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Investigation of an elutable N-propylphosphonic acid chitosan derivative composition with a chitosan matrix prepared from carbonic acid solution

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A B S T R A C T

Porous chitosan composites using CO_2 dissolution procedure and including water soluble N-propylphosphonic chitosan derivative (p-CHI) were obtained and characterized. In contrast to the control material, composites containing modified chitosan distinguished by a rapid moisture absorption and good adhesion to the skin. The FTIR analysis confirmed the presence of propylphosphonic group in the structure of the polymer. The porosity of the materials was in the range 55–77% and decreased with increasong amount of modified chitosan in materials. Solubility of composites was dependent on the content of p-CHI in scaffolds (40%, 25% and 15%) and reached values 11%, 9% and 6,5%, respectively. The values of other parameters like swelling degree (30 g/g) good antioxidant and antimicrobial properties (almost 100% reduction of S.aureus, E.coli and C. albicans growth) and low in vitro cytotoxicity against fibroblasts were highly advantageous for possible biomedical applications of the composites.

Keywords: Chitosan, Propylphosphonic acid adhydride Physico-chemical properties Biocompatibility, Antioxidant properties

1. Introduction

Chitosan is a polysaccharide composed of randomly distributed β(1 → 4)-linked D-glucosamine and N-acetyl-D-glucosamine units in a linear polymer structure. It is produced by deacetylation process of chitin occuring in the exoskeleton of shellfish and insects, as well as in the cell walls of fungi (Roberts, 1992; Muzzarelli, 2009). Its biocompatibility, biodegradability, antimicrobial properties and non-toxicity offer potential in a range of applications in the biomedical, cosmetic, drug, environmental and food industries (Dutta, Dutta, & Tripathi, 2004; Kumar, 2000; No, Meyers, Prinyawiwatkul, & Xu, 2007; Pokhrel, Yadav, & Adhikari, 2015). Chitosan has been used in many applications, such as targeted cancer therapies (El-Sayed et al., 2016), drug delivery systems (Valente, Gaspar, Antunes, Countinho, & Correia, 2013), as a component of packaging materials (Davidivich-Pinhas, Danin-Poleg, Kashi, & Bianco-Peled, 2014) and dietary supplements component (Haider, Majeed, Williams, Safdar, & Zhong, 2017; Tylingo, Mania, & Szwacki, 2016). A modified chitosan hydrogels can also be used as adsorbents in wastewater treatment due to their great metal ion sorption and flocculation capabilities (Piątkowski, Bogdał, Radomski, & Jarosiński, 2010). Chitosan has been used in cosmetic and personal care industries as a moisturizer, acne treatment, toothpaste and chewing gum component, and many others (Thanou & Junginger, 2005). Recent advances have also shown that a highly degradable, complex, large 3D objects can be fabricated using chitin-based materials (Fernandez & Ingber, 2014).

Chitosan and its oligomers are also beneficial in tissue regeneration. Chitosan activates some elements of the immune system and accelerates blood coagulation. Oligosaccharides, formed from chitosan in the wound due to the activity of lysozyme, strongly attract granulocytes and macrophages (Uragami and Tokura, 2010; Gorczyca, 2015). These and many other advantages of chitosan are the reason for using this biopolymer in obtaining the wound dressings. For example, chitosan hydrogel coated grafts, crosslinked upon ultraviolet light irradiation, exhibited a resistance against *E. coli in vitro* and *in vivo*. The chitosan hydrogel directly acted as antibacterial biomaterial on a Dacron graft, and was effective to inhibit the local infection (Fujita et al., 2004).

The biocompatible chitosan/polyethylene glycol diacrylate blend films were successfully prepared by Michael addition reaction with different weight ratios as wound dressing. Indirect cytotoxicity assessment of films with mouse fibroblasts (L929) indicated that the material showed nocytotoxicity toward growth of L929 cell and had good *in vitro*

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biocompatibility having the potential to be used as wound dressing material (Zhang et al., 2008).

The sponge-like dressings based on chitosan glutamate (high molecular weight) and sericin was developed for the treatment of chronic skin ulcers. The amount of sericin in the optimized dressing is suitable to exert a protective effect on human fibroblasts against oxidative damage. Moreover, the optimized dressing is able to improve fibroblast proliferation, that is, to promote wound healing (Michela et al., 2016).

The availability of amino and hydroxyl groups in chitosan makes the biopolymer susceptible to chemical modifications (Mourya & Inamdar, 2008). Their main purpose is possibility of controllably altering the properties of the polymer for a particular application, so-called material functionalization. Although chitosan is insoluble in organic solvents, neutral and alkaline solutions, it is readily soluble in dilute aqueous solutions of organic acids and some mineral acids such as hydrochloric acid below pH 6.5 (Roberts, 1992). Solubility of chitosan at neutral pH can be achieved by introduction by derivatization of appropriate hydrophilic functionality into the chitosan structure (Roberts, 1992; Ahmed & Ikram, 2015). One of the examples of such modification is introduction of phosponate groups into chitosan. First technique of obtaining phosphate derivatives of chitosan were based on heating chitosan with orthophosphoric acid and urea in dimethylformamide (DMF) or by the reaction of chitosan with phosphorous pentoxide in methanesulfonic acid (MSA). It was found that only the modified chitosan with low substitution degree was water soluble (Alves & Mano, 2008). The above-described methods allow to attach only one phosponate group to the free amino group of chitosan. Kumar et al. (Kumar, Isloor, Ismail, & Matsuura, 2013) proposed a new method of synthesis of water soluble chitosan derivative using N-propylphosphonic anhydride (T3P) which allowed solubility in water under neutral pH. T3P is a mild and efficient water removal agent with low toxicity and low allergenic potential. It is easy to work with and offers a broad funcional-group tolerance, high efficiency and yield and low epimerization, as well as a high purity of the end product (Garcia, 2007). T3P has been used as a peptide coupling reagent (Augustine, Atta, Ramappa, & Boodappa, 2009) and the synthesis of 4-thiazolidinones from alcohols. Kumar et al. (2013) presented an alternative method for the synthesis of those heterocyclic compounds with T3P, dimethylsulfoxide (DMSO) and various alcohols in a one-pot three-component synthesis. It was found this method offered mild reaction conditions and excellent yields of the synthesized 4-thiazolidinones.The other advantages of this method were: short reaction time, easy isolation of products, broad functional group tolerance and ease of product purification (Kumar et al., 2013).

In this work, a new method of preparation of chitosan composite films with T3P derivative was presented. Composites were formed from two components: unmodified chitosan dissolved by CO_2 saturation method described by Gorczyca and co-authors (Gorczyca, Tylingo, Szweda, Sadowska, & Milewski, 2014), and chitosan modified by T3P reagent according to method proposed by Kumar and co-authors (Kumar et al., 2013).

The use of CO_2 in chitosan processing has been already shown by the Japanese research group in 2001 (Sakai, Hayano, Yoshioka, & Yoshioka, 2001) but to date it was only applied for coating of cellulose-derived materials (Sakai et al., 2002). The use of the CO_2 saturation method eliminates the possibility of presence the residual acids in prepared chitosan materials that could react with the phosphorus groups of the new polymer derivative and affect the properties of the formed composites. The another reason of application this method in our work is obtaining chitosan dressings characterized by greater biocompatibility (Gorczyca, Tylingo, Szweda, Sadowska, & Milewski, 2014).

