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Synthesis, characterization and antibacterial evaluation of cotton fiber coated with chitosan-agar/tannin derivative/polypyrrole composites

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Abstract: The development of chemically modified fibers for biomedical applications can be conveniently addressed through the production of new materials for sutures. Herein, it is proposed the mutual coating of chitosan (sample C) and a tannin-derivative (Tanfloc) – sample T for the following polymerization of polypyrrole – sample P in a complex structure that combines the outstanding performance of components as antibacterial and antibiofilm agents. The characterization of modified fibers was provided by SEM images, FTIR spectrum, and mechanical assays that were conducted and confirmed the adequate deposition of coating layers on fibers. The best antibacterial activity of the modified fiber was observed for the system prepared in the presence of three components (sample CTP) that returned a complete elimination of *Staphylococcus aureus* after 60 min of contact and a reduction in the biofilm formation in order of 99.38%.

Keywords: Antibacterial. Antibiofilm. Polypyrrole. Chitosan. Wearables. Cotton.

Introduction

Suture threads are essential components for clinical treatments (prevention of the spread of infections)[1] making the adequate choice of materials for use in wound treatment[2]. Materials based on cotton and silk have excellent properties such as good knot security[2], requiring additional treatment to acquire antibacterial properties (against *Staphylococcus aureus* and *Escherichia coli)*[3]. The typical procedure is based on the coating of the fibers, which can adversely affect the intrinsic physical and handling (mechanical properties) of the resulting material[4]. As a standard procedure to avoid these drawbacks and to reduce the risk of surgical site infections it is explored the incorporation of antibacterial agents such as triclosan, which has been used as an antiseptic for *in vitro* and *in vivo* applications with direct reduction in the adherence of selective clinical strains of Gram positive, Gram negative, antibiotic-resistant and biofilm-forming bacteria to the surface of surgical sutures[5]. Polyglactin is another essential compound that has been applied in association with triclosan as a coating layer for the prevention of *in vivo* bacterial colonization[6].

Despite the extensive use of triclosan, it has been reported the limited action of this compound against antibiotic-resistant bacteria[7]. On the other hand, the use of metallic nanoparticles (MNPs) has been progressively considered in related applications due to their non-toxic behavior for mammalian cells[8]. Based on these aspects, combinations of silver nanoparticles, antimicrobial peptides, and chitosan have attracted interest in the medical field due to their characteristic biodegradability, biocompatibility, bioadhesiveness, and non-toxicity behavior[9]. Alternatively, materials such as chitosan[10], tannin derivatives[11] and conducting polymers such as polypyrrole[12] can be associated with cotton fiber to improve its physical, handling, and antibacterial properties.

Chitosan (a derivative of chitin) is an edible, biodegradable, and biocompatible compound that has been widely used for several medical purposes, taking advantage of its antibacterial activity[13] against *Staphylococcus aureus*[14] which can be controlled by deacetylation degree, molecular weight, and pH[15]. Different mechanisms have been proposed to explain the antibacterial properties of chitosan: the change in the cell permeability due to the interaction between polycationic chitosan with electronegative charges on the bacterial cell surface[16] and the chelating activity that selectively binds essential metals and nutrients, inhibiting bacterial growth[17].

On the other hand, tannin derivatives possess remarkable properties such as antimutagenic and antitumor agents[18]. In addition, antiviral and antibacterial responses have been reported for tannins applied as additives in biopolymers, collagen and chitosan, agarose, and starch as a result of their

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unique characteristics[13], involving the inhibition in the proliferation of contaminants, tissue regeneration, and wound healing[19]. In addition, tannin-derivative compounds have been successfully applied in the green synthesis of metal nanoparticles and as antibacterial agents, as reported in Ref. [20].

Another important building block applied in the improvement of the antibacterial activity of compounds is the conducting polymer polypyrrole, which has been considered one of the most studied polymers, synthesized by electrochemical or chemical methods[21], and characterized by excellent antibacterial properties for application in wearable devices[22–25], odor control, textile-based medical devices such as bandages and wet wipes[21] being indicated for disease and infection control such as in multiple infections from resistant bacteria - methicillin-resistant *S. aureus* (MRSA) as well as in textile products for domestic use[21].

