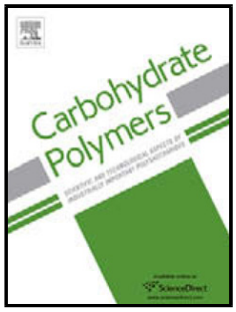

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New antimicrobial chitosan derivatives for wound dressing applications

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Abstract

Chitosan is a non-toxic, biocompatible, biodegradable natural cationic polymer known for its low imunogenicity, antimicrobial, antioxidant effects and wound-healing activity. To improve its therapeutic potential, new chitosan-sulfonamide derivatives have been designed to develop new wound dressing biomaterials. The structural, morphological and physico-chemical properties of synthesized chitosan derivatives were analyzed by FT-IR, ^{1}H-NMR spectroscopy, scanning electron microscopy, swelling ability and porosity. Antimicrobial, *in vivo* testing and biodegradation behavior have been also performed. The chitosan derivative membranes showed improved swelling and biodegradation rate, which are important characteristics required for the wound healing process. The antimicrobial assay evidenced that chitosan-based sulfadiazine, sulfadimethoxine and sulfamethoxazole derivatives were the most active. The MTT assay showed that some of chitosan derivatives are nontoxic. Furthermore, the *in vivo* study on burn wound model induced in Wistar rats demonstrated an improved healing effect and enhanced epithelialization of chitosan-sulfonamide derivatives compared to neat chitosan. The obtained results strongly recommend the use of some of the newly developed chitosan derivatives as antimicrobial wound dressing biomaterials.

KEYWORDS: chitosan derivative, membrane, biomaterial, healing activity

Chemical compounds studied in this article:

Chitosan (PubChem CID: 21896651); Sulfadiazine (PubChem CID: 5215); Sulfamethoxydiazine (PubChem CID: 5326); Sulfamerazine (PubChem CID: 5325); Sulfadimethoxine (PubChem CID: 5323); Sulfisoxazole (PubChem CID:5344); Sulfamethoxazole (PubChem CID:5329); Chloroacetyl chloride (PubChem CID: 6577);

Acetic acid (PubChem CID: 176); Sodium hydroxide (PubChem CID: 14798); Sodium tripolyphosphate (PubChem CID: 24455).

Abbreviations used

ANOVA, analysis of variance; CS MMW, chitosan with medium molecular weight; DA, degree of acetylation; DS, degree of substitution; DMFA, dimethylformamide; FT-IR, Fourier transform infrared spectroscopy; ^{1}H-NMR, Proton Nuclear Magnetic Resonance; MSR, membrane swelling ratio; PBS, phosphate buffered saline; SEM, scanning electron microscope; TLC, layer chromatography; TPP, sodium tripolyphosphate.

1. Introduction

The wound is a type of injury in which dermis of the skin is damaged by burn, trauma, and cut. Often a serious wound can cause death and should be treated with specialized wound dressing materials. Although there is a high demand for wound dressing materials, wound dressing technology is still far behind, due to the intrinsic complexity of the wound healing process. Ideal wound dressing materials need to have good tissue conformity, easy application, moist environment, low risk of infection, proper removal of exudates and accelerated tissue healing rate. There are various types of wound dressing biomaterials that are commercially available, generally manufactured from natural or synthetic polymers or a combination of both. In recent years, porous membranes are considered as the best wound dressing materials since they provide a moist and occlusive environment, which significantly facilitates the wound healing process (Willi & Chandra, 2004). However, the moist conditions, provided by membranes, are equally necessary for pathogen proliferation and colonization. The impregnation of the wound dressing material with an antimicrobial agent is

also highly preferred (Jones, Grey & Harding, 2006; Sudheesh Kumar et al., 2012; Mallick et al., 2012).

Another desirable function of the wound dressing is to accelerate hemostasis and tissue regeneration. A cationic dressing is known to fulfill this requirement. Chitosan is a naturally derived cationic polysaccharide consisting of N-acetyl glucosamine and D-glucosamine sugars (Yang et al., 2008; Batista, Pinto, Gomes & Gomes, 2006). During chitosan biodegradation, it produces N-acetyl glucosamine, which is used to accelerate the re-epithelialization process. Its non-toxicity, versatile biological activities as antimicrobial, low immunogenicity, wound-healing activity, antioxidant properties, and low cost have provided ample opportunities for further development of biomaterials for various therapeutic applications. Due to its polycationic nature the chitosan inhibits the growth of a wide variety of bacteria and fungi, showing a broad spectrum of antibacterial activity, high killing rate against bacteria, and low toxicity toward mammalian cells (Xie, Liu & Chen, 2007). Chitosan and its derivatives received increasing attention in diverse areas such as food preservation (Aider, 2010), water purification (Kołodyńska, 2012; Wang, Chen, Yuan, Sheng & Yu, 2009; Gupta, Chauhan & Sankararamakrishnan, 2009), paper industry (Dutta, Ravikumar & Dutta, 2002), pharmacy or medicine (Dash et al., 2011; Gallaher et al., 2000; Xie et al., 2007; Ma et al., 2008; Pillai, Willi & Chandra, 2009; Yang, Chou & Li, 2005; Li et al., 2012; Dias et al., 2013; Öztürk, Agalar, Keçeci & Denkbas, 2006; Xing, Lie, Zhengwei & Changyou, 2011).

Chitosan possesses three reactive sites including a primary amine and two primary or secondary hydroxyl groups per glucosamine unit readily subjected to chemical modification (Okamoto et al., 2003; Samal et al., 2012). The structural characteristics of chitosan mimic glycosaminoglycan components of the extracellular matrix, while the biocompatibility, biodegradability, antibacterial (Samal et al., 2014), antioxidant activities and mucoadhesive properties impart versatility. Utilization of chitosan in biomedical applications is limited due

to its low solubility at physiological pH of 7.4, althoughit is soluble in acidic aqueous media. Moreover, the antimicrobial effects of chitosan are improved in acidic conditions due the interactions between protonated amino groups of chitosan and anionic components of bacteria (Martins et al., 2014). In order to overcome these limitations and enhance antimicrobial properties, various chitosan derivatives have been designed (Yang et al., 2008). Many efforts have been made to introduce hydrophilic groups by covalent attachment to reactive amino groups at the C_2 position. Various kinds of modification of chitosan have been investigated in recent years using acylation, alkylation, carboxymethylation and quaternization (Ma et al., 2008). Recently, the antibacterial and antifungal activities of chitosan have been followed with great interest. Furthermore incorporation of antibacterial agents into the chitosan backbone offers excellent antibacterial properties (Samal et al., 2014; Ignatova, Manolova & Rashkov, 2013; Sashiwa, Yamamori, Ichinose, Sunamoto & Aiba, 2003).

On the other hand, sulfonamides and their different derivatives are extensively used in medicine due to their pharmacological properties such as antibacterial activity (Kremer et al., 2006; Zahid, 2009). Sulfonamide derivatives were successfully employed as effective chemotherapeutic agents for the prevention and cure of bacterial infections in humans. These drugs act on the bacteria and either prevent their growth (bacteriostatic effect) or act as germicides (bactericides) and have no effect on the smooth muscles, heart, blood pressure or respiration (Gomes & Gomes, 2005). Therefore, it is essential to have an ideal membrane which can combine all the above mentioned features of chitosan and sulfonamides into one single design, to address the different aspects of a dynamic wound dressing biomaterial.

Herein, we report on the preparation, physico-chemical characterization and biological evaluation of the novel chitosan-sulfonamide derivatives as membranes which have all theoretical premises to be useful in the therapy of wounds, focused on burn wounds. New sulfonamide drug-functionalized chitosan membranes were proposed to reduce bacterial

infection risks as well as promote re-epithelialization of damaged tissue and thus be effective as wound dressing biomaterials.

2. Material and methods

2.1. Materials

Chitosan with medium molecular weight (CS MMW, 425 kDa, deacetylation degree of 85%), sulfonamides (sulfamethoxydiazine, sulfadiazine, sulfamerazine, sulfadimethoxine, sulfisoxazole and sulfamethoxazole), chloroacetyl chloride, acetic acid, sodium hydroxide, sodium tripolyphosphate (TPP) and organic solvents (p.a.) were purchased from Sigma Aldrich Company. All solvents and reagents were used without further purification. Thin layer chromatography (TLC) plates (aluminum foil covered with 0.25 mm thick silica gel 60) from Merck (VWR International) were used.

2.2. Chemistry

2.2.1. Synthesis of sulfonamide-chitosan derivatives (3a-f)

To a stirred solution of chitosan (11 mmol) in 1% acetic acid (100 mL), a solution of N-chloroacetylsulfonamide derivative (**2a-f)** (13.2 mmol) in dimethylformamide (DMFA) (50 mL) was added. The reaction mixture was stirred for about 24 h at room temperature and then the pH was adjusted to 9 with 15% NaOH solution, resulting in a precipitate (Batista, Pinto, Gomes & Gomes, 2006; Xie, Liu & Chen, 2007; Feng & Xia, 2011). The products were washed five times with double-distilled water until the pH of the filtrate was 7. The final compounds were purified by dialysis against deionized water for 5 days and then freeze-dried on Alpha 1-2 LD Plus Freeze Dryer.

Chitosan-aminoacetyl-sulfamethoxydiazine **(3a).** IR (KBr, cm^{-1}): 1640 (C=O amide I), 1595 (NH amide II), 1543, 834 (phenyl), 1258, 1165 (SO_2-N); ^{1}H-NMR (300 MHz, CD_3COOD, δ):

2.32 (3H, $COCH_3$); 2.32 (3H, OCH_3); 3.47 (H-2 chitosan); 4.00-4.18 (H-3,4,5,6 chitosan); 5.15 (H-1 chitosan); 3.15-3.35 (NH), 7.03 (2CH, aromatic), 7.79 (2CH, aromatic), 8.09 (2CH, pyrimidine).

Chitosan-aminoacetyl-sulfadiazine **(3b).** IR (KBr, cm^{-1}): 1629 (C=O amide I), 1595 (NH amide II), 1546, 839 (phenyl), 1259, 1168 (SO_2-N); ^{1}H-NMR (300 MHz, CD_3-COOD, δ): 2.18 (3H, $COCH_3$); 3.67 (H-2 chitosan); 3.85- 4.53 (H-3,4,5,6 chitosan); 5.16 (H-1 chitosan); 3.12 (NH); 6.86-6.88 (2CH, aromatic); 7.01-7.02 (2CH, aromatic); 7.65 (1CH, pyrimidine); 8.42-8.43 (2CH, pyrimidine).

