation of the Action of Microorganisms" and the antimicrobial properties were assessed using Gram-negative bacterium *Escherichia coli* and the fungi *Candida albicans* in an agar diffusion test. Bioassays showed an antimicrobial effect in direct contact with the material.*

Key words: *chitosan, fatty acids, dilinoleic acid, antimicrobial films.*

1. Introduction

Chitosan is a polysaccharide derived from renewable sources, with unique characteristics such as film-formation, biodegradability, biocompatibility, nontoxicity, and antimicrobial activity, as well as numerous functional groups (amine and hydroxyl) able to react with other compounds [1 - 4]. Therefore, to improve the physical, chemical, or biological properties of chitosan and to widen its applications, different chitosan derivatives have been obtained [5 - 7]. For example, incorporating hydrophobic compounds, such as garlic oil, into chitosan can improve its mechanical properties and antimicrobial efficacy, however this methodology still have some limitations [8]. Modifying chitosan with fatty acids may be more valuable; however, when fatty acids are physically incorporated into chitosan, the derivatives have unstable mechanical properties, despite better antimicrobial action [9].

The aim of our study was to obtain new chitosan derivatives by its acylation with fatty acids, linoleic (LA) and dilinoleic acids (DLA), in order to increase flexibility and enhance antimicrobial action. The antimicrobial properties of chitosan derivative films were assessed against the bacteria *Staphylococcus aureus* and *Escherichia coli* and the fungus *Candida albicans*.

2. Materials and methods

2.1. Materials

Chitosan derivatives were synthesized using chitosan (CH), linoleic acid (LA) and acetic acid (AA) purchased from Sigma Aldrich, as well as 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl) supplied by GenScript Corporation and dilinoleic acid (Pripol 1009) kindly supplied by Croda. Chitosan was refined by dissolving it in 1% AA solution, filtered, precipitated with dilute ammonia, and dried for 24 hours under the vacuum at 40 °C. The degree of deacetylation (determined by the conductometric titration) was approximately 80%, and the average viscosimetric molecular weight was 40 kDa [10].

2.2. N-acylation of chitosan with fatty acids

N-acylation of chitosan with linoleic acid was carried out according to a previously reported procedure [10]. Briefly, a methanol solution of linoleic acid and catalyst EDC.HCl (1:1 mol/mol) was added dropwise to dissolved chitosan in 1% of AA. The reaction was carried out for 24 hours at room temperature and, afterwards, the product was precipitated, washed with distilled water and methanol, and then dried for 24 hours under vacuum at 40 °C.

N-acylation of chitosan with dilinoleic acid was conducted differently, due to the higher viscosity of the dimer compared to acid. In a round-bottom flask equipped with a reflux condenser, the DLA was diluted with methanol at 60°C and EDC.HCl (1:1 mol/mol) was added. Next, the DLA solution was added dropwise to dissolved chitosan in 1% of AA and the reaction was carried out for 24 hours at 60 °C. The product was precipitated in ammonia solution, centrifuged, and washed with distilled water (until the pH was approximately

Table 1. Molar ratios of chitosan to LA/DLA.

Sample code	chitosan, mol NH ₂	LA, mol	Sample code	chitosan, mol NH ₂	DLA, mol
CHLA 0.16	1	0.16	CHDLA 0.16	1	0.16
CHLA 0.34		0.32	CHDLA 0.34		0.32
CHLA 0.52		0.54	CHDLA 0.52		0.54
CHLA 1.00		1.00	CHDLA 1.00		1.00

neutral) and methanol. The precipitates were then dried for 24 hours under the vacuum at 40 °C.

N-acylated derivatives with different degrees of substitution of LA and DLA were prepared by using different chitosan/fatty acid molar ratios, as shown in **Table 1**.

