CHARACTERIZATION AND *IN VITRO* EVALUATION OF THE BEHAVIOUR OF 6-CARBOXYCELLULOSE/AG(I) IN THE PRESENCE OF MICROORGANISMS

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A new type of an antimicrobial material based on silver salt (chloride) adsorbed onto 6-carboxycellulose (OC/Ag(I)) was characterized using solid-state carbon-13 cross-polarization–magic angle spinning nuclear magnetic resonance (^{13}C CP/MAS NMR) spectroscopy, flame atomic absorption spectrometry (FAAS), X-ray diffraction (XRD) and size exclusion chromatography (SEC). *In vitro* investigations of the behaviour of the material were carried out in buffered nutrient media (pH 7.0 or 5.4) in the presence of several human pathogens, such as *Aspergillus fumigatus*, *Candida albicans*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* at 37 °C within a period of one week. The concentration of Ag released into the liquid nutrient media was measured by FAAS. Further, the composition of the resulting liquid and solid phase was characterized by different chromatographic methods, Fourier transform infrared spectroscopy (FTIR), FAAS, and inductively coupled plasma orthogonal acceleration time-of-flight mass spectrometry (ICP-oa-TOF-MS) and scanning electron microscopy (SEM). The results showed that none of the investigated microorganisms have shown significant degradation potential of OC structure. Only a partial release of Ag was observed, compared to the initial samples, regardless of the presence of the microorganism strain and both investigated pH values. Other noticeable changes observed in the molecular structure of OC were due to the increasing neutralization degree upon cationization by the ions present in the nutrient media.

Keywords: oxidized cellulose, silver salt of oxidized cellulose, degradation, antimicrobial materials, analysis of polymer, silver

INTRODUCTION

Silver plays an important role in promoting wound healing and in preventing infection of a wound[1-6] and thus, silver-containing dressings are well-established in the medical industry as safe and effective antimicrobial agents.[5,7-11] Many types of these dressings are nowadays commercially available for this purpose.[10,11] These materials are mainly designed: (i) to release Ag

into the wound bed for antimicrobial action (Acticoat 7, Smith & Nephew), (ii) to absorb wound fluid and exudates containing infectious organisms into the dressing fabric, where the silver exerts its antibacterial action (Aquacel Ag, ConvaTec and Actisorb Silver, Johnson & Johnson), (iii) both to absorb wound exudates and to release Ag into the wound bed (Contreet Foam, Coloplast) or (iv) to release Ag sulphadiazine which liberates, in the presence of wound fluid, Ag and sulphadiazine acting against silver-resistant bacteria (Urgotul Ag, Urgo).[8,10,11]

Numerous approaches for the development of these materials were used by individual producers[10,11] relating to the usage of different types of dressing materials (polyethyelene, polyester, sodium carboxymethylcellulose, nylon, etc.) and silver coating (nanocrystalline silver, silver in an ionic form, silver sulphadiazine, etc.). The present research performed by VUOS, Synthesia Inc., the Czech Republic, is focused on the development of these types of silver-releasing agents based on a combination of Ag salts and oxidized cellulose (OC/Ag(I)). These materials are designed to release Ag ions to exert a sustained antimicrobial effect of oxidized regenerated cellulose (OC), previously demonstrated by several authors[12-15] and attributed to the significant acidity of OC solutions,[14] and to take other advantages of OC, such as its bio absorbability or hemostatic action.[16]

The concentration of silver in commercially available silver-containing dressings of different types varies considerably and different factors like the distribution of silver in a dressing, its chemical and physical form and dressing structure influence the amount of silver released from the dressing and the efficacy of silver as an antimicrobial agent.[11] An improved antimicrobial activity of OC/Ag(I) towards the different tested bacteria when compared to unmodified OC samples was previously reported.[12] However, the reduction of the silver ion accompanied by changes of surroundings to brown and/or black colors in contact with some bacteria and additionally the changes in sample composition previously reported[12] needed to be resolved. Information about the behaviour of OC/Ag(I) during the *in vitro* studies in the presence of several human pathogens has not been reported to this date, similarly as the information about the material structure. This is due to the fact that the characterization of the original material with Ag content of only about 1% (w/w), and of the resulting complex sample of a high molecular weight obtained after the *in vitro* degradation study is still an analytical challenge.

The objective of this study was thus to evaluate the *in vitro* degradation of a novel type of antimicrobial material based on a silver salt of 6-carboxycellulose (OC/Ag(I)) caused by the metabolic action of several human pathogens under different experimental conditions, as well as to characterize the material.

EXPERIMENTAL

Samples and sample preparation

The samples of OC/Ag(I) were prepared at VUOS Synthesia Inc. (Pardubice, Czech Republic) using the following procedure. The amount of 100 g of oxycellulose was dispersed in 1 L of 0.205% aqueous silver nitrate. After stirring the mixture for 12 h in the dark, the sample was filtered and placed into 1 L of 0.5% solution of NaCl in 50% ethanol for 1 h for photostabilization. After that the samples were wrung out and placed into 96% ethanol for 1 h in order to remove water and dried under the laboratory conditions.