Chitosan-aminoacetyl-sulfadimethoxine **(3c).** IR (KBr, cm^{-1}): 1656 (C=O amide I), 1592 (NH amide II), 1545, 896 (phenyl), 1262, 1164 (SO_2-N); ^{1}H-NMR (300 MHz, CD_3-COOD, δ): 2.18 (3H, $COCH_3$); 1.24-1.38 (6H, OCH_3); 3.54 (H-2 chitosan); 3.60- 3.87 (H-3,4,5,6 chitosan); 4.79 (H-1 chitosan); 3.14 (NH); 7.70-7.72 (2CH, aromatic); 7.95-7.98 (2CH, aromatic); 8.26 (1CH, pyrimidine).

Chitosan-aminoacetyl-sulfamethoxazole **(3d).** IR (KBr, cm^{-1}): 1650 (C=O amide I), 1594 (NH amide II), 1548, 897 (phenyl), 1261, 1167 (SO_2-N); ^{1}H-NMR (300 MHz, CD_3-COOD, δ): 2.68 (3H, $COCH_3$); 2.19 (3H, CH_3); 3.13 (H-2 chitosan); 3.52-3.86 (H-3,4,5,6 chitosan); 4.75 (H-1 chitosan); 2.82-2.97 (NH); 6.89-6.91 (2CH, aromatic); 7.67-7.70 (2CH, aromatic); 6.03 (1CH, izoxazole).

Chitosan-aminoacetyl-sulfamerazine **(3e).** IR (KBr, cm^{-1}): 1654 (C=O amide I), 1596 (NH amide II), 1548, 896 (phenyl), 1259, 1165 (SO_2-N); ^{1}H-NMR (300 MHz, CD_3-COOD, δ): 2.63 (3H, $COCH_3$); 2.40 (3H, CH_3); 3.14 (H-2 chitosan); 3.65-3.87 (H-3,4,5,6 chitosan); 4.88 (H-1 chitosan); 2.82-2.98 (NH); 6.87-6.89 (2CH, aromatic); 7.68-7.71 (2CH, aromatic); 7.96 (1CH, pyrimidine); 8.19 (1CH, pyrimidine).

Chitosan-aminoacetyl-sulfisoxazole **(3f).** IR (KBr, cm^{-1}): 1654 (C=O amide I), 1565 (NH amide II), 1546, 897 (phenyl), 1261, 1164 (SO_2-N); ^{1}H-NMR (300 MHz, CD_3-COOD, δ):

174 2.50 (3H, $COCH_3$); 1.54 (3H, CH_3); 1.61 (3H, CH_3); 3.14 (H-2 chitosan); 3.54-3.87 (H-
175 3,4,5,6 chitosan); 4.77 (H-1 chitosan); 2.79-2.99 (NH); 6.82-6.84 (2CH, aromatic); 7.72-7.81
176 (2CH, aromatic).

177 ***2.3. Preparation of chitosan derivatives membranes***

178 Chitosan (CS MMW) and its sulfonamide derivatives (**3a-f**) (2%, w/v), in 2% acetic acid
179 solutions, were kept at -20 °C overnight and after that the frozen samples were lyophilized for
180 24 h, crosslinked with a solution of 5% TPP and then washed several times with double-
181 distilled water in order to obtain porous chitosan membranes (Feng & Xia, 2011; Anisha et
182 al., 2013; Arpornmaeklong, Pripatnanont & Suwatwirote, 2008; Sionkowska & Płanecka,
183 2013). The resulting membranes were stored in Falcon tubes at 4 °C.

184 ***2.4. Characterization of Chitosan Derivatives***

185 ***2.4.1. ATR -FTIR Spectroscopy***

186 Chloroacetylsulfonamide derivatives (**2a-f**) and chitosan derivatives (**3a-f**) were
187 characterized by ATR-FTIR measurements using a Biorad FT-IR spectrometer FTS 575C.
188 Spectra were recorded in the range of 4000- 500 cm^{-1} with 32 scans at a resolution of 4 cm^{-1}.
189 Spectral processing was carried out with Horizon MBTM FTIR Software and GRAMS 32
190 Software (Galactic Industry Corporation, Salem, NH), Version 6.00 (Samal et al, 2014).

191 ***2.4.2. ^{1}H-NMR Spectroscopy***

192 ^{1}H-NMR spectra of chitosan derivatives were recorded in D_2O/CD_3COOD with a Bruker
193 Avance 300 MHz instrument. The chemical shifts were expressed in ppm downfield of
194 tetramethylsilane (TMS) as an internal standard. Nuclear magnetic resonance spectral analysis
195 (^{1}H-NMR) was used to determine the acetylation degree of chitosan, and the substitution
196 degrees of various chitosan derivatives.

Degree of Acetylation (DA) of chitosan it was calculated by using ratio between the integral of the peak of the three protons from acetyl group and integral of the characteristic peak of proton from -C<u>H</u>-NH_2 group of deacetylated monomer, using the following formula:

$$DA = ([Ia]/3)/ [Ib] \times 100 \quad (1)$$

where: Ia=integral of the peak of the three protons of acetyl group, Ib = integral of the characteristic peak of proton from -C<u>H</u>-NH_2 group.

Degree of Substitution (DS) of chitosan derivatives (3a-f) was calculated by using the ratio between the integral of the peak of the four aromatic protons from sulfonamide part and integral of the characteristic peak of proton from -C<u>H</u>-NH_2 group of deacetylated monomer, using the following formula:

$$DS = ([Ia]/4)/ [Ib] \times 100 \quad (2)$$

where: Ia = integral of the peak of the four aromatic protons from sulfonamide part, Ib = integral of the characteristic peak of proton from -C<u>H</u>-NH_2 group.

2.5. Characterization of chitosan derivatives membranes

2.5.1. Morphology

The morphology of chitosan membrane was examined by using a Fei Quanta 200F (field emission gun) scanning electron microscope (SEM). The dried samples were coated with gold before observation in a scanning electron microscope.

2.5.2. Porosity test

The porosity of prepared crosslinked chitosan derivatives membranes was determined using the method of immersing in absolute ethanol until saturation. The samples have been weighed before and after immersion in alcohol and the porosity degree (P) was calculated according to the following formula (3):

$$P = (W_2\text{-}W_1)/\rho V_1 \quad (3)$$

where: W_1 and W_2 = the weight of membrane before and after immersion in absolute ethanol, V_1 and ρ are the volume and density of alcohol.

2.5.3. In vitro biodegradation

The degradation of the membranes was studied in a phosphate buffered saline (PBS) (pH 7.4) containing lysozyme at 37°C. Membranes were equally weighed and immersed first in PBS until swelling equilibrium was reached, then the PBS solution was changed to a PBS medium containing lysozyme (10000 UI/mL) and incubated at 37°C for 7 days. The membranes were taken out and weighed after 1, 4 and 7 days. The percentage biodegradation (D%) was calculated using the following formula (Baran, Tuzlakoğlu, Mano & Reis, 2012):

$$D\% = (W_0\text{-}Wx)/W_0 \times 100 \quad (4)$$

where: W_0 = wet weight before incubation, W_X = wet weight after incubation.

2.5.4. Swelling ratio

The crosslinked chitosan derivative membranes were cut into small pieces that had equal weights (W_d) and then were immersed in double-distilled water and acetate buffer solution respectively. The membranes were taken out at specified periods of time, the excess of water was removed by gentle wiping with a filter paper and immediately weighing them (W_w). The membrane swelling ratio (MSR) was calculated using the following formula (Lin, Tan, Marra, Jan & Liu, 2009):

$$MSR\ (\%) = (W_w\text{-}\ W_d)/\ W_d x100 \quad (5)$$

where: W_d, W_w are the weight of membranes before and after immersion in double-distilled water.

2.5.5. Surface free energy estimation by contact angle measurement

Surface free energy (SFE) can be calculated by measuring contact angles of various fluids with different known surface tension characteristics. To obtain the components of the surface

free energy and the total surface free energy of chitosan and chitosan derivative membranes, the contact angles at equilibrium between the film surface and three pure liquids: double-distilled water, formamide and diiodomethane were measured. The contact angle was determined by the sessile drop method, at room temperature and controlled humidity, within 10 s, after placing 1 μL of pure liquids on the film surface, using a CAM-200 instrument from KSV-Finland. Contact angle was measured at least 10 times on different sites of the surface, the average value being considered. It was calculated using Young-Laplace equation (Rotta et al., 2009).

SFE and the components were calculated using the acid-base approach which divides the total SFE into dispersive Lifshitz–van der Waals interaction (γ_s^{LW}) and polar Lewis acid–base interactions (γ_s^{AB}) according to the equation (6) (Shabalovskaya, Siegismund, Heurich & Rettenmayr, 2013). The acid base interactions are subdivided into electron donor γ_s^- (Lewis base) and electron acceptor γ_s^+ (Lewis acid) parts.

$$(1+\cos\theta)\,\gamma_s^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^+\gamma_l^-} + \sqrt{\gamma_s^-\gamma_l^+}\right) \quad (6)$$

where: θ is the contact angle, γ_l^{TOT} is the liquid's total surface tension, γ_l^{LW} and γ_s^{LW} are the apolar Lifshitz–van der Waals components of the liquid and the solid, respectively, and $\gamma_s^+\gamma_l^-$ and $\gamma_s^-\gamma_l^+$ are the Lewis acid–base contributions of either the solid or the liquid phase.

2.6. Biological evaluation

2.6.1. Antimicrobial assay

Diameter of inhibition area

Antibacterial activity measured as the diameter of inhibition area was evaluated by agar disc diffusion method (CLSI, 2012) using the following bacterial strains: *Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Bacillus cereus* ATCC 14579, *Bacillus*

subtilis, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* CIP 82118.The antifungal effect was evaluated on *Candida albicans* ATCC 10231, *Candida glabrata* and *Candida sake*. All antimicrobial strains were obtained from the Culture Collection of the Department of Microbiology, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania.

