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Hernia-repair prosthetic devices functionalized with chitosan and ciprofloxacin coating: controlled release and antibacterial activity

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Abstract

Polypropylene nets are widely used as hernioplasty prostheses. The reproduction of bacteria within the net fibers intersections can occur after the application of the prosthesis causing infections. For this reason, bacteria have to be removed in the very early stage of surgical implantation. Activation of the prosthesis surface was done by an innovative oxidizing plasma treatment (APP-DBD) working under atmospheric conditions in order to favor the deposition of an antibacterial coating of chitosan (biocompatible carbohydrate) and ciprofloxacin (broad spectrum antibiotic). Two different coating mixtures were realised and the antibacterial properties of such functionalised nets were investigated, together with their effectiveness. Physico-chemical characterisations of meshes were carried out before and after functionalisation by SEM-EDS and infrared spectroscopy. The release of both chitosan and ciprofloxacin, under controlled experimental conditions, was followed respectively by colorimetric determination (using UV-Visible spectroscopy) and chromatographic analysis (using HPLC). *In vitro* tests allow verifying antimicrobial activity (inoculation of specimens in a *Staphylococcus aureus* suspension).

1. Introduction

Polypropylene (PP) is the most widely used non-absorbable material for hernia-repair. After the Natta's discovery,¹ PP has been used in hernioplasty thanks to its bulk properties observed by F. Usher in the 1960s² and since then a lot of different types of meshes have been developed.³ The first evolution of this biomaterial for surgery applications comprehended the production of lightweight implants, of very small thicknesses and more comfortable for patients compared to standard-weight ones. Despite post-surgery infection being a rare and unexpected complication in hernioplasty and urologic surgery, the consequent failure of implants cannot be underestimated.^{4,5} Infections resulting from surgical implants are generally difficult to treat because they require long periods of antibiotic therapy and sometimes repeated surgical procedures. Infections could have a lot of different causes; one above all is the choice of the prosthetic mesh.⁶⁻⁸ In fact, some authors report evidence of chronic infections with formation of fistulas occurring after abdominal hernia-repair using PP meshes.⁹ Bacteria, causing the infection (mostly *Staphylococcus aureus*), have a medium size of about 1 mm, thus they may infiltrate all surgical meshes, whereas macrophages (cells of our body whose function is to phagocytize bacteria) present a higher medium size (*ca.* 10 mm) compared to the pores of many prosthetic meshes. Therefore, bacteria are shielded by the intervention of macrophages. It follows that the infection in progress, and not resolved, leads to a continuous and excessive recruitment of macrophages in the site surrounding, with formation of an infected non-vascularised tissue, which is often annoying for the patient. Sometimes the resulting diseases lead to removal of implants. Some authors reported that an adequate system of draining and irrigation of the surgical wound, with or without antibiotic administration, could be sufficient to prevent the infection development, but not always this procedure is sufficient.¹⁰ For all these reasons the prevention from bacterial colonization in hernioplasty is mandatory.

The discussion concerning how to prevent the post-surgery infections is still open. The oral and/or systemic antibiotic administration gives quite satisfying results.¹¹ On the other hand, other authors reported either positive or negative effects after local antibiotic treatment during the surgery.¹² Only few authors suggest the application of preventive treatment of the meshes with broad-spectrum antibiotics.^{13–16}

Recently, an interesting study has been realised by Fernandez-Gutierrez *et al.*:¹⁷ it focused on the functionalisation of lightweight PP meshes coated by a casting procedure with a biodegradable poly(HEMA-*co*-AMPS) layer, which acts as a drug-carrier, and vancomycin as an antibiotic agent. The results presented attested that the application of polymeric supports as controlled delivery systems of antibiotics could help surgeons in solving inflammatory problems due to bacteria proliferation and external body reactions. Moreover, it was highlighted how the design of functionalised hernia-repair implants (paying particular attention to the selection of appropriate drug/carrier couples) will be the future strategy for the development of novel and various biologically active biomaterials. This consideration stimulates the study of *in vitro* tests to extend the number of data concerning drug/carrier couples to be tested for *in vivo* applications.

In this paper, we extend our previous work¹⁸ considering a possible functional device achieved by coating deposition onto the plasma activated surface of lightweight PP meshes (30–70 g m⁻²). The coating, following the indications contained in ref. ¹⁷, is made of a drug and a carrier, where the drug is a commercial broad spectrum antibiotic (ciprofloxacin) and the carrier possesses also antimicrobial properties (chitosan).