In particular, it is critical to the production of suture threads that can be able to inhibit bacterial growth at the surgical site without inducing resistance to antibiotics. Herein, a simple strategy to chemically modify cotton fibers by successive coating with chitosan, tannin-derivatives (Tanfloc), and polypyrrole is proposed to provide a material that not only preserves the intrinsic properties of the pristine fibers but also introduces the multifunctional activity with high antibacterial activity, fast kinetics for kill time assays and antibiofilm activity.

Materials and Methods

Materials

Bacteriological granulated agar (Merck, Darmstadt, Germany), pyrrole (Sigma-Aldrich, Saint Louis, USA), anhydrous ferric chloride (Exodus, Sumaré, Brazil), chitosan (Exodus, Sumaré, Brazil), hydrochloric acid (Sigma-Aldrich, Saint Louis, United States), bacteriological tryptone soy broth (TSB) and broth plate count agar (Merck, Darmstadt, Germany) and acetic acid (Vetec Química, Rio de Janeiro, Brazil) were used as received. Tanfloc® is a cationic compound from the tannin extract of *Acacia mearnsii* that is produced from the Mannich method that is based on the incorporation of imine cation in structure (responsible by cationic behavior of tannin derivative). Tanfloc® is produced by Tanac S.A. Brazil and more details about structure and applications are available in Refs. [26,27]. Pyrrole was distilled under reduced pressure before each experiment. The saline solution was prepared by dissolving 0.85 g of sodium chloride in 100 mL of an aqueous solution. All solutions were made using Milli-Q water with a resistivity of 18.2 MΩcm.

Methods

Cotton threads (Linhas Extra Forte, São Paulo, Brazil) with 0.5 mm in diameter and 10 cm in length were immersed in a 1% acetic acid solution for 12 h to activate the surface of fibers to be coated by additives[28]. Following this step, the successive coating of fiber by different additives was conducted to evaluate the influence of fillers on the overall response of fiber coated with chitosan (sample C), fiber coated with Tanfloc (sample T), and combined activity of fibers coated with chitosan, Tanfloc and polypyrrole (sample CTP) in comparison with negative control – pristine cotton fiber.

Characterization techniques

The morphology of fibers and the aggregation level induced by the progressive incorporation of fillers were evaluated from scanning electron microscopy carried out using an SEM Vega 3XM - Tescan (Brno – Kohoutovice, Czech Republic) at an acceleration voltage of 10 kV. The chemical composition of coating layers was scrutinized by the Fourier transform infrared spectrum (FTIR) - KBr method in an IR Prestige-21 Fourier transform infrared spectrometer Shimadzu (Japan). Mechanical evaluation of the changes in the response of the fibers under chemical treatment was analyzed from an electromechanical universal machine (EMIC model DL 10000, Instron, São José dos Pinhais, PR, Brazil) with output processed from TESC software.

D. Preparation of sample C

The solution was prepared by dissolving 500 mg of chitosan in 49 mL of water and 1 mL of acetic acid (10 mg mL^{-1}). After the complete dispersion of chitosan, 500 mg of agar was added. After that, the cotton fibers were immersed for 30 minutes in the resulting solution, washed with Milli-Q water, and dried in an oven at 90°C for 5 minutes. This procedure was repeated three times to ensure uniformity of the chitosan coating layer on cotton fiber[28].

Preparation of sample T

The sample T was prepared by dissolving 500 mg of Tanfloc in 50 mL of water (10 mg mL^{-1}). After complete dispersion of the Tanfloc in the solution, the cotton fibers were immersed in this solution for 30 minutes, washed with Milli-Q water, and dried in an oven at 90 °C for 5 minutes. This procedure was repeated three times to ensure uniformity of the coated cotton fiber.