Chemical and physical characteristics of both OC and OC/Ag(I) samples were given by *p*H value of 1% (w/w) solutions of these samples (*p*H was measured at 25 °C), concentration of free carboxyl groups (–COOH), carbonyl groups content (aldehyde and ketone)[17] and ash level were 2.9, 19.3% (w/w), 1.7% (w/w), 0.1% for OC samples, respectively 3.2, 18.1% (w/w), 0.60% (w/w), 1.8% (w/w) for OC/Ag(I). The concentration of Ag in OC/Ag(I) samples was 1.2% (w/w).

All samples were stored in non-transparent PE sachets in a refrigerator.

Bacterial cultures

Two bacterial (*Pseudomonas aeruginosa* CCM 1960 and *Staphylococcus epidermidis* CCM 4418), one yeast (*Candida albicans* CCM 8180), and one fungal (*Aspergillus fumigatus* CCM 373) strains were used throughout this work. All the strains were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic). A suspension of bacterial and yeast strains was prepared from fresh colonies on blood agar plates (HiMedia, India) (for bacteria) and malt agar (HiMedia, India) (for yeast) after 24 h incubation for all strains. The concentration of cells was adjusted to 10^8 cfu mL^{-1} using the 0.5 McFarland standard turbidity scale and serially diluted to initial inoculum of 10^5 cfu mL^{-1}. A suspension of fungal spores was prepared from cultures grown for 7 days on malt agar at 24 °C and the density of spores was adjusted to 10^5 spores mL^{-1}.

FT-IR method

The FT-IR spectra of KBr pellets were taken by the Thermo Scientific Nicolet 6700 spectrometer at room temperature. Spectra were recorded in an absorbance mode and thoroughly analyzed for all absorption peaks from wavenumbers 4000 to 800 cm^{-1}. The resolution was of 4 cm^{-1} and 64 scans were collected. The reported spectra of the OC and OC/Ag(I) were obtained by subtraction of the spectra of pure KBr from the raw data.

Solid state NMR spectroscopy

Solid-state ^{13}C CP/MAS (cross polarization/magic angle spinning) NMR spectra were measured at ambient temperature by a Bruker Avance 500 spectrometer at 125.8 MHz with spinning frequency 11 kHz and contact time 2 ms, i.e., under conditions that allowed quantitative analysis. Chemical shifts in the ^{13}C NMR spectra were referred to the carbonyl line of glycine (with a signal at 176.0 ppm from TMS) by sample replacement.

X-ray diffraction (XRD)

Powder X-ray diffraction data (Cu Kα, λ = 1.5418 Å) were collected on a D8 Advance diffractometer (Bruker AXS, Germany) with Bragg-Brentano Θ-Θ goniometer (radius 217.5 mm) equipped with a secondary beam curved graphite monochromator and Na(Tl)I scintilation detector. The generator was operated at 40 kV and 30 mA. The scan was performed at room temperature from 5 to 60° (2 Θ) in 0.02° step with a counting time of 10 s per step.

Chromatographic analyses

HPLC method

The HP Agilent 1100 HPLC system (Agilent Technologies, USA), equipped with a vacuum degasser, quaternary pump, autosampler and evaporative light scattering detector (ELSD), was used for the chromatographic experiments. All separations were carried out on a LiChrospher Diol 100 column (4×150 mm; 5 µm). Samples (10 µL) were eluted by a method that was optimized for the separation of either oligomers or monomers of the saccharide units of the original materials. Oligomers and monomers were eluted using a mobile phase comprised of 85% acetonitrile (A) and 15% water (B) used with one of the following gradient conditions: 75% (A), 25% (B) until 15 min, 50% (A), 50% (B) until 30 min, 0% (A), 100% (B) until 31 min, 0% (A), 100% (B) until 35 min. Flow rates and column temperatures for the separation of oligosaccharides and monosaccharides were of 1.0 mL min^{-1} and 40 °C.

Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) measurements were carried out using the Agilent 1100 Series system equipped with a vacuum degasser, isocratic pump, manual injector (100 µL sampling loop), thermostatted column compartment, refractive index (RI) detector. Data evaluation was performed with standard ChemStation GPC data analysis software (GPC-Addon).

The following parameters were used in the SEC measurements: flow, 1.00 mL min^{-1}; mobile phase 0.2 mol L^{-1} $NaNO_3$ and 0.01 mol L^{-1} NaH_2PO_4 in demi water, column temperature 25 °C, optical unit temperature 35 °C, columns, PL aquagel OH guard 8 µm (50x7.5 mm), PL aquagel-OH MIX 8 µm (300x7.5 mm), PL aquagel-OH 40 8 µm (300x7.5 mm); injection volume, 20 µL; run time, 27 min. Samples were filtered through a 0.45 µm nylon syringe filter (Whatman Autovial) prior to the analysis. A set of analytical pullulan polysaccharide standards, for GPC, Mp 5900–708 000 g mol^{-1} was used for calibration purposes.

Scanning electron microscopy

The morphology of the samples was examined using a scanning electron microscope JEOL-JSM 5600 LV manufactured by Jeol, Japan. The samples were mounted on metal stubs using double sided adhesive tapes. Scanning electron micrographs of the samples were obtained at an acceleration voltage of 15 kV. The samples were previously sputter coated with gold for 30 s, using a Cresingtor plasma sputter coater.