According to the research hypothesis, the increase in the proportion of modified derivative in chitosan composites will result in increase their solubility. In the future, it allows to easily program the solubility of such materials. The work also aims to check if the incorporation of a propylphosphonic domain in chitosan molecule will influence the basic parameters of composites such as: welling ability, solubility, flexibility, hardness or antimicrobial, antioxidant and cytotoxic properties.

So far, the possibility of modifications has been determined, and there is no data related to the physico-chemical characteristics of the composites, and the opportunities afforded by the modified chitosan soluble in solvents at neutral pH. There was noticeable effect of gaining composites having antioxidant and antimicrobial properties, which has high application potential. The another advantage of this approach is the possibility of making homopolymer composites with desirable properties

2. Experimental

2.1. Materials

Chitosan (Medium Molecular Weight, Deacetylation Degree: 75–85%, 200–800 cP, 1 wt.% in 1% acetic acid, 25 °C, Brookfield), Phosphate Buffer Saline (PBS), 1-Hydroxybenzotriazole, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt [ABTS (NH4)2], iron(II) chloride and ferrozine were purchased from “Sigma–Aldrich (St. Louis, MO, USA)”. Propylphosphonic anhydride (T3P, 50% solution in DMFA) was purchased from “Merck KGaA” (Darmstadt, Germany). Acetone, Acetic acid, Calcium Chloride, Lactic acid, Sodium Hydroxide, Sodium Chloride, Methanol, Ethanol and Triethylamine were purchased from “Avantor Performance Materials Poland” (Gliwice, Poland). Carbon dioxide was bought from “Linde” (Gdansk, Poland). *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *C. albicas* ATCC 10231 were provided by “TCS Bioscience Ltd” (BotolphClaydon, Bucks, UK). TSB, TSA and Sabouraud agar and peptone were purchased from “Biocorp” (Warsaw, Poland). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), medium, antibiotics and supplements necessary for cell culture were obtained from Sigma-Aldrich (St. Louis, MO, USA). MilliQ water was used for the preparation of all aqueous solutions. All other reagents were of analytical grade or higher.

2.2. Preparation of chitosan materials

2.2.1. Chitosan modification with T3P

Chitosan modifications were prepared according to the method proposed by Kumar and co-workers, with a slight modification (Kumar et al., 2013). To the mixture of propylphosphonic anhydride − T3P (15,89 g; 0,0486 mol) and hydroxybenzotriazole (3,72 g; 0,0243 mol) added triethylamine (3,68 g; 0,0364 mol). The mass of the reactants was calculated from stoichiometric mole ratios. The mixture was stirred for 2 h at 30 °C (thermostatic chamber POLEKO ST 1 BASIC). Distilled water was added (20 ml^3) followed by chitosan (5 g; 0,0243 mol) and stirred for 24 h at 30 °C. The modified chitosan was precipitated with an excess of acetone and then washed with a mixture of acetone − methanol in 1:1 ratio under vacuum until clear.

2.2.2. Preparation of chitosan solution by CO_2 method

The unmodified chitosan solution was prepared by using a method of dissolving an aqueous chitosan suspension with gaseous CO_2. (Gorczyca, Tylingo, Szweda, Sadowska, & Milewski, 2014). Chitosan (1.5 g) was dissolved in 100 g of 0.1 M lactic acid and then 0.5 M sodium hydroxide was slowly added with constant agitation until a pH of 8.5 was reached.

In next step chitosan precipitate was separated by centrifugation (9000 × *g*, 30 min), washed three times with distilled water, and centrifuged again. The chitosan precipitate was weighed and added distilled water, so that the final mass of the mixture, together with the precipitate, has a mass of 150 g. The solution was saturated with CO_2 until fully dissolved with the use of an analog overhead stirrer (BIOMIX BMX-10).

Table 1
The composition of materials and description of their use in tests.

Sample	Content of p-CHI_{100}in the dry composite [% w/w]	Mass ratio of the dry polymers in composite CHI:p-CHI_{100} [% w/w]	Determined properties of composites:
CHI (control)	0	1:0	- mechanical properties,
p-CHI_{25}	25	3:1	- morphology (porosity and pore size − SEM)[a],
p-CHI_{50}	50	1:1	- structural properties (FTIR)[b],
p-CHI_{75}	75	1:3	- antioxidant properties[c],
p-CHI_{100}	100	0:1	- Fe^{2+} chelating ability[c],
			- biocompatibility[c]
CHI (control)	0	1:0	- antimicrobial properties,
p-CHI_{15}	15	1:0.18	- swelling degree,
p-CHI_{25}	25	1:0.33	- solubility
p-CHI_{40}	40	1:0.67	

[a] Withoutp-CHI_{50}sample.
[b] Withoutp-CHI_{25} and p-CHI_{75} samples.
[c] Only CHI and p-CHI_{100}samples.

2.2.3. Preparation of a dry composites

The T3P modified chitosan was mechanically stirred in distilled water until fully dissolved to obtain a 2% solution. For the unmodified chitosan, a 1% solution was used, obtained according to point 2.2.2. The resulting solutions were mechanically stirred in the ratios presented in Table 1.,then inserted in 12-well plates, frozen and freeze-dried (Christ Alpha 1–4 LD Plus; 0.94 mbar, sample temperature: 20 °C, condenser temperature: − 50 °C).

2.3. Morphological examination

The cross section images of the phosphorylated chitosan materials were examined with a scanning electron microscope, equipped with a field emission gun (Dual Beam Versa 3D, FEI with FEG). The surface images of the surface were taken in standard, high vacuum at 5 kV and probe currents of 1.6 or 3.3pA.The porosity of the chitosan scaffolds were calculated using image − processing tools of the Photoshop CS5 software.

2.4. FTIR measurement

The FTIR spectra of dry collagen preparation were measured using the FTIR spectrometer (Nicolet 8700; Thermo Electron Corp., Waltham, MA) equipped with the GoldenGate (Specac Corp., Oprington, UK) ATR accessory with a single reflection diamond crystal. The temperature of the crystal was maintained at 25.0 ± 0.1 °C by using an automatic temperature controller (Specac Corp.) coupled with the ATR accessory. In each measurement, 64 scans were collected with a resolution of 4 cm^{-1} and the range of 4000–750 cm^{-1}. The spectrum of collagen of the plate was measured and later subtracted from every measured spectrum as the background. After measuring all FTIR spectra corresponding to a selected strain and background subtraction, the average spectrum was calculated. The spectrometer was purged with dry nitrogen to diminish the negative influence of water vapor.

2.5. Swelling/dissolution tests

The swelling capacity of the scaffolds was evaluated by gravimetric determination the scaffolds before (W) and after (W_1) placing the scaffolds in an aqueous solution containing a salt composition similar to that of wound exudate according to EN 13726-1, prepared by adding 8.298 g of NaCl and 0.368 g of $CaCl_2{\cdot}2H_2O$ to 1 dm^3 of distilled water. The samples were incubated at 37 °C for 24 h, withdrawn from the medium, and weighed after removal of the surface fluid using a filter paper. The samples were then freeze-dried (CHRIST Alpha 1–4 LD plus) and weighed again (W_2). The percentage of fluid absorption was defined as the ratio of the weight increase ($W_1 - W_2$) relative to the weight remaining after freeze-drying (W_2). Each value was calculated as the mean of three independent measurements. To show the influence of the ionic strength of the solution, a similar test was performed using distilled water as the reference medium. The dissolution of the scaffold (in percent) is defined as the ratio of the weight decrease of the scaffold ($W - W_2$) relative to the initial weight (W).