Preparation of sample CT

The steps described in sections D and E were conducted in sequence from which the chitosan was firstly deposited on cotton fibers for the following deposition of the Tanfloc resulting in the sequence of

deposition of layers of chitosan and Tanfloc – sample CT.

Preparation of sample CTP

Cotton fibers modified with chitosan and Tanfloc were immersed in a solution of pyrrole (0.36 M) and HCl (0.1 M) and left under stirring for 30 minutes [28]. After this step, the fibers were polymerized by adding a solution of ferric chloride (0.36 M) at 4°C followed by agitation for 12 hours.

Agar diffusion test

Agar diffusion assays were performed according to Ref.[27]. The Gram-positive *S. aureus* bacterial inoculum (ATCC 25923) was prepared from a culture kept in agar at 4°C. An aliquot of bacterial inoculum was inserted into a saline solution with a concentration of 0.85%, leaving the bacterial suspension with a turbidity of 0.5 on the McFarland scale (10^8 CFU mL^{-1}). With the help of a swab, this bacterial suspension was dispersed in a Petri dish containing plate count agar (PCA) culture medium. Different samples (C, T, CT, and CTP) were incorporated into the plates to evaluate their antibacterial activity. After this step, the plate was incubated at 37 °C for 24h[27].

Kill time assays

The shortest time required for modified fibers to actuate in the elimination of the bacteria was determined from kill time assays, conducted as follows: 5 mL of 10^8 CFU mL^{-1} bacterial solution was placed in a test tube, which was later diluted to 10^6 CFU mL^{-1}. After homogenizing the bacterial solution, the different fiber-based samples (C, T, CT, and CTP) were placed in four tubes. The positive control and the blank were performed to compare with the response of four different systems. Then, 100 µL aliquots were removed from the tubes at fixed intervals of time (1 h, 2 h, 3 h, and 4 h) and placed in Petri dishes containing plate count agar (PCA). The plates were kept at 37 °C for 24 h[29] for the following counting of the viable cells.

Biofilm assays

Using a bacteriological loop, the inoculum of *S. aureus* (ATCC 25923) was dispersed in 10 mL of tryptic soy broth (TSB) in different tubes. After preparing the bacterial solutions, different samples (C, T, CT, and CTP) were added to four tubes. The positive and negative controls were performed to compare with the results of the modified fibers. Then, the tubes were incubated at 37 °C for 24h.

After this process, the fibers were transferred to new tubes containing 10 mL of saline solution. The resulting solutions (with samples) were sonicated in a bath (f = 40 kHz) for 15 min to remove species trapped in the wall of the tubes (adhered biofilms). Then, 100 µL aliquots were removed in triplicate from each system and seeded in a PCA medium. After that step, the plates were incubated at 37°C for 24h, and the colonies were counted to determine the number of viable cells in the biofilms [27,30,31].

Toxicity assays

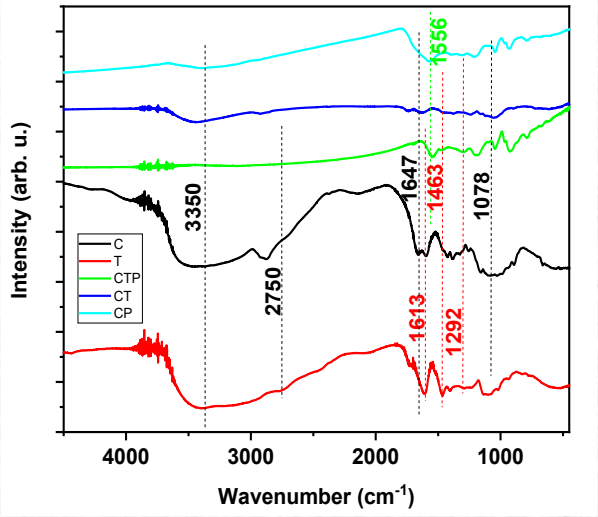
The toxic potential of cotton fibers associated with different materials (chitosan, Tanfloc, chitosan, and Tanfloc, chitosan, Tanfloc, and polypyrrole) was evaluated against *Artemia Salina*. For this, the fibers with different compositions were placed in test tubes vigorously shaken, and subsequently sonicated for 30 minutes to improve the dispersion of impregnated compounds in the solution.