Microwave digestion

In order to study the homogeneity of Ag distribution in OC/Ag(I) samples, the microwave digestion of the samples was carried out in Speedwave™ MWS-3+ (Berghof, Germany) microwave system with the maximum total output of the microwave generator 1450 W. For this purpose, a 100-500 mg portion of the powdered sample was weighed and placed into a 100-mL pressure resistant PTFE vessel, whereupon 6 mL of HNO_3 (65%, w/v) was added. The samples were digested in 5 steps: (i) 5 min at 80% power and 160 °C (ramp 3 min), (ii) 10 min at 90% power and 210 °C (ramp 10 min), (iii-v) 5 min at 10% power and 100 °C (ramp 1 min). The resulting solution was transferred into 100 mL volumetric flask and diluted to approximately 50 mL with deionized water. After that, 5.5 mL of ammonia solution (25%, w/v) was added to dissolve AgCl and the volume was adjusted with deionized water to 100 mL.The solutions were analysed by F-AAS along with blank sample containing the same amount of individual reagents and prepared in the same way as the samples. Before the analysis, the samples were diluted with deionised water.

AAS method

The determination of K, Na and Ag was carried out with an Avanta P double beam atomic absorption spectrometer (GBC Scientific Equipment Pty. Ltd.,

Australia) equipped with deuterium arc background correction in the flame emission and absorption mode, respectively. Sample solutions were introduced to the spectrometer using a standard GBC pneumatic nebulizer. The hollow cathode lamps obtained from Photron Pty. (Australia) were used throughout. The wavelength was set at 766.5 nm for K, 589.0 nm for Na, and 328.1 nm for Ag with a lamp current of 4 mA. Spectral resolution of 0.2 and 0.3 nm was used for Na, K and Ag, respectively. The gas flame mixture was formed with 10.0 L min^{-1} air and 1.5 L min^{-1} acetylene (99.5%, from Linde Gas, Inc., Czech Republic).

In order to evaluate the silver release from the OC/Ag(I) samples, an amount of 0.20 g of powdered sample was placed in sterile vials in an aseptic manner and Brain Heart Infusion broth (BHI) (HiMedia) (for bacteria) or Malt Extract Broth base (MEB) (HiMedia) (for yeast and fungi) was then added to each vial to make up to a total volume of 10 mL. An aliquot (1 mL) of the bacterial, yeast or fungal suspension was added to each vial containing the sample. Control broths with and without bacterial, yeast or fungal inoculation were also included. The vials were then incubated under different experimental conditions. Aliquots of 1 mL bacterial broth were sampled from each vial at specific time intervals and serial 25 fold dilutions for each aliquot were used, which allowed Ag quantification by the applied analytical technique.

Before the determination of Na and K content in OC and/or OC/Ag(I), the samples were reconstituted in cadoxene. The concentration of solutions ranged from 0.5 to 10 mg L^{-1} for Ag and 0-20 mg L^{-1} for Na and K. A single calibration graph was constructed for each element, from which all calculations were made in one analytical run. Five aqueous standards were used to obtain the calibration curves. Five replicates at 3 seconds integration time were used. All the calibration plots were linear in the investigated concentration ranges with the correlation coefficients better than 0.999.

ICP-TOF-MS

ICP-MS measurements were performed by the Optimass8000 ICP-oa-TOF-MS instrument (GBC Scientific Equipment Pty. Ltd., Australia). Details of the instrument and the operating conditions are summarized in an earlier study.[18]

Determination of carboxyl content

The carboxyl content was determined by the well-established volumetric method described previously.[19, 20]

Statistical analysis

The calculations and statistical evaluation were carried out using commercial software Statistica 6.1 (StatSoft., Inc. Tulsa, USA), and Origin 7.0 (OriginLab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSION

Characterization of OC/Ag(I) samples

^{13}C CP/MAS NMR

^{13}C CP/MAS NMR method, although less accessible to routine laboratories, was used for characterization of the samples as FTIR method, i.e. the most frequently used tool for obtaining rapid information about oxycellulose structure and physical and chemical changes taking place with various treatments,[20, 21] is unable to reveal changes among the spectra of OC and OC/Ag(I) for the samples with the content of Ag of about 1% (w/w).

^{13}C CP/MAS NMR spectra of oxidized cellulose and modified oxidized cellulose samples were measured and are presented in Fig. 1. The solid-state ^{13}C CP/MAS spectrum of oxycellulose has been previously reported by several authors.[19] Thus, analogically, in the spectrum of oxidized cellulose (Fig. 1a), the 170 ppm peak is assigned to the carboxyl carbon, that is $C^6_{(COOH)}$ in the oxidized anhydroglucose unit of the cellulose chain, the peak at 93 ppm is attributed to C^1 of the terminal α-D-glucose unite, the 63 ppm component of $C^6_{(CH2OH)}$ resonance, the peak at 104.7 ppm is assigned to C^1-carbon of basic cellulose chain, the peaks at 87.3 and 82.9 ppm are due to carbons on the chains located in the crystalline (C^4_a) and amorphous interiors (C^4_b), respectively, the cluster of peaks in the region 70-76 ppm due to C^2, C^3, and C^5 resonances, from both amorphous and crystalline part, shows a maximum at 75 ppm, the signal at 14 ppm is due to the CH_3 group.