Chitosan is a carbohydrate polymer that attracted great interest as a new functional biomaterial because of its excellent biological properties such as non-toxicity, biocompatibility and biodegradability.^{19–21} It is commonly used in the biomedical field as a carrier for biological active species in drug delivery systems.²² Moreover, chitosan possesses its own antimicrobial activity against many Gram positive bacteria (e.g. *Staphylococcus aureus*) and Gram-negative ones (e.g. *Pseudomonas aeruginosa* and *Escherichia coli*). The exact mechanism of chitosan antimicrobial activity is not yet fully understood, even if some hypotheses have been formulated in the literature.^{23,24}

In the present work, the chitosan layer, dispersing ciprofloxacin, was deposited, by means of the fouldard method, directly onto the PP devices soon after a controlled oxidation process of the fiber surface, carried out by atmospheric pressure plasma (APP-DBD) in an oxidizing atmosphere, in order to favor the interactions between chitosan and the PP surface.²⁵

Once the meshes were prepared and physico-chemically characterised, the kinetics of release of both the active principles (chitosan and ciprofloxacin) was determined in order to evaluate the device as a drug delivery system. In addition, the test of the antibacterial efficacy of the functionalised prosthesis was carried out by means of *in vitro* tests (inoculation of specimens in a suspension of *Staphylococcus aureus*).

Among other aspects, the preparation approach here described contains some novel aspects which are noteworthy to point out. All the mesh functionalisation chain (mesh activation via plasma and active coating deposition) was thought to make possible an industrialization of the final product, considering a continuous passage of the mesh from the plasma apparatus to the fouldard system. The plasma apparatus used was of the APP-DBD type: it allows to work continuously at atmospheric pressure and this eliminates the complication of producing the plasma phase under vacuum; the fouldard system is known to be a versatile method to depose coatings on the support under controlled conditions: the combination of the two approaches should predispose towards a mesh functionalisation industrial scale-up.

2. Experimental

2.1 Materials

Monofilament sterilized polypropylene meshes for surgical applications were provided by Herniamesh[®] S.r.l. (Chivasso, Italy). Lightweight (around 30 g m⁻²) meshes probes were 6×11 cm, with 0.32 ± 10% mm of thickness, fibers diameter of 80 ± 10% μm and 0.2024 g average weight. Chitosan (medium molecular weight), ciprofloxacin (CFC in the following, purity ≥ 98.0%), cibacron brilliant red 3B-A, sodium dihydrogen phosphate, sodium chloride and glycine were supplied by Sigma Aldrich. Tween 80 from Merck, acetonitrile AC0331 Supergradient Eluent HPLC grade from Scharlau, and phosphoric acid, hydrochloric acid and lactic acid from Carlo Erba were used.

All materials used for the antibacterial properties testing are reported in the ESI.

All aqueous solutions were prepared using ultrapure water Millipore Milli-Q[™].

2.2 Plasma apparatus

Both sides of mesh samples were treated by Plasma Nano Tech (Torino, Italy) in an open-air atmospheric plasma pressure glow dielectric barrier discharge (APP-GDBD system from Grinp S.r.l.): apparatus following an already tested procedure.^{18,25}

The system consists of two stainless-steel parallel plates of 80 cm × 23 cm × 3.5 cm, available for sample treatment and five electrodes of 80 × 1 cm, providing self-plasma impedance adapting glow discharge, and generating plasma phase. The type of discharge (filamentary or glow mode) is determined from the space between electrodes and composition of dielectric. The maximum attainable process power is 2500 W (corresponding to 3.75 W cm⁻²). An energy loss of about 40% is expected. A rotary pump and a heating box were used to produce water vapor for treatments. The system is a lab scale roll to roll version of an industrial size system, it allows to work continually and to develop dedicated functionalisation processes directly transferable to industrial scale production. An oxidizing mixture of He–O₂–H₂O (1050 W of nominal power applied) was selected for the surface plasma functionalisation. Each treatment was conducted for 30 s per mesh side.

2.3 Preparation of the antibacterial solution and coating deposition

Plasma activated meshes were functionalised by the deposition of an antibiotic solution using a foulard impregnation process. A foulard system (**Scheme 1**) is positioned in line after the plasma apparatus roll to roll set up. Meshes passed between the two foulards impregnated by the coating solution two times (3 seconds per time) by applying a pressure of 2 bar.

The coating solution was prepared by adapting the procedure described in ref.²⁶. Chitosan was dissolved in deionized water solution together with lactic acid and magnetically stirred for 7 hours at room temperature. Then CFC was added into the whole solution and homogenised by stirring for 2 hours. The final concentrations in aqueous solution were: 2.0 vol% of lactic acid, 2.0 wt% of chitosan, and 0.18 wt% of CFC for the mesh specimens named MA. For the sake of comparison, also meshes with the same contents of lactic acid and chitosan but containing 0.09 wt% of CFC (specimens named MB) were prepared.

The average weight of the deposited coating mixture for MA and MB is 0.0063 ± 0.0004 g (95% confidence interval) for each mesh. This value corresponds to a functionalisation amount of *ca.* 3 wt% with respect to the average weight of the mesh.

2.4 Physico-chemical characterization

Scanning electron microscopy (SEM) analyses were carried out by using a ZEISS EVO 50 XVP with a LaB₆ source, equipped with detectors for secondary electron collection and an EDS probe for elemental analyses, using 10 kV as the accelerating voltage. Samples insulate, thus they were covered with a gold layer of *ca.* 15 nm thick before the analysis to avoid any charging effect (Baltec SCD050 sputter coater). The presence of gold in EDS spectra (principal signal at 2.2 keV) is due to this step and will not be evidenced in the following data discussion.