Results and Discussion

Structure and morphology

The characteristic groups of additives incorporated into cotton fibers were scrutinized by the FTIR spectrum, summarized in Fig. 1. As can be seen, the FTIR spectrum of the fiber/chitosan (sample C) shows peaks attributed to the chitosan structure in the range of 500 cm^{-1} to 1084 cm^{-1} with bands attributed to the amine group at 1374 cm^{-1}. A wide absorption band between 3500 cm^{-1} and 2750 cm^{-1} is observed for chitosan components, due to the stretching of the primary amine (NH_2) and hydroxyl groups (OH^-) of chitosan. The characteristic bands at 1647 cm^{-1} and 1078 cm^{-1} are attributed to the twisting of the —NH group and the stretching of C—O, respectively. The characteristic band around 1660 cm^{-1} corresponds to the C=O stretching vibration of the amide group of chitosan[32].

Figure 1 - FTIR spectrum for samples C, T, CT, CP and CTP.



Source: Own authorship, 2023.

These shifts in the characteristic bands can be

attributed to the response of the amine groups ($-NH_2$) from chitosan at protonated cationic form ($-NH_3^+$) in an acidic solution, that remains in contact with the nucleophilic surface of the cotton fiber by hydrogen bonds and/or dipole-dipole interactions[33].

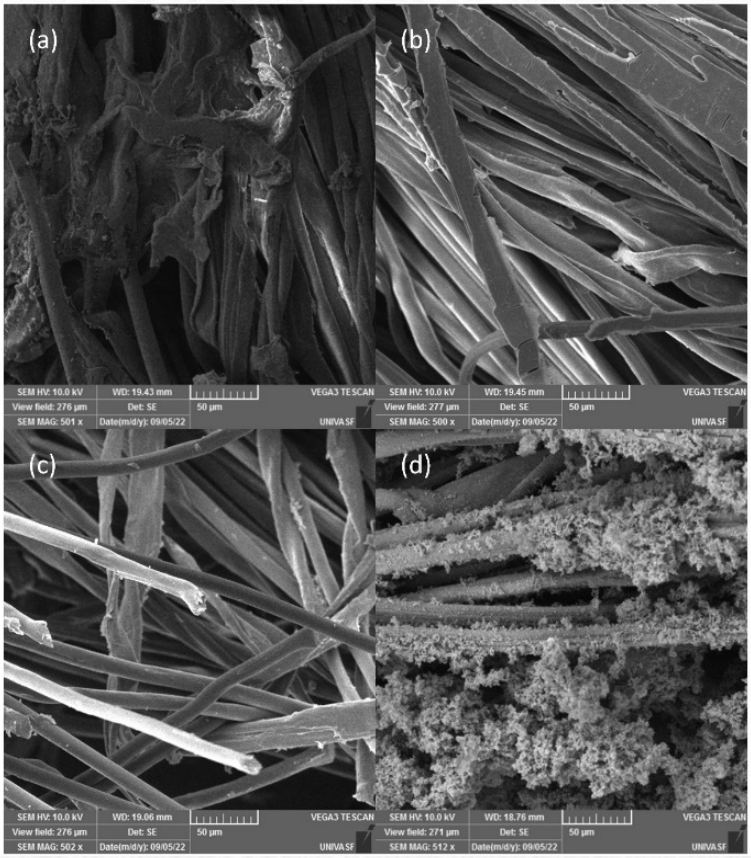
For sample T, the peak at 3350 cm^{-1} is attributed to the –OH elongation of the phenolic tannin. The small peak near 2943 cm^{-1} has been associated with the bond stretching vibration between aromatic C–H groups[34]. The peak at 1795 cm^{-1} belonged to the carboxyl-carbonyl group[35] while the peak at 1613 cm^{-1} corresponds to the –C=C– aromatic ring[34] and the band at 1463 cm^{-1} is assigned to methylene groups. The peak at 1292 and 1039 cm^{-1} was mainly due to the plane strain of the phenolic –OH group [36,37]. A band attributed to the –OH in the plane strain of the carboxylic acid group can be seen at 1141 cm^{-1} [38]. The off-plane vibrations from the deformation of the C-H bond in benzene rings were represented by small absorption bands at 936, 841, and 771 cm^{-1} [39].

