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ANTIBACTERIAL PERFORMANCE OF CHITOSAN BASED MEMBRANES LOADED WITH TETRACYCLINE FOR WOUND HEALING APPLICATIONS[1]

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Abstract: *In this study, the possibility of immobilizing an antibiotic (tetracycline) onto chitosan (CS) and chitosan/zeolite (CSZ) composite membranes for wound healing applications was investigated. To study the loading capacity of tetracycline onto the CS/CSZ membranes UV-spectroscopy was employed. The main challenge was to provide antibacterial properties through a local delivery of antibiotics in order to prevent infection in wounds during the wound treatment procedures. The antibacterial activity against Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 29213 strains of the developed membranes was assessed trough disk-diffusion method by means of Mueller-Hinton agar. The obtained results showed that chitosan/zeolite membranes loaded with tetracycline exhibited better antimicrobial properties compared to other studied objects.*

Keywords: *Chitosan, Chitosan/zeolite composite membranes, Zeolite, Tetracycline, Escherichia coli, Staphylococcus aureus.*

INTRODUCTION

Skin wounds are the result of disruption of normal tissue anatomy and are classified according to the type of healing process (acute and chronic wounds). There are many factors that can affect wound healing at different stages of this process, resulting in incorrect or impaired tissue repair (Guo & DiPietro, 2010). One of the most significant problems in the treatment of wounds is the development of infection caused by pathogenic bacteria. The presence of bacterial infection adversely affects angiogenesis, granulation tissue formation and epithelialization (Archana, Dutta, & Dutta, 2015). Bessa et al. (Bessa, Fazii, Di Giulio, & Cellini, 2013) indicated that bacterial infections are most commonly caused by *Staphylococcus aureus* (37%), followed by *Pseudomonas aeruginosa* (17%), *Proteus mirabilis* (10%), *Escherichia coli* (6%) and *Corynebacterium spp.* (5%). Wound dressings play a vital role in wound healing process as they protect against exogenous microorganisms (Ambekar & Kandasubramanian, 2019). In recent years, the type of these materials has been constantly changing, with particular interest being pointed at biopolymer-

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based dressing materials, i.e chitosan (Anitha et al., 2014; Barbosa, Debone, Severino, Souto, & Da Silva, 2016; Gupta, Agarwal, & Sarwar Alam, 2014; Liu et al., 2018; Patrulea, Ostafe, Borchard, & Jordan, 2015)

Chitosan is a linear aminopolysaccharide composed of glucosamine and N-acetyl glucosamine linked by β (1-4) glycosidic bonds. As a natural biopolymer, chitosan has been widely studied as a means of preventing and treating infections due to its antimicrobial properties, biocompatibility, biodegradability and non-toxicity (Ma et al., 2017). It has been shown that bandages containing the biopolymer as a component have good antibacterial properties and haemostatic effect (Noel, Courtney, Bumgardner, & Haggard, 2008). The presence of 2-amino-2-deoxy-β-D-glucopyranose residues (D-glucosamine-$GlcNH_2$) in the chitosan macromolecule plays an important role in improving and enhancing the reepitalization process and the repair of cellular tissue (Singh & Ray, 2000). Although in highly infected wounds, antibiotic administration is almost inevitable, medications have many disadvantages, such as: low stability, low water solubility, toxicity and side effects (Taghizadeh, Ashassi-Sorkhabi, Afkari, & Kazempour, 2019). The use of harmless carriers for antibiotics to be administered in the area of infection is an option for reducing their toxicity, since the planned therapeutic concentrations of the drug are delivered to the site of the infection without the need for large doses of medication. Controlled drug delivery to the site of the wound provides continuous healing action, reducing the frequency of dressing change. The ability of chitosan to form nanostructured film-layered forms allows it to be used as a carrier of compounds with antibacterial activity directly in the infected area of the wound (N. Mengatto, M. Helbling, & A. Luna, 2012).

In modern practice, antibiotics such as gentamycin, minocycline, tetracycline, streptomycin and others are commonly used in treatment of wounds. Tetracycline has been found to have a broader antimicrobial spectrum than any other antibiotic and has exceptional chemotherapeutic efficacy against both Gram-positive and Gram-negative bacteria (Chopra & Roberts, 2001; Gupta et al., 2014).

The purpose of the current study is to develop novel chitosan-zeolite-tetracycline nanocomposite (CSZT) membranes and to determine their antibacterial sensitivity against Gram-positive and Gram-negative bacteria for wound healing applications.

