PREPARATION OF ANTIMICROBIAL PAPER SHEETS USING CHITOSAN

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The purpose of this study was the antimicrobial functionalization of paper pulp with natural biopolymer chitosan. Laboratory paper sheets were prepared for this purpose from paper pulp with incorporated chitosan. The prepared paper sheets were analysed regarding amino group content, mechanical properties and hydrophilicity. All of these parameters are important for hygienic applications of paper pulp (wipes, towels, toilet paper, *etc.*). The antimicrobial efficiency of functionalised pulp was examined regarding Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*), Gram-negative bacteria (*Escherichia coli*), and fungi (*Candida albicans* and *Candida glabrata*). The presence of chitosan in the paper pulp conferred antimicrobial properties to the paper sheets, leading to the inhibition of *Staphylococcus aureus*, and both fungi: *Candida albicans* and *Candida glabrata*. Such antimicrobial activity is extremely important for the application of paper products in the hygiene and medical sectors.

Keywords: chitosan, adsorption, paper pulp, paper sheets, hydrophilicity, antimicrobial activity

INTRODUCTION

Hygienic tissue paper commonly includes products such as toilet paper, kitchen paper, napkins, handkerchiefs, bathroom tissue, etc. The market for these products is continually expanding, being among the world's largest ones, due to higher living standards and increased awareness of healthy lifestyles. In addition, an increased rate of infections, more bacterial and viral illnesses and epidemics result in an increased need for high standards of hygiene products.[1,2] The mentioned facts are the driving force for hygienic tissue paper functionalization. There is, thus, a significant interest to incorporate antimicrobial ingredients into tissue products.

Nowadays, there are only very a few European tissue products with antimicrobial properties. One of the inventions (United States Patent Application 20030143372) consists in antibacterial toilet tissue composed of upper and intermediate planar-shaped absorbent layers and a dry antibacterial layer that is activated by moisture.[3] Kleenex recently produced anti-viral tissues that have 3 soft layers (Patent No. US7115273B2).[4] The central layer has an antiviral formula that is moisture-activated and it was proven to kill 99% of cold and flu viruses within the tissue before they spread. Some antimicrobial paper products based on silver nano-particles are also available on the market. The study of Ben and Westerhoff[5] reported that both colloidal and ionic silver leaches from the tissue paper material incorporating nano-silver as an antimicrobial agent. It has been concluded that this issue requires further investigations to better understand the fate and transport of colloidal and ionic silver, which is important for estimating short- and long-term environmental impacts.

Nowadays, the priority is given to the use of natural and biodegradable substances, such as, for example, less employed polysaccharides and their derivatives possessing antimicrobial and antifungal properties. Among the various polysaccharide products, cationic functional polysaccharides are the most promising from the biomedical perspective, as they possess antimicrobial activity, which is useful for many applications. These cationic functional polysaccharides contain protonated amino groups, which interact with the cell surface of pathogenic microorganisms and in this way destroy them by

several possible mechanisms.[6-8] One of the most popular cationic polysaccharides is chitosan obtained by the alkaline deacetylation of chitin. Chitosan's positive charge, the degree of *N*-deacetylation, the mean polymerization degree and the nature of chemical modifications are properties that strongly influence the antimicrobial effectiveness of chitosan. It has well-documented biological activities, including the ability to improve resistance to viral infections in plants, to inhibit viral infections in animal cells, or prevent phage infections in microbial cultures, to mention just a few.[6-10] It is also known as an immunological enhancer. As such, it is an ideal substrate for material functionalization in order to obtain multifunctionalities.[11]

However, to our knowledge, no studies have been reported yet regarding the usage of chitosan for paper pulp tissue products in order to simultaneously inhibit bacteria and fungi, thus reducing the transmission of infectious diseases, *e.g.* at home, job, school, during travel and, in healthcare establishments. The main objective of this investigation was to use biodegradable polysaccharide chitosan as an additive for pulp and paper in order to obtain different functionalities, as for example antimicrobial properties, giving higher added value to the final tissue paper products. At the same time, sorption properties were analysed because this property is essential for final hygienic and sanitary product applications. It is known that sensitive body parts, such as the nose, need soft, high-quality tissues.

EXPERIMENTAL

Preparation of the chitosan solution

A solution of 1% chitosan was prepared. Lactic acid (85%) was used to achieve complete dissolution of chitosan in distilled water within 24 hours at pH 4. The high molecular weight chitosan was obtained from the producer Mahtani Chitosan, India (chemical grade purity; degree of deacetylation ≅90%).