The response of samples prepared with the mutual incorporation of additives into the fibers (sample CTP) is characterized by the presence of bands at 1600 cm^{-1} (attributed to the double bond between carbons (C=C) of the stretching of the pyrrole ring) and the peak at 1033 cm^{-1} which is assigned to the in the –C–H plane deformation of the pyrrole unit [32] while bands at 1463 and 1556 cm^{-1} are assigned to the C-C and C=C stretching vibrations of the polypyrrole ring confirming the incorporation of polypyrrole on fibers after their chemical polymerization.

SEM images were performed to evaluate the changes in the morphology of cotton fibers under successive steps of coating by antibacterial additives. As shown in Fig. 2a, the incorporation of chitosan into fibers results in a thick film that results in the aggregation of fibers due to the adhesive properties of chitosan. On the other hand, the impregnation of Tanfloc on cotton fibers (see SEM image in Fig. 2b) preserves the integrity of the pristine material and produces a thin layer on fibers, maximizing the surface area of material with the minimum aggregation of fibers by agglomerates of additives. An interesting finding is observed for samples prepared in the mutual presence of Tanfloc and chitosan. The typical behavior of the Tanfloc coating prevails and a coating on individual fibers is established (see Fig. 2c) preserving the intrinsic morphology of the fibers (minimal aggregation of fibers in agglomerates).

The polymerization of polypyrrole was provided on the CT samples (see Fig. 2d) in which is possible

Figure 2 - SEM images at the same magnification (500 x) for (a) cotton fibers coated with chitosan (sample C), (b) Tanfloc (sample T), (c) mutual deposition of chitosan and Tanfloc (sample CT), and (d) after polymerization of polypyrrole on chitosan/Tanfloc layer (sample CTP).



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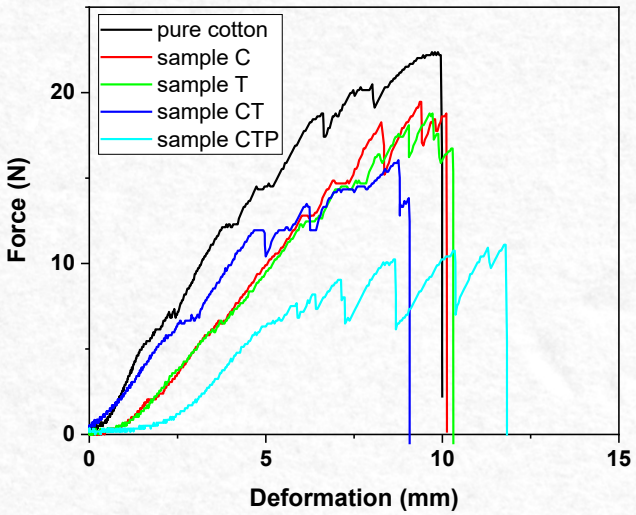
to identify the presence of grains of the conducting polymer dispersed on the surface of modified fibers.

Another important characterization technique evaluated to provide information about the modification in the structure of fibers is the mechanical characterization of pure and modified fibers from the force-deformation assays. As shown in Fig. 3, in comparison with the cotton thread, it is observed a general trend of reduction in the maximum force applied in the fibers applied before the rupture, with a more pronounced effect observed for the sample modified with polypyrrole, following the order sample CTP> sample CT> sample T> sample C> pure cotton.

Figure 3 - Force-deformation curves for samples prepared with different coating layers (sample C, sample T, sample CT, sample CTP, and pure cotton – negative control).