2.3. FTIR-ATR analysis

The chemical structure of N-acylated chitosan derivatives was assessed by Fourier transform infrared-attenuated total reflection spectroscopy (FTIR-ATR) on Nexus spectrometer with Golden Gate (ATR) (Thermo Nicolet Corp.). Samples were dried at 40 °C under vacuum for 24 hours and 32 scans were averaged across the spectral range of 400 - 4000 cm⁻¹.

2.4. Film preparation

Polymer films were prepared by solution casting and drying. Chitosan derivatives were dissolved in 1% AA and methanol in a 3:1 volume ratio, poured on petri dishes, and left at room temperature overnight. Next, samples were placed in a vacuum dryer at 40 °C to ensure complete evaporation of the solvent.

2.5. Antimicrobial tests

First, the resistance of prepared samples of chitosan derivatives to bacterial degradation was tested according ISO 846 standard: “Plastic-Evaluation of the Action of Microorganisms.” Disc shaped samples, 10 mm in diameter, were cleaned prior to the test via dipping in 70% ethanol for 30 min and dried under constant airflow. In accordance with ISO846 method C, non-nutrient salt agar was inoculated with *Staphylococcus aureus* or *Escherichia coli* (10⁶ colony forming units (CFU)/mL) and sample discs were placed on top of the agar and then covered with more agar. After incubation for 28 days at 29 °C, bacterial colonies were observed and counted an aCOLyte Super Colony Counter (Synbiosis).

The antimicrobial properties of chitosan derivatives were assessed using Gram-negative bacterium *Escherichia coli* (strain ACCT 25922, 4,5·10⁴ CFU/mL) and the fungi *Candida albicans* (strain CaCa3, isolated from a clinical sample) (1·10³ CFU/mL) in an agar diffusion test. Bacteria were plated on Plate Count Agar, while the fungi were plated on Sabouraud agar, and sterile samples (discs) were placed on top. The duration of the test after inoculation was 48 h for bacteria and 24 h for the fungi, at 37 °C.

3. Results and discussion

A new series of chitosan derivatives, with different degrees of fatty acid substitution, was synthesized by acylation of the primary amine groups of chitosan with the carboxylic groups of a fatty acid to form an amide bond. Our goal was to obtain linear, soluble derivatives of chitosan modified with fatty acid. We wanted to avoid cross-linking during the reaction with the difunctional DLA and, as a result, the molar ratio was calculated as acid compound (not carboxylic group) to amine group of CH. While the possibility of chitosan cross-linking remains, all of the obtained derivatives were soluble in dilute AA. The chemical structures of linoleic, dilinoleic acid, and the CH-fatty acid derivatives are given in **Figure 1**.

FTIR spectra of chitosan and its derivatives, CHLA 0,34 and CHDLA 0,34, are shown in **Figure 2**. The peaks at 2924 and 2857 cm^{-1} (assigned to the aliphatic C–H stretch band) can be attributed to successful conjugation of the long alkyl chain of fatty acid. The peak at 1740 cm^{-1} , assigned to C=O bond, is observed to increase in CHDLA derivatives, due to the occurrence of not only an amide bond, but also free carboxyl groups of DLA.

For both LA and DLA chitosan derivatives, we observed noticeable changes in the intensity of the individual absorption bands associated with a higher fraction of acid in the obtained derivatives, as shown in **Figure 3**. A change in the spectrum at 1703 cm^{-1} represents the acetylated amino group, indicating a decrease in the degree of deacetylation along with a higher molar ratio of acid. The absorption at 1555, 1540 and 1375 cm^{-1} is stronger, confirming the presence of carboxylic acids (C–O, C–H stretch, respectively).