Sterile stainless steel cylinders (50 mm internal diameter; 100 mm height) were applied on the agar surface in Petri dishes and test samples (**3a-f**), prepared as disc-shaped membranes (2.5 mg/disc), were added. The Petri dishes were incubated at 37°C for 24 h (for bacteria) and at 24°C for 48 h (for yeasts). After incubation, the diameter of inhibition area was measured. Commercially available discs containing nitrofurantoin (300 μg/disc) and ciprofloxacin (5 μg/disc) were used as positive controls.

Minimum inhibitory/bactericidal concentrations (MICs/MBCs)

The MICs and MBCs for two of the most common bacterial strains (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922) were evaluated according to the the guidelines of EUCAST Def 3.1 (2012). Briefly stock solutions were prepared by dissolving the chitosan derivatives (**3a-f**) in the concentration of 1% in acetic acid 1%. Using these solutions, series of two-fold dilutions were subsequently obtained. In a 9 cm diameter Petri dish, one milliliter of each dilution was mixed throughly with Mueller-Hinton agar (19 mL), sterilized by autoclaving and cooled to 50°C. After this, the concentrations of the chitosan derivatives inside the medium were 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.62 mg/mL, 0.31 mg/mL, 0.15 mg/mL, 0.07 mg/mL and 0.03 mg/mL respectively. A blank plate (control of growth) was also prepared by mixing acetic acid 1% (1 ml) with molten agar (19 mL). For each bacterial strain, a 0.5 McFarland suspension was prepared in 0.85% saline solution and after that, the inoculum was standardized in order to assure 10^4 colony-forming units (CFU) per spot (5 μL). All inoculated plates were incubated for 18 h at 36°C. The MIC was

interpreted as the lowest concentration of the membrane that completely inhibits the growth of bacteria in the spot area and the MBC, as the lowest concentration of the membrane that completely kill the bacteria in the spot area. Each determination was performed in triplicate.

2.6.2. MTT cell proliferation assay

Biocompatibility of the polymeric materials was assessed using MTT Cell Proliferation Assay (ATCC® 30-1010K^{TM}). Mouse fibroblasts (L929) were cultured in T-25 tissue culture flasks and incubated overnight at 37°C and 95% relative humidity in air atmosphere containing 5% CO_2. The cell suspension was seeded at a density of $5x10^4$ cells/mL and incubated for 24 h until a monolayer was formed and exposed to polymeric extracts. In order to prepare the polymeric extract the samples (25 mm^2) of chitosan derivative and chitosan (as negative control) were sealed in polyethylene foils and sterilized by exposure to UV radiation for 8 h. After that the samples were placed in the culture medium with or without fetal bovine serum in the closed tubes of 15 mL and incubated at 37^{o}C for 24±2 h. The medium was filtered and used immediately.

Morphology of cells was assessed using inverted microscope and quantitative evaluation of cytotoxicity was done using tetrazolium salt (MTT).

The fibroblasts cultured in the presence of polymeric samples were washed with PBS, fixed in methanol, stained with Hematoxylin and Eosin (H&E) dyes and pictures were taken using an inverse-phase microscope (Nikon Japan).

After the desired time exposure (24 h, 48 h, 72 h), the culture medium was replaced with fresh medium containing 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution, in a 10:1 (v/v) ratio, and the plates were incubated at 37°C, for 3 h. Then, 500 µL of isopropanol was added to each well to dissolve the formazan crystals by gently shaking on a platform, for 3 h. The coloured solution was transferred to a 96-well plate and

the optical density (OD) was read at 570 nm using a Sunrise microplate reader (Tecan, Austria). Cell viability was evaluated using the following formula (7):

$$\text{Cell viability (\%)} = OD_{sample} / OD_{control} \times 100 \quad (7)$$

where: OD_{sample} = optical density of the sample (chitosan and chitosan derivatives), $OD_{control}$ = optical density of the cell culture without polymeric materials).

The test was performed also in the presence of hydrogen peroxide (0.03%) as positive control.

2.6.3. Wound healing assay

The study related to burn wound protocols was approved by the Animal Research Committee of the "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania. Eighteen male Wistar rats of 300 grams were placed prone, under deep inhalation anesthesia (Isoflurane 2L/min), and the dorsal areas were shaved with electric clippers to ensure even burn wounding. High-pressure steam at 114°C was applied for 2 seconds through controlled electro-valve to inflict intermediate thickness burn wound in the rat back. After debridement, the rats were randomly divided in 3 groups (of 6 rats each) and the burn surface was covered with standard gauze dressing (control rats group), chitosan (chitosan rats group) and chitosan-sulfadiazine (chitosan-sulfadiazine rats group) membranes. During the test the membranes were periodically removed and replaced. It is important to replace the membranes for cleaning the wound of dead cells, damaged tissue, fibrin and excess of exudates. All these aspects lead to the necessity of changing dressings in 8^{th}, 11^{th} and 14^{th} day. During every dressing renewal, visual scale analysis, photographs and punch biopsies of the burn surface were performed. ImageJ software assessed the area of wound surface and the level of wound contraction. Paraffin-embedded Hematoxylin and Eosin (H&E) staining was used to compare wound healing processes between groups. At day 14, after the punch biopsy, the rats were euthanized with 1-2 cc KCl via intracardiac injection, while in deep inhalation anesthesia.

2.7. Statistical analysis

All tests were performed in triplicate and the data are expressed as means ± SD. The statistical software package StatView was used for data analysis of biological assays. Experimental results were analyzed by 3 (groups) x 3 (time sample points) repeated measures ANOVA and Fisher's post hoc test to compare the burn surface area between control, chitosan rats group and chitosan-sulfadiazine rats group at Day 8, 11 and 14. The criterion for significance was $P< 0.05$.

3. Results and Discussion

3.1. Chemistry

In order to obtain functionalized chitosan derivatives, several sulfonamides - sulfamethoxydiazine, sulfadiazine, sulfadimethoxine, sulfamethoxazole, sulfamerazine and sulfisoxazole (**1a-f**) were reacted with chloroacetyl chloride in dry acetone in the presence of anhydrous potassium carbonate to obtain N-chloroacetyl sulfonamides (Fig. 1a).

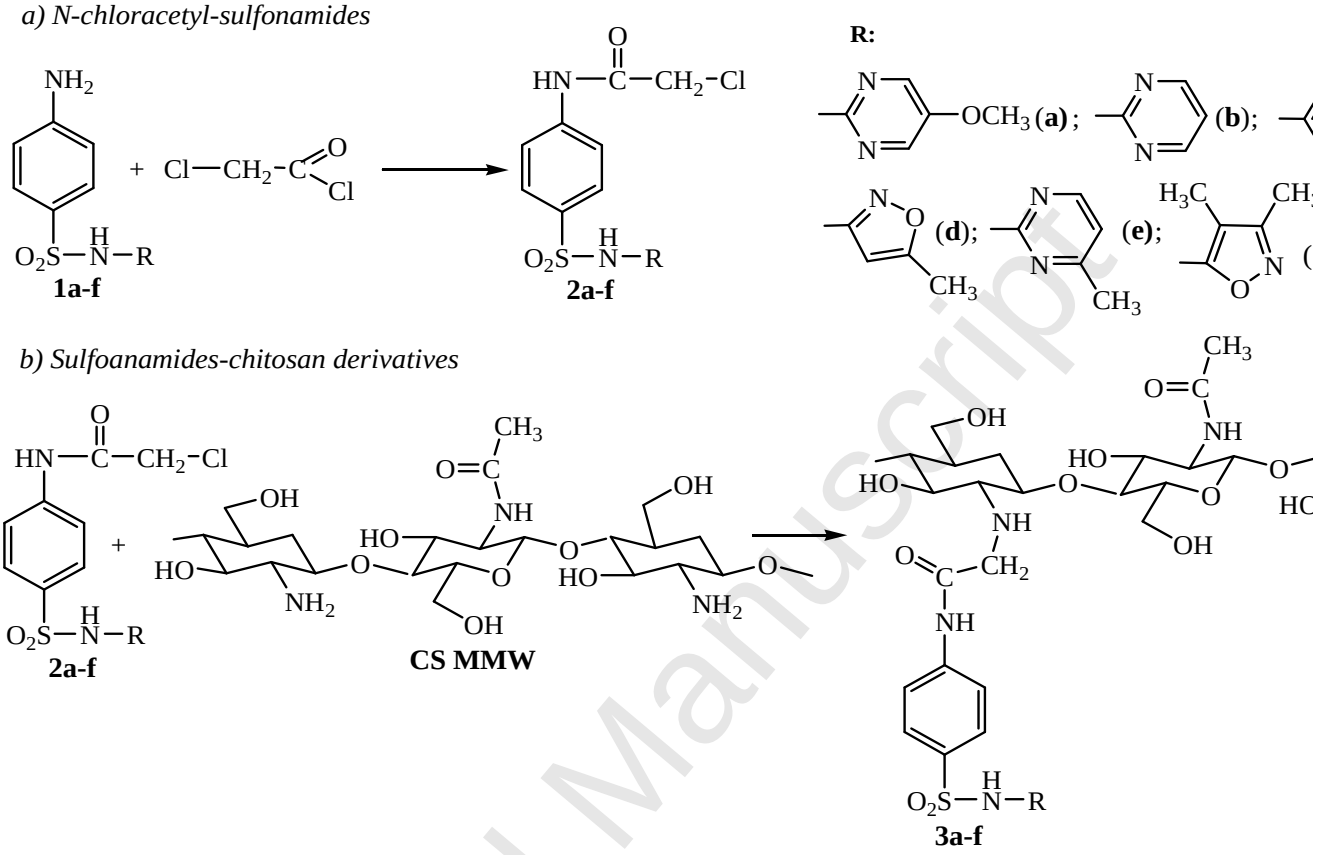


Fig. 1. Two steps synthesis of chitosan derivatives with sulfonamide structures.

In the next step by reaction of chitosan with N-chloroacetyl sulfonamides (**2a-f**) six new chitosan sulfonamide derivatives were obtained denoted with **3a-f** (Fig. 1b).

3.2. Characterization of chitosan derivatives

3.2.1. Spectral data

In the IR spectra of chitosan derivatives (**3a-f**), appear characteristic peaks from chitosan and sulfonamide moieties (Fig 2). New peaks appeared at around 1540 cm^{-1} and 830 cm^{-1} attributed to phenyl group and two other peaks attributed to SO_2-N sulfonamide group appeared at around 1250 cm^{-1} and 1160 cm^{-1}. Increased intensities of the amide I and II bands of the chitosan are evident due to the new amide moieties arising from the amide-linked sulfonamide substituents.