2.6. Mechanical properties

The mechanical properties of the scaffolds were characterized using a universal testing machine (Instron model 5543, USA controlled using the "Merlin" software V 4.42.) according to previously described method (Bi et al., 2011; Hoyer et al., 2012). Five cylindrical samples of the test scaffolds (Ø 25 mm × 4 mm) were compressed up to 50% deformation at a test speed of 0.5 mm/s and the compression load (kPa) was measured. Based on the obtained results, the texture profile values were determined: hardness and flexibility.

2.7. Antioxidant activity

2.7.1. DPPH test

The ability of the scaffolds to react with oxygen-containing free radicals was assessed by the diphenylpicrylhydrazyl (DPPH) test adapted from Cullen et al. (2002). Briefly, different amounts of the materials tested were placed in 5 ml aliquots of a methanol solution containing DPPH (the solutions were prepared fresh, and the absorbance at 540 nm was adjusted to 1.0), and the samples were shaken in the dark at 37 °C. The change in color was monitored by spectrophotometric measurement (Spectrophotometer UV–vis Thermo Spectronic Helios Alpha) at 540 nm over a 2 h period. Measurements were performed hourly, and the scavenging effect was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = [1\text{-(absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}})] \times 100$$

The EC_{50} value is calculated by linear regression and is defined as the mass of sample material needed to reduce the initial absorbance at 540 nm of 1 ml of the DPPH solution by 50% after 2 h of incubation. The control consisted of the reagent solution without the material tested. Each determination was conducted in triplicate.

2.7.2. ABTS assay

The total antioxidant capacity was evaluated according to the modified ABTS assay (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999). The ABTS•+ radical cation solution [(2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid))] was generated from ABTS salt (potassium persulfate (2.45 mmol) was reacted with 7 mmol

of ABTS salt in 0.01 M PBS, pH 7.4, for 15 h at room temperature in the dark). The solution containing the resultant ABTS radical cations was diluted with methanol to give an absorbance of 1.0 at 734 nm. Different amounts of the material tested were suspended in 5 ml aliquots of the ABTS•+ solution and the samples were shaken in the dark at 37 °C. The change in color was monitored by spectrophotometric measurement (Spectrophotometer JENWAY 6300, U.K.) at 734 nm over a 2 h period. The scavenging effect and the EC_{50} value were calculated as described above.

2.7.3. Fe (II)-chelating ability

The Fe(II)-chelating activity of the chitosan–protein scaffolds was measured according to the modified method by Yen, Yang, & Mau (2008),where the Fe(II)-chelating ability was monitored by the absorbance of the ferrous iron–ferrozine complex at 562 nm (Spectrophotometer JENWAY 6300, U.K.). Briefly, different amounts of the material tested were suspended in 4 ml aliquots of a methanolic solution containing $FeCl_2$ (2 mM) and ferrozine (5 mM, the absorbance (562 nm) of which had been adjusted to 1.0). The samples were shaken at 37 °C for 2 h. The chelating effect and EC_{50} values were calculated as described above.

2.8. Antimicrobial properties

Antimicrobial activities were tested according to the method described in JIS L 1902 Standard with slight modifications. Colonies of bacteria were first subcultured on Triptic Soya Broth agar plates and yeast colonies on Sabouraud agar plates and incubated for 24 h in 37 °C. Microbial suspensions were prepared in test medium by adjusting the number of bacterial cells between $1,5 \times 10^7$ to 5×10^7 CFU/ml and yeast cells to 10^6 CFU/ml with spectrophotometer by measuring the absorbance at 600 nm and 660 nm wavelengths respectively (optical density 0.1). The test medium were prepared by dissolving 1 g of pepton and 8,5 g of NaCl in 1000 ml of water and further sterilization. For each genus of microorganisms 6 samples of each dressing material were prepared and 6 controls materials (a total of 12 samples; 3 samples and 3 controls for testing at time 0 h and 3 samples and 3 controls for testing at time 24 h). Samples were cut in 2,5 cm side squares under aseptic conditions and sterilized under UV light. Next, each material was pre-wetted with water for 1 h prior to the addition of inoculum. Composites were exposed to test organisms on separate dishes by spreading 0,2 ml of prepared inoculum across the surface of the sample. Immediately after inoculation (time 0) and after 24 h samples were placed in 10 ml of test medium and vortexed intensively for 25 s. Next ten-fold serial dilutions were plated on TSA plates and incubated for 24 h in 37 °C. After the incubation, only plates where 15 CFU to 300 CFU were counted. When there were no colonies recovered the number was recorded as “10” and the number of viable counts was established (CFU/ml). Obtain the viable count of the bacteria according to the following formula:

$$Vc = N \times D,$$

where:

Vc is the bacteria concentration, in colony forming units per ml (CFU/ml),

N is the average value, in colony forming units (CFU), from Petri dishes,

D is the dilution factor from the plates counted.

Antimicrobial activity was calculated according to the formula:

$$R = log\frac{B}{C}$$

where:

R − value of antimicrobial activity,

B − average of the number of viable cells on the control piece after 24 h,

C − average of the number of viable cells on the test piece after 24 h.

Percentage reduction of bacteria/fungi was calculated by assuming that R = 1 corresponds to reduction of 90%, R = 2 corresponds to reduction of 99%, and R = 3 corresponds to reduction 99.9%.

2.9. Biocompatibility tests

2.9.1. Cell culture

L929 cells (adult mouse fibroblast cell line) were obtained from ATCC (American Type Culture Collection). Cells (mycoplasma free) were maintained in monolayer culture at 37 °C in a humidified 5% CO_2 atmosphere in Low Glucose Dulbecco’s modified Eagles’ medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mM glutamine (Sigma-Aldrich, St. Louise, CA, USA). Under these conditions the cell-doubling time was 24 h.

2.9.2. Cell viability and morphology assessment

The cytotoxicity of the composite chitozan was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the ISO 10993-5:2009(E). L929 cells at a density of 1×10^4 cells/well were seeded on 96-well tissue culture microtitre plate and 100 µl culture medium only (blank) was dispensed into peripheral wells. The cells were incubated for 24 h (5% CO_2, 37 °C, > 90% humidity) to form a half-confluent monolayer. After 24 h incubation, culture medium was aspirated from the cells and 100 µl of treatment medium containing either the appropriate concentration of samples, or the positive control, or blank was added. After 24 h of incubation the cell viability and morphology were examined. Photos (magnification, ×125) of control cells and chitosan composites were taken using light microscope (TELAVAL 3 (Carl Zeiss, Jena, Germany). Then, attached and proliferated cells were quantified using MTT assay. Briefly, the culture medium was removed and 50 µl of MTT solution (1 mg/ml in medium without supplements and phenol red) was added to each well and incubated for 2 h at 37 °C in a humidified 5% CO_2 atmosphere. After that, the media with MTT solution was removed and crystals of formazan from each well were suspended in 100 µl of isopropanol. The plate was left on shaking platform for 10 min. The absorbance was recorded on a Microplate Reader (Bio-Rad, Hercules, CA, USA) at the length of 570 nm. The experiments were repeated at least three times. A decrease in number of living cells correlates to the amount of blue-violet formazan formed, as monitored by the optical density at 570 nm. To calculate the reduction of viability compared to the blank the equation was used:

$$\text{Viability\%} = 100x OD_{570e}/OD_{570pc}$$

where: $OD_{570e}$is the mean value of the optical density of the samples; OD_{570pc} is the mean value of the optical density of the positive control (L929 cells in culture medium).