Paper pulp

Paper pulp was obtained from Paloma d.d. Company Sladki Vrh, Slovenia, with the following composition: 20% Conifer, 20% Hallein and 60% Eucalyptus. Such pulp is used by the mentioned company for the production of wipes and towels.

Functionalization of paper pulp

Paper pulp was modified with chitosan. Sample B was chosen as an optimal one from the preliminary research, where 10-50 mL additions of 1% chitosan solution were made to 50 g of paper pulp, or the same amounts of 2% chitosan solution to 50 g of paper pulp. On the basis of the results obtained by goniometry (contact angle; estimation of hydrophilicity) and by the standard method for determining the pH for paper pulp extract (standard ISO 3071), the optimal sample was sample B (containing 10 ml of 1% of chitosan solution). In contrast to other samples, this sample was the only one that maintained a hydrophilic character and the pH of its extract was similar to the physiological pH of skin (pH≅5.5). Thus, this sample was further subjected to analyses as described below. Two samples were studied: *i.e.* sample A as reference paper pulp (50 g) and sample B as sample modified by chitosan (10 ml of 1% of chitosan solution).

Preparation of paper sheets

Paper sheets were prepared from paper pulp conventionally. using laboratory equipment according to standard ISO 5269/1-1998(E) Pulps – Preparation of laboratory sheets for physical testing – Part 1 Conventional sheet-former method.[12]

Methods

Potentiometric titration

This method was used to determine the charges of paper sheets. For paper pulp functionalised by chitosan, the content of protonated amino groups was determined. For each measurement, approximately 1 g of paper pulp (from the paper sheet form) was used. Measurements were performed using a satin burette instrument (Mettler T-70) upgraded to an MT T90, equipped with a combined glass electrode (DG 117, Mettler Toledo, Switzerland). Measurements were performed in an inert environment, by blowing nitrogen (Merck) through the solution.

Afterwards, the burettes were filled with 0.1M HCl (318965, Sigma Aldrich) and 0.1M KOH (Baker, Dilut-it). All the solutions were prepared with deionized water with low carbonate content (less than 10^{-6} M), which was achieved by boiling, and subsequent cooling, in a nitrogen atmosphere. The fibres were titrated forward and backward between pH 3-10.

In order to avoid any sticking to the electrode and interferences by the stirrer, the samples were kept in a stainless steel container. Prior to the titrations, the ionic strength was set at 0.1M, by adding pure solid KCl (Kemika, Zagreb). The ionic strength therefore remained within 2% of the initial value upon the additions of HCl and KOH. Blank HCl-KOH titrations were performed under the same conditions as described above. The equilibrium criteria for the timed addition were set at dE/dt = 0.1 mV/min. The total amount of weak acidic groups was calculated from the difference (ΔV) in the added KOH volume between the fibre sample (V_t) and the blank ($V_{t,Blank}$), and any given pH. The molar concentration Q related to the overall charge of the weak ions, was calculated from the charge balance according to Equation 1, where square

brackets and c_i denote molar concentrations of ionic species, and z_i is the charge number of the species i.[13-15] All reported values are the mean values of triplicate determinations.

$$Q = \sum_i c_i z_i = [OH^-] - [H^+] + [Cl^-] - [K^+] \quad (1)$$

ATR FT-IR

ATR FT-IR analysis was performed on a Perkin Elmer Spectrum GX spectrometer, equipped with a diamond crystal ATR attachment. Only dry samples were used for all the measurements. The depth analysis showed a value of approximately 0.75 µm. Spectra were recorded at a 4 cm^{-1} spectral resolution within the range of 4000-650 cm^{-1}, using an average of 16 scans.

Water absorption

The water absorption ability of the paper sheets was determined according to standard DIN 54540-4.[16] The paper sheets were cut into sizes of 100 x 100 mm (surface area of 0.01 m^2 and weighed to 0.01 g precision). These samples were transferred into flasks, where required amounts of distilled water were added, and kept for for 60±3 s. After some time, the samples were strained for 120±3 s and weighed again to 0.01 g precision. Sorption ability was expressed by unit g/m^2 and estimated by the following Equation (2):

$$WA = m_m - \frac{m_s}{P} \quad (2)$$

where *WA* is water absorption ability [g/m^2], m_m [g] is the mass of the wet sample, m_s [g] is the mass of the dry sample and *P* [m^2] is the surface area (*P* = 0.01 m^2).

Capillary rise of water

The absorbency of the paper samples was also determined according to standard DIN 53106 – Testing of paper and board; determination of the capillary rise of the water.[17] For this purpose, the lower ends of the samples of 200±0.1 mm in length and 15±0.1 mm in width were immersed perpendicularly 25+/-1 mm into distilled water of 23+/-1 °C, and the distances that the water migrated within 10 min +/-5 s were measured in mm.