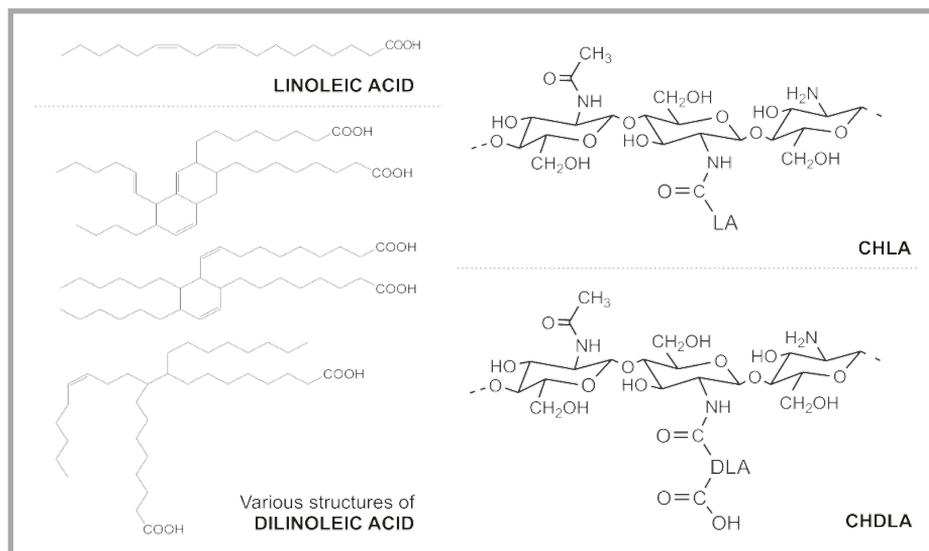


Figure 1. The chemical structures of fatty acids and schemes of chitosan-based derivatives.

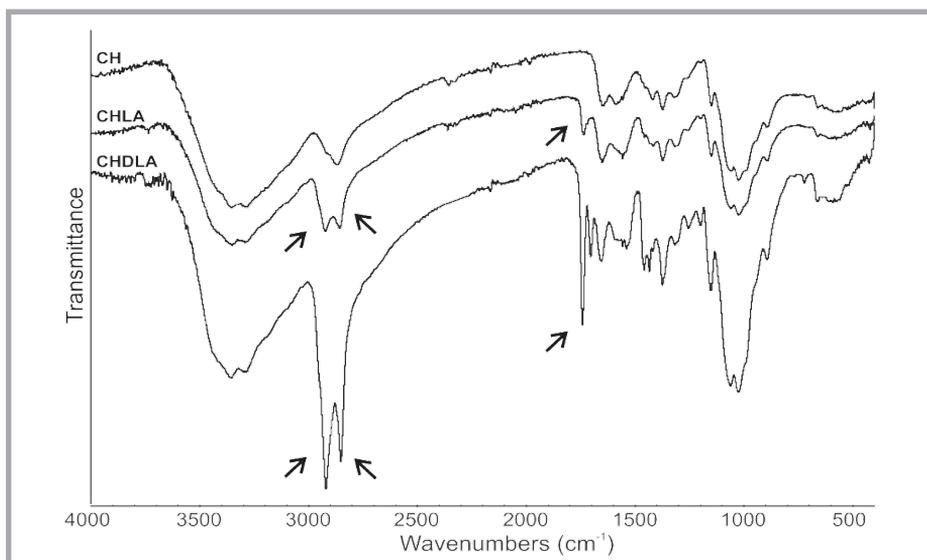


Figure 2. FTIR spectra of chitosan and its LA and DLA derivatives with molar ratio 0,34.

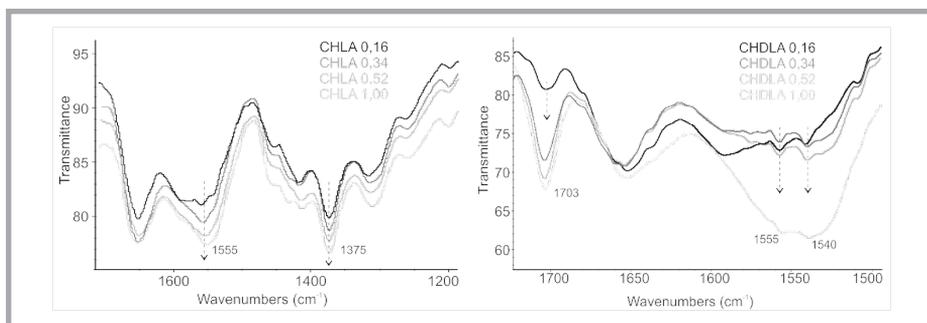


Figure 3. The relationship between absorption intensity and fatty acid fraction in CHLA and CHDLA derivatives.