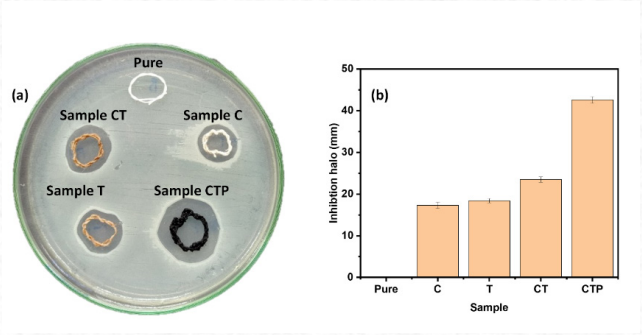
Source: Own authorship, 2023.

This process can be explained by the disruption in the interactions of modified fibers induced by the successive incorporation of chitosan, Tanfloc, and the polymerization of polypyrrole. However, it is worth mentioning that this reduction in resistance does not affect the functionality or the main purpose of the fibers for specific applications in sutures, since the skin is considered a non-linear elastic material with low sensitivity to the rate of deformation [40] and that the forces applied in the force-deformation tests are higher than the required forces involved in the skin/muscle set that is under treatment.

Antibacterial assays (agar diffusion assays)

The agar diffusion assays were conducted for pure (negative control experiment) and modified fibers that were separated in a length of 5 cm and disposed of as circles to make possible the identification of the corresponding inhibition haloes for each modified fiber. All of the modified fibers were disposed of as circles on plates that were inoculated with S. aureus for 24 h at 37 oC. Fig. 4a summarizes the inhibition zones obtained for negative control (pure cotton fiber) and samples C, T, TP, and CTP. As can be seen (and as expected) the pure fiber returned negligible activity while the isolated and combined incorporation of additives returned different antibacterial activity. The quantitative evaluation of the inhibition haloes is shown in Fig. 4b (as a result of experiments in triplicate) indicating that overall performance follows the order: sample CTP> sample CT> sample T > sample C. The antibacterial activity observed for chitosan-modified fiber is associated with the amino group, which binds to the surface components of bacteria and inhibits their growth[41].

Figure 4 - Inhibition halo assays (a) image of the plate containing circles of pure and modified fibers (sample C, sample T, sample CT, sample CTP) and (b) measured values for inhibition haloes.



Source: Own authorship, 2023.

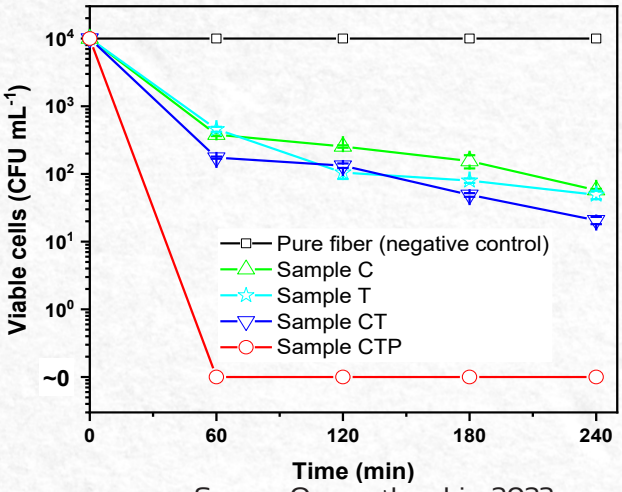
On the other hand, the inhibition zones observed as a consequence of the incorporation of the Tanfloc can be explained by its characteristic ability to form complexes with macromolecules, in addition to its polyphenolic nature of material[42]. In terms of the outstanding performance of the polypyrrole, this process is attributed to the formation of positive charges along the PPy chain, resulting in a strong electrostatic interaction between oppositely charged species (cationic surface of polypyrrole and negatively charged surface of bacteria) as a main source of antibacterial activity of the material.

Kill time assays

The kinetics of death of *S. aureus* provided by different experimental systems (coating layers) were determined from the direct measurement of the number of viable cells after a fixed interval of time of interaction of bacteria and antibacterial agent. As can be seen in Fig. 5, for samples C, T, CT, and CTP, the general trend is observed with the progressive reduction in the number of viable cells with the increasing time of treatment, while the negative control (as expected) returned negligible variation in the number of viable cells of *S. aureus*.

The higher value in the slope of the curves preserves a direct relationship with the effectiveness of the antibacterial agent. By direct comparison of the responses, it can be seen that fiber CTP eliminates the viable cells after 60 min of interaction. Intermediate performance is observed for samples C, T, and CT, an indication that polypyrrole is extremely important to provide outstanding performance in terms of bacterial inhibition in association with chitosan and Tanfloc layers.

Figure 5 - Results of the kill-time assays with the number of viable cells as a function of the time of interaction with the antibacterial agent for sample C, sample T, sample CT, sample CTP, and negative control.



Source: Own authorship, 2023.

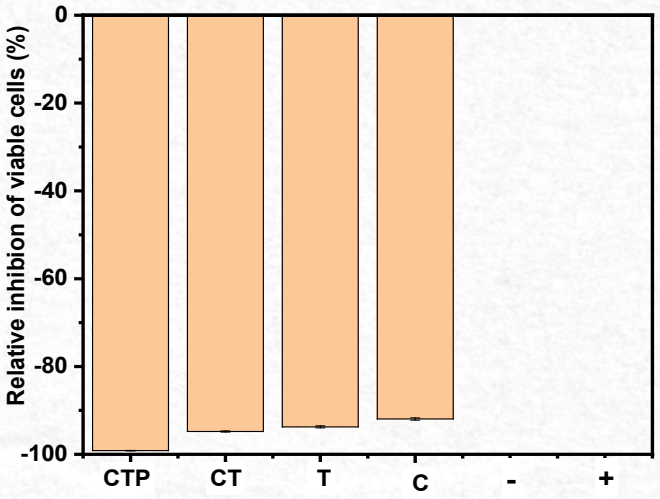
The general mechanism for the fast inhibitory activity of sample CTP can be attributed to the cationic behavior of the synthesized compound, which electrostatically interacts with negatively charged cell walls of bacteria attracting the material to the modified cotton surfaces. This process is combined with the release of reactive species from all of the additives that inhibit bacterial development, as reported in previous studies[30,43,44] that considered the isolated activity of additives.

Antibiofilm activity

The antibiofilm activity of compounds was evaluated from the direct counting of viable cells adhered in biofilm structures on cotton fibers (pure and modified). Results summarized in Fig. 6 indicated that all of the modified fibers are characterized by strong antibiofilm activity for all of the compositions that are compared with negative and positive controls (in which negligible activity is observed for cotton fibers – no growth of bacteria in negative control and complete growth of biofilm on cotton fibers – positive control). For all of the coating treatments, the antibiofilm activity of materials is higher than 91%, following the order sample CTP (99.38% of inhibition)>sample CT (95.32% of inhibition)>sample T(93.98% of inhibition)>sample C (91.18% of inhibition).

As can be seen, Fig. 6 shows a reduction in biofilm formation with the unmodified fiber applied as the control group. The fiber treated with chitosan, Tanfloc, and polypyrrole showed the greatest reduction (96.9%) in biofilm formation compared to the group of modified fibers. The electrostatic interaction of bacteria (as planktonic form and as biofilm) is followed by the migration of reactive species along with the cell wall that inhibits vital processes in bacteria[45].

Figure 6 - Results for the relative biofilm inhibition against *S. aureus* for sample C, sample T, sample CT, and sample CTP in comparison with the positive control.



Source: Own authorship, 2023

These results confirm the relevance of the polypyrrole in the overall antibacterial activity of the coating, due to the combined diffusive activity of reactive species in polypyrrole and the intrinsic antibacterial activity of the material, which provides outstanding performance in terms of the planktonic and biofilm inhibition of modified cotton fibers. The successive coating of additives (chitosan and Tanfloc) before the chemical polymerization of polypyrrole improves the activity of the resulting material.