Infrared spectra were recorded in attenuated total reflection mode (ATR-FTIR, using a diamond cell for single reflection) in a Bruker IFS28 spectrophotometer equipped with a Globar source, DTGS

detector and working with 128 scans at 4 cm^{-1} resolution in the range $4000\text{--}400\text{ cm}^{-1}$. ATR spectra were obtained on single fibers repeating the acquisition three times.

2.5 Analytical procedure for chitosan and CFC release determination

Functionalised PP meshes were cut into two square samples ($5 \times 5\text{ cm}$). The samples were weighted, wrapped on themselves and immersed in a closed tube falcon containing 10 mL of physiological solution (aqueous solution containing 0.9 wt% NaCl and 0.1 wt% Tween 80, PHY in the following). Samples were then placed inside an incubator (FOC225I Refrigerated Incubator – Velp Scientifica) at $35\text{ }^{\circ}\text{C}$ and subsequently sampled after 1, 2, 7 and 14 days. For each release test three replicas were performed. The same conditions were applied for antibacterial activity tests.

Cibacron Brilliant red 3B-A was selected as the dye for the colorimetric quantification of chitosan as reported by Muzzarelli.²⁷ The UV-Vis spectra were recorded with a CARY 100 Scan-Varian spectrophotometer and the wavelength selected for the colorimetric determination was 575 nm. An external calibration was made in order to quantify the chitosan release. The standard solutions were prepared by dilution of a chitosan stock solution (500 mg L^{-1}), prepared by dissolving the powder in PHY aqueous solution containing lactic acid 0.05 vol%. The calibration curves showed $r^2 \geq 0.9950$ and good reproducibility.

CFC quantification was performed by using a High Pressure Liquid Chromatography (HPLC) system (Merck-Hitachi) equipped with the following components: L-6200 pumps, Rheodyne injector, UV-Visible L-4200 detector (the wavelength selected was 274 nm) and column Polaris C18-A 3 mm Varian, $150\text{ mm} \times 2.0\text{ mm}$. CFC samples were eluted by employing isocratic conditions: phosphate buffer pH = 2.8 (85%) and acetonitrile (15%), with a flow rate of 0.2 mL min^{-1} . An external calibration curve in PHY solution was made in order to quantify the CFC release; the calibration curves showed $r^2 \geq 0.9991$ and good reproducibility.

2.6 Antibacterial activity evaluation

The evaluation of the antibacterial activity on the PP meshes was performed according to the International Standard ISO 22196.

Staphylococcus aureus ATCC 6538, Manufacturer TCS Biosciences, was the bacterium used for the test:²⁸ it is a first generation derivative from the original freeze dried vial of the ATCC culture.

The test was performed after inoculation and subsequent incubation for 1, 2 and 7 days, on three uncoated meshes (defined as Blank) and six functionalised (three MA and three MB) meshes after sterilization with ethylene oxide. Three untreated test specimens were used to measure viable cells 1 hour after inoculation.

The procedure details concerning the antibacterial test are reported in the ESI.

The data concerning the viable bacteria amount (and consequently the antibacterial effectiveness) were determined as follows.

Blank and functionalised samples after incubation were processed as described in the ESI section and the viable bacteria were immediately counted.

The enumeration of viable bacteria was carried out by performing 10-fold serial dilutions of the polyoxyethylene sorbitan mono-oleate broth (SCDLP in the following) in phosphate buffered physiological saline solution by placing 1 mL of each dilution, as well as 1 mL of the SCDLP broth recovered from the test specimen, into separate sterile PCA dishes. All platings were performed in duplicate. Dishes were incubated at $35 \pm 2\text{ }^{\circ}\text{C}$ for 48 hours.

After incubation, a count of the number of colonies was done if the number of colonies in the plates containing the 1 mL aliquots of SCDLP was less than 30. Vice versa, if no colonies were recovered from any of the PCA plates for a dilution series, the number of colonies was counted as <10 (where 10 is the volume of the SCDLP broth added to the specimen). The number of viable bacteria recovered for each set of test specimens was expressed as a geometric mean. It is noteworthy that for calculating the average when there were no viable bacteria recovered from a dilution series, 10 was considered as the number of viable bacteria.

3. Results and discussion

3.1 Prosthesis physico-chemical characterisation

SEM images of the MA sample are reported in **Figure 1** at low (section A) and high (section B) magnifications whereas a section of the coating is shown in **Figure 2**. The fibers appear almost regular in size, smooth on the surface, with some shallow incisions along the fiber length. The coating thickness, determined as indicated in **Figure 2**, ranges between 250 and 750 nanometers. Under experimental conditions used the EDS analysis should reach the depth of ca. one micrometer, therefore revealing the composition of both the coating and the support. The EDS spectra of meshes only plasma-activated and functionalised with the active coating are reported in **Figure 3** (sections A and B, respectively). Only carbon was detected for plasma activated fibers, whereas the composition of the outermost layer of the functionalized samples, carried out in different positions along the fibers, indicates the presence of carbon, as expected for PP, and oxygen, which indicates the presence of a continuous layer of chitosan. Only a small contribution due to nitrogen (indicative of CFC presence) is visible in the spectra, probably because the amount of the molecule in the coating is very low.