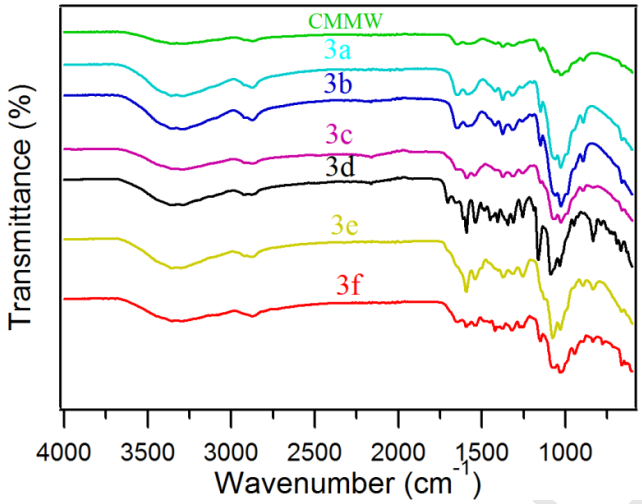


Fig. 2. IR spectra of chitosan (CS MMW) and its sulfonamide derivatives (**3a-f**).

In ^{1}H-NMR spectra of chitosan derivatives (**3a-f**) appeared the characteristic signals for both units: chitosan and sulfonamide (Batista, Pinto, Gomes & Gomes, 2006), that prove the substitution took place. Sulfonamide residue appeared in the range of 6.82-7.72 ppm and 7.65-7.95 ppm (aromatic protons); 7.65-8.43 ppm (pyrimidine - 3a, 3b, 3c, 8e) and 6.03 ppm (isoxazole - 3d).

For physical and spectral characterization of *N*-chloroacetyl-sulfonamides (**2a-f**) see supplementary data (Dragostin et al., 2015).

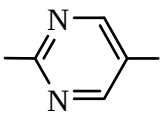
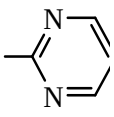
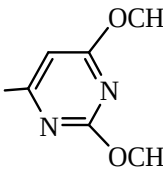
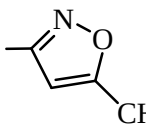
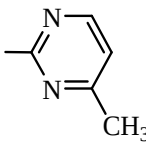
3.2.2. Degree of acetylation (DA) and degree of substitution (DS)

The values of substitution degree of chitosan derivatives (**3a-f)** was found to vary between 9.61 % and 34.24 % - Table 1. That means that chitosan derivatives still have between 67.95% and 43.32 % free amino groups.

Table 1

Degree of substitution (DS%) of chitosan derivatives (**3a-f**).

Compound	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**

R_1						
DS%	15.45	31.64	12.50	9.61	34.24	10.27

3.3. Characterization of Chitosan Derivative Membranes

Chitosan derivative membranes were prepared using sodium tripolyphosphate(TPP) as a crosslinking agent. It is known that non crosslinked chitosan membranes have poor chemical stability and will dissolve in an acid environment and therefore need to be crosslinked. Free amine groups of chitosan derivatives are protonated in acid conditions and consequently are positively charged. In the presence of TPP, a network is produced based on electrostatic interactions between negatively charged crosslinking agent and positively charged glucosamine chains (Giri, Thakur, Ajazuddin, Badwaik & Tripathi, 2012).

3.3.1. Morphology and porosity analysis

The morphology and porosity degree of polymeric membranes as wound dressing materials are important features, because they influence the absorption capacity of exudates, the colonization rate and cellular organization but also the process of angiogenesis (Xing, Lie, Zhengwei & Changyou, 2011). It has been shown that the porosity degree could be influenced by the concentration of polymeric solution (2%, w/v), by freezing temperature (-20°C), but also by the cross-linking method (chemical cross-linking) and cross-linking agent (sodium tripolyphosphate) (Kumirska, Weinhold, Thöming & Stepnowski, 2011; O'Brien, Harley, Yannas & Gibson, 2005). It was observed that in the series of chitosan-sulfonamide derivatives (**3a-f**), the membrane porosity was comparable with the porosity of the chitosan membrane (84.42%). The highest porosity degree was recorded for chitosan-sulfadiazine (**3b**), for which the value of the porosity degree was 94.49% (Fig. 3a). These results are supported also by the SEM images - Fig. 4, for chitosan (A and B) and chitosan-sulfadiazine (3b) (C and

D) membranes. For SEM images of other chitosan-sulfonamide membranes see Fig. 2 (Dragostin et al., 2015).

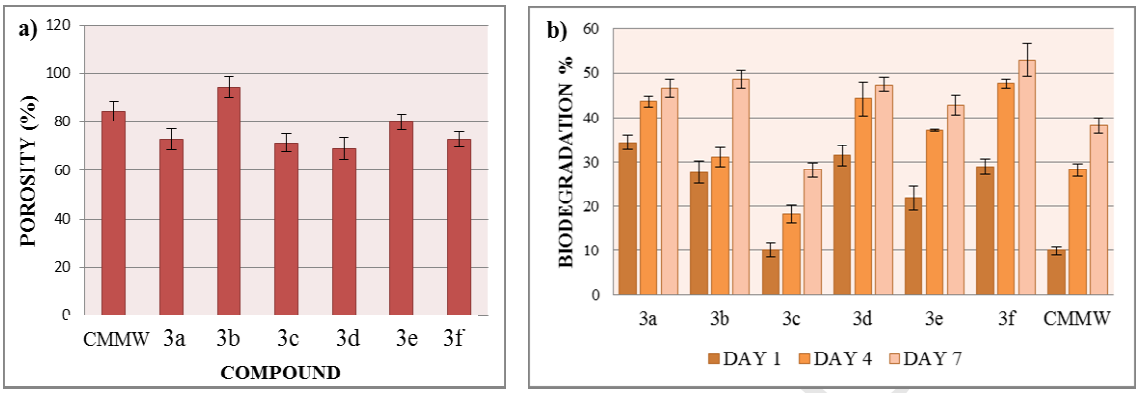


Fig. 3. a): porosity degree of chitosan derivatives (**3a-f**),**b)**: percentage biodegradation of chitosan derivatives membranes (**3a-f**), in respect with that of chitosan.

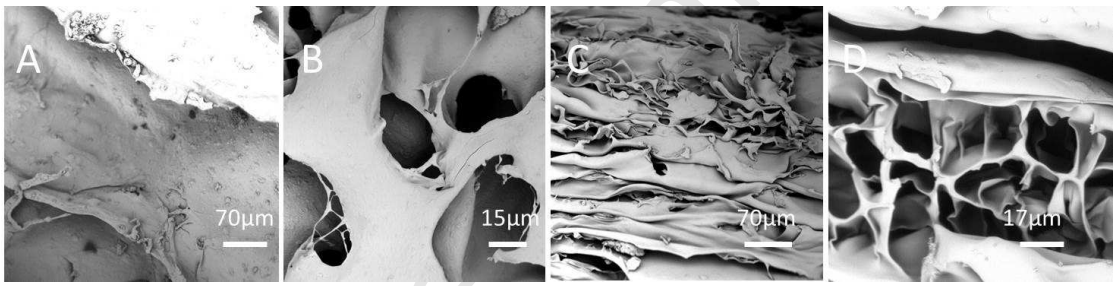


Fig. 4. SEM images of chitosan (**CSMMW**) (A and B) and chitosan-sulfadiazine (**3b**) (C and D) membranes.

3.3.2. In vitro biodegradation

The biodegradation study of chitosan, and the chitosan-sulfonamide derivatives under the action of lysozyme revealed considerable differences between them (Fig. 3b). The percentage biodegradation of the chitosan derivatives was higher than chitosan, excepting chitosan-sulfadimethoxine (**3c**). The highest percentage biodegradation was recorded for chitosan-sulfisoxazole (**3f**), for which, at the end of the experiment (7^{th} day), the biodegradation was 52.89% while the biodegradation of chitosan was 38.19%. According to the literature data the lysozyme biodegradation products of chitosan could be D-glucosamine and glycosaminoglycan which are nontoxic for cells (Baran, Tuzlakoğlu, Mano & Reis, 2012).

3.3.3. Swelling degree

According to Gethin's study, the pH of intact skin is about 5, while in the case of wounds this value increases to 7 or even more, depending on the type of wound (Gethin, 2007). In this study, we have performed a comparison between swelling behavior at physiological environment of intact skin (pH 5) and wounded environment (pH 7), at 37°C (Fig. 5). Although there are no significant differences between the swelling ratio recorded at both pH values, it is to be noted that at pH 5 the swelling degree is a little bit higher than at that of pH 7.

Among the chitosan derivatives the highest swelling ratio was recorded for **3c** (chitosan-sulfadimethoxine) derivative, for which swelling capacity was 2407 % after 60 min, at pH 7 and 2675 % after 30 min, at pH 5. A very good swelling ratio in comparison with chitosan at both pH values was also recorded for **3b** (chitosan-sulfadiazine), **3d** (chitosan-sulfamethoxazole) and **3f** (chitosan-sulfisoxazole) derivatives. For example in case of **3b** the thermodynamic equilibrium was reached after 60 min, with an increased swelling capacity of 2070 % at pH 7 and 2217 % at pH 5 respectively.

In the similar conditions for chitosan (CS MMW) thermodynamic equilibrium was installed after 60 min with an increased uptake capacity of 1823 %, at pH 7 and after 30 min at pH 5 (2107 %).

These results support that, excepting **3a** (chitosan-sulfamethoxidiazine derivative), all other chitosan derivatives showed a higher swelling ratio in respect with to neat chitosan, which means they could have a higher absorption capacity of exudates in the wound healing process.