2.10. Statistical analyses

The STATISTICA software (StatSoft, Inc., Tulsa, USA) was used for analyses. Significance was determined by at $P < 0.05$. All data reported is based on the means of three replicates (n = 3)

Experimental results were expressed as mean ± standard deviation (SD). Student’s t-Test and one-way analysis of variance (ANOVA) were applied. Differences were considered to be statistically significant at $p < 0.05$.

3. Results & discussion

In this work, a porous chitosan material with a high potential in various applications was proposed. Our focus was on creating a

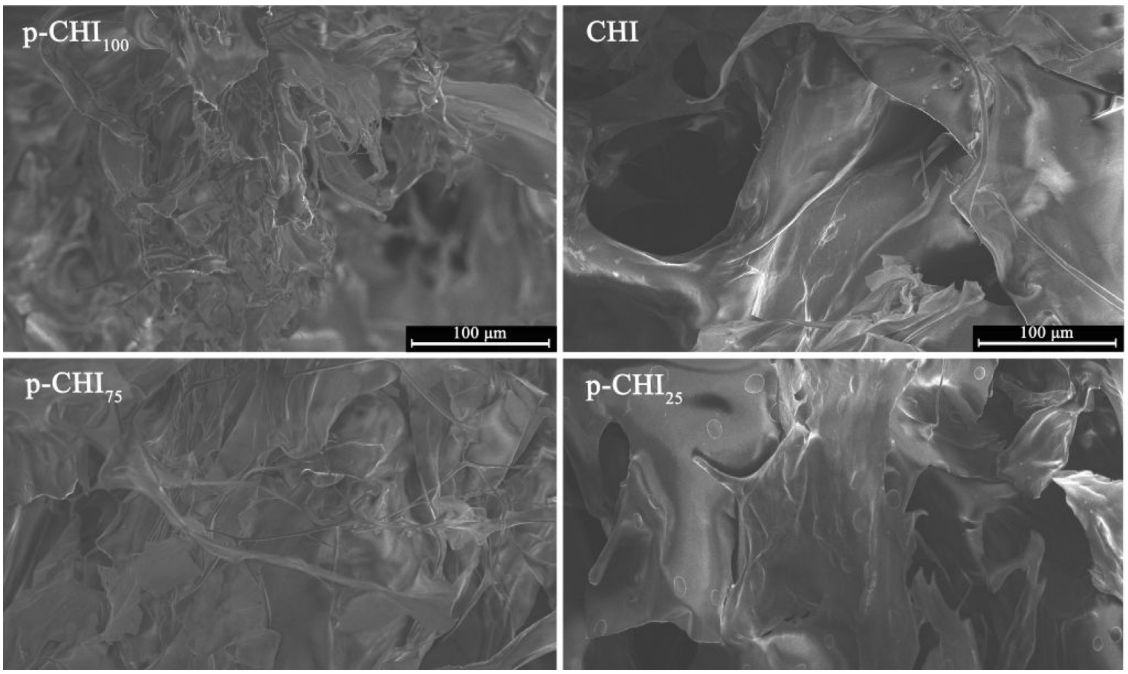

Fig. 1. Structure of chitosan composites obtained using Scanning Electron Microscopy (CHI- control material, *p*-CHI_X – composite containing X% by weight of the modified polymer.).

potential wound dressing material for different types of wounds that would absorb excessive wound drainage, maintain a moist wound environment, provide thermal insulation, adequate pH level and allow gaseous exchange.

The solubility of chitosan in dilute organic acids depends on the degree of deacetylation. Introduction of phosphate groups made it possible to obtain soluble forms in aqueous solutions at pH 7. The use of crystalline forms, e.g. by precipitation of chitosan from organic acid solution with NaOH solution, only facilitates the formation of soluble forms at pH below 6 by acidifying solutions with very weak acids like carbonic acid, as confirmed in the publications (Gorczyca, Tylingo, Szweda, Sadowska, & Milewski, 2014; Sakai, Hayano, Yoshioka, & Yoshioka, 2001; Sakai et al., 2002).

Chitosan derivatives can be used to create beads, films, fibers, membranes and hydrogels. Chitosan materials prepared in presence of acids, if not reduced by washing or evaporation, may have compromised biocompatibility. Except for evaporative reduction in the cases of acetic acid, formic acid and carbonic acid, wound dressing materials containing chitosan typically require the additional step of acid extraction by rinsing with neutral pH water or alcohol with additional drying.

Solvent extraction of dry chitosan material is less applicable for chitosan hydrogel wound dressing preparation making evaporative extraction a more attractive proposition especially if the acid is carbonic acid (Gorczyca, Tylingo, Szweda, Sadowska, & Milewski, 2014; Sakai et al., 2001, 2002). Solvent extraction can be time consuming, costly and complicates the introduction of pharmaceutical components into the chitosan if the acid component is present.

In order to avoid this problem, a growing number of studies have been focused on the preparation of water-soluble chitosan derivatives. One of the alternative methods was proposed by Gorczyca et al. (2014), where chitosan was dissolved in acetic acid and then precipitated with 0.5 M sodium hydroxide. The precipitate was then dissolved in water saturated with gaseous CO_2, until the pH 6.0 was reached. Such obtained chitosan can be used for hydgrogels preparation. This approach not only simplifies and optimizes the preparation of chitosan for wound dressing materials but also allows an addition of protein/peptide groups during the materials formulation.

Functional wound dressing materials have to be capable of providing the optimal moist environment necessary for wound healing in direct contact with the wound bed. As it was shown in our previous research (Tylingo, Gorczyca, Mania, Szweda, & Milewski, 2016) the solubility of the chitosan materials made with the use of CO_2, that were not modified with collagen or fish gelatin, was not sufficient to spontaneously develop hydrogels on a wound surface. Since the wound exudate pH is around 7.4, an alkaline chitosan was needed to form a hydrogel in contact with wounds, which was achieved by modifying the chitosan material with the propyl-phosphonate groups.

In order to determine the viability of phosphonated chitosan derivatives in tailoring of new materials with controlled solubility, a number of dry sponges made out of modified and unmodified chitosan have been prepared and analyzed. Several parameters of these composites like: morphology, swelling ability, solubility, texture profile and antibacterial activity have been analyzed.

3.1. Morphology of the chitosan composites

The surface color of the examined dry films varied from white to brown depending on their composition. The control samples (CHI) were white, while the samples prepared with phosphonated chitosan developed a brown color (p-CHI_{100}) (Silverstein, Webster, & Kiemle, 2016) from light to dark intensity, depending on the concentration of the modified chitosan. This is probably due to the electron configuration of the reagent's functional groups (chromophores). Both the reagent and modified chitosan show the highest absorbance at the same wavelength of 298 nm (own research). Similar maximum absorbance is observed for the C=O and N=O groups, which are 279 nm and 278 nm, respectively (Silverstein et al., 2016). All chitosan samples retained a shape of the die in which they were freeze-dried and maintained their integrity during the visual analysis.