Goniometry: contact angle measurements

The contact angle measurements were carried out on the surface of the reference and chitosan paper sheets with dimensions of 0.5 cm x 0.5 cm, by the sessile drop method, using a Data Physics OCA 35 goniometer (DatPhysics, Germany) apparatus. Contact angles were measured using MilliQ water (Millipore, USA), the volume of the drops being fixed to 3 µL (Fig. 1). Measurements were done at room temperature with at least five replications within an experimental error ± 2%.

Antimicrobial characterization

The antimicrobial properties of the untreated and treated paper sheets were evaluated according to ASTM E2149-01: Standard test method for determining the antimicrobial activity of antimicrobial agents under dynamic contact conditions.[18] Gram-positive and Gram-negative bacteria, as well as fungi, were used as test organisms. The incubated test culture in a nutrient broth was diluted using a sterilized 0.3 mM phosphate buffer (KH_2PO_4; pH 6.8) to give a final concentration of 1.5-3.0 × 10^5 colony forming units (CFU)/mL. This solution was used as a working bacterial dilution.

Paper sheet samples (2 g) were cut into small pieces (1 × 1 cm) and transferred to a 250 mL Erlenmeyer flask containing 50 mL of the working bacterial dilution. All flasks were capped loosely, placed on the incubator, and shaken for 1 h at 37 °C and 120 rpm using a Wrist Action incubator shaker. After a series of dilutions using the buffer solutions, 1 mL of the diluted solution was plated in nutrient agar. The inoculated plates were incubated at 37 °C for 24 h, and the surviving cells were counted. The average values of the duplicates were converted to CFU/mL by multiplying with the dilution factor. The antimicrobial activity was expressed as R [%] reduction of the organism after contact with the test specimen, compared to the number of microorganism cells surviving after contact with the control.

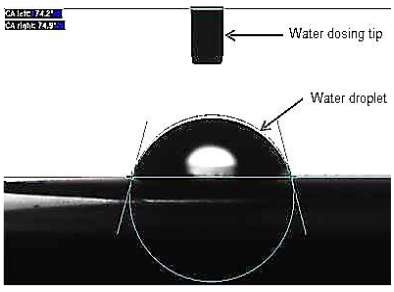

Figure 1: Captured water droplet (3 µL) when measuring the contact angles on paper sheets

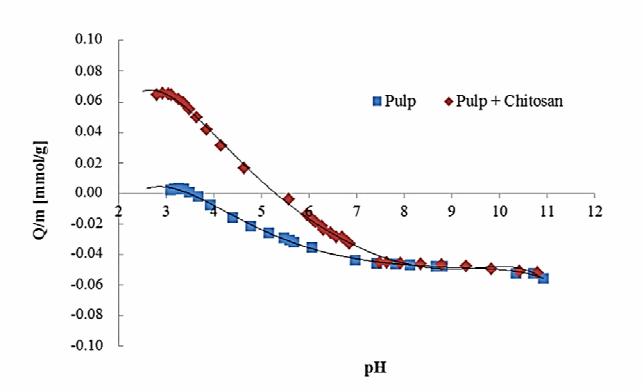

Figure 2: Potentiometric titration curve[19]

RESULTS AND DISCUSSION

Charge behaviour

Figure 2 shows the potentiometric titration curve of paper sheets (reference sample and that functionalised by chitosan). It represents the charge Q/m (mmol/g) as a function of pH.

It can be seen from the potentiometric titration that the functionalization of paper pulp with chitosan was successful due to the fact that the titration curve shifted to a positive charge (due to the presence of protonated amino groups). Also, the isoelectric point shifted towards a higher pH, thus proving the presence of chitosan. The adsorption of chitosan provided to the paper pulp around 65 mmol/kg of amino groups, which are responsible for an antimicrobial behaviour.[6] A negative charge can be estimated from the negative plateau values of the titration curves. Those charges originated from deprotonated acidic fibre groups (carboxylic acids) and accounted for both samples, the reference and modified ones of around 50 mmol/kg. This clearly suggests that electrostatic interactions were not responsible for the chitosan binding onto the paper pulp. Obviously, the chitosan attached to the paper pulp by physical forces and thus did not alter the fibre carboxyl groups.