EXPOSITION

Materials and methods

The main chemicals used in membrane preparation are as follows: chitosan (CS, obtained from the Black Sea shrimp shells) with a deacetylation degree of 83%, clinoptilolite (Z, Beli Plast, Kardzhali District, Bulgaria), acetic acid (99%, Sigma-Aldrich Co.) and tetracycline (> 98%, Sigma-Aldrich Co.).

The procedure involves pre-preparation of chitosan-zeolite membrane (CSZ) by the addition of a 1% chitosan solution (1 g of chitosan in 100 ml of 1% acetic acid at 52°C and continuously magnetic stirring for 24 hours) to 0.5% aqueous zeolite (clinoptilolite) suspension. Thus obtained chitosan – zeolite suspension was then transferred to petri dishes and dried at 60°C.

A 100 ppm aqueous solution of tetracycline was used to adsorb the drug onto the CSZ membrane surface. The impregnation was performed in dark conditions at 25 ° C under magnetic stirring (600 rpm) for 72 hours. For comparison, a membrane of pure chitosan was prepared and the adsorption of tetracycline on its surface followed the sequence described above. The amount of adsorbed tetracycline on CS and CSZ membranes was determined through UV spectrophotometer (Evolution 300, Thermo Scientific).

The obtained membranes were characterized by means of Nicolet iS 50 FTIR infrared spectrometer in the range of 4000-400 cm^{-1}.

The antibacterial sensitivity of the membranes was determined by the Kirby-Bauer disc diffusion method against Gram-positive (*Staphylococcus aureus* ATCC29213) and Gram-negative (*Escherichia coli* ATCC25922) bacteria.

Membrane characterization by means of FTIR

Infrared spectra of CSZ and CSZT are shown in Figures 1 and 2, respectively. The FTIR spectra of pure zeolite, chitosan and tetracycline are presented for comparison purposes.

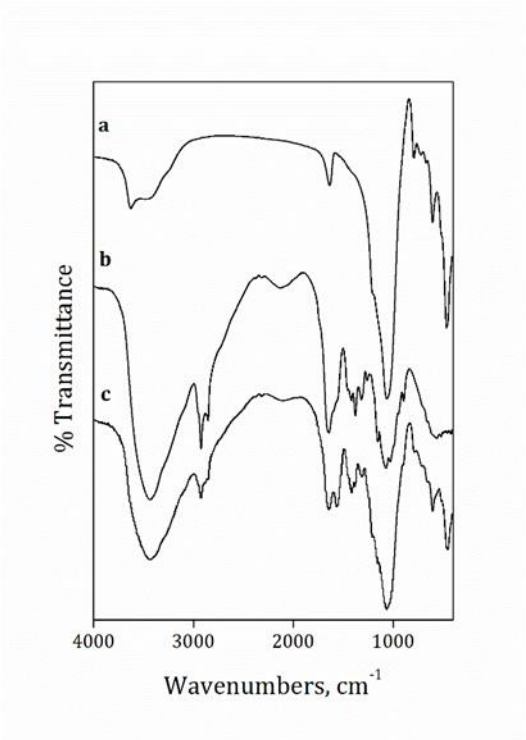

Fig. 1. IR spectrum of: a) zeolite; b) chitosan; c) CSZ;

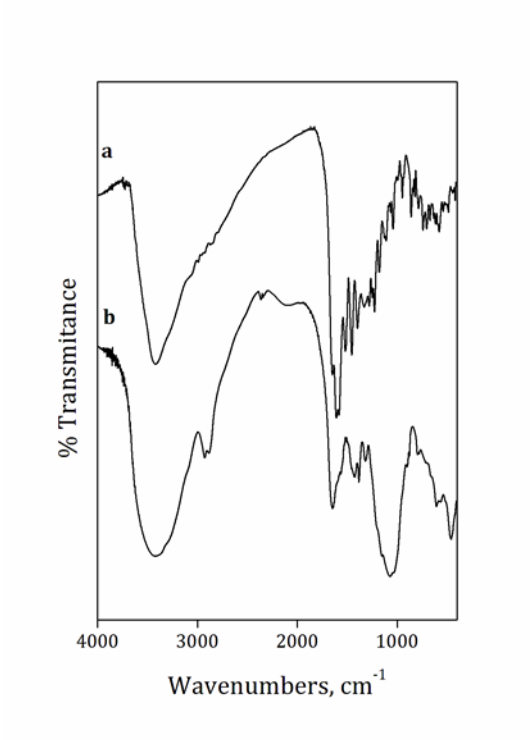

Fig. 2. IR spectrum of: a) tetracycline; b) CSZT;

The main bands in the zeolite spectrum (Figure 1a) are those at 3628 cm^{-1} (stretching of the hydroxyl groups of water adsorbed by the zeolite); 3447 cm^{-1} (stretching of hydrogen bonded hydroxyl groups); 1636 cm^{-1} (H-O-H bending); 1065 cm^{-1} (M - O asymmetric stretching vibration, M = Al, Si); 794 cm^{-1} (symmetric stretching vibration of O-M-O bond); 467 cm^{-1} (deformation vibration of M-O bond) (Mansouri, Rikhtegar, Ahmad Panahi, Atabi, & Shahraki, 2013; Perraki & Orfanoudaki, 2004).