The SEM analysis of the chitosan composites showed that their surfaces were highly porous (Fig. 1). All the scaffolds samples were studied at the same setting of the microscope. The average pore diameter for the control samples of unmodified chitosan was around 80 µm. In the case of modified chitosan (p-CHI_{100}) the morphology of the surface changed and became more fibrous, which made the average pore size difficult to determine. However, the overall porosity decreased in comparison with unmodified chitosan. Compared to the

Table 2
Porosity of the chitosan composites.

Sample	Porosity [%]
CHI	77.5 ± 1.8
p-CHI$_{25}$	72.1 ± 2.3
p-CHI$_{50}$	65.5 ± 1.3
p-CHI$_{75}$	61.4 ± 1.9
p-CHI$_{100}$	55.2 ± 1.9

Values represent means ± standard deviation (n = 3).

modified chitosan p-CHI100, the structure of the sample p-CHI$_{75}$ didn't change significantly. Similarly, the structure of the sample p-CHI$_{75}$was similar to unmodified chitosan. (Fig. 1).

The freezing processing conditions have a significant effect on morphology of the scaffolds. Number of variables in the process can affect the structure: the polymers concentration in the suspension prior to freeze-drying, the coupling reagents used, the interface composition and the reaction conditions (temperature, drying process parameters). The impact of these variables was minimized during the experiment as the modified chitosan was completely dissolved in water within the defined concentration ranges. Thus, it can be assumed that the morphology changes of the composites were only related to the chemical composition of used biopolymers (Fig. 1). The samples were frozen at −24 °C and then freeze-dried under vacuum (0.94 mbar). All of the samples were place in equal distance to the freeze drying condenser to maintain a lower temperature gradient.

With increasing concentration of modified chitosan the porosity of the chitosan composites decreased. The modified chitosan p-CHI$_{100}$ had the lowest porosity values, while the control sample porosity values were the highest (Table 2).

Our previous work had shown that the pore sizes for chitosan-protein composites increases with the increase in their porosity (Tylingo, Gorczyca et al., 2016; Tylingo, Mania et al., 2016). The same effect can be observed for the xerogel presented in this work. Porosity is an important parameter in wound dressing product as it affects fluid transfer, gas exchange and allows the material to be used for nutrient and drug delivery. According to Ikeda, a porosity above 80% desired for an ideal wound care material (Ikeda et al., 2014). Ikeda et al. (2014) also found that the porosity decrease of the chitosan sponges was observed as the concentration of the chitosan increased. The pore size of scaffolds can be regulated by optimizing the processing conditions, lowering the concentration of the polymer, altering the freezing temperature and slowing the cooling process to produce larger ice crystals.

3.2. FTIR studies

Fig. 2 shows FTIR spectra of chitosan materials, where the wide absorption band at

3600–3100 cm^{-1}is assigned to the stretching vibration of N−H i O−H. The chitosan spectrum showed aliphatic C−H stretching at 2879 cm^{-1}. The bands at 1635 cm^{-1}, 1522 cm^{-1} and 1315 cm^{-1}, assigned to the amide I, amide II and amide II bands, respectively confirmed, degree of N-acetylation, for chitin and chitosan (Kumirska et al., 2010). The C=O stretching band in amide I band revealed the presence of CH3−C=O in chitosan. According do Kasaai (2010) the band at 1590 cm^{-1} assigned to $-NH_2$ displayed a greater intensity than the one at 1655 cm^{-1}, assigned to CH 3−C=O as the deacetylation of chitin progressed. The N−H stretching of the amino groupat 1522 cm^{-1} has slightly higher intensity than the amide I band but at a lower wave number that for stretching vibrations of $-NH_2$ of chitosan. The spectra for the modified chitosan showed that amide I and II bands shifted to lower wave numbers, and their bands intensities dropped (Fig. 2). Also, an additional band at 2934 cm^{-1} for the propyl group from the coupling agent can be observed, which was confirmed by Kumar (Kumar et al., 2013). A strong band at 928 cm^{-1}is assigned to P-O-P (Heras, Rodriguez, Ramos, & Agullo, 2001). The intensity of that increased with an increased concentration of modified chitosan in the composites (Fig. 2). The band at 1160 cm^{-1}is assigned to the stretching vibrations of −P=O which confirmed the presence of T3P in the polymer structure (Iliewa et al., 2001).Apart from the amide bands, a wide band in the carbohydrate region of 800–1200 cm^{-1} is observed.

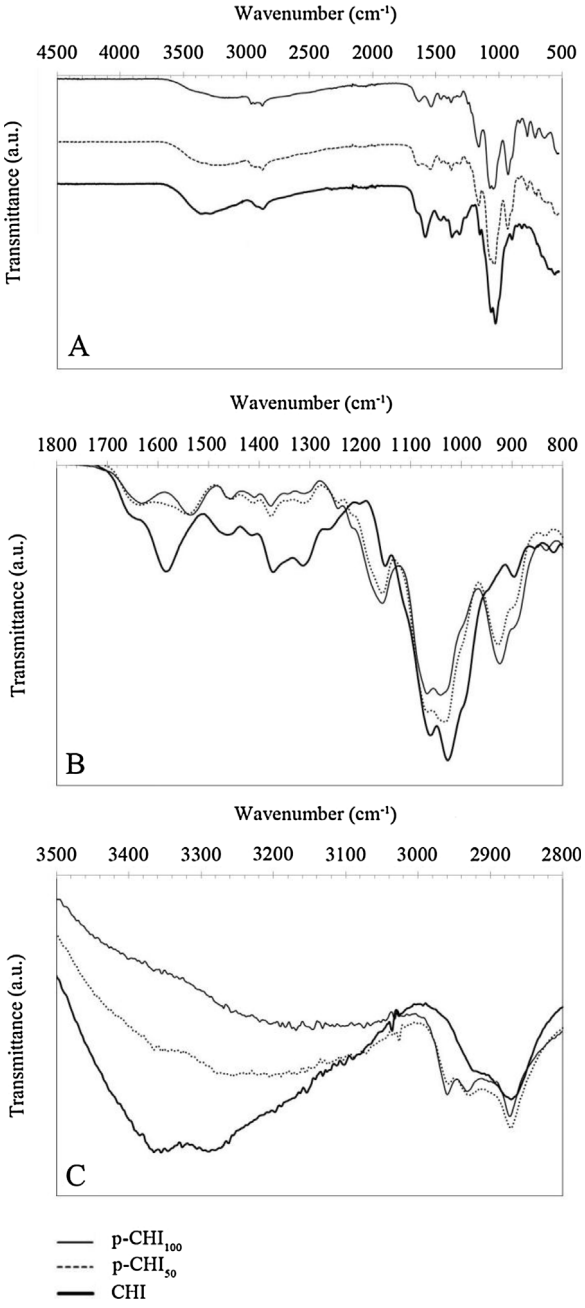

Fig. 2. FTIR spectra of chitosan materials (CHI − unmodified chitosan sample, p-CHI$_{50}$ −chitosan composite including 50% (w/w) of modified chitosan polymer, p-CHI$_{100}$ − modified chitosan sample; part A, B and C differ only in the length of the wave number ranges).