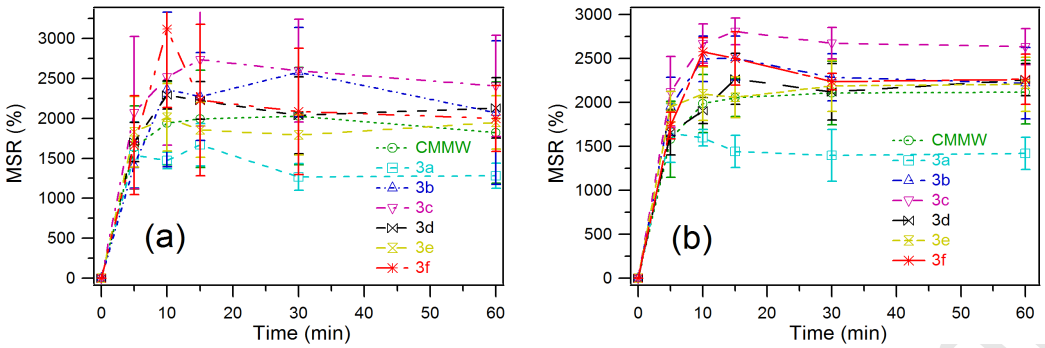


Fig. 5. Swelling degree profiles of chitosan and chitosan derivatives (**3a-f**) membranes at pH 7 (a) and pH 5 (b).

3.3.4. Surface free energy estimation by contact angle measurement

As concerns the biocompatibility several parameters should be considered, such as surface free energy (SFE). The SFE is an important property for cell attachment which determines the quality of the material surface and its possible biomedical applications (Yang, Huang, Shen & Yeh, 2010). It has been demonstrated a strong connection between the total surface energy and the cell attachment: a higher energy surface supports a greater attachment than a lower energy surface (Hallab, Bundy, O'Connor, Moses & Jacobs, 2001). The chemical functionalization of chitosan with different sulfonamides does not negatively impact on total surface free energy -Table 2, which means that the biocompatibility of chitosan derivatives is similar to the chitosan one. For data referring to the values of the surface free energy components see Table 3 (Dragostin et al., 2015).

Table 2

The contact angle and total surface free energy values for chitosan and chitosan-derivatives (**3a-f**) membranes.

Samples	Contact angle (degree)			γ_S^{TOT} (mN/m)
	water	formamide	diiodomethane	
CS MMW	73.28±1.32	69.86±0.72	65.16±1.16	29.09
3a	47.81±0.89	40.55±0.75	38.45±1.45	23.57
3b	58.45±1.07	60.65±1.71	59.80±0.96	26.49
3c	78.96±1.45	73.25±0.80	68.28±0.79	26.76
3d	71.58±1.05	66.47±0.99	60.88±1.12	28.71

3e	71.89±1.62	67.43±1.45	61.05±1.03	32.45
3f	78.11±1.33	73.98±1.22	67.98±0.78	33.13

3.4. Biological evaluation

3.4.1. Antimicrobial assay

The antimicrobial activity results of chitosan derivatives (**3a-f**), expressed as diameters of inhibition area, are presented in Table 3.

Table 3

Diameter of inhibition area (mm) of chitosan derivatives (**3a-f**).

Compound	Diameter of inhibition area (mm)					
	S.a.	***S.l.***	***B.c.***	***B.s.***	***E.c.***	***P.a.***
CS MMW	0	0	0	0	0	0
3a	0	15	0	0	0	0
3b	11	26	15	12	11	0
3c	10	32	18	19	17	0
3d	0	0	0	0	0	0
3e	12	26	12	9	12	0
3f	0	0	0	0	0	0

S.a. - *Staphloccocus auresus* ATCC 25923, S.l. - *Sarcina lutea* ATCC 9341, B.c. - *Bacillus cereus* ATCC 14579, B.s. - *Bacillus subtilis*, E.c. - *Escherichia coli* ATCC 25922, P.a. - *Pseudomonas aeruginosa* CIP 82118.

For all bacterial strains, chitosan (CS MMW) was inactive at a concentration of 2.5 mg/disc. In the same experimental conditions, **3b** (chitosan-sulfadiazine), **3c** (chitosan-sulfadimethoxine) and **3e** (chitosan-sulfamethoxazole) showed a good antimicrobial activity, for tested bacterial strains, excepting *Pseudomonas aeruginosa* CIP 82118, to which the compounds were inactive.

Compared with positive controls (Table 4), the tested compounds showed a higher activity than ciprofloxacin (5 µg/disc) and similar with that of nitrofurantoin (300 µg/disc).

Table 4

Diameter of inhibition area (mm) of Nitrofurantoin and Ciprofloxacin.

Control	Diameter of inhibition area (mm)					
	S.a.	***S.l.***	***B.c.***	***B.s.***	***E.c.***	***P.a.***
N 300µg/disc	19	8	12	20	20	-
C 5 µg/disc	-	-	-	-	-	33

N - nitrofurantoin, C - ciprofloxacin, *S.a. - Staphloccocus auresus* ATCC 25923, *S.l. - Sarcina lutea* ATCC 9341, *B.c. - Bacillus cereus* ATCC 14579, *B.s. - Bacillus subtilis*, *E.c. - Escherichia coli* ATCC 25922, *P.a. - Pseudomonas aeruginosa* CIP 82118, " - " no effect.

Evaluation ofantifungal activity of chitosan derivatives (**3a-f**), showed that all the compounds are inactive at 2.5 mg/disc to *Candida albicans* ATCC 10231, *Candida glabrata* and *Candida sake*.

The values of MICs and MBCs for chitosan-derivatives (**3a-f**) evaluated using the broth micro dilution method are listed in Table 5.

Table 5

The MICs and MBCs values for chitosan and chitosan derivatives.

Chitosan derivatives	*S. aureus* ATCC 25923		*E. coli* ATCC 25922	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
CS-sulfametoxidiazine (**3a)**	5	>5	0.62	1.25
CS-sulfadiazine (**3b)**	1.25	2.5	0.03	0.15
CS-sulfadimetoxine (**3c)**	2.5	5	2.5	5
CS-sulfametoxazol (**3d)**	2.5	5	0.15	0.31
CS-sulfamerazine (**3e)**	5	>5	2.5	5
CS-sulfizoxazol (**3f)**	1.25	2.5	2.5	5
CS MMW	5	>5	2.5	5
Ampicilin(µg/ml)	0.25	0.5	0.5	1

According to the results, all tested compounds are more active than chitosan, but less active than ampicillin used as positive control. It was observed that the activity is closely related to the sulfonamide moiety that substitute the glucosamine unit of chitosan. The most active derivative was chitosan-sulfadiazine (**3b**). It was active on *Staphylococcus aureus* ATCC 25922, with MIC value of 1.25 mg/ml and MBC value of 2.5 mg/ml. More than that this compound was more active on *Escherichia coli*, a Gram-negative bacterial strain, MIC and MBC values being less (0.03 and 0.15 mg/ml respectively) than values recorded for Gram-positive bacterial strain - *Staphylococcus aureus*.

In conclusion, the antimicrobial effects of chitosan derivatives are more intense than chitosan and comparable with other derivatives reported in the literature. For example, the MIC and MBC of diethyl methyl chitosan (DEMC), against *Escherichia coli* was lower than chitosan (Avadi et al, 2004). The antibacterial effects of the acyl thiourea derivatives of chitosan were also much better than chitosan, the value of MIC and MBC against *Escherichia coli* being 15.62 µg/mL and 62.49 µg/mL, respectively (Zhong et al, 2008). In our research, MIC value of pure chitosan against *Escherichia coli* was found to be 2.5 mg/mL, while the most active chitosan derivative of sulfadiazine (**3b**) shows an MIC value of 30 µg/mL.

Considering all obtained results, derivative **3b** was selected for further testing *in vivo* wound healing test.

3.4.2. MTT cell proliferation assay

It was observed that the cell viability (%) evaluated using MTT assay decreases in presence of chitosan derivatives in comparasion with chitosan - Table 6. Even if the cell viability for these derivatives is less than chitosan, some of these compounds are considered nontoxic because the viability is higher than 70%, according to the literatute data (Lönnroth, 2005).