The characteristic bands in the infrared spectrum of chitosan (Figure 1 b)) are: 3442 cm^{-1} (- OH and -NH_2 stretching); 2923 cm^{-1} and 2854 cm^{-1} (- CH_3 and - CH_2 asymmetric stretching); 1643 cm^{-1} (deformation vibration of C=O in $NHCOCH_3$ due to incomplete deacetylation of chitosan); shoulder at 1553 cm^{-1} (deformation vibrations of N-H); 1418 and 1378 cm^{-1} (deformation vibrations of - CH_3 and - CH_2 groups); 1152 cm^{-1} (symmetric stretching vibrations of the C-O-C bond); 1073 cm^{-1} (stretching vibrations of the C-O bonds) (Zvezdova, 2010).

The analysis by infrared spectroscopy confirmed the formation of chitosan - zeolite composite (CSZ). Figure 1 c) shows that the CSZ spectrum contains both chitosan and zeolite bands. The appearance of a pronounced peak at 1563 cm^{-1} in the CSZ spectrum corresponds to the deformation vibration mode of NH_3^+, formed as a result of the formation of hydrogen bonds between the - OH groups of the zeolite and the - OH and - NH groups of chitosan (Wan Ngah, Teong, Wong, & Hanafiah, 2012). The adsorption of tetracycline onto the CSZ surface was found to alter the intensity and shape of some of the bands in the IR spectrum of the CSZ membrane (Figure 2 b)). The band intensities at 1065 cm^{-1} (asymmetric stretching vibration (M-O)) and 794 cm^{-1} (symmetric stretching vibration (O-M-O)) decreased, while the intensity of the bands at 3444 cm^{-1} corresponding to the hydrogen bonded hydroxyl groups, increased. This implies that tetracycline is adsorbed onto the CSZ membrane surface by forming hydrogen bonds between the two functional groups (–NH_2 and –OH) present in its molecule and the functional groups of zeolite and chitosan.

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Tetracycline adsorption onto CS and CSZ membranes

The amount of adsorbed tetracycline on the membranes was determined by measuring the concentration of tetracycline solution before and after the adsorption experiment, via UV spectrophotometer, using a calibration curve built in the range of 20 to 100 ppm, at a wavelength of 276 nm. The resulting calibration curve is presented in Figure 3.

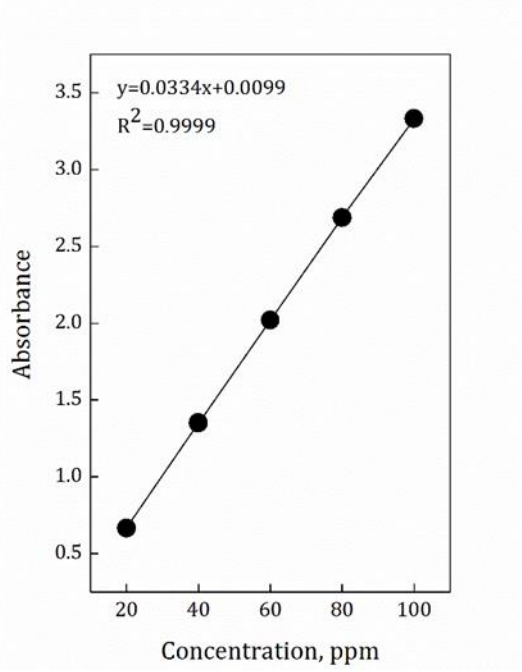

Fig. 3. Calibration curve (λ_{max} = 276 nm)

The concentration of tetracycline in the aqueous solution after adsorption was calculated by the calibration equation (Fig. 3). The amount of tetracycline adsorbed onto the membrane was determined based on the difference in drug concentrations before and after adsorption.

In order to estimate the adsorption capacity of the prepared membranes, several experiments of different duration (12, 24, 48 and 72 hours) were performed. These experiments did not exceed 72 hours since hydrolysis of tetracycline in aqueous solution may occur (Taghizadeh et al., 2019). The obtained results are presented in Figure 4. The data in Figure 4 indicate that the highest adsorbed tetracycline amount per milligram membrane is in 72 hours. It was also found that chitosan and zeolite composite membranes absorbed about twice as much tetracycline (47 μg/mg) as compared to those prepared from chitosan only (23 μg/mg). The incorporation of zeolite into the chitosan membranes provides additional adsorption centers, which allows larger amount of tetracycline to be adsorbed.

It has been shown that the minimum tetracycline amount required to inhibit the growth of 90% of the strains is less than 6 μg/ml (Ahmed, Charyulu, Harish, & Prabhu, 2009). Therefore, it could be concluded that the amount of adsorbed tetracycline onto the membranes is sufficient to be applied against infection causing bacteria.

Antibacterial activity of the obtained membranes

The antibacterial activity of the membranes was evaluated against Gram-positive (*Staphylococcus aureus* ATCC 29213) and Gram-negative (*Escherichia coli* ATCC 25922) bacteria, and the results for bacterial inhibition zone are shown in Figure 5. The data in Figure 5 show that CSZT membranes have a greater inhibitory effect against *Staphylococcus aureus* and *Escherichia coli* than CST membranes, due to the larger amount of tetracycline on zeolite-containing membranes. The bacteriostatic action of tetracycline is due to its ability to bind to the 30S ribosomal subunit of bacterial ribosomes and inhibit protein synthesis by blocking the binding of aminoacyl-mRNA to the mRNA-ribosome complex. This blocks the addition of amino acids to the newly formed polypeptide chain.

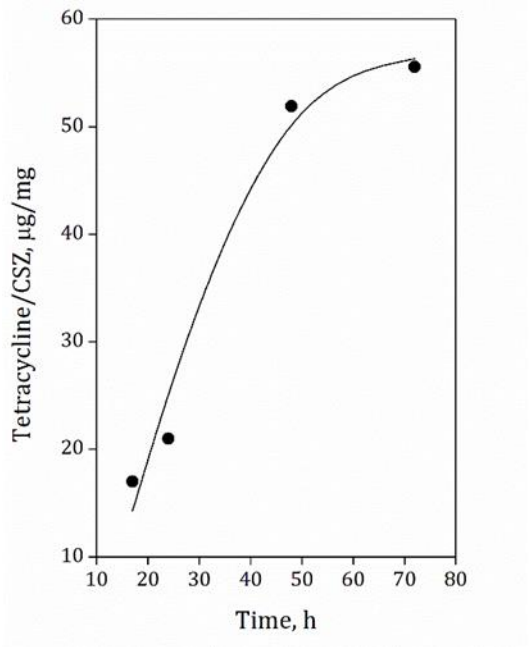

Fig. 4. Amount of tetracycline adsorbed over time

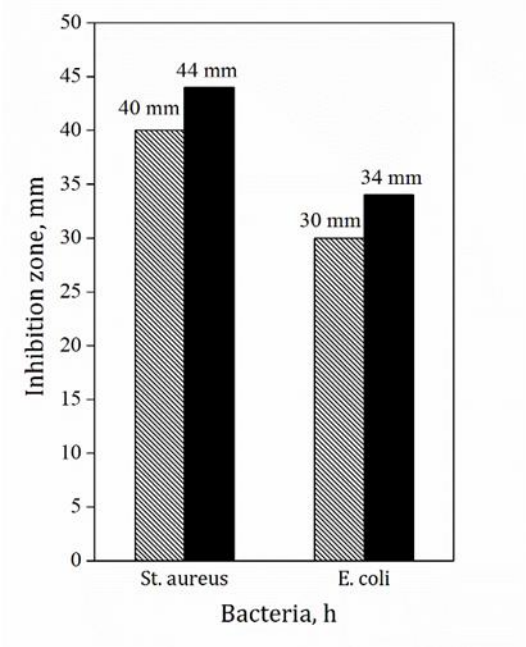

Fig. 5. Antibacterial activity of CST and CSZT membranes

CONCLUSION

A series of new tetracycline adsorbed chitosan-zeolite nanocomposite membranes have been prepared. The FTIR spectra of the studied membranes confirm the retention of tetracycline on the carrier surface by hydrogen bonding. The antibacterial activity of the obtained membranes was also investigated. The results show that both CST and CSZT nanocomposite membranes exhibit good antibacterial activity against Gram-positive (*Staphylococcus aureus* ATCC 29213) and Gram-negative (*Escherichia coli* ATCC 25922) bacteria. This, in turn, would allow the obtained membranes to be considered as a good opportunity for the preparation of dressings used in wound treatment.

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