When comparing the spectra of OC and OC/Ag(I) (Fig. 1), a difference in the chemical shift for $C^6_{(COOH)}$ can be seen, most probably due to the chemical influence by Ag salt. In addition, two signals for the CH_3 group were observed in the spectra of OC/Ag(I) (Fig. 1b) having the same overall intensity, probably due to the fact that the modification by Ag silver salts also impacts the CH_3 group. Another notable feature in the spectra of modified oxidized cellulose, compared to the spectrum of the non-modified sample, is the twice higher increase in sensitivity at the 93 ppm peak (C^1_t), showing that the OC/Ag(I) sample contains shorter chains.

AAS method

Atomic absorption spectrometry was applied both to determine the content of silver and to evaluate its distribution in the sample. The

homogeneity of a sample is an important prerequisite for attaining a desirable therapeutical effect and obtaining accurate results during the studies on Ag release from OC/Ag(I) samples. A total number of 16 OC/Ag(I) with the same Ag concentration obtained from different places of the original samples were analysed in order to check the homogeneity of these materials. The values for Ag content (mean ± S.D.) in OC/Ag(I) were found to be 1.2 ± 0.1% (w/w). Based on these observations, it is evident that the analytical values for silver content will not depend strongly on the sampling area.

The results of AAS measurements additionally revealed that the total content of silver corresponded to its halide form and no detectable part was present as Ag(0) or as a silver salt of oxycellulose. As the halide compound was easily soluble in aqueous ammonia, it was expected that it was most probably AgCl. However, in order to prove this assumption X-ray diffraction was further employed.

X-ray diffraction

A typical X-ray diffraction spectrum obtained for a powdered OC/Ag(I) is shown in Fig. 2. All of the diffraction peaks marked in Fig. 2 can be attributed to the structure of AgCl for which the lattice parameters were in a good agreement with those of the standard 04-002-8237 Chlorargyrite, syn in the database (PDF-4+/ICDD). There is no evidence of additional silver containing phases.

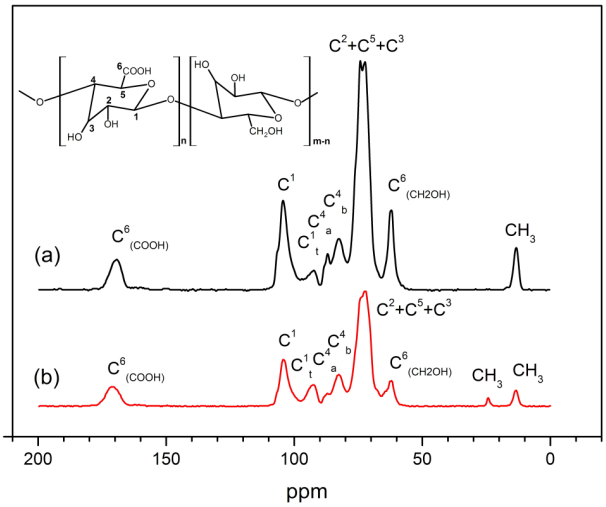

Figure 1: ^{13}C CP/MAS NMR spectra of (a) oxidized cellulose and (b) oxidized cellulose with Ag salt

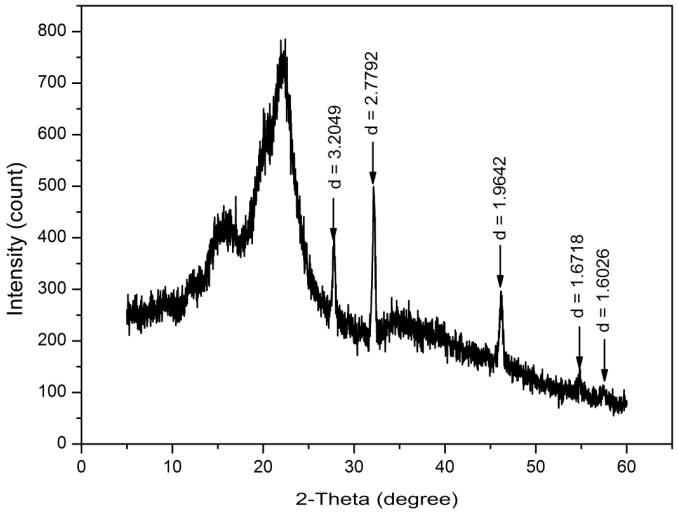

Figure 2: X-ray diffractogram of oxidized cellulose modified with Ag salt

Studies on OC/Ag(I) *in vitro* behaviour

To evaluate the amount of silver being released from the OC/Ag(I) sample and to study the possible metabolic versatility of several human pathogens for the degradation of these samples, different chromatographic methods, FTIR, AAS, ICP-TOF-MS and SIMS were applied to the analysis of both nutrient media and the resulting solid sample. Experiments were carried out under similar conditions as those previously applied during the evaluation of antimicrobial activity of OC salts,[12] including the period of one week and the temperature of 37 °C, both being optimum for the cultivation of bacteria and corresponding to the conditions found *in vivo*, since the material is aimed to be used as an adhesion barrier to assist natural wound repair. In order to prevent the photoreduction of silver ions from the OC/Ag(I) samples, dark conditions were maintained throughout the study.

AAS determination of Ag

Silver was determined in nutrient media containing OC/Ag(I) without and in the presence of different kinds of microorganisms at an incubation temperature of 37 °C in the dark for a period of one week. To find whether there were any differences between the results depending on the factor selected, the analysis of variance (ANOVA) was used. Finally, a total number of 20 samples was analysed and two-way ANOVA was used for the data treatment. "Microorganism strain" with five levels (no microorganism, *A. fumigatus*, *C. albicans*, *P. aeruginosa*, and *S. epidermidis*) was selected as the first factor and "time" with two levels (2 or 7 days) was selected as the second factor. Both factors, as well as the interaction between factors, were found to be insignificant ($p = 0.100$, 0.631, 0.879) at the significance level of 0.05. This means that the silver concentration in the analysed solutions was not dependent on the presence of the investigated microorganism strain and did not significantly change during the period of one week. In addition, silver release was not influenced by the interaction between microorganism strain and time. Mean Ag concentration in the analysed solutions varied from 6.7 to 22.6 mg L^{-1}, which means that approximately 3.4-11.3% of Ag from OC/Ag(I) was released. These values are significantly higher than those expected based on the solubility constant of AgCl. This fact may be attributed to the creation of different complexes like $AgCl_2^-$, $AgCl_3^{2-}$ and $AgCl_4^{3-}$. If the solution contains excess chloride due to the presence of, say, sodium chloride, there is a tendency toward complex formation.[22] For BHI media, the value of 5 g L^{-1} of NaCl is declared by the producer; an important concentration of NaCl to the experiments is also introduced via OC/Ag(I) as will be discussed later. As chlorides may act by forming complexes, they influence both the solubility of AgCl and the microbiological effectiveness of Ag^+.[6] In general, silver release was not dependent on the type of the culture medium (MEB or BHI) with various pH values of solutions (5.4 and 7.0 for MEB and BHI, respectively). Under the conditions where no microorganism was present, no significant changes were indicated using paired *t*-test ($p = 0.129$) for silver concentration values determined in both types of media for a period of 7 days.

The appearance of both liquid media and OC/Ag(I) did not change because of the reduction of silver ions during all of the incubation experiments as long as dark conditions were set even in the presence of bacteria, as previously reported,[12] which is attributed to the self-protection mechanism of bacteria[23] as silver in its elemental form is purely absorbed by mammal and bacterial cells. Visual changes were observed however in the light due to the photoreduction of silver ions.

Chromatographic analysis

HPLC

As shown previously,[24,25] during the solubilization and degradation of regenerated cellulose under conditions which approximate those found *in vivo*, the oxidized polymer readily undergoes chain shortening to give oligomers which, in the presence of plasma or serum, are further hydrolyzed to smaller fragments, including glucuronic acid and glucose. In order to monitor possible oligosacharides and monosacharides products from OC/Ag(I) degradation processes in nutrient media, high performance liquid chromatography coupled with ELSD detection was employed.

Fig. 3 shows a comparison of the ELSD-HPLC profiles of solutions obtained after incubation of OC/Ag(I) in MEB and BHI in the absence and presence of the investigated microorganisms at 37 °C for 7 days. It can be seen from Fig. 3 that in the presence of the investigated microorganisms no significant differences were observed in the

area below 30 min, i.e. elution times of oligosaccharides and monosaccharides, with the exception of the conditions including *C. albicans* (see Fig. 3a vs Fig. 3b). In the presence of *C. albicans*, a significant decrease in glucose concentration at retention times of 4 min (Fig. 3b) was observed in comparison with those observed in nutrient medium (Fig. 3a). These findings may be attributed to the different inhibition action of OC/Ag(I) observed previously for bacteria, yeast and fungi. It was reported[12] that the minimal inhibitory concentration of the OC/Ag(I) sample for bacteria is lower than those for fungi and no inhibition by the OC/Ag(I) sample was observed for yeasts. Based on these findings, it may be expected that a higher cell count in the solution containing *C. albicans* will result in the consumption of saccharide and thus in the decrease of the signal. The oligosaccharides and monosaccharides observed in Fig. 3 originate from nutrient media and they are not products of solubilization and degradation of OC/Ag(I).

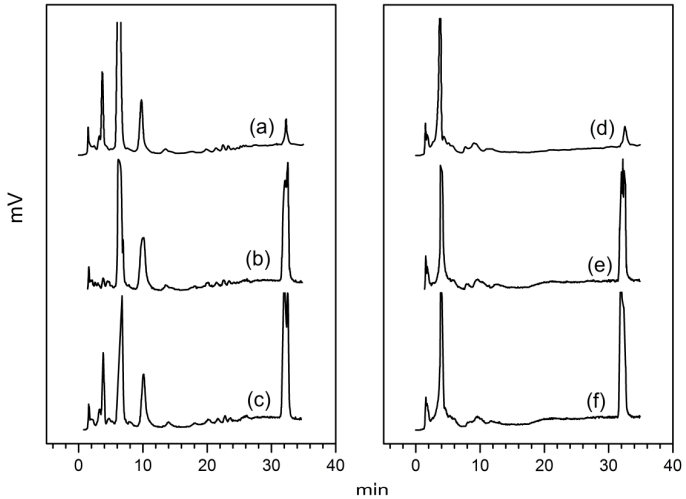

Figure 3: ELSD-HPLC profiles of (a) MEB and (d) BHI background and those of OC/Ag(I) partially solubilized with these media and after incubation with (b) *C. albicans*, (c) *A. fumigatis*, (e) *P. aeruginosa* and (f) *S. epidermidis* for 7 days

Table 1
Results of SEC analysis for peak molecular weight (Mp, g mol^{-1}), weight-average molecular weight (Mw, g mol^{-1}), number-average molecular weight (Mn, g mol^{-1}), polydispersity (PDI) and peak areas (P_A, %) of OC/Ag(I) for initial samples and after application of different microorganisms at 37 °C for 7 days

	Peak 1					Peak 2				
Microorganism	Mp	Mw	Mn	PDI	P_A	Mp	Mw	Mn	PDI	P_A
No	6.5×10^5	5.7×10^5	4.3×10^5	1.32	10	1.3×10^4	1.6×10^4	0.9×10^4	1.82	89
A. fumigatus	6.0×10^5	5.6×10^5	4.6×10^5	1.22	4	1.3×10^4	1.7×10^4	1.0×10^4	1.61	97
C. albicans	5.9×10^5	5.2×10^5	4.2×10^5	1.24	3	1.3×10^4	1.7×10^4	1.0×10^4	1.62	97
P. aeruginosa	6.4×10^5	5.9×10^5	4.8×10^5	1.22	7	1.4×10^4	1.7×10^4	1.0×10^4	1.67	93
S. epidermidis	6.5×10^5	5.9×10^5	4.8×10^5	1.23	7	1.3×10^4	1.7×10^4	1.0×10^4	1.55	93

SEC

In order to separate and characterize higher molecular fractions, SEC was additionally employed. Molar mass distributions (MMDs) determined for initial samples and those stored at 37 °C for 7 days in the absence and presence of different microorganisms revealed two similar peaks in all cases. The left peak (Peak 1) was a high molecular peak with an Mp (molecular weight at peak maximum) of about 6.5×10^5 g mol^{-1}; for the right peak (Peak 2), the Mp was of about 1.3×10^4 g mol^{-1}. The Mp values were determined by comparing the elution time of the peaks of the sample with the elution times of the pullulan standards. Table 1 presents a summary of the experimental results for the OC/Ag(I) with respect

to number-average molecular weight (Mn), weight average molecular weight (Mw), and polydispersity index (PDI) at different experimental conditions. In addition, peak areas (P_A) for all identified compounds were normalized so that the total peak area added up to 100 and is presented in Table 1. After that, quantitative results are shown by expressing the area of a given peak as a percentage of the sum of the areas of all the peaks. As follows from the data in Table 1, the values of Mn, Mw and PDI of both Peak 1 and Peak 2 did not show a significant decrease, Peak 2 being predominant in all cases; also, the polydispersity remained quite constant. The results suggest that the treatment of OC/Ag(I) with the investigated microorganisms under the set experimental conditions did not result in significant degradation of the OC structure.

FTIR analysis

Figure 4 shows the infrared spectra of OC/Ag(I) and of the samples after the treatment with different microorganisms. The analysis of the spectra was focused on the region 2000-800 cm^{-1}, which is the most valuable for unambiguous spectral changes. This region contains several distinct absorption bands at 1736, 1636, 1420, 1160, 1031 and 1063 cm^{-1}. Furthermore, there is a number of low-intensity absorption bands at 1383, 1282, 925, 895 and 855 cm^{-1}. The peak around 1740 cm^{-1} is due to the stretching of C=O, and the peak at 1636 cm^{-1} is related to the O–H bending vibration of the water molecules adsorbed onto OC and also to the carboxylate anion COO^-. The bands around 1420 and 1380 cm^{-1} are assigned to COO^- and/or to O–H bending in COOH and O–H in plane deformation, respectively. The 1160 cm^{-1} band is assigned to the asymmetric vibration of C–O–C stretching, the band at 1063 cm^{-1} is attributed to skeletal vibrations involving stretching of C–O and C–C bonds attached to the glucose rings and the band at 900 cm^{-1} is attributed to the asymmetric out-of-plane ring stretching in cellulose due to the β-linkage.[20, 21]The analysis of OC/Ag(I) spectra evaluated before and after the degradation experiment (Fig. 4) shows that the main spectral changes are the redistribution of the absorption intensity at 1800-1550 cm^{-1} (see Fig. 4). A significant decrease in intensity was observed for the characteristic carbonyl peak (–COOH) at 1740 cm^{-1} in all cases, compared with the original OC/Ag(I) sample, consistent with an increase in the intensity of the band of the carboxylate anion at 1636 cm^{-1} in the products. This effect was more pronounced in BHI media at pH 7.0. This fact may be attributed to the creation of sodium and/or potassium salts of OC from nutrient media as BHI contains high concentrations of potassium and especially of sodium ions, in contrast to MEB (see Table 2). Lower absorbance intensity ratio for higher substituted samples due to the blocking of the carboxyl group by the reaction was previously observed by different authors[20,21] upon cationization of oxidized cellulose.

Carboxyl content

In order to evaluate the neutralization degree of the OC/Ag(I) samples before and after the treatments under different experimental conditions, the residual carboxyl content has been determined. Carboxyl content reduced from 18.1% for initial OC/Ag(I) to 14.1, 13.6, 11.4 and 12.1% for conditions with *A. fumigatus* (pH = 5.4), *C. albicans* (pH = 5.4), *S. epidermidis* (pH = 7.0) and *P. aeruginosa* (pH = 7.0), respectively. The decrease of free carboxyl groups being more pronounced at pH 7 corresponds well with the results obtained by the FTIR method and confirms the further cationization of the OC/Ag(I) samples by the ions present in the nutrient media.

ICP-TOF-MS qualitative analysis and AAS determination of Na and K

ICP-TOF-MS spectra of all investigated samples were evaluated within the range of 6-260 amu to identify the main elemental composition changes among the original OC/Ag(I) sample and those after different experimental treatment (data not shown here). The visual inspection of the spectra revealed the significant differences in Na and K ions concentration. Due to this, the concentration of both K and Na in all investigated samples, i.e. in OC/Ag(I) before and after the treatment with different microorganisms and MEB and BHI media, was determined by AAS and the data are included in Table 2. A significant increase in K concentration from 0.09 mg g^{-1} for the initial OC/Ag(I) sample up to 2.4-3.5 mg g^{-1} for the samples incubated under different experimental conditions (see Table 2) was observed. Higher levels of K in the OC/Ag(I) samples were due to its presence in both nutrient media. Along with an increase of K concentration in the OC/Ag(I) samples, a decrease was observed regarding the K content in both MEB and BHI

(see Table 2). As to the presence of Na in the OC/Ag(I) sample, it can be seen from the data presented in Table 2 that relatively high Na content was present already in the initial OC/Ag(I) sample. This is due to the use of NaCl for photostabilization during the preparation of OC/Ag(I). The content of Na in OC used for the preparation of the OC/Ag(I) sample was lower than 0.05 μg g^{-1} (see Table 2).

During the treatment of the OC/Ag(I) sample in MEB media, the concentration of Na in the sample decreased and the content of Na in the nutrient media increased, which may be due to the dissolution of NaCl from OC/Ag(I) into the solutions. In BHI media, where significantly higher concentrations of Na were present in contrast to MEB (2917 versus 117 mg L^{-1}), a decrease of the Na concentration was observed accompanied by an increase in Na concentration in the OC/Ag(I) samples. Based on the computation of the analyzed data, a significant correlation of r = –0.924 between the amount of K and the content of carboxylic groups was noted. No significant correlation at the significance level of 0.05 was remarked in the case of Na with r = –0.382.

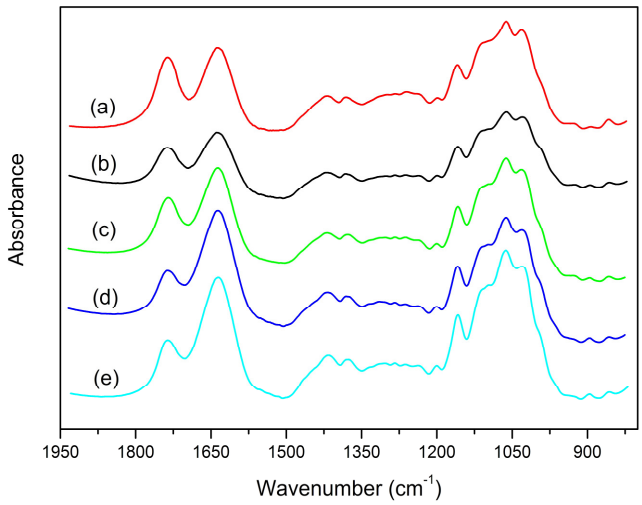

Figure 4: FTIR spectra of (a) control OC/Ag(I) sample (red) and those submitted to a 7 day incubation with (b) *A. fumigatus* (MEB, pH = 5.4) (black), (c) *C. albicans* (MEB, pH = 5.4) (green), (d) *P. aeruginosa* (BHI, pH = 7.0) (blue) and (e) *S.epidermidis* (BHI, pH = 7.0) (cyan) in the region 2000-800 cm^{-1}

Table 2
Concentrations[a] of Na and K determined in original OC[b] and in OC/Ag(I) and nutrient media (MEB, pH = 5.4, and BHI, pH = 7.0) before and after incubation with different microorganisms for 7 days

	K (mg g^{-1})[c]	K (mg L^{-1})[d]		Na (mg g^{-1})[c]	Na (mg L^{-1})[d]	
	OC/Ag(I)	MEB	BHI	OC/Ag(I)	MEB	BHI
No microorg.	0.089 ± 0.004	153 ± 1	413 ± 2	6.1 ± 0.8	117 ± 6	2917 ± 53
A.fumigatus	3.3 ± 0.2	129 ± 1	[e]	2.6 ± 0.4	276 ± 11	[e]
C. albicans	2.4 ± 0.4	133 ± 1	[e]	2.5 ± 0.3	201 ± 11	[e]
P. aeruginosa	3.3 ± 0.2	[e]	396 ± 2	8.8 ± 0.1	[e]	2115 ± 107
S. epidermidis	3.5 ± 0.1	[e]	370 ± 2	10.6 ± 0.8	[e]	1848 ± 53

[a] Mean ± 2SD (n = 3); [b] Concentration of K and Na in OC was 0.079 ± 0.003 mg g^{-1}, respectively < 0.05 μg g^{-1}; [c] Concentration in mg g^{-1} determined in the original OC/Ag(I) sample or in OC/Ag(I) analyzed after incubation with different microorganisms in MEB or BHI media after dissolving the separated solid phase in cadoxene. Cadoxene solution contained 0.126 ± 0.005 mg L^{-1} of K and < 0.2 mg L^{-1} of Na; [d] Concentration in mg L^{-1} determined in original MEB and BHI media or those separated after incubation of OC/Ag(I) with different microorganisms; [e] Not determined

However, it may be also expected that in both MEB and BHI media OC-Na salts were partially created as a significant decrease in free carboxylic groups was observed within 18.1-11.4% for different experimental conditions and only the content of K is not satisfactory for these changes. On the other hand, the imperfect separation of the solid sample from the remaining liquid media may also be responsible.

SEM

SEM photographs of the starting OC/Ag(I) and those after incubation with *C. albicans* and *S. epidermidis*, respectively, are shown as examples in Fig. 5. Under *in vitro* conditions in both buffered nutrient media, i.e. in neutral or slightly acidic pH at 37 °C, within one week, all OC/Ag(I) samples undergo gradual physical changes and lose their fibrous nature and no noticeable solubilization takes place. Although chemical modification of these polymers can also significantly affect their rate of degradation, in this special case the observations are similar to those previously reported[26] for unmodified OC, whose structure does not break down at 37 °C even after several weeks unless the pH is higher than 7, or cellulase enzyme is used.

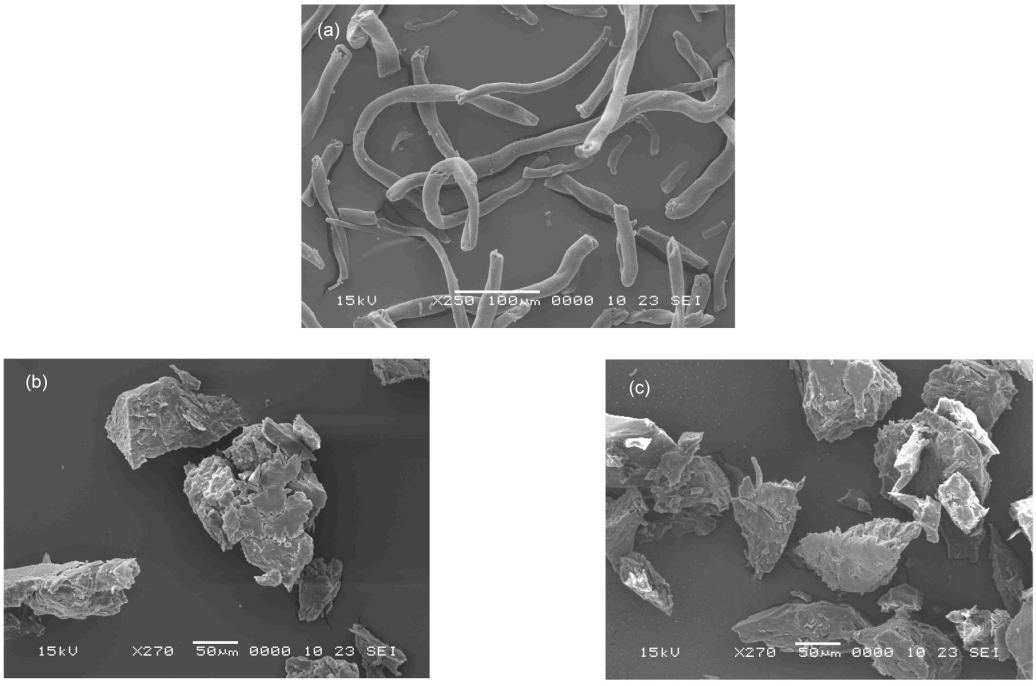

Figure 5: SEM images of (a) initial OC/Ag(I) and of the samples treated with (b) *C. albicans* in MEB (pH = 5.4) and (c) *S. epidermidis* in BHI (pH = 7.0) at 37 °C for 7 days

CONCLUSION

In vitro investigations of a novel type of antimicrobial material based on 6-carboxycellulose containing silver chloride salt (OC/Ag(I)) under the influence of *A. fumigatus*, *C. albicans*, *P. aeruginosa* and *S. epidermidis* at 37 °C in the dark, in buffered nutrient media at neutral or slightly acidic pH for the period of 7 days revealed that significant silver release into the liquid nutrient media is not dependent on the presence of the investigated microorganism strain and did not significantly change during the observation period. Additionally, Ag release was not dependent on the pH of the culture medium, being 5.4 and 7.0. However, if the solution contains an excess of chloride, there is a tendency toward different complex formation between silver chloride and chlorides, which influences the solubility of AgCl, resulting in an increase in the concentration of Ag in the analyzed solutions. As follows from the FTIR and chromatographic analysis data, none of the investigated microorganisms promoted any significant breakdown of the OC structure under the set

experimental conditions. The only noticeable changes observed in the material structure were due to the creation of K and Na salts of OC from ions in the nutrient media, as confirmed by the evaluation of the neutralization degree by the titrimetric method and by qualitative and quantitative analyses of both the liquid and the resulting solid samples by ICP-TOF-MS and AAS, respectively. The content of Na and K was strongly dependent on the composition of the nutrient media. Additionally, physical changes as a consequence of the loss of the fibrous character were noticed. However, exposing the samples to light conditions may result in changes of the colour of both OC/Ag(I) and liquid nutrient media due to the photoreduction of silver ions. For the intended clinical use of OC/Ag(I), it must be taken into consideration that the wound surface contains blood, while physical fluid contains chlorides and some enzymes. This may then influence both the microbiological effectiveness of the silver ions and the degradation of OC. These investigations need to be continued in a further study.

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