3.3. Swelling and dissolution tests

The swelling and dissolution behaviors of the scaffolds were evaluated with an adjusted concentrations range of modified chitosan shown in Table 1. This was required due to the fact that the chitosan materials with modified chitosan content above 40% would completely

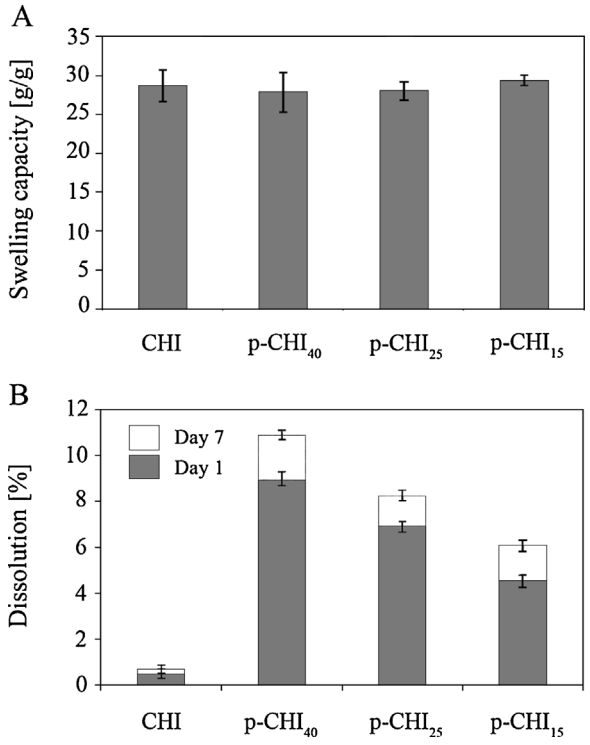

Fig. 3. Swelling capacity and dissolution of obtained chitosan composites compared to the unmodified sample (CHI- control material, p-CHI_X – composte containing X% by weight of the modified polymer.) Values represent means ± standard deviation (n = 3).

dissolve in contact with liquid, which would affect their mechanical properties and disqualify them as a wound care material. The swelling capacity was similar, regardless of the modification type (Fig. 3A). It was determined the scaffolds containing up to 40% of modified chitosan were capable of holding an amount of liquid about 30 times their own weight. According to Tiğh and Karakecili (2007),water absorption rate of the chitosan sponges is in the range of 27–40 g/g and increases with the polymer deacetylation level. Good liquid absorption properties of the chitosan materials can be attributed to the presence of highly hydrophilic cross-linked polysaccharides able to form stable xerogels. The deacetylation process increases the number of hydrophilic amine groups. In order to improve the swelling capability of the examined materials, chitosan with a higher deacetylation degree should be used (Tiğh and Karakecili, 2007).

Chitosan solubility depends on the amount of protonated amino groups and N-acetyl group in its polymeric chain. Unmodified chitosan is readily soluble in dilute acidic solutions at pH below 6.0. The chitosan modification with propylphosphonic anhydride results in replacing the amino groups with more hydrophilic groups, thus improving its solubility in neutral pH. Dissolution degrees of obtained chitosan composites modified with T3P compared to the unmodified sample are presented in Fig. 3B. The loss of weight for the control sample (CHI) was minimal. The highest reduction in weight was observed for the p-CHI_{40} sample, then for the samples CHI_{25} and CHI_{15}, respectively. The most significant loss of weight for the composites was observed after 24 h, declining thereafter. Then, in the following 6 days, a decrease of 1.5% per day was observed. Since chitosan is not water-soluble, the weight loss was primarily related to the dissolution of the modified chitosan fractions of the polymers. Thus, the solubility of the composites, adjusted for the concentration of modified chitosan in the samples p-CHI_{40}, p-CHI_{25}, p-CHI_{15} were 27.5%, 34% and 40%, respectively. These results suggest that, even after 7 days, the soluble fractions of the polymers had not been fully dissolved. The correlation between the increase in reducing the amount of the modified chitosan in the composites and the decrease of its concentration was observed (Fig. 3). The viscosity of phosphorylated chitosan is about 1000 times higher than for water, in the same conditions, which makes it more difficult to fully dissolve in the scaffolds. However, this does not affect its functionality, because the ability of creating a xerogel on the composite surface is

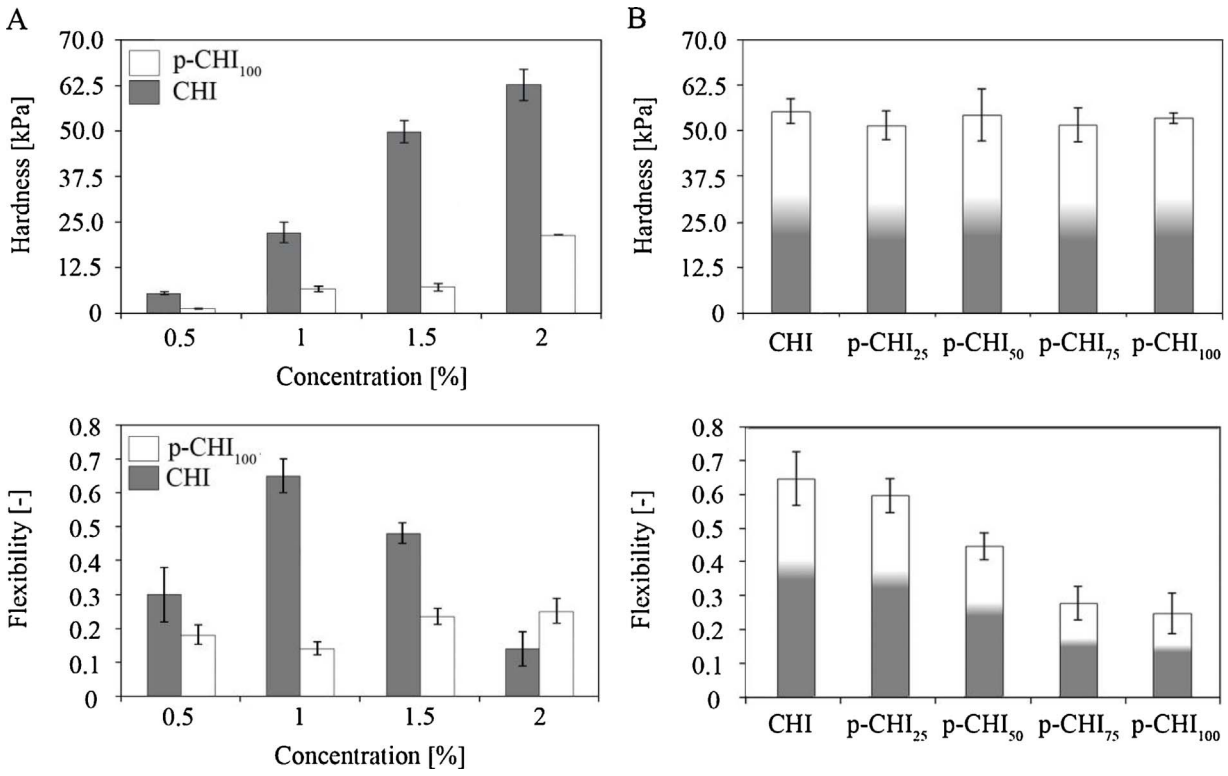

Fig. 4. Hardness and flexibility of chitosan materials: A – depending on the concentration of the polymer in solution intended to drying. B – after formation of composites by mixing two solutions of chitosans in appropriate amounts and drying. Values represent means ± standard deviation (n = 3).

beneficial for wound protection/healing properties. These results suggest that changes in the preparation methods of the modified chitosan composites, could improve their functionality. For example, modified chitosan xerogel grafts/scaffolds, would improve their availability and reduce the amount of the modified polymer in the sponge.