Producing chitosan films by solution casting is relatively easy; however, the resultant films are often difficult to handle, due to their tendency to curve via rapid drying. Further, thick chitosan films become overly stiff, resulting in cracks during manufacturing. However, the use of long chain fatty acids (linoleic and dilinoleic) as chitosan modifiers should not only change the chemical properties of the films, but also act as a plasticizer, increasing flexibility and, thereby, facilitating further processing of films.

In the first microbiological test, according to ISO 846, method C, the resistance of the chitosan derivatives to microbial degradation was assessed by visual examination. Briefly, chitosan and derivatives were exposed to Gram-positive bacteria *S. aureus* and Gram-negative bacteria *E. coli* in non-nutrient agar for 28 days and all of the tested materials

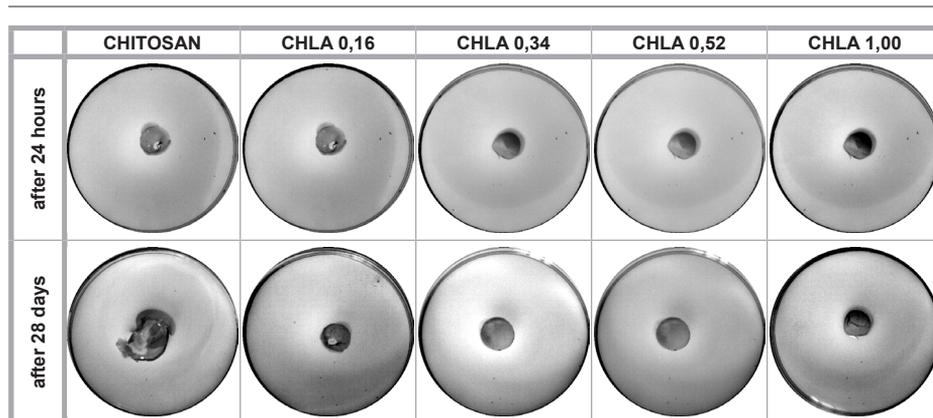


Figure 4. Chitosan and linoleic derivatives exposure to *S. aureus* – ISO 846 test

exhibited a lack of bacteria colonization. No difference was observed between samples exposed to bacteria for 24 hours or 28 days, as it is shown for LA-chitosan derivatives in **Figure 4**. We conclude that the chitosan and its derivatives are not readily degraded by these bacteria and, thus, do not act as a carbon and energy source. Further, the plasticizing effect of fatty acid modification, both LA and DLA, can also be observed in the photos (**Figure 4**); at higher fatty acid, sample deformation was minimal.

The antimicrobial properties of the chitosan derivatives were assessed in the second test, which was conducted with growth agar. After incubation, none of the materials exhibited a clear inhibition zone. This finding is not surprising, as this effect has been observed previously [3] and can be explained by the lack of active release and poor diffusion of chitosan in the agar [2], resulting in only organisms in direct contact being affected [8]. However, even though our FA-chitosan films did not result in an inhibitory zone, a closer examination of the samples does indicate that the films do possess antimicrobial activity. Figure 5 shows the results of the agar diffusion test for samples with the highest fatty acid content. In Figure 5A, no inhibition zone is visible around the sample disc in the plate. However, when the film is removed (Figure 5B), there is a noticeable lack of microorganisms where the film was present, indicating an antimicrobial effect of direct contact with the material, both at the edge of the sample and underneath it. For all tested materials, we obtained similar antimicrobial results, with no significant differences being observed between linoleic and dilinoleic derivatives.

4. Conclusions

Chemical modification of chitosan by acylation of its primary amine groups with the carboxylic groups of fatty acids (LA and DLA) was performed. For both LA and DLA chitosan derivatives, noticeable changes in the IR spectra associated with the fatty acids present in the derivatives were observed. The use of long chain fatty acids (linoleic and dilinoleic) as chitosan modifiers acted as plasticizers, increasing flexibility and, thereby,

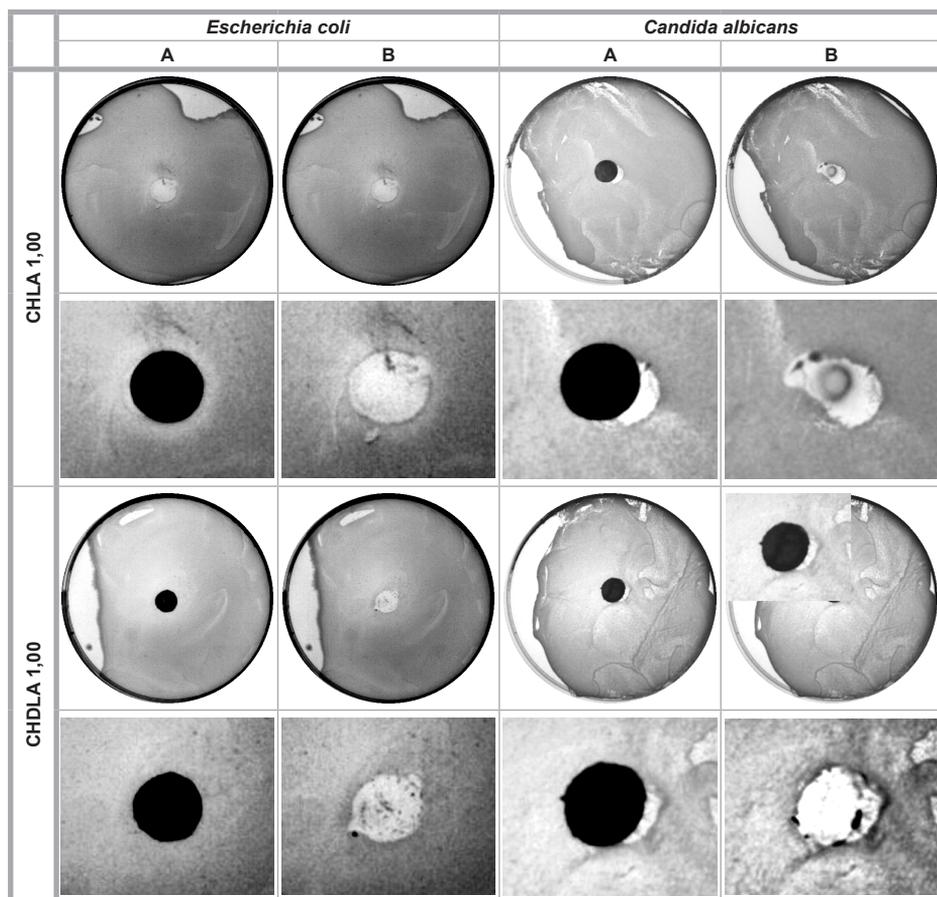


Figure 5. The agar diffusion test for CHLA 1,00 and CHDLA 1,00

facilitating further processing of films. Antimicrobial studies indicated that the chitosan and its derivatives are not readily degraded by *S. aureus* and *E. coli* bacteria and, thus, do not act as a carbon and energy source. Finally, a noticeable lack of microorganisms beneath polymeric films indicated an antimicrobial effect of direct contact with the material. Collectively, these findings suggest that modified chitosans may be well suited as antimicrobial coatings for medical devices, e.g. catheters.

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6. References

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