ATR FT-IR

Using ATR FT-IR spectroscopy, typical functional groups of chitosan were evaluated after chitosan incorporation into paper pulp. Typical peaks for paper sheets (a) and chitosan powder (b) can be seen in the FT-IR spectrum (Fig. 3), both as reference samples. The FT-IR spectrum of the chitosan powder (b) shows peaks at 1660 cm^{-1} and 1590 cm^{-1}. These two wavenumbers are assigned to the carbonyl stretching vibration (amide I), and the N-H bending vibration (amide II) of a primary amino group, respectively. On the FT-IR spectrum of the paper functionalised with chitosan, the same typical signals of chitosan's functional groups appeared within the area between 1660 and 1590 cm^{-1}, which proved the presence of chitosan in the paper.

Sorption capacity

Figure 4 shows the water sorption ability of a paper sample in comparison with that of paper functionalised with chitosan.

The addition of chitosan into paper pulp decreased the water absorption ability (water mass in g/m^2 of the paper sheet) of the paper sheets. Thus, the sorption ability of the functionalised paper sheet decreased by 40%. It can also be seen from Figure 4 that the capillary rise of water for sample B was by around 30% lower than for sample A. Even more, the measurements of the contact angle proved the decrease of the water sorption ability of the paper sheets after the addition of chitosan. The contact angle for the reference sample (sample A) was on average of 20°, which showed the extremely hydrophilic character of this sample. When chitosan was added to paper pulp, the contact angle of the paper sheet increased up to fourfold to 88° (average value of 5 measurements). However, this sample still showed a hydrophilic character (contact angle less than 90°), which is welcome for practical usage. By the interaction of chitosan with the paper pulp, an obviously new structure was formed, in a way that the packaging densities of the fibres were strengthened and thus

the amorphous regions were less available for water penetration. Moreover, due to its conformation (extended chain) and high molecular weight, chitosan can also cover fibre surfaces (film formation) and thus prevent absorption of water into the internal part of fibres.

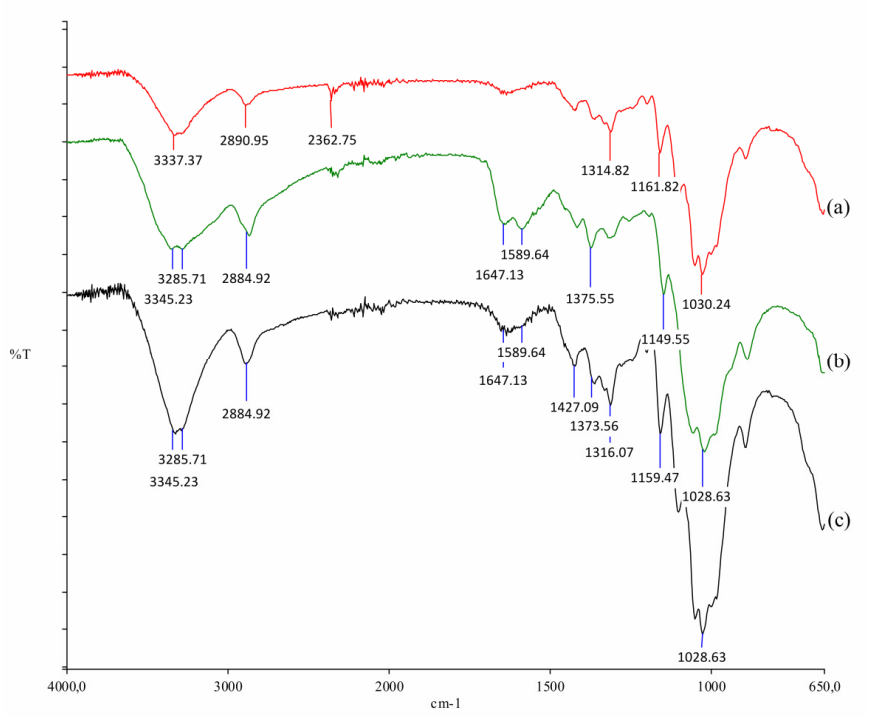

Figure 3: FT-IR spectra for paper sheets (a), chitosan (b) and paper sheets functionalised by chitosan (c)

The decrease in the sorption capacity of paper sheets after addition of chitosan is not advantageous from the practical point of view, since sanitary products, such as handkerchiefs, towels, wipes, *etc.*, need to be highly absorptive. In the future, the addition of chitosan needs to be optimised so as not to alter the sorption properties to such an extent. Thus, chitosan nanoparticles may be used instead of chitosan macromolecules (acidic chitosan solution): chitosan in the nanoparticle form could be targeted to attach in some places only, without covering the fibre surfaces or being absorbed into their internal part.