3.4. Texture profile

The mechanical properties (hardness and elasticity) of the prepared polymers were investigated. This experiment was divided into two parts. The purpose of the first part was to determine the hardness and elasticity of the polymers made out of unmodified or modified chitosan at concentrations of 0.5%, 1.0%, 1.5% and 2%, first frozen and then freeze-dried (Fig. 4A). The purpose of the second part was to determine the hardness and elasticity of the polymers made out a mixture of unmodified/modified chitosans at predetermined ratios (Table 1), prepared by the same method as in part 1. For both unmodified and modified chitosan polymers, their hardness increased with an increase in concentration of polymer. The values were higher for the control sample (CHI), than for modified chitosan (p-CHI_{100}), at the same concentrations in solution. For example, a sponge made out of the 1% solution of unmodified chitosan had a comparable hardness to a sponge made out of the 2% solution of modified chitosan (Fig. 4). The elasticity results did not follow the same trend. Elasticity is defined as the ratio of the distance required to achieve maximum force on the second compression cycle to that on the first compression cycle. The values of the compressive stress were the highest for the polymer prepared from the 1% solution of unmodified chitosan.

The lower elasticity of the sponge obtained from the 0.5% solution of unmodified chitosan was caused by a smaller amount of the polymer per the sponge volume. The force required to compress the sponge was smaller and the porous sponge structure was affected during the compression cycle, preventing its recovery to the initial shape.

The decrease of elasticity could also be attributed to the increase of the dispersed polymer concentration for the control sample. Similar results were presented by Ikeda et al. (2014). Kim et al. observed the composites hardness increase with an increase in concentration of polymer (Kim, Park, Kim, Wada, & Kaplan, 2005), which was confirmed in this research.

It can be concluded, that above certain concentration level of chitosan (1% for unmodified chitosan), with an increased hardness of the composite, its elasticity decreases.

In the second part, composites prepared out a mixture of 1% unmodified chitosan and 2% modified chitosan were tested (Table 1). These concentration levels were chosen due to the fact that the sponges made out these polymers were equally hard (~ 55 kPa). This allowed to determine the hardness and elasticity of the composites prepared from both polymers (Fig. 4B). The results showed that the composites with modified chitosan had the same hardness, regardless of its amount in the mixture, as the composites with either 100% unmodified chitosan or 100% modified chitosan. These results indicated there were interactions between these two types of chitosan that could affect their mechanical properties.

This is due to the fact that the T3P modification doesn't change the polycationic character of chitosan, even with the introduction of the negatively charged phosphonate groups. This can be observed when combining modified chitosan with polyanionic substrates such as gelatin, sodium alginate and collagen creates gels formed by ionic forces (Shen et al., 2008). Flexibility of the obtained composites was in the range from 0.25 (100% modified chitosan only) and 0.65 (100% unmodified chitosan), increasing with the increase of the modified chitosan ratio in the mix (Fig. 4B). These results showed that the hardness and flexibility of the sponges obtained with the T3P modified chitosan can be controlled with the changes in the polymers concentrations in the hydrogel, prior to the freeze-drying.

3.5. Antioxidant activity

To determine the antioxidant activity of the modified chitosan scaffolds, three frequently used tests were performed: DPPH, ABTS and metal (Fe^{2+}) chelating activity (Fig. 5A–C). The evaluation of antioxidant capacity was measured through the determination of EC_{50} (Fig. 5D), which is concentration required to obtain a 50% antioxidant effect, after 2 h from the beginning of the test. In the first two tests, a free radical scavenging activity thru the color reduction in time is monitored. In the third test, measurement of the color reduction determines the chelating activity of the ferrous ions. This process is more rapid and after 2 h there is no visible change in color of the solution (Gorczyca et al., 2014).

The EC50 values were calculated by linear regression of the results of the free radical scavenging activity (or the chelating activity of the ferrous ions) at different materials concentrations. Higher EC_{50} values represent lower ability to remove free radicals or chelate ferrous ions.

Each performed test demonstrated the ability of chitosan to remove free radicals or chelate ferrous ions (Fig. 5). In each case, free radical scavenging activity (or Fe^{2+} chelating activity) increased with increasing concentration of the chitosan material. As it is shown in Fig. 5D. the difference in the ability to neutralize ABTS+ for unmodified and modified chitosans is minimal. The highest difference in free radical scavenging activity for both materials was achieved with the DPPH assay where the materials with modified chitosan exhibited strong antioxidant DPPH radical scavenging activity with EC_{50} value three times higher than for the control sample. The chitosan ability to chelate ferrous ions was minimally affected by the modification. Chitosan has the ability to chelate with various metal ions, due to the presence of −OH and −O groups of D-glucosamine and amino groups. However, the deacetylation of chitin doesn't directly translate into chitosan's ability to chelate with metal ions. The chelating properties of chitosan can be improved cross-linking, controlled N-acetylation or binding with other polymers (Piątkowski et al., 2010). The introduction of additional −OH groups via T3P did not significantly affect the EC_{50} value. The antioxidant activity of chitosan can be explained through various mechanisms. One of them is the free radical scavenging activity and another is reducing power ability and ability to chelate with metal ions, due to the presence of hydroxyl and amino groups. The chitosan's antioxidant activity is limited in terms of the diversity of the available functional groups but the number of the groups in the structure compensates this (Rajalakshmi, Krithiga, & Jayachitra, 2013). The additional hydroxyl groups introduced to the polymer with the propylphosphonic units could be the reason of an increased antioxidant activity in the DPPH test. Also, the research on antioxidant activity of chitosans with different molecular weights showed a comparable free radical neutralizing ability of chitosan with a low molecular weight (12 kDa) with ascorbic acid solution (Chien, Sheu, Huang, & Su, 2007). Yen et al. reported than antioxidant properties of fungal chitosan harvested from shiitake mushrooms increased of deacetylation was increased (Yen, Tseng, Li, & Mau, 2007). The same researchers reported that chitosan's chelating abilities on ferrous ions were similar to chelating effect of EDTA, and several times higher than for citric acid. The exact mechanisms of the chitosan–metal ions interactions are far from being completely understood. There are reports in the literature that metal (II) ions are chelated thru the interactions with the hydroxyl groups at the C6 position and amino group at the C2 position of glucosamine (Inoue, Yoshioka, & Hotta, 1988).