In order to have a confirmation of the chitosan deposition onto PP fibers, spectroscopy in the medium infrared range was carried out, since chitosan main absorptions fall in the range of 1150–890 cm^{-1} . These signals consist of vibrations of both glycosidic bond and C–O–C moieties.²⁹

ATR-FTIR spectra of plasma treated fibers before and after chitosan/CFC deposition are reported in **Figure 4**. Also in this case, the analysis was performed in different points along the fibers with the same results, confirming the homogeneity of the coating. The presence of a wide band centered at 1050 cm^{-1} in the spectrum of functionalised fibers is certainly due to chitosan finger print bands,¹⁸ whereas no signals certainly assignable to CFC molecules are visible in the spectra. However, the presence of CFC onto the meshes will be demonstrated in the following paragraph, by means of release tests in solution.

3.2 Kinetics of chitosan and CFC release in physiological solution

The analyte (chitosan and CFC) release from the meshes in PHY solution is reported as the percentage obtained by the ratio of the amount of analyte detected in solution and the amount of the coating mixture deposited onto the mesh (assuming a homogeneous coating). For each mesh subjected to the release test, the amount of the coating mixture deposited was calculated as the weight difference before and after the deposition treatment.

The chitosan and CFC release from MA meshes as a function of the residence time in the PHY solution is reported in **Figure 5A** and **6A**, respectively. The experimental data were reported as the average percentage calculated on three replicas and the error bars were reported in the plots. The error bar should comprehend also the coating deposited weight change due to the high hydrophilicity of chitosan which is able to adsorb high amounts of water from the environment. Nevertheless, although the meshes after preparation were not stored in closed vessels to control the relative humidity of the environment, they were kept in air-conditioned laboratories and this did not affect the drying of the deposited coating.

The amount of chitosan released in PHY solution reaches the value of 37% in 14 days, otherwise the release of CFC slightly increases with the immersion time up to 4% after 14 days.

These data demonstrate that the foulard method used to impregnate the meshes with antibacterial coating was effective. Otherwise, although the trend of kinetic curves relative to chitosan and CFC release is very similar and indicates a homogeneous dissolution of the coating, unexpectedly the percentages of chitosan and CFC released in PHY solution are considerably different (i.e., chitosan release is much higher than the CFC one). Srinatha *et al.*³⁰ already observed this phenomenon, in particular, with samples with a higher loading of chitosan with respect to CFC, explaining the experimental data through considerations related to the chitosan matrix dissolution behaviors

(solubility, tortuosity of the macromolecular film and so on). In their discussion the release of CFC was favored by approaching a chitosan/CFC ratio of 1. The analysis of release from the meshes MB with a higher chitosan/CFC ratio (results reported in **Figure 5B** for chitosan and **Figure 6B** for CFC) allows us to confirm this trend, since, in this case, the amount of chitosan released in PHY solution reaches the value of 57% in 14 days, whereas the release of CFC achieved 0.4% in the same period of time. In other words, the higher the chitosan/CFC ratio in the mesh coating, the lower is the release of CFC in PHY solution.

Considering the effect of release in antimicrobial application, the data indicate that the system studied is a good candidate to be used in surgical contest, since the maximum release of antibiotic/antibacterial molecules is observed in the first few hours from the implant, i.e., when it is more important to realise the action against the bacterial attack. Moreover, the fact that chitosan and CFC are still present on the surface of the meshes after 14 days of immersion in PHY solution suggests that the same situation could occur in biological media. If this hypothesis is correct, the antibiotic activity could occur directly on the mesh surface and this should avoid the growth of the bacterial biofilm on it.³¹

3.3 Testing of antibacterial properties

Antibacterial activity tests were performed on both treated meshes, MA and MB, and on untreated PP meshes (blank A and blank B, respectively) in order to highlight the efficacy of the smart coating functionalisation. For the sake of brevity, only results obtained after 1, 2 and 7 days of incubation are presented in **Tables 1, 2 and 3**, respectively, whereas all the other data are presented in the ESI.

The number of viable colonies decreases for the blank A material during incubation and the average number of viable bacteria (calculated using **eqn (SI-1)** reported in the ESI) decreases from 2.2×10^4 after 1 hour of incubation to 1.6×10^4 cells per cm^2 after 7 days of incubation.

For the MA material, the viable bacteria count on the plates containing SCDLP broth (where chitosan and CFC are present at the highest concentrations) and on those relative to dilutions (where the antibacterial agent is diluted by phosphate-buffered PHY) indicates that no colonies of *Staphylococcus aureus* were detected in all the specimens analysed after the three times of incubation studied (1, 2 and 7 days).

In the second experiment carried out to examine the antibacterial properties of the MB material, the number of viable colonies decreases for the blank B material during incubation and the average number of viable bacteria decreases from 2.1×10^4 after 1 h incubation to 2.0×10^4 cells per cm^2 after 7 days of incubation.

The experiment followed in the presence of the MB material evidences no colonies of *Staphylococcus aureus* detected neither in the plates containing SCDLP broth, nor in plates relative to 10^{-1} dilution with phosphate-buffered PHY for 1, 2 and 7 days. Vice versa, microbial growth was detected on 10^{-2} and 10^{-3} diluted plates for all the incubation times (1, 2 and 7 days). The fact that the number of colonies in these plates is more limited with respect to the blank tests which indicates that the device is able to control the bacterial growth.

Summarizing, MA and MB materials evidence two different situations. In the first case, the bioactive solutions obtained from the MA material, also the diluted ones, kill the bacterial colonies, therefore in no cases it is possible to observe the bacterial formation: the MA material shows bactericidal properties. In the second case, the bioactive solution obtained from the MB material stops bacteria reproduction without killing the microorganisms, which can continue growing when the bioactive solution is diluted and the active agent is present in lower concentrations: the MB material possesses bacteriostatic behavior.

4. Conclusions

This work describes and discusses the entire line of production of polypropylene meshes for hernia repair, starting from the preparation, passing through their physico-chemical characterisation, and concluding with the evaluation of their antibacterial properties.

The carrier/drug couple chosen for this study is chitosan/ciprofloxacin, which shows the double advantage of extending the knowledge about the joint use of drugs against bacterial growth in surgical sites, and collecting data using a drug/carrier couple where the chosen carrier itself possesses antimicrobial properties. This last aspect of the work should contribute to increase the number of studies concerning the evaluation of drug/carrier couple activity.

The study of two samples with different amounts of antibiotic indicates a concentration which produces a bactericidal effect (MA conditions) versus a simple bacteriostatic action (MB conditions) against *Staphylococcus aureus*. Moreover, the loading of different chitosan/CFC ratios affects the kinetics of release and the amount of drugs released in PHY solution, since higher the chitosan/CFC ratio, lower the quantity of CFC released.

The analysis of chitosan and CFC contents in physiologic medium provided evidence that only part of the antibacterial coating is dissolved in the physiological solution. This suggests that the antibacterial activity, after prostheses implant, can take place in biological fluids around the surgical site, but also at the surface of the device, avoiding the growth of the bacterial biofilm on it.

These results encourage new experiments devoted to verifying the usefulness of the smart device developed for *in vivo* applications.

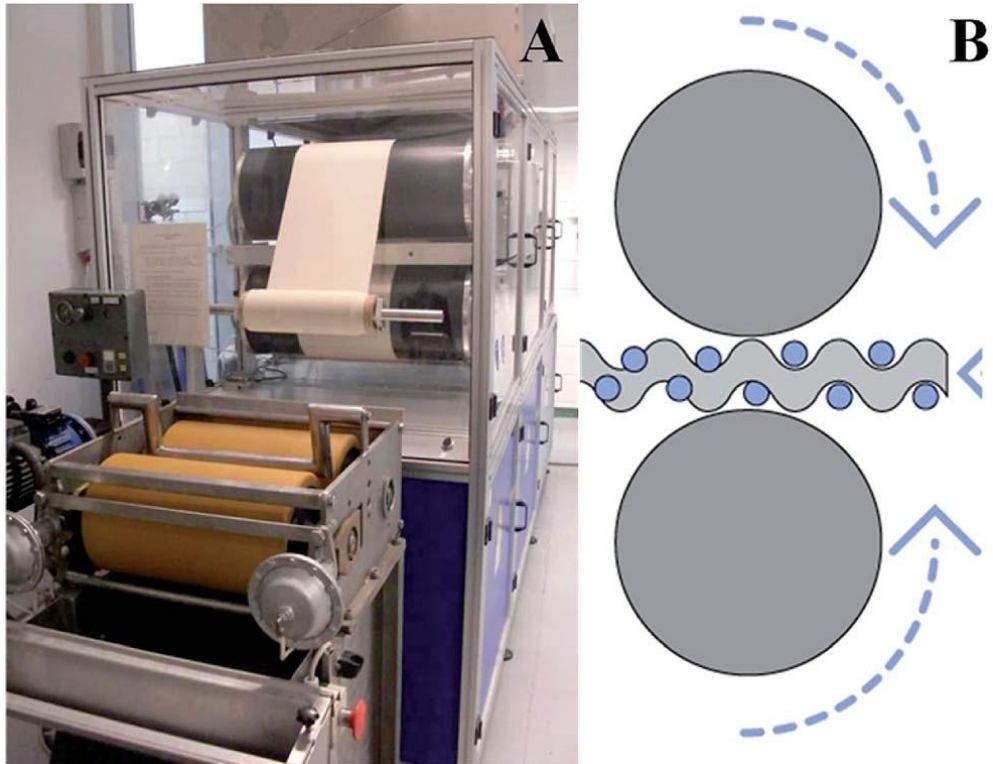
5. Acknowledgements

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Scheme 1. Experimental set-up relative to the lab scale roll to roll plasma apparatus followed by the foulard system (A) and foulard deposition mechanism (B).

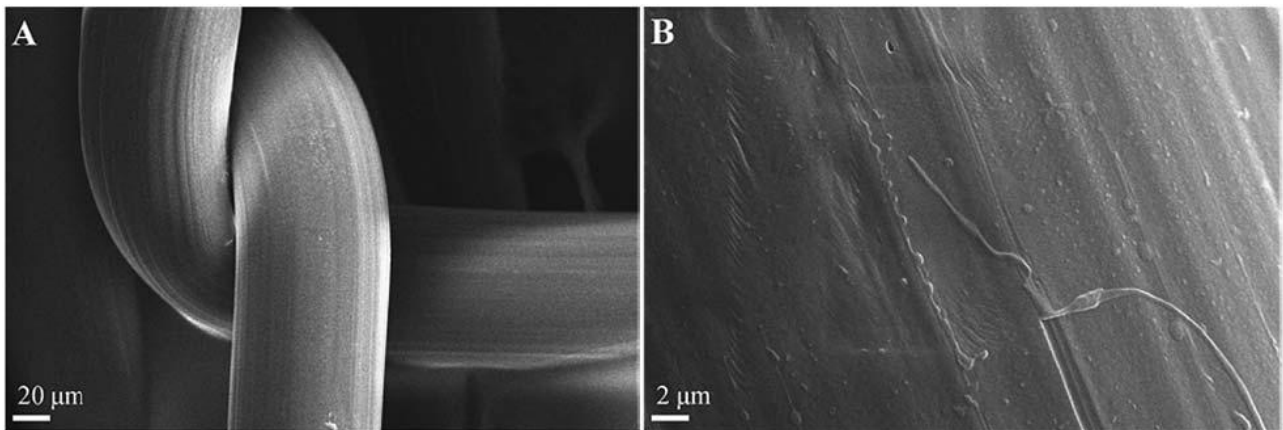


Figure 1. SEM micrographs of the PP fiber functionalised with the chitosan/CFC antibiotic coating at low (A) and high (B) magnifications.

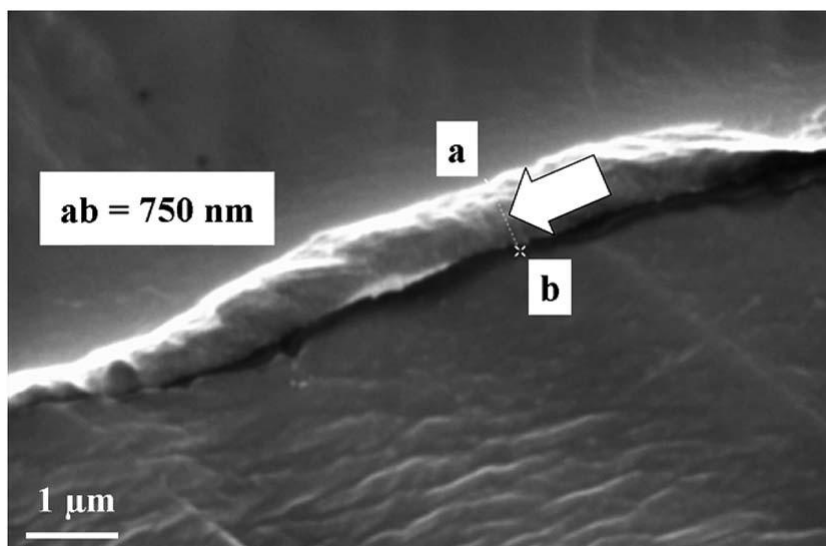


Figure 2. Cross-section of the PP fiber functionalised with chitosan/CFC coating. The arrow indicates the maximum thickness of the coating between the points a and b.

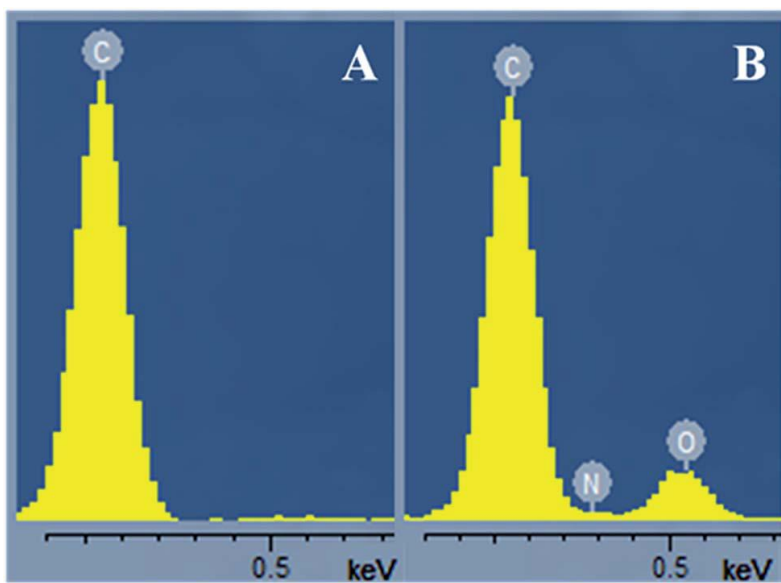


Figure 3. EDS spectra of plasma-activated PP fibers (section A) and chitosan/CFC coated fibers (section B).

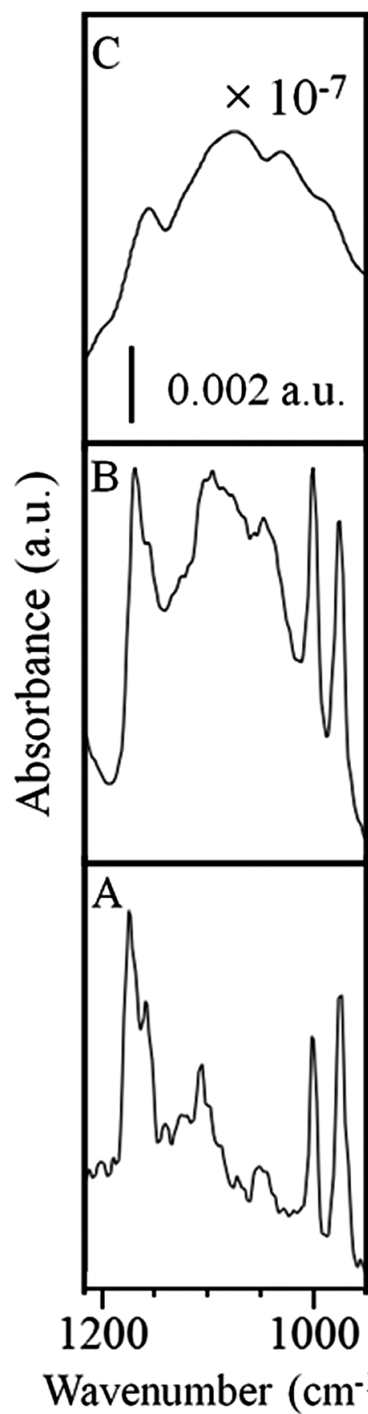


Figure 4. Absorbance FTIR spectra in the range of 1200–950 cm^{-1} of: (A) pristine PP fiber; (B) PP fiber functionalised with antibiotic chitosan/CFC coating and (C) chitosan reference powder. Spectra A and B were collected in the ATR mode, whereas spectrum C in transmission mode.

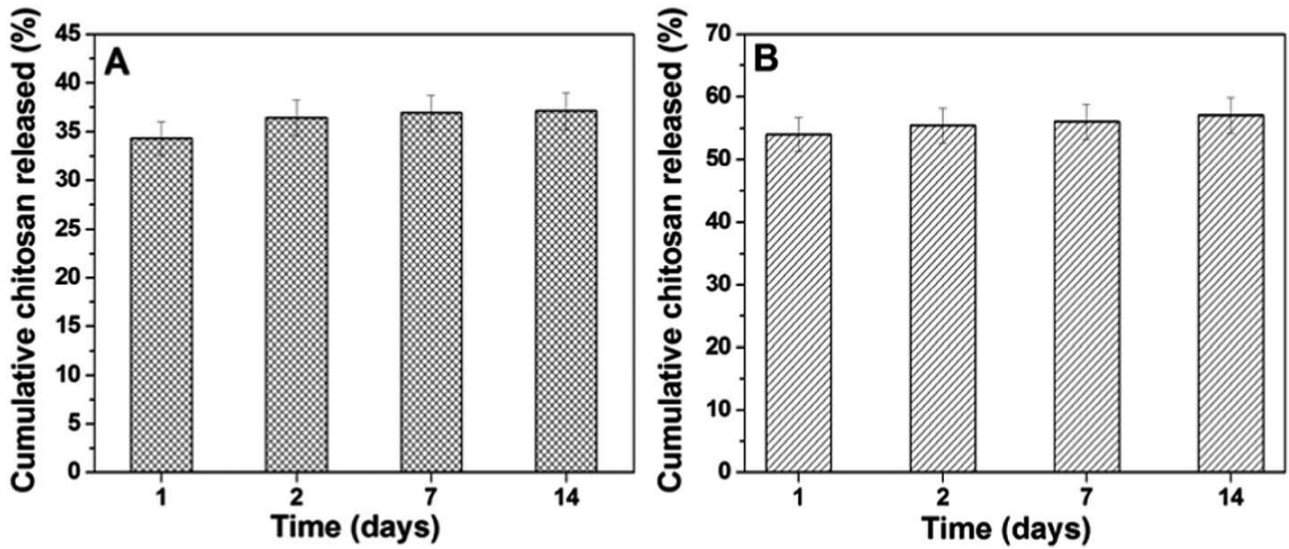


Figure 5. Chitosan release (%) from MA (section A) and MB (section B) meshes vs. residence time (days) in PHY solution.

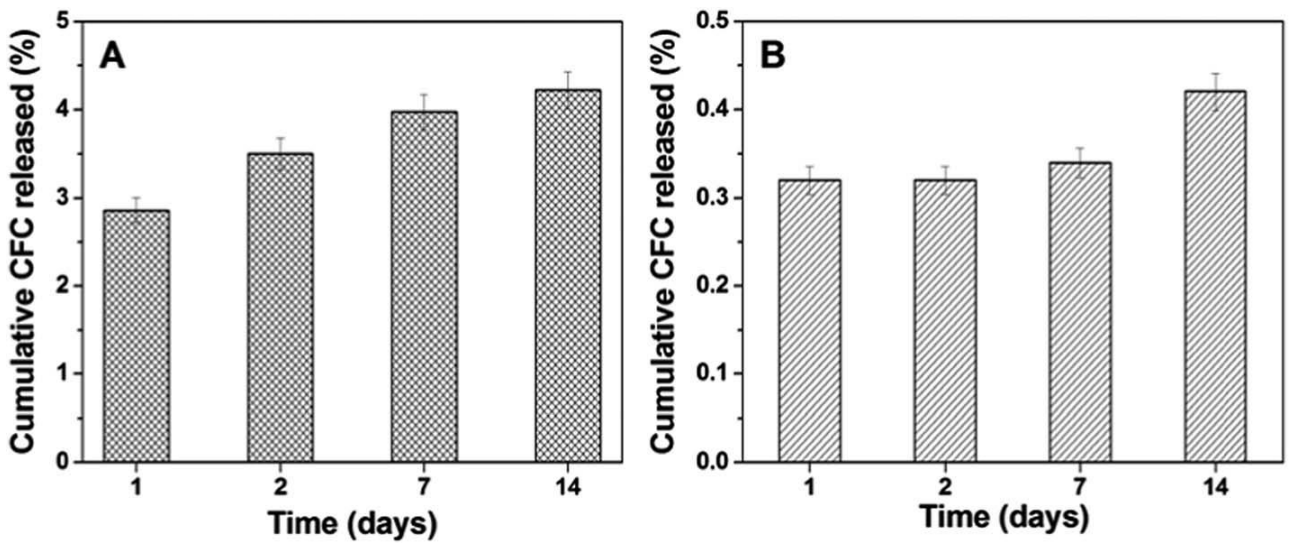


Figure 6. CFC release (%) from MA (section A) and MB (section B) meshes vs. residence time (days) in PHY solution.

Table 1. Untreated and treated test specimens after inoculation and subsequent incubation for 1 day.

Samples	Inoculum SCDLP	10 ⁻¹ dilution ^{a)}	10 ⁻² dilution	10 ⁻³ dilution
Blank A1	>300	>300	>300	39
Blank A2	>300	>300	>300	38
Blank A3	>300	>300	>300	38
MA1	<10	<10	<10	<10
MA2	<10	<10	<10	<10
MA3	<10	<10	<10	<10
Blank B1	>300	>300	>300	35
Blank B2	>300	>300	>300	41
Blank B3	>300	>300	>300	43
MB1	<10	<10	23	15
MB2	<10	<10	27	13
MB3	<10	<10	21	16

a) Dilutions are reported in CFU per mL.

Table 2. Untreated and treated test specimens after inoculation and subsequent incubation for 2 days.

Samples	Inoculum SCDLP	10 ⁻¹ dilution ^{a)}	10 ⁻² dilution	10 ⁻³ dilution
Blank A1	>300	>300	>300	47
Blank A2	>300	>300	>300	54
Blank A3	>300	>300	>300	49
MA1	<10	<10	<10	<10
MA2	<10	<10	<10	<10
MA3	<10	<10	<10	<10
Blank B1	>300	>300	>300	39
Blank B2	>300	>300	>300	34
Blank B3	>300	>300	>300	37
MB1	<10	<10	22	28
MB2	<10	<10	17	21
MB3	<10	<10	19	23

b) Dilutions are reported in CFU per mL.

Table 3. Untreated and treated test specimens after inoculation and subsequent incubation for 7 days.

Samples	Inoculum SCDLP	10 ⁻¹ dilution ^{a)}	10 ⁻² dilution	10 ⁻³ dilution
Blank A1	>300	>300	>300	29
Blank A2	>300	>300	>300	35
Blank A3	>300	>300	>300	32
MA1	<10	<10	<10	<10
MA2	<10	<10	<10	<10
MA3	<10	<10	<10	<10
Blank B1	>300	>300	>300	26
Blank B2	>300	>300	>300	28
Blank B3	>300	>300	>300	23
MB1	<10	<10	12	9
MB2	<10	<10	11	15
MB3	<10	<10	16	13

c) Dilutions are reported in CFU per mL.