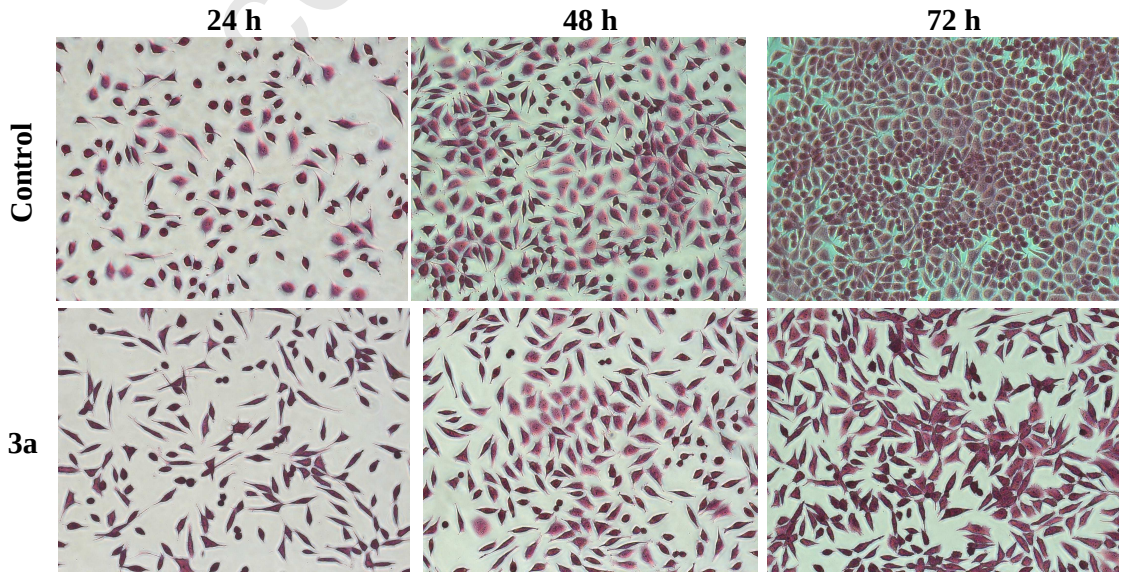
Table 6

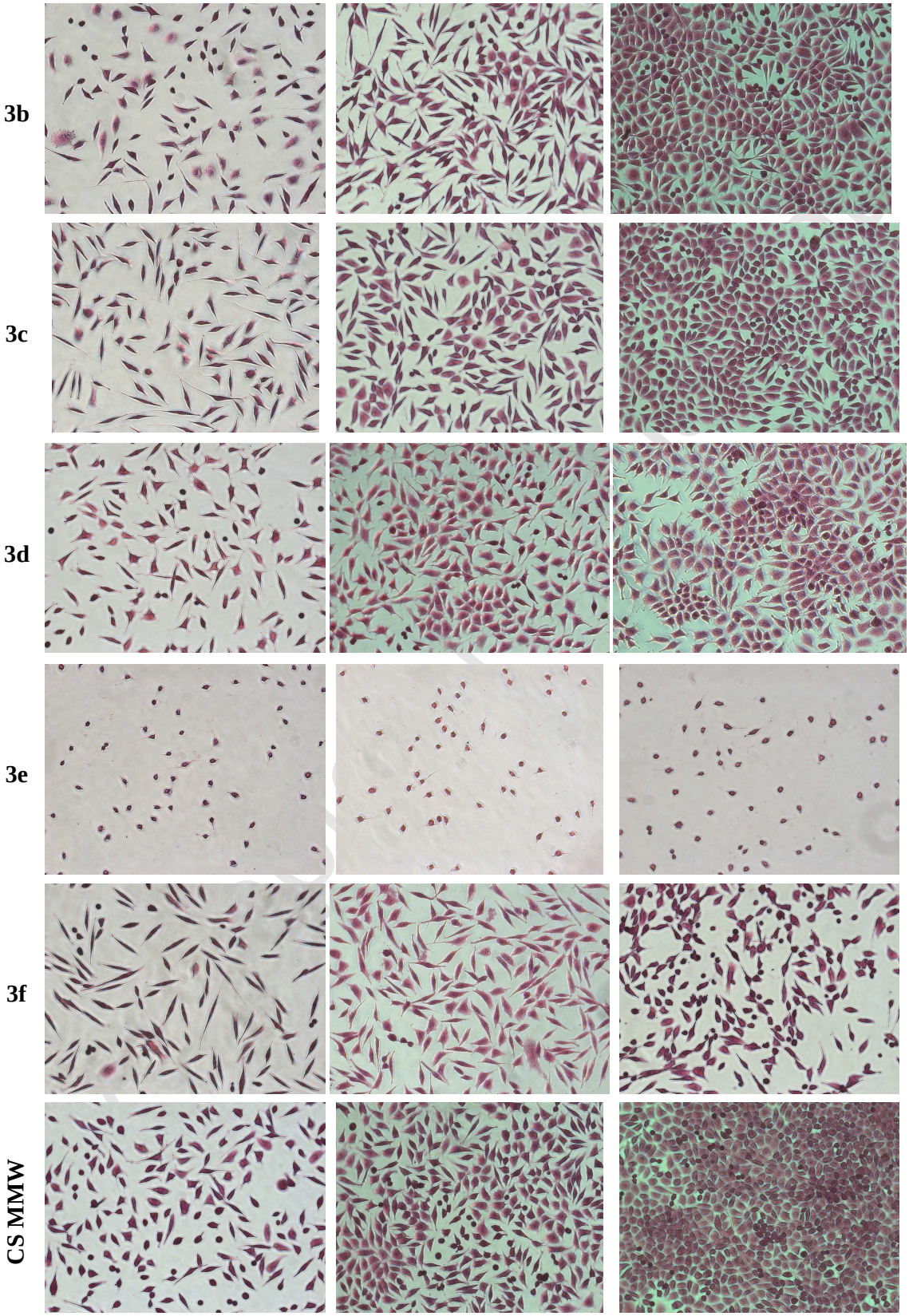
The cell viability value (%) for chitosan derivatives at 24 h, 48 h and 72 h.

Samples	Cell viability (%)		
	24 h	48 h	72 h
CS-sulfametoxidiazine (**3a)**	89.58	69.25	62.00
CS-sulfadiazine (**3b)**	80.08	77.65	96.18
CS-sulfadimetoxine (**3c)**	87.33	74.34	98.73
CS-sulfametoxazol (**3d)**	82.67	74.34	62.79
CS-sulfamerazine (**3e**)	3.45	2.65	0.64
CS-sulfizoxazol (**3f)**	80.62	56.86	52.78
CS MMW (negative control)	124.60	113.94	114.94
H_2O_2 0.03% (positive control)	1.49	0.66	0.00
Control	100.00	100.00	100.00

The least toxic are CS-sulfadiazine (**3b**) and CS-sulfadimetoxine (**3c**), for which the cell viability was higher than 70% at all exposed periods (24 h, 48 h and 72 h). After 72 h of incubation the viability of these derivatives was 96.18% (**3b**) and 98.73% (**3c**), the values being comparable with those of chitosan (CS MMW) (114.94%).

Microscopic images of the cell culture incubated in the presence of chitosan derivatives are presented in Fig. 6. In comparison with the control, in which the cells have round and polygonal shape, in the cell culture incubated in the presence of chitosan derivative **3c** and **3b,** the cells are elongated, some of them becoming spindly, their shape being similar to the cells incubated in presence of chitosan (CS MMW).





538 **Fig. 6.** Cell cultures incubated in the presence of chitosan derivatives (**3a-f**) in reference with

539 chitosan (CS MMW) and control.

3.4.3. Wound healing assay

Burn degree was evaluated by skin wounds punch-biopsy and Hematoxylin and Eosin staining protocol of the samples harvested at 48 hours post-infliction. Intermediate burn wounds resulted at 2 seconds exposure to high-pressure hot steam.

The chitosan-sulfadiazine derivative (**3b)** was evaluated for its wound healing properties vs. chitosan (CS) as a reference. The polymeric membranes (CS, 3b) were stapled to gauze and fixed on top of the burn wound with tie-over suture.

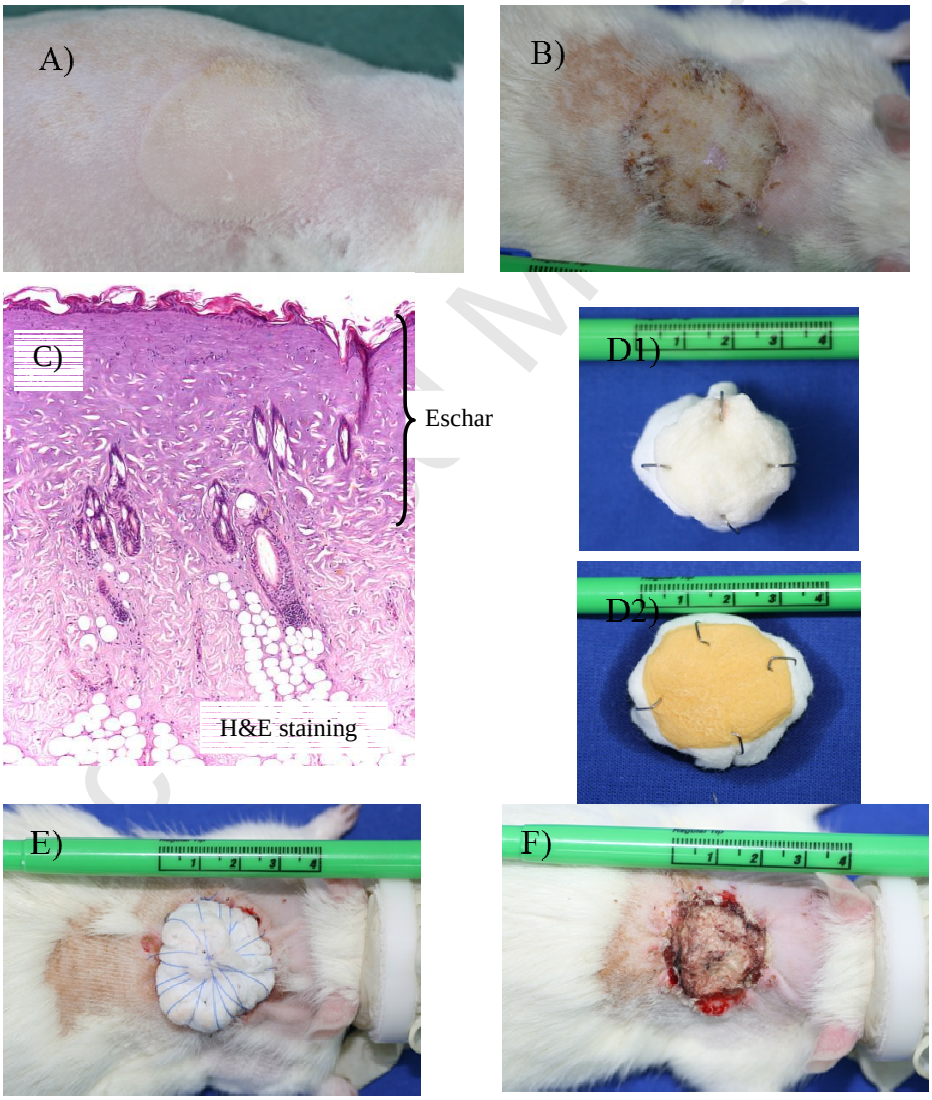


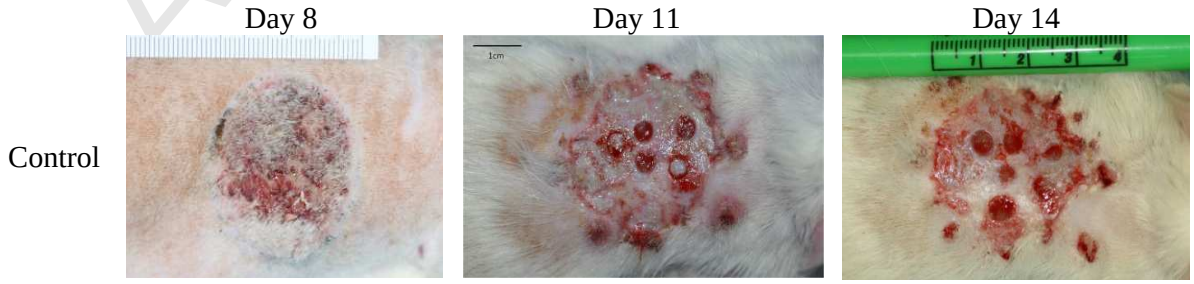
Fig. 7. Burn wounds, histological exam and topical treatment. A: acute burn injury inflicted by high-pressure steam at 2 seconds exposure (day 0), B: intense edema of the burn wound

549 will subside and the eschar will form, C: at 48 hours the histology shows necrosis and
550 detachment of the epidermis and the superior half of the dermis together with skin appendages
551 are necrosed, D: shows CS membrane (D1) and 3b membrane (D2) underneath (of equal
552 sizes), stapled on gauze, E: tie-over suture fixed the membrane on the burn wound, F: shows
553 dehydrated, contracted membrane after 4 days, before changing to the new one.
554
555 The burn wound healing was evaluated at 8, 11 and 14 days after the start of the
556 experiment. The average value and standard deviation of burn surface area in each group at
557 different days during recovery phase are shown in Table 7.
558 The burning surface in all groups at day 0 is not different, showing the burn infliction
559 methods consistency. The burning surface in rats group treated with chitosan-sulfadiazine
560 (3b) was significantly smaller than rats group treated with chitosan (CS) ($p<0.01$) and control
561 group ($p<0.01$) at day 8, 11 and 14, and the burn surface in rats group treated with chitosan
562 was also significantly smaller than control group ($p=0.03$) at day 8, 11 and 14. Both CS and
563 3b dressing materials were more effective for wound healing than the control group (with
564 standard gauze dressing), and 3b dressing in particular, had the best healing effect among all
565 the groups. In addition, wound healing progressed with time of all three groups, i.e. the longer
566 recovery duration, smaller the burn wound area ($p<0.01$).
567
568 **Table 7**
569 Burn surface area and standard deviation (mm^2) for control, chitosan (CS) and chitosan-
570 sulfadiazine (3b) rats groups at day 0 (burn infliction) and at 8, 11 and 14 day.

Group	Day	Mean ± SD (mm^2)
Control	Day 0	955±16
	Day 8	858±10
	Day 11	758±19
	Day 14	742±26

CS rats group	Day 0	945±11
	Day 8	742±27
	Day 11	699±20
	Day 14	556±51
3b rats group	Day 0	951±13
	Day 8	589±52
	Day 11	474±25
	Day 14	374±35
P value	Group	*<0.01*
	Day	*<0.01*
	Interaction	*0.2*

Macroscopic aspect of the burn wounds was documented by standard photographs shown in Fig. 8. Macroscopic evaluation of control group shows subsequent biopsies and the surface of the burn wound after eschar detachment is whitish, pearly appearance with weak signs of healing even at day 14. CS rats group shows color turning over slightly pink appearance as a sign of improved healing and better local vascularization, yet it retains raw non-epithelialized dermis in the upper quadrants of the wound (approx. 40% of the wound surface). Chitosan-sulfadiazine (3b) rats group shows no more raw appearance, the shiny areas are the new epithelial cell layers formed due to better wound healing, and fully cover the previous wounded area at day 14, compared to control and CS rats group, respectively. All burn wound areas significantly decrease over time.



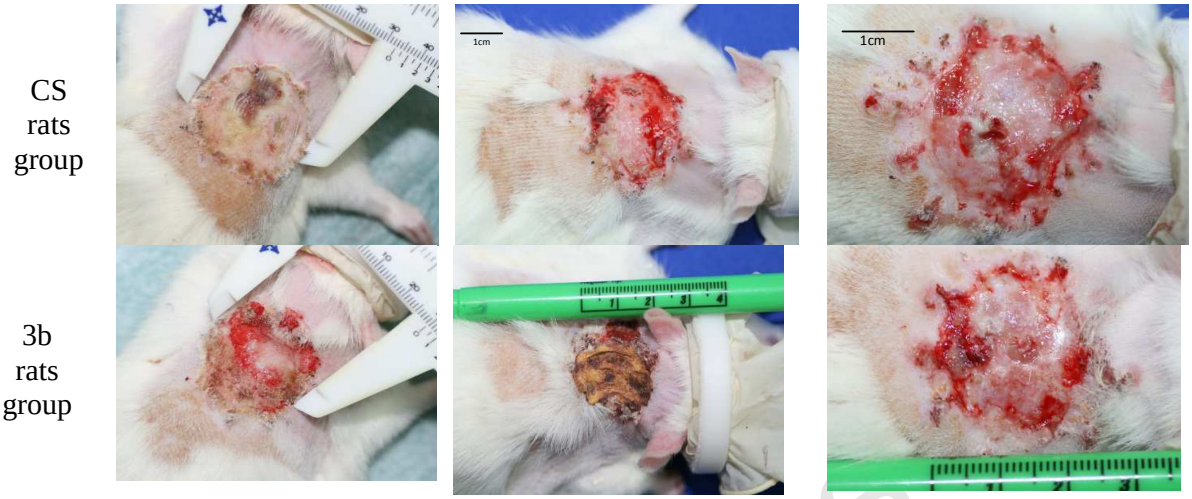


Fig. 8. Macroscopic evaluation of burn wound area of the control and samples (CS rats group, 3b rats group) at various timelines: 8 days, 11 days and 14 days.

Microscopic evaluation (see Fig. 5, Dragostin et al, 2015) of the control group, in the 8th and 11th day of evolution showed that the wounded area was completely ulcerated with fibro-vascular tissue in the upper half of the reticular dermis, abundant polymorphous inflammatory infiltrates with no signs of re-epithelization. In the 14th day, beneath the necrotic debris (eschar), small, isolated nests of immature squamous epithelium were observed, while the upper dermis was replaced by fibro-vascular tissue with mild chronic inflammatory infiltrate and congestion. Microscopic evaluation of the histological samples showed intermediate burn at day 8 with immature but continuous epidermal layer for 3b rats group compared to initial sample, discontinuous re-epithelialization by an overgrowth of the epithelial buds from the residual viable pilosebaceous units for CS rats group. For CS rats group the re-epithelialization resulted in a thin, immature epidermal layer and underneath repairing of the dermis by fibro-vascular tissue showed moderate inflammatory infiltrate, with mosaic of non-healed and healed areas. At day 11 and 14 group treated with 3b exhibited complete re-epithelialization resulting into the mature epidermal layer and underneath repairing of the dermis by fibro-vascular tissue with reduced chronic inflammatory infiltrate and reduced congestion.

4. Conclusions

New chitosan-sulfonamide derivatives were synthesized and characterized with regard to structural, physico-chemical properties, swelling capacity, biodegradability, biocompatibility and tested in respect with antimicrobial and antifungal activities. It has been found that all six chitosan-sulfonamide derivatives exhibited better antimicrobial activity than the pristine chitosan, which indicated that the antimicrobial ability of chitosan was strengthened by the introduction of sulfonamide part to chitosan. The chitosan derivatives showed improved swelling and biodegradation rate and are biocompatible and most of them are not cytotoxic. *In vivo* test proved that among the chitosan-sulfonamide derivatives, the chitosan-sulfadiazine showed also improved healing effects. It can conclude that these new chitosan derivatives could be useful in application as potential new dressing materials for wound, especially for burn wounds.

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References

Aider, M. (2010). Chitosan application for active bio-based films production and potential in the food industry: Review. *LWT- Food Science and Technology, 43,* 837-842.

628 Anisha, B. S., Sankar, D., Mohandas, A., Chennazhi, K. P., Nair, S. V., & Jayakumar, R.
629 (2013). Chitosan–hyaluronan/nano chondroitin sulfate ternary composite sponges for
630 medical use. *Carbohydrate Polymers*, *92*, 1470-1476.
631 Arpornmaeklong, P., Pripatnanont, P., & Suwatwirote, N. (2008). Properties of chitosan–
632 collagen sponges and osteogenic differentiation of rat-bonemarrow stromal cells.
633 *International Journal of Oral and Maxillofacial Surgery*, *37*, 357-366.
634 Avadi, M. R., Sadeghi, A. M. M., Tahzibi. A., Bayati. Kh., Pouladzadeh. M., Zohuriaan-
635 Mehr, M. J., Rafiee-Tehrani. M. (2004). Diethylmethyl chitosan as an antimicrobial
636 agent: Synthesis, characterization and antibacterial effects. *European Polymer Journal*,
637 *40(7)*, 1355-1361.
638 Baran, E. T., Tuzlakoğlu, K., Mano, J. F., & Reis, R. L. (2012). Enzymatic degradation
639 behavior and cytocompatibility of silk fibroin–starch–chitosan conjugate membranes.
640 *Materials Science and Engineering*, *32*, 1314-1322.
641 Batista, M. K. S., Pinto, L. F., Gomes, C. A. R., & Gomes, P. (2006). Novel highly soluble
642 peptide chitosan polymers: Synthesis and spectral characterization. *Carbohydrate*
643 *Polym*ers, *64*, 299–305.
644 Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards.
645 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-second
646 Informational Supplement. (2012). *32*(3).
647 Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan - A versatile semi-
648 synthetic polymer in biomedical applications. *Progress in Polymer Science*, *36*, 981-
649 1014.
650 Dias, A. M. A., Rey-Rico, A., Oliveira, R. A., Marceneiro, S., Alvarez-Lorenzo, C.,
651 Concheiro, A., Júnior, R. N. C., Braga, M. E. M., & De Sousa, H. C. (2013). Wound
652 dressings loaded with an anti-inflammatory jucá (Libidibiaferrea) extract using
653 supercritical carbon dioxide technology. *Journal of Supercritical Fluids*, *74*, 34-45.
654 Dragostin, O. M., Samal, S. K., Dash, M., Lupascu, F., Pânzariu, A., Tuchilus, C., Ghetu, N.,
655 Danciu, M., Dubruel, P., Pieptu, D., Vasile, C., Tatia, R. & Profire, L (2015). Data on
656 synthesis and characterization of new chitosan derivatives. *Carbohydrate Polymers*,
657 *Data in Brief* "*submitted"*)
658 Dutta, P. K., Ravikumar, M. N. V., & Dutta, J. (2002). Chitin and chitosan for versatile
659 applications. *Journal of Macromolecular Science-polymer Reviews*, *C42*, 307-354.
660 Feng, Y., & Xia, W. (2011). Preparation, characterization and antibacterial activity of water-
661 soluble O-fumaryl-chitosan. *Carbohydrate Polymers*, *83*, 1169-1173.

662 Gallaher, C. M., Munion, J., Hesslink, R. Jr., Wise, J., & Gallaher, D. D. (2000). Cholesterol
663 reduction by glucomannan and chitosan is mediated by changes in cholesterol
664 absorption and bile acid and fat excretion in rats. *Journal of Nutrition, 130,* 2753-2759.
665 Gethin, G. (2007). The significance of surface pH in chronic wounds. *Wounds* UK, *3(3),* 52-
666 57.
667 Giri, T. K., Thakur, A., Ajazuddin, A. A., Badwaik, H., & Tripathi, D. K. (2012). Modified
668 chitosan hydrogels as drug delivery and tissue engineering systems: present status and
669 applications. *Acta Pharmaceutica Sinica* B, *2(5):* 439-449.
670 Gomes, J. R. B., & Gomes, P. (2005). Gas-phase acidity of sulfonamides: implications for
671 reactivity and prodrug design. *Tetrahedron*, *61*, 2705-2712.
672 Gupta, A., Chauhan, V. S., & Sankararamakrishnan, N. (2009). Preparation and evaluation of
673 iron-chitosan composites for removal of As(III) and As(V) from arsenic contaminated
674 real life groundwater. *Water Research, 43,* 3862-3870.
675 Hallab, N., Bundy, K., O'Connor, K., Moses, R. L., & Jacobs, J. J. (2001). Evaluation of
676 metallic and polymeric biomaterial surface energy and surface roughness characteristics
677 for directed cell adhesion. *Tissue Engineering, 7,* 55-7.1
678 Ignatova, M., Manolova, N., & Rashkov, I. (2013). Electrospun antibacterial chitosan-based
679 fibers. *Macromolecular Bioscience,13,* 860-872.
680 Jones, V., Grey, J. E., & Harding, K. G. (2006). Wound dressings. *The British Medical*
681 *Journal*, *332*, 777-780.
682 Kołodyńska, D. (2012). Adsorption characteristics of chitosan modified by chelating agents of
683 a new generation. *Chemical Engineering Journal*, *179*, 33-43.
684 Kremer, E., Facchin, G., Estévez, E., Alborés, P., Baran, E. J., Ellena, J., & Torre, M. H.
685 (2006). Copper complexes with heterocyclic sulfonamides: synthesis, spectroscopic
686 characterization, microbiological and SOD-like activities: crystal structure of [Cu
687 (sulfisoxazole) 2 (H_2O) 4]· $2H_2O$.*Journal of Inorganic Biochemistry*, *100*, 1167-1175.
688 Kumirska, J., Weinhold, M. X., Thöming, J., Stepnowski, P. (2011). Biomedical Activity of
689 Chitin/Chitosan Based Materials - Influence of Physicochemical Properties Apart from
690 Molecular Weight and Degree of *N*-Acetylation. *Polymers*, *3*, 1875-1901.
691 Lin, Y. C., Tan, F., Marra, K. G., Jan, S. S., & Liu, D. C. (2009). Synthesis and
692 characterization of collagen/hyaluronan/chitosan composite sponges for potential
693 biomedical applications. *Acta Biomaterialia*, *5*, 2591-2600.
694 Lönnroth, E. C. (2005). Toxicity of Medical Glove Materials: A Pilot Study. *International*
695 *Journal of Occupational Safety and Ergonomics* (*JOSE*), *11(2),* 131–139.

Ma, G. P., Yang, D. Z., Zhou, Y. S., Xiao, M., Kennedy, J. F., & Nie, J. (2008). Preparation and characterization of water-soluble N-alkylated chitosan. *Carbohydrate Polymers, 74*, 121-126.

Mallick, S., Sharma, S., Banerjee, M., Sankar Ghosh, S., Chattopadhyay, A., & Paul, A. (2012). Iodine-stabilized Cu nanoparticle chitosan composite for antibacterial applications. *ACS Applied Materials & Interfaces, 4*, 1313-1323.

Martins, A. F., Facchi, S. P., Follmann, H. D. M., Pereira, A. G. B., Rubira, A. F., Muniz, E. C. (2014). Antimicrobial Activity of Chitosan Derivatives Containing N-Quaternized Moieties in its Backbone: A Review. *International Journal of Molecular Sciences,15*, 20800-20832.

Mazyala, E. J. (2008). Dermal Fibroblast: A Histological and Tissue Culture Study, MSc thesis, Faculty of Health Sciences, University of Stellenbosch, South Africa.

O 'Brien, F. J., Harley, B. A., Yannas, I. V., Gibson, L. J. (2005). The Effect of Pore Size on Cell Adhesion in Collagen-GAG Scaffolds. http://epubs.rcsi.ie/.

Okamoto, Y., Yano, R., Miyatake, K., Tomohiro, I., Shigemasa, Y., & Minami, S. (2003). Effects of chitin and chitosan on blood coagulation. *Carbohydrate Polymers, 53*, 337-342.

Öztürk, E., Agalar, C., Keçeci, K., & Denkbas, E. B. (2006). Preparation ad characterization of ciprofloxacin loaded alginate/chitosan sponge as a wound dressing material. *Journal of Applied Polymer Science, 101*, 1602-1609.

Pillai, C. K. S., Willi, P., & Chandra, P. S. (2009). Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Progress in Polymer Science, 34*, 641-678.

Rotta, J., Ozório, R. A., Kehrwald, A. M., Mariz de Oliveira Barra, G., Dias de Melo Castanho Amboni, R., & Barreto, P. L. M. (2009). Parameters of color, transparency, water solubility, wettability and surface free energy of chitosan/ hydroxypropyl-methylcellulose (HPMC) films plasticized with sorbitol. *Materials Science and Engineering C, 29*, 619–623.

Samal, S. K., Dash, M., Chiellini, F., Wang, X., Chiellini, E., Declercqe, H. A., & Kaplan, D. L. (2014). Silk/chitosan biohybrid hydrogels and scaffolds via green technology. *Royal Society of Chemistry Advances, 4*, 53547-53556.

Samal, S. K., Dash, M., Van Vlierberghe, S. Kaplan, D. L., Chiellini, E., Van Blitterswijk, C., Moroni, L., & Dubruel, P. (2012). Cationic polymers and their therapeutic potential. *Chemical Society Reviews, 41*, 7147–7194.

729 Samal, S. K., Dash, M., Declercq, H. A., Gheysens, T., Dendooven, J., Voort, P. V. D. (2014).
730 Enzymatic Mineralization of Silk Scaffolds. *Macromolecular bioscience, 14* (7), 991-
731 1003.
732 Sashiwa, H., Yamamori, N., Ichinose, Y., Sunamoto, J., & Aiba, S. (2003). Chemical
733 Modification of Chitosan, 17[a] Michael Reaction of Chitosan with Acrylic Acid in
734 Water. *Macromolecular Bioscience,3,* 231-233.
735 Shabalovskaya, S. A., Siegismund, D., Heurich, E., & Rettenmayr, M. (2013). Evaluation of
736 wettability and surface energy of native Nitinol surfaces in relation to
737 hemocompatibility. *Materials Science and Engineering* C, *33*, 127–132.
738 Sionkowska, A., & Płanecka, A. (2013). Preparation and characterization of silk
739 fibroin/chitosan composite sponges for tissue engineering. *Journal of Molecular*
740 *Liquids*, *178*, 5-14.
741 Sudheesh Kumar, P. T., Lakshmanan, V. K., Anilkumar, T. V., Ramya, C., Reshmi, P.,
742 Unnikrishnan, A. G., Nair, S. V., & Jayakumar, R. (2012). Flexible and microporous
743 chitosan hydrogel/Nano ZnO composite bandages for wound dressing: in vitro and in
744 vivo evaluation. *ACS Applied Materials & Interfaces,4*, 2618-2629.
745 Wang, J. P., Chen, Y. Z., Yuan, S-J., Sheng, G. P., & Yu, H. Q. (2009). Synthesis and
746 characterization of a novel cationic chitosan-based flocculant with a high water-
747 solubility for pulp mill wastewater treatment. *Water Research*, *43*, 5267-5275.
748 Willi, P., & Chandra, P.S. (2004).Chitosan and alginate wound Dressings: A short Review.
749 *Trends in Biomaterials & Artificial Organs*, *18*(1), 18-23.
750 Xie, Y., Liu, X., & Chen, Q. (2007). Synthesis and characterization of water-soluble chitosan
751 derivate and its antibacterial activity. *Carbohydrate Polymers*, *69*, 142-147.
752 Xing, L., Lie, M., Zhengwei, M., & Changyou, G. (2011). Chitosan-based biomaterials for
753 tissue repair and regeneration. *Advances in Polymer Science*, *244*, 81–128.
754 Yang, J. M., Yang, S. J., Lin, H. T., Wu, T.-H., & Chen, H.-J. (2008). Chitosan containing
755 PU/Poly (NIPAAm) thermosensitive membrane for wound dressing. *Materials Science*
756 *and Engineering: C*, *28*,150–156.
757 Yang, T. C., Chou, C. C., & Li, C. F. (2005). Antibacterial activity of N-alkylated
758 disaccharide chitosan derivatives. *International Journal of Food Microbiology*, *97*, 237-
759 245.
760 Yang, C. Y., Huang, L. Y., Shen, T. L., &Yeh, J. A. (2010). Cell adhesion, morphology and
761 biochemistry on nanotopographic oxidized silicon surfaces. *European Cells and*
762 *Materials*, *20*, 415-430.

763 Zahid, H. C. (2009). Metal-based antibacterial and antifungal sulphonamides: synthesis,
764 characterization, and biologica properties. *Transition Metal Chemistry, 34*, 153-161.
765 Zhong, Z., Xing, R., Liu, S., Wang, L., Cai, S., Li, P. (2007). Synthesis of acyl thiourea
766 derivatives of chitosan and their antimicrobial activities *in vitro*. Carbohydrate
767 Research, 343(3), 566-70
768 Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards.
769 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-second
770 Informational Supplement. (2012). *32*(3).
771 European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European
772 Society for Clinical Microbiology and Infectious Diseases (ESCMID). EUCAST
773 Definitive Document E. Def 3.1. (2000). Determination of minimum inhibitory
774 concentrations (MICs) of antibacterial agents by agar dilution. *Clinical Microbiology*
775 *Infectious,6,* 509–515.
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778 **HIGHLIGHTS**
779 • New sulfoanamide-chitosan derivatives have been synthesized and characterized
780 • Sulfoanamide-chitosan derivatives membranes have been prepared and characterized
781 • Chitosan derivatives membranes have improved swelling and biodegradation rate
782 • Chitosan-sulfadiazine membrane has good antimicrobial effect and healing properties
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