Antimicrobial testing

Figure 5 shows the results of antimicrobial testing performed in accordance with the standard ASTM E 2149-01. The samples were considered antimicrobial when the reduction was higher than 75%.[20,21] The following microorganisms were chosen for antimicrobial testing: Gram-positive *Staphylococcus aureus* and *Streptococcus agalactiae*, Gram-negative *Escherichia coli*, and fungi *Candida albicans* and *Candida glabrata*.

It is obvious from Figure 5 that sample B inhibited *Staphylococcus aureus* (inhibition increased from 45% to 78%). *Streptococcus agalactiae* (sample A) showed 24% antimicrobial efficiency, while the addition of chitosan (sample B) surprisingly decreased this inhibition and thus showed no efficiency regarding this microorganism. The addition of chitosan increased the inhibition for *Escherichia coli* by about 6% (from 35 to 41%). However, this small increase means that the paper sheet is not efficient as an antimicrobial agent for *Escherichia coli*.

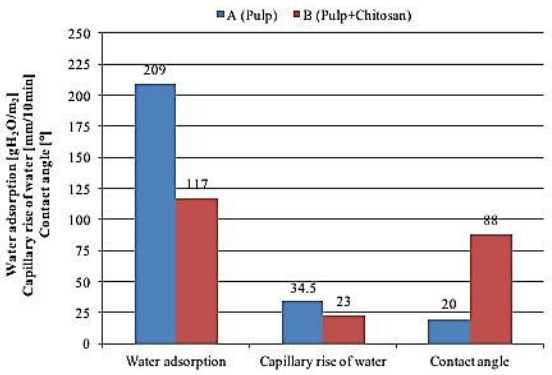

Figure 4: Water absorption, capillary rise of water and contact angels for paper pulp sheets

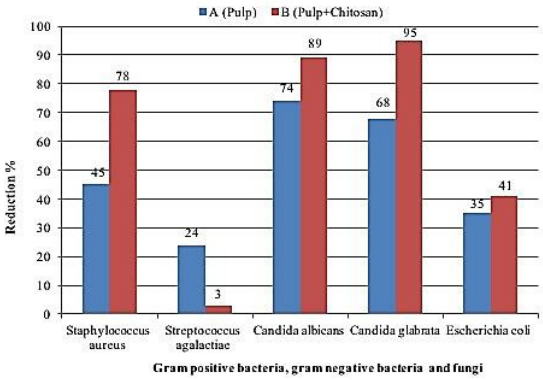

Figure 5: Antimicrobial testing regarding gram positive bacteria (*Staphylococcus aureus*, *Streptococcus agalactiae*), gram negative bacteria (*Escherichia coli*) and fungi (*Candida albicans, Candida glabrata*)

Chitosan-functionalised paper sheets exhibited increased antimicrobial efficiency against *Candida albicans* with a reduction from 74% to 89%. The same situation was observed in the case of *Candida glabrata*, where the paper sheets functionalised by chitosan showed a 95% reduction. The results indicate that paper functionalised by chitosan presents antimicrobial efficiency against both fungi *Candida albicans* and *Candida glabrata*. It can be thus concluded that chitosan imparts antifungal properties to functionalised paper sheets.

It can be summarised that functionalised paper sheets inhibited successfully the growth of the following microorganisms: *Staphylococcus aureus*, *Candida albicans* and *Candida glabrata*. All of these microorganisms may cause skin problems or intimate care problems (urogenital problems), thus paper pulp functionalised by chitosan could be an ideal basis for producing toilet paper, wipes and intimate papers or handkerchiefs. For a transfer of this technology to a production line, the addition of chitosan would be economically acceptable and the technology is transferable, but all economic and environmental factors must be studied and respected.[22-24] Another advantage of such products would be that their antimicrobial efficiency may positively influence their storage process.

CONCLUSION

Potentiometric titration and ATR FT-IR showed that chitosan can be successfully introduced into paper pulp. Chitosan binding to pulp fibres worsens the water sorption ability of the produced paper. This may be explained by the fact that chitosan attaches to fibres changing the molecular structure: *i.e.* amorphous regions are reduced, or the sorption ability is restricted because chitosan covers the fibre surfaces. However, the presence of chitosan in the paper pulp imparted antimicrobial properties, leading to the inhibition of *Staphylococcus aureus*, and both fungi: *Candida albicans* and *Candida glabrata*. These results make such paper products extremely important for application in the hygiene and medical sectors.

In our opinion, this kind of functionalised paper (with some optimisation steps) may find application as toilet paper, wipes, and intimate handkerchiefs.

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