The role of free radicals and antioxidants in wound healing process has recently been receiving heightened interest in developing new wound dressing and drug delivery materials. (Fitzmaurice, Sivamani, & Isseroff, 2011). Multiple factors can cause impaired wound healing. One of them is oxidative stress, which can significantly delay healing by causing oxidative damage. It is of great importance that the modern wound dressing materials should maintain balance between oxidants and antioxidants in the wound environment in comparison

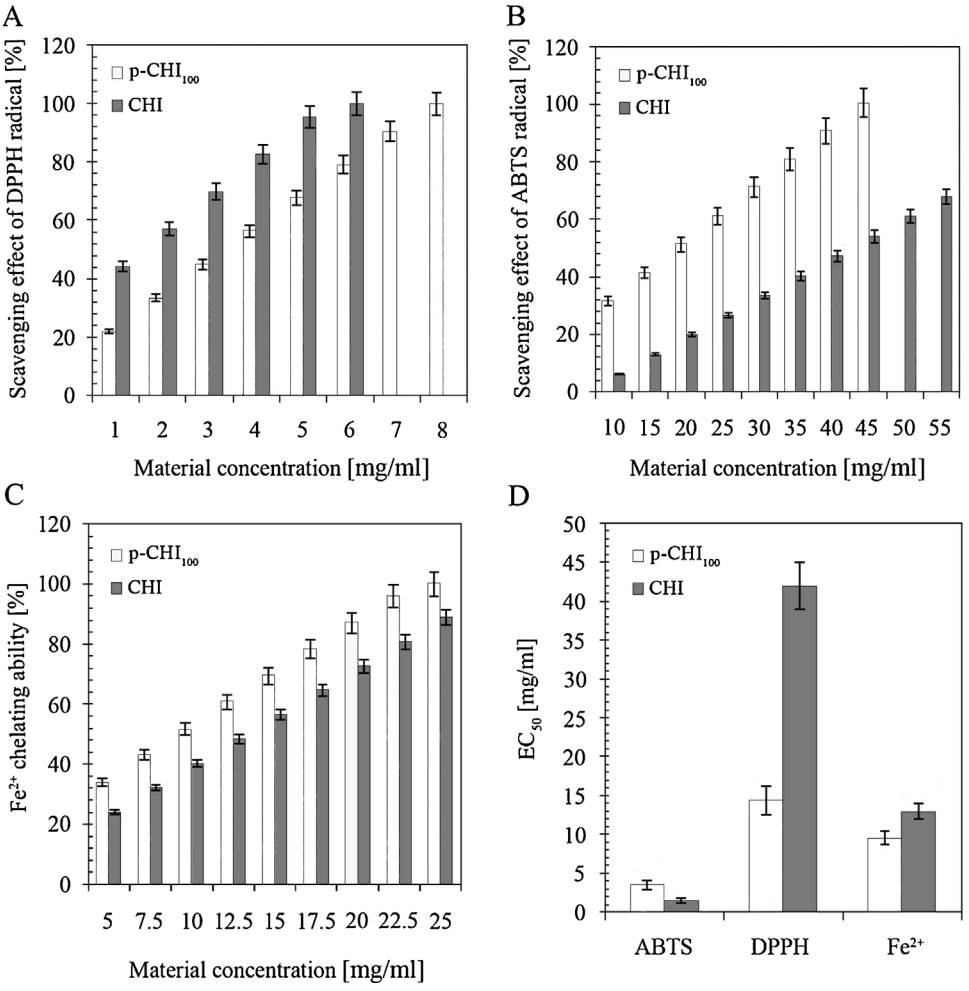

Fig. 5. The antioxidant properties and iron ions chelating ability of chitosan composites. Values represent means ± standard deviation (n = 3).

with traditional. It was reported that the excess but also a very low level of reactive oxygen species could affect the wound healing process (Roy, Khanna, Nallu, Hunt, & Sen, 2006).

The chitosan composites presented in this work exhibit free radical scavenging ability at much lower level than natural antioxidants like glutathione, vitamin C, vitamin E and flavonoids, which have the EC_{50} values in the range of μg/ml. A sponge made of modified chitosan had a comparable free radical scavenging ability in the DPPH test to epigallocatechin gallate (EGCG). EGCG is a polyphenol found in tea leaves and a powerful antioxidant, about 100 times stronger than vitamin C (80% of inhibition of DPPH radicals at 5.8 mg/ml). (Moreno-Vásquez et al., 2017).This value is higher that reported by other reports (Puoci et al., 2008), where the effect of ferulic acid grafted onto methacrylic acid on the inhibition of DPPH radicals was evaluated. However, a 30 and 80% inhibition of DPPH radicals by 8 mg/ml blank chitosan and ferulic acid grafted onto methacrylate was reported. A sponge obtained from modified chitosan (p-CHI_{100}) shows 100% inhibition of ABTS radicals at 102 mg/ml. EGCG-grafted chitosan was showing 100% inhibition of ABTS radicals at 4 mg/ml, which was caused by introduction of the hydroxyl groups to the chitosan backbone (Moreno-Vásquez et al., 2017).

3.6. Antimicrobial properties

Antimicrobial tests showed that chitosan materials exhibited intense antimicrobial activities. Control chitosan material (CHI) itself does not prevent the growth of the microorganisms. Three tested materials (p-CHI_{40}, p-CHI_{25}, p-CHI_{15}) were active against gram-positive *S. aureus* and nearly as well toward the yeast of the genius *Candida*. The three tested chitosan samples present stronger antimicrobial activity against the *S. aureus* than *E. coli* for all composites studied, as displayed in Table 3.

Antimicrobial activities were presented as a logarithmic reduction rate of bacteria/yeast R (Table 4) and as a reduction of cell number induced by tested materials in relation to the number of cells in control after 24 h.

Several models suggested that the antimicrobial activity of chitosan results from its cationic nature. The electrostatic interaction between positively charged polymer and negatively charged microbial cell membranes is predicted to be responsible for cellular lysis and assumed as the main antimicrobial mechanism (Goy, Morais, & Assis, 2016). The antimicrobial activity of chitosan is higher at lower pH as it depend on the amount of protonated amino groups in the polymeric chain. At the pKa in the range of 6.3-6.5 its antimicrobial activity decreases, as pH is a crucial factor affecting chitosan's solubility and biocompatibility. The

Table 3
The average number of microorganisms cells for controls and tested composite materials after 24 h contact of microorganisms with samples.

Sample	Time of interaction [h]	Average number of cells [CFU/ml]		
		S. aureus	*E.coli*	*C. albicans*
CHI	0	4900000	820000	44000
CHI	24	10000000	42000000	1000000
p-CHI_{15}	24	50	5000000	50
p-CHI_{25}	24	50	3000000	2200
p-CHI_{40}	24	50	30000	50

Table 4
Comparison of the antimicrobial activities of chitosan composites in respect to the control sample.

Sample	*S. aureus*		*E. coli*		*C. albicans*	
	R	Reduction [%]	R	Reduction [%]	R	Reduction [%]
p-CHI$_{15}$	5,3	99,99	0,9	88,10	4,3	99,99
p-CHI$_{25}$	5,3	99,99	1,2	92,86	2,7	99,78
p-CHI$_{40}$	5,3	99,99	3,2	99,93	4,3	99,99

research results presented in our work show that for the chitosan composite prepared with propylphosphonic anhydride, its antimicrobial activity is maintained in neutral pH.

3.7. Biocompatibility

The *in vitro* cytotoxicity of chitosan composite was investigated by the MTT assay using adult mouse fibroblast L929 cells according to the ISO 10993-5:2009(E). This is a standard *in vitro* method for biological evaluation of medical devices, which relies on the mitochondrial activity of vital cells and represents a parameter for their metabolic activity (Pