ANTIMICROBIAL PROPERTIES OF COATINGS BASED ON CHITOSAN DERIVATIVES FOR APPLICATIONS IN SUSTAINABLE PAPER CONSERVATION

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It is a pleasure and honour to be invited to contribute to the special issue of Cellulose Chemistry and Technology, dedicated to its fiftieth anniversary. Congratulations on this important anniversary and we wish CCT would continue its ascent among high quality journals in the field of natural polymers.

This paper analyses the potential of coatings based on chitosan derivatives (ChDs) to provide antimicrobial protection on paper surface, and also to substitute cellulose ethers used as consolidation/resizing materials in paper heritage conservation. The chitosan derivatives (alkyl chitosan – ACh, quaternary chitosan – QCh and carboxymethyl chitosan – CCh) were applied as one or multilayer coatings of a single or a combination of two derivatives, using different support paper types. The assessment of the antimicrobial activity of coated paper samples has shown different effectiveness among the ChDs: QCh and CCh inhibited Gram-positive and Gram-negative bacteria and had moderate effectiveness on fungi, while ACh completely inhibited fungal growth, but was less effective on Gram-positive bacteria. Finally, it was concluded that optimal combinations of two ChDs in coating formulas could confer antimicrobial protection on paper surface against a broad spectrum of pathogenic microorganisms.

Keywords: paper conservation, chitosan derivatives, coating formulas, antimicrobial activity

INTRODUCTION

Biodeterioration of paper heritage

A large part of our history is recorded in documents and art works made on paper support, making the preservation of these materials a matter of great importance. Because of its high bioreceptivity, paper is very susceptible to biodeterioration, which is an unwanted alteration process caused by the action of biological agents.[1,2] Biodeterioration phenomena represent a complex of physical and chemical spoilage processes caused by the growth of very different organisms, generically called 'biodeteriogens', characterized by the saprotrophic ability of using cellulose substrates to sustain their growth and reproduction. Printing and writing paper may be characterized as a composite material with many simultaneously active variables, among which are:[3] the basic raw material consisting of the cellulose or lignocellulose fibres, sizing material, filler type and different chemical additives. In addition, the printed or written paper of heritage objects may contain other substances (such as inks, pigments, etc.), as well as materials used in previous conservation treatments, which make it a

more complex and heterogeneous medium. Therefore, paper is basically an organic material, which could be a source of nourishment for many microorganisms, whose development affects not only the appearance of the objects, causing stains, patinas, etc., but often deeply modifies their chemical and physical properties.[4]

The most important biological agents involved in the biodeterioration of paper documents are microorganisms (fungi, gram-positive and gram-negative bacteria) and insects. The bacteria responsible for the degradation of paper are generally of the aerobic type and their development is favoured by the heat (usually temperature below 40 °C), moisture (relative humidity higher than 65%) and neutral/slightly alkaline pH.[5] The nutrients consumed by bacteria from paper could be cellulose, starch, gelatine, animal glue, etc.[6] Fungi, in particular those producing moulds, are biological agents most commonly found in deposits, archives and libraries, because, unlike bacteria, they require rudimentary conditions (humidity and temperature lower than in the case of bacteria and a large range of pH, including the acid domain) to develop and are more difficult to remove. Currently, more than 180 species of fungi that biodeteriorate cellulose have been recorded.[7]

Current treatments for stopping biodeterioration of paper heritage objects

Conservation of paper documents involves curative and restoration treatments, such as disinfection, wet cleaning, de-acidification and application of resizing and/or consolidation materials, which should also have a protective function. The disinfection is the first step of a conservation process and it aims to kill all viable microorganisms and disintegrate the biofilms formed on paper surface, which are removed together with other contaminants during the second step of wet cleaning. These two operations are requested before curative and preventive treatments, such as de-acidification and resizing/consolidation.[8]

Currently, the disinfection treatments are based on chemical and physical methods. Chemical methods involve the use of biocides of organic nature mainly, such as alcohols, alkylating agents, azole compounds, phenol derivatives and quaternary ammonium compounds. Among physical methods, the dehydration, gamma irradiation, high frequency current treatment, ultraviolet radiation, freezing and high temperature are applied. The physical methods do not have a long term action since they leave no residues; their biocidal action is only immediate. In addition, the irradiation or HF current treatments can have unwanted side effects on paper properties, such as the reduction of folding endurance and tear resistance, increased yellowing, and general embrittlement.[9] The majority of chemical compounds, even in gaseous state, as ethylene oxide (EtO) fumigation, leave residues that can prolong the antimicrobial effect during a limited period of time.[2,10] Although EtO fumigation is a successful method of disinfecting books already infested by microorganisms, it is not a preventive technique, as it has been shown that previously fumigated material may become infested again.[11] Moreover, the toxic residues left on the objects could entail negative effects on the paper itself and health hazards to people who have to deal directly with treated materials.[9]

Preventive and curative solutions against microbial attack of paper heritage objects

Microclimate control: According to the literature,[12] microclimate control through monitoring the environmental conditions, to keep the water activity of paper under 0.60 and the temperature under 20 °C, can be used to avoid microbial germination and the colonization of organic materials. However, these measures are not completely effective in solving dangerous microbial infestations, because the latter can occur even in rooms with climate control. In addition, the technology needed for an efficient climate control is not available in all the heritage repository institutions in the world, especially in developing countries.[2]

Resizing and consolidation: The resizing of old paper documents is a conservation treatment, which consists in the application of a film-forming material to the surface of the paper sheet. The purpose of resizing is to reintroduce the characteristics lost through the effects of degradation factors or previous conservation treatments. Resizing also serves frequently as consolidation and stabilizing treatment.[13]

Cellulose ethers are the materials commonly used for resizing/consolidation processes, because they have structural compatibility with cellulose (the main component of the paper) and can complete the consolidation by hydrogen bonding and adhesion forces, thereby enhancing the mechanical strength of paper and the ability to manipulate it without producing noticeable

changes in its appearance. Among the cellulose ethers, the methyl-cellulose (MC) and carboxymethyl-cellulose (CMC) are the most widely used in the current practice of resizing/consolidation on paper documents, because they are the most stable cellulose derivatives over time.[14] Nevertheless, due to their hygroscopic nature and susceptibility to microbial attack, which create conditions for chemical and biochemical degradation of documents and accelerate the aging time, cellulose ethers have limited effectiveness in the long term preservation of paper documents.

Synthetic polymers (polyethylene, polypropylene, polyvinylchloride, cellulose acetate, polyesters), which became popular during the 20th century, could be effective for both resizing/consolidation and antimicrobial protection. For example, polyethylene-coated paper shows no evidence of microorganism growth, and parylene (*in-situ* formation of poly-p-xylene film on fibre surface) has been shown to protect paper and improve its biostability.[15] However, all synthetic polymers create irreversible changes in the internal structure and surface of the paper, like thickness increase, cross-linking and discolouring over time.[16]

Alternative materials for antimicrobial protection of paper heritage objects

The limits of current resizing/consolidation materials for paper heritage objects show a real need for an interdisciplinary approach to the conservation processes by developing new materials, which could offer effective protection of paper against biodeteroration factors, along with an improvement of its strength, and serve as a barrier to water and gases.

Chitosan, a linear polysaccharide composed of randomly distributed β(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units, appears as an attractive compound to substitute cellulose derivatives in paper conservation. The main chemical and structural features of chitosan, which present interest for papermaking and conservation of heritage objects on paper support, are as follows: structural similarity and compatibility with cellulose and capacity to form hydrogen bonds acting as a paper strengthening agent; natural cationic charge providing antimicrobial properties without showing toxicity; very good film properties reducing the absorption capacity of paper for water and gases.[17-26]

Currently, research on the potential applications of chitosan in paper manufacture relates to various technological processes, but mainly to the improvement of paper properties (e.g. high physical and mechanical properties, good print characteristics) or the development of new properties, such as barrier to gases and water vapours, or antimicrobial properties. Allan and co-workers[17] studied the effects of chitosan on the mechanical and optical properties of paper and found that the best wet resistance and best dry resistance were obtained by spraying a chitosan solution on dry paper. Other studies, which involve the use of chitosan in papermaking, refer to: obtaining a carbonless copying paper assortment;[27] in-mass application of chitosan grafted with acrylic monomers to improve the paper mechanical strength;[28] coating formulations containing an acid salt of chitosan to obtain antistatic properties, recommended for photographic paper;[29,30] coating formulations containing chitosan dissolved in acetic acid, along with other additives (oxidized starch, cationic starch, polyvinyl alcohol, polyacrylamide), to improve the printing properties of paper;[31] the use of chitosan along with carboxymethyl-cellulose (CMC), starches and vegetable gum in paper coating formulations in order to improve the strength of cigarette paper and the absorption capacity of toxic substances in tobacco smoke.[32]

In paper conservation, among the first researches on chitosan used in resizing/conservation applications are those made by Ponce-Jiménez and co-workers.[33] In these studies, the effects of chitosan on the physico-mechanical and antifungal properties of paper were evaluated, compared with those of cellulose ethers. The results indicated that the paper surface treated with the acid salts of chitosan exhibited a considerably higher resistance to fungi, compared to the surfaces treated with cellulose ethers, but at the same time the acid salts of chitosan had a negative effect on mechanical strength indices compared with cellulose ethers. Also, a decrease in the whiteness and pH of paper was noted, which led to the conclusion that these negative effects are caused by the acid pH (around 4.0) of the chitosan solution. This conclusion was supported partially by another study, in which the samples of old paper sized in acidic medium were immersed in an acidic solution of chitosan, followed by precipitation with sodium silicate. It was found that this treatment led to both

strengthening of the deteriorated paper structure and increase of the aging resistance due to acidity neutralization by sodium silicate alkalinity.[34]

However, despite the extensive research done in recent years, chitosan has not yet known notable applications in papermaking, nor in the conservation of heritage objects on paper support, its main limitation being the lack of water solubility under neutral/slightly alkaline pH. Chitosan is only soluble in dilute solutions of some organic acids (acetic acid, citric acid, lactic acid, etc.). Therefore, the use of acidic chitosan solutions comes in contradiction with paper restoration processes, which require de-acidification of paper supports and not supplementation of acidity. However, the studies presented above[33,34] point out that the use of chitosan in document conservation could be of great interest if it were available in the form of derivatives with high purity and water solubility at neutral pH.

Currently, water soluble chitosan derivatives are intensively studied as antimicrobial additives in order to obtain a material (paper, textile, bio-materials, etc.) with minimal potential microbial infection. However, a recent review of the research on chitosan and chitosan derivatives includes only a few studies regarding the interactions with cellulose materials. The only research that mentions the use of water-soluble chitosan derivatives as consolidation and conservation materials for archive documents refers to carboxymethyl chitosan.[35] This study has shown that paper consolidated with carboxymethyl chitosan has strength properties similar to those treated with methyl-cellulose and much better than those treated with chitosan, but if it is applied alone it does not cause paper resizing. In conclusion, one can appreciate that chemical modification of chitosan can lead to water soluble derivatives at neutral pH, with multiple functions in paper heritage conservation or surface treatments of specialty papers.

The objective of the research work presented herein is to evaluate the antimicrobial effectiveness of three water soluble chitosan derivatives (ChDs) when used as paper coating materials. The ChDs were laboratory synthesized with particular functionalities, having in view their potential applications in paper heritage conservation. In this respect, the synthesis conditions for each ChD were chosen considering our previous research, which demonstrated that optimum coating formulas can improve strength properties simultaneously with decreasing the hydrophilicity of coated paper.[36]

EXPERIMENTAL

Chitosan and chitosan derivatives

Unmodified chitosan was provided by Sigma Aldrich, with medium molecular weight (MW) of $2.34 \cdot 10^5$/mol, and degree of acetylation (DA) of 18.5%.

Chitosan derivatives with specific functionalities, as described below, (Table 1) were synthesized according with our previous research, and were evaluated as antimicrobial materials for paper coating.

N-alkyl-chitosan derivative (ACh)[37] was designed especially to provide barrier to water and water vapour and thus, in this simplest and harmless way, to increase the resistance to microbial attack. The most important functionality of this type of derivative is the length of the hydrophobic alkyl chain (R = C3 - C18) and the degree of substitution. They form hydrophobic films on the paper surface, limiting the interaction with water and atmosphere humidity and the alkyl groups could also enhance the interaction with the hydrophobic cell membrane of microorganisms.[2,38,39]

N,O-quaternary-chitosan derivative (QCh)[40] was synthesized in order to increase both the solubility and positive charge density of chitosan by the introduction of quaternary ammonium groups in the chitosan macromolecule, and thus enlarging the spectrum of action as antimicrobial agent due to its cationic charge over the whole pH range[41]. These quaternary derivatives also have hydrophobic groups (alkyl or aryl groups) in their structures, which can enable better interaction with the microbial cells.[42]

N,O-carboxymethyl-chitosan derivative (CCh)[43] has an amphoteric character (anionic and cationic groups) with high complexation and excellent metal-binding capacity, it is easily soluble in water at neutral and alkaline pH and has very good film-forming properties. In this case, the chelation of metals is the accepted mechanism for the antimicrobial action associated with the presence of -COOH groups.[44]

The cellulose derivative, methyl-cellulose (MC), was selected as a reference since it is a conventional consolidation material for paper heritage conservation.[14,45,46] MC was applied in the same manner as chitosan derivatives, not by the conventional method, which consists in a single layer application by spraying or brushing.

Paper substrates

The experimental protocol was designed to assess the antimicrobial effectiveness of the ChDs with different features, applied by coating on the paper surface in simplified models, in which some of the variables could be easily controlled. Thus, the tests were carried out on two types of "model" paper:

Laboratory pulp sheets (M_1 model paper) obtained only from fibre pulp mixture, consisting of softwood

and hardwood Kraft pulp (30/70), at a grammage of 65±1 g/m^2. This simple composition was chosen to have a "model paper", which would make it possible to understand the interactions between cellulose fibres and chitosan derivatives without interference of other chemicals, commonly used in the papermaking.

Table 1
Main characteristics of chitosan derivatives

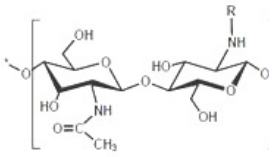
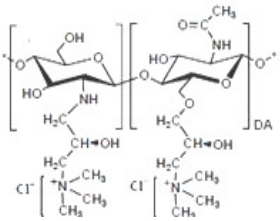
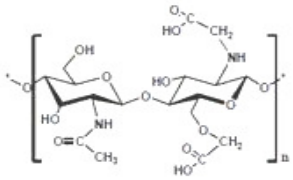
Derivative	N-alkyl chitosan	N,O-quaternary chitosan	N,O-carboxymethyl chitosan
Chemical structure			
Abbreviation	ACh	QCh	CCh
Substitution site	Primary amino groups	Primary amino groups, primary hydroxyl groups	Primary amino groups, primary hydroxyl groups
Substitution degree	0.02-0.05	0.85-0.95	0.80-0.90

Table 2
Main characteristics of "model papers"

Characteristics	M_1 model paper	M_2 model paper
Origin	Laboratory paper	Commercial paper
Age	3 months	26 years
Grammage, g/m^2	65	80
Ash content, %	0.35	11.5
$Cobb_{60}$ index, g/m^2	91	51
pH of water extract	7.1	8.2

Table 3
One layer coatings – different concentrations of ChD solution

Sample	Conc., g/L	Layer number	pH	Coating weight, g/m^2/side
Ch	5.2	1	4.1	0.495
ACh	5.6	1	6.9	0.510
QCh	4.5	1	6.8	0.495
CCh	4.8	1	7.7	0.485

Table 4
Multiple layer coatings – constant concentration of ChD solution

Sample	Conc.,	Coating weight per layer, g/m^2/side			
	g/L	1	2	3	Total
ACh	5	0.715	0.700	0.655	2.070
QCh	5	0.735	0.586	0.580	1.901
CCh	5	0.795	0..885	0.785	2.365

Commercial printing paper (M_2 model paper) with a more complex composition than the laboratory-made paper (filler, sizing agent and other additives) and with properties close to those of old documents (books and manuscripts), which are found routinely in storehouses or libraries. Thus, an assortment of printed paper with a certain degree of natural aging (26 years old) was selected.

The main characteristics of the "model" papers are presented in Table 2.

Paper coating method

Coating formulas were applied with a spiral bar using an automatic film applicator and the total amount of solid deposition on paper surface was adjusted by polymer concentration or by the number of polymer layers. Thus, in the first series of experiments (Table 3), the polymers were applied in a single layer, but with different concentrations (established after preliminary tests) in order to obtain the same coating weight of about 0.5 g/m^2 for each side of a paper sheet. In the second series of experiments (Table 4), the polymers were applied on the paper surface in the same concentration, but the number of polymer layers was varied from 1 to 3.

After application of the polymer solution, the paper samples were dried first under ambient conditions until the disappearance of free water on the paper surface and then on a photo dryer (5 min), which enables a slight tensioning of the paper sheet and eliminates the tendency of local deformation or curling.

Antimicrobial activity assessment

The test microorganisms used for assessing antimicrobial activity were cosmopolitan species of bacteria and fungi, isolated from the museum archive documents (dating from 18th century and provided by "Moldova" National Museum Complex of Iasi). The sampling was performed by the fingerprinting method, from different areas presenting biological attacks. The procedure consisted of the following steps: inoculation on specific culture media, incubation at 37 °C for 24-48 hours (for bacteria) and 7-14 days (for fungi), isolation and taxonomic identification according to their macro- and micro-morphological characters. The share of isolated species is presented in Table 5.

The culture media used were: Tryptic Soy Broth Agar (TSBA) for bacterial growth; Czapek-Dox agar (20 g agar-agar, 1000 ml distilled water and 30 g of sucrose as supplementary carbon source) and Sabouraud agar, both for fungal growth. All culture media were purchased from Merck Company.

The antibacterial activity of the chitosan derivatives was tested using a modified and adapted method of the SR EN ISO 846/2000 standard. The method consists in the following steps: paper samples coated with different ChD formulas, previously UV sterilized, were sprayed with bacterial inoculums (18 hours aged) and placed onto culture medium surface (TSBA medium); the samples were incubated in a thermostat, at 37 °C and analyzed after 24 hours; the resistance of the polymer film to bacteria is appreciated according to the mentioned standard by giving scores from 1 to 5, correlated with the degree of development of bacterial culture onto/around the paper samples.

Antifungal activity was tested by two methods. The first one (Method A) consists in the pulverization of conidia suspension (10^{-8} dilution) onto paper samples and placing them on artificial nutrient medium; the spore suspension was prepared by adding the 14-day mould spore cultures, maintained at 25 ± 1 °C and 80% relative humidity, in mineral salt solution; paper samples were irradiated for sterilization at a minimum dose of 20-25 kGy using SVST industrial irradiator with a source Co_{60} 100 000 Ci; then, the paper samples were placed on the Sabouraud medium culture and inoculated by pulverization with spore suspensions of the tested species. The second method (Method B) implies the flooding of the nutrient medium surface with conidia suspension (10^{-10}) and placing the paper fragments on the medium surface. In both methods, the samples were incubated in a thermostat, at 25±1 °C and 90-100% relative humidity, for a period of 21 days, and analyzed after 7, 14 and 21 days, respectively, and photographed. The resistance of the polymer film to mould is appreciated by giving scores from 1 to 5, according to SR EN ISO 846/2000.

RESULTS AND DISCUSSION

Antimicrobial activity of ChD coatings on laboratory paper (M1 model paper)

Single layer coatings

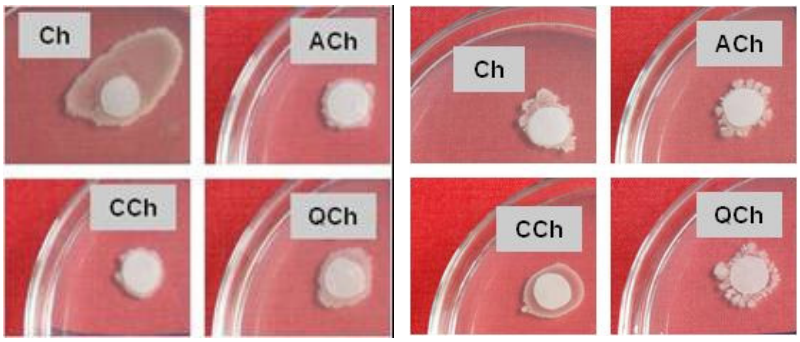
In the first series of experiments, a single layer of polymer (ChDs and native chitosan) has been applied on both sides of the paper at constant coating weight of 0.50 ± 0.03 g/m^2/side (Table 3), aiming a direct comparison of the antibacterial activity of the ChDs and chitosan.

Antibacterial activity: Four different bacterial strains (I_4, II_1, III_3 and IV_2) of *Bacillus* sp. (Gram-positive bacterium) were tested. The selection of this type of bacterium was made because it is frequently isolated from the old papers analyzed (Table 5) and considering the increased resistance of Gram-positive bacteria to the bactericidal action of chitosan, compared to Gram-negative ones.[47,48] The results presented in Fig. 1 show a relatively good development of bacterial strains on all coating types. However, bacterial growth is slightly weaker for ChD coatings, compared with unmodified chitosan (Ch), but no notable difference between the three chitosan derivatives can be seen.

Antifungal activity: Two fungal strains – *Penicillium notatum* and *Penicillium* sp.4 – were used to evaluate the antifungal effect of Ch/ChDs films. The results have shown that none of the coating types presents inhibitory effects against the growth of fungal strains.

Table 5
Bacterial and fungal species isolated from old papers

Bacteria	%	Fungi	%
Bacillus	62	*Penicillium* sp.1	67
Clostridium	21	*Penicillium* sp.2	22
Pseudomonas	13	*Alternaria*	11
Micrococcus	4		



I_4 bacterial strain II_1 bacterial strain

Figure 1: Development of *Bacillus* sp. strains on paper coated with chitosan and ChDs

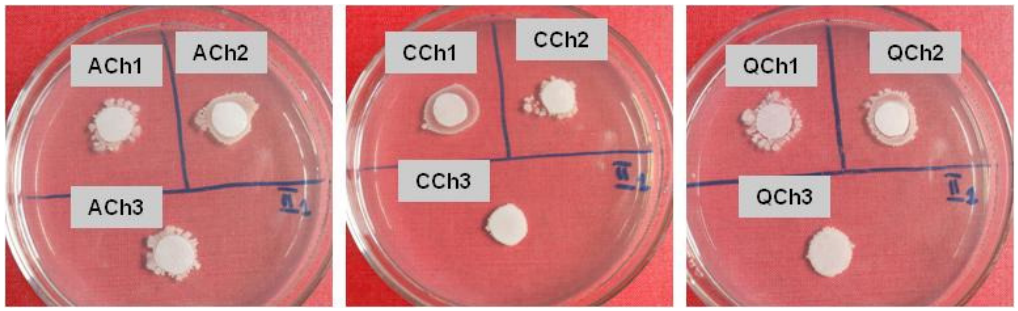


Figure 2: *Bacillus* sp. (strain II_1) growth on paper coated with 1, 2 or 3 layers of ChDs

Concluding remarks: Generally, it has been demonstrated that the antimicrobial properties of Ch/ChDs are due to the bacteriostatic rather than bactericidal effects, which implies a direct contact between the film and the membrane cell.[49,50,51] Thus, microbe inhibition will depend not only on Ch/ChD activity, but also on the high degree and uniformity of the coverage of the paper surface by the polymer film, which can prevent the contact of cellulose fibres with microbe spores and their growth. Consequently, the lack of inhibitory effects at low coating weight (~0.5 g/m^2/side) could be explained by migration of the polymer into the internal porous structure of the paper, which thus prevents the formation of a uniform film on the paper surface. Fast migration of the polymer solution is due to the lack of sizing and high porosity of laboratory-made paper.

The migration of the polymer solution into the paper structure was confirmed by SEM images of the paper surface and by the results obtained from testing the physical and mechanical resistance of the paper at different coating weights, obtained by multilayer application. Thus, it was noticed that all chitosan derivatives led to substantial increases of strength properties (in particular, the double fold number and elongation at break) after the first layer application, while the strength increase was not significant after applying the second and third layers.[52] Therefore, the idea of multiple successive layers was adopted to obtain more uniform films and better coverage of the paper surface.

Multi-layer coatings

In order to obtain paper samples with increasing coating weight, in the second series of experiments chitosan derivatives (ACh, QCh, CCh) were applied in one, two or three layers on each side of the paper sheet. Depending on the

viscosity of the derivative solution and the number of applied layers, coating weight varied between 0.75 and 2.25 g/m^2 on each side (Table 4). The antimicrobial activity against bacteria and fungi was evaluated after each derivative layer application.

Antibacterial effects: The photos presented in Fig. 2 show that *Bacillus* sp. growth (strain II_1) depends on both number of layers and derivative type. The inhibition effect of the ACh derivative does not increase significantly with an increase in coating weight, while it became consistent in the case of the QCh and CCh derivatives, which produce total inhibition of bacterial strain II_1 at maximum coating weight (CCh3 and QCh3).

Antifungal effects: Like in the case of single layer coatings, the *Penicillium notatum* and *Penicillium* sp.4 were used to evaluate the antifungal effect of coated paper samples. The increase of coating weight by the application of two or three layers of ChDs led to the inhibition of fungal growth, which depended on both inoculation method (spraying or flooding) and *Penicillium* strain. Generally, no differences were found between two- and three-layer coatings; therefore, only the results for the two-layer coatings are summarized in Tables 6 and 7. One can note that the treatment with chitosan derivatives can significantly inhibit fungal growth compared with the blank sample; but the most effective compound for both fungal species is alkyl-chitosan (ACh).

Concluding remarks: The preliminary tests made on laboratory sheets consisting only of cellulose fibres (with open structure and high porosity) have shown the chitosan derivatives have inhibitory effects on the microorganism growth only when the coating weight is enough to obtain a continuous and uniform covering of the paper surface, as in the case of the two- or three-layer coatings.

At a coating weight of 1-1.5 g/m^2/side, when a uniform coverage of the paper surface was achieved, the inhibition effect depended on the microorganism type. ACh was very effective for fungal inhibition and produced only a slight effect in the case of Gram-positive bacteria. The high effectiveness of ACh in reducing fungal growth could be explained by the hydrophobic character of the formed films.[37,38] CCh and QCh were very effective in limiting bacterial growth, but had less effect on fungal development.

Table 6
Degree of coverage (DC, %): Method A*

Coating type	*Penicillium notatum*	*Penicillium* sp.4
Reference (M1)	100	100
ACh	68	85
QCh	79	60
CCh	97	98

*Method A – pulverization

Table 7
Degree of coverage (DC, %): Method B*

Coating type	*Penicillium notatum*	*Penicillium* sp.4
Reference (M1)	25-30	35-40
ACh	0	0
QCh	8	20
CCh	6	30

*Method B – flooding

Antimicrobial activity of chitosan derivative coatings on commercial printing paper

The results obtained for the coatings applied on laboratory paper have shown that the application of a third layer does not contribute to a significant antimicrobial effect, in addition to the second layer. For this reason and considering that commercial paper (M2 model paper) is characterized by lower porosity and better barrier to water than laboratory paper, these experiments

were performed only with two-layer coatings, at a total coating weight of 1 ±0.05 g/m^2/side. Since it was noted that each ChD presented different antimicrobial effectiveness as a function of the bacterial or fungal species, the derivatives were combined in various ways in order to obtain antimicrobial barriers against a broad spectrum of pathogenic microorganisms. Five different combinations of chitosan derivatives were tested, consisting in the same type of ChD (ACh/ACh, QCh/QCh and CCh/CCh) or two different types of chitosan derivatives (QCh/ACh and CCh/ACh).

Table 8
Development of bacterial cultures

Coating type	*Bacillus* sp.	*Pseudomonas* sp.
Reference (M2)	+++	+++
ACh/ACh	+--	---
QCh/QCh	---	---
CCh/CCh	+--	---
QCh/ACh	---	---
CCh/ACh	+--	---

Development level: +++ Very good; +-- Weak; --- Absence

Table 9
Degree of fungal coverage (%) for different combinations of ChDs

Coating type	*Penicillium notatum*		*Penicillium* sp.4	
	Method A*	Method B**	Method A*	Method B**
Reference (M2)	100	25-30	100	35-40
ACh/ACh	68	0	85	0
QCh/QCh	79	8	60	20
CCh/CCh	97	6	98	30
QCh/ACh	74	0	73	0
CCh/ACh	84	0	96	0

*Method A – pulverization; **Method B – flooding

Antibacterial effects were evaluated against both Gram-positive bacteria (*Bacillus* sp. IV_2) and Gram-negative bacteria (*Pseudomonas* sp. VII_B). Table 8 presents the bacterial culture development onto chitosan derivative coatings, compared with the reference paper, without coating.

Both types of bacteria develop very well on the reference sample (uncoated), while on ChD coatings, with few minor exceptions, the inhibition of growth is evident. Slight developments are observed in the case of Gram-positive bacteria (*Bacillus* sp.), which could be due to the influence of bacterium type on the inhibition mechanism developed by the antimicrobial film. Generally, despite the distinction between the cells of Gram-negative and Gram-positive bacteria, the antibacterial activity begins with interactions at the cell surface and continues with the cell wall disruption. In the case of Gram-negative bacteria (*Pseudomonas* sp.), the negative charge on the cell wall is significant, leading to a strong adsorption and adhesion on cationic charged coatings by electrostatic interactions.[53] In this way, one can clearly explain the high effectiveness of the chitosan derivatives for this type of bacteria. The process is more complex in the case of Gram-positive bacteria (*Bacillus* sp.), when it is presumed that the attachment of bacteria on the surface of the film could be made through lipotechoic acid (LTA).[50] This mechanism could explain the total inhibition of *Bacilllus* sp. development only onto quaternary chitosan derivative films (QCh/QCh) or its combination with alkyl-chitosan (QCh/ACh), since it can complexate LTA acid via quaternary nitrogen.

Antifungal tests were performed by pulverization and flooding methods, using two strains of *Penicillium* (*P. notatum* and *Penicillium* sp.4) isolated from old papers. The results are not very conclusive if both testing methods are considered. However, the flooding method (method B) gives more conclusive results, highlighting the strong antifungal effect of alkyl-

chitosan, which produces a total inhibition in the development of both species (Table 9). This effect is observed especially when alkyl-chitosan derivative is applied as a second layer in (ACh/ACh), (CCh/ACh) and (QCh/ACh) formula combinations, respectively.

The significant reduction in the coverage of the film with fungal mycelium after different treatments can be seen as an effective protection against fungal attack, considering the optimal conditions created for fungal development (humidity, optimal temperature, rich nutrient medium), which are not valid in common storage spaces for paper materials (deposits). The high antifungal effectiveness of alkyl-chitosan could be partly due also to the hydrophobic character of the ACh layer, as it is known that fungi develop even at low environment humidity.

CONCLUSION

Chitosan is a viable alternative to current antimicrobial agents due to its specific structure and functionalities, but the lack of water solubility restricts its use as antimicrobial agent at neutral/slightly alkaline pH. Chemical modification of chitosan provides the means to overcome its limitations related to water solubility. The synthesised chitosan derivatives proposed in this paper have characteristics targeted for particular purposes in restoration/conservation based on their specific functional groups: quaternary nitrogen groups (QCh) confer good consolidation ability and antimicrobial activity over the whole pH range due to their permanent and independent cationic charge; alkyl groups (ACh) form hydrophobic films with a hydrophobic character and antifungal properties; carboxymethyl groups (CCh) confer amphoteric nature to chitosan, allow the formation of uniform films on cellulose fibre surfaces and complex structures with cellulose.

Chitosan derivatives applied in two successive layers have antibacterial and/or antifungal activity, which varies according to the species of bacteria or fungi investigated: quaternary-chitosan completely inhibits Gram-positive and Gram-negative bacteria, while alkyl-chitosan completely inhibits the development of fungi; combined coatings based on these chitosan derivatives represent a viable solution to achieve antimicrobial barriers against a broad spectrum of pathogenic microorganisms.

By their multiple functions – strength improvement, development of barrier to water and inhibition of microbial growth – the chitosan derivatives studied here could provide sustainable alternatives to conventional materials, particularly cellulose derivatives for paper heritage conservation.

ACKNOWLEDGEMENT: The Romanian Government is acknowledged for supporting this research by financing the synthesis of chitosan derivatives in the framework of PN II-PCCA project – Developing Non-conventional Materials and Cold Plasma Technique for Sustainable Solutions in Paper Heritage Conservation – (PAPHERCON), Grant Agreement n° 221/2012.

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