Toxicity test with Artemia Salina

Artemia Salina nauplii have been explored in the evaluation of the toxicity of a large number of different compounds, including nanoparticles as a biological model in toxicology given its small body size, short life cycle, and relatively simple cultivation setup[46].

The toxicity test using the microcrustacean *Artemia salina* is one of the most common methods that

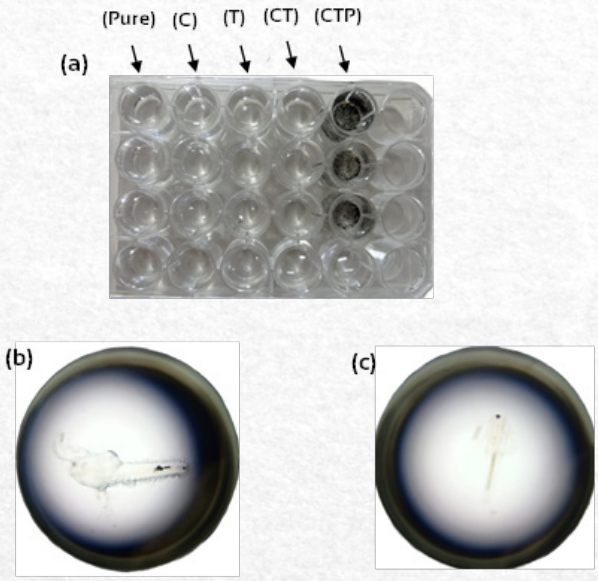
are associated with the use of animals to evaluate the toxicity of substances, acting as a bioindicator due to its reduced degree of tolerance and clear response to small environmental variations [47].

The results of the assays for the mortality of *Artemia Salina nauplii* returned a percentage of zero immobilization, and there was no death for the samples analyzed in the triplicates. All *Artemia Salina nauplii* were alive after 48 h of the cytotoxicity test, showing that there is no toxicity of the material under study.

Accumulation of material in the guts of *Artemia Salina nauplii* was observed in each triplicate and for all materials using a stereomicroscope equipped with a camera. Compared to controls, the exposed *Artemia Salina* entrails were full of particles (which were detached from the fibers after strong agitation and sonication).

No nauplii of *Artemia Salina* from a set of 120 (10 in each triplicate and 30 for each type of fiber, four different associations tested) that interacted with the different fibers died within 48 h of the toxicity test, as shown in Figs. 7a to 7c. These results suggest that the different fibers tested are not toxic to *Artemia Salina*. The ingested particles appeared as a long dark streak within the bowels. This can be explained by the lack of food intake and absorption and the filling of the intestine with particles.

Figure 7 - Toxicity assay in (a) pure fiber, fiber with chitosan, fiber with Tanfloc, fiber with chitosan and Tanfloc, fiber with chitosan, Tanfloc, and polypyrrole using *Artemia Salina*. In (b) a nauplius without contact with the materials in (c), in contact with the materials showing the animal's abdomen with the material.



Source: Own authorship, 2023.

Conclusions

The mutual incorporation of chitosan, Tanfloc, and polypyrrole as coating layers on cotton fibers preserves the mechanical properties of the pure material (due to the plastic properties of the Tanfloc coating layer) enriched by cationic behavior of chitosan and the outstanding antibacterial properties of polypyrrole that renders a quasi-complete biofilm removal for modified cotton fibers, a characteristic time for complete bacterial elimination of 60 minutes and a good degree of diffusion of reactive species that improves the antibacterial properties of the compound with negligible toxicity to *Arthemia Salina*. An important aspect to be considered is the prevention of critical changes in the mechanical resistance of fibers due to the self-aggregation of polypyrrole grains. To avoid these critical effects, the previous incorporation of chitosan and Tanfloc as coating layers protects the fibers against aggressive conditions of polymerization, maintaining the mechanical resistance of the resulting fiber at an acceptable limit for biomedical applications. These results confer to this multilayer coating procedure a promising application in antibacterial-modified fiber-based surfaces.

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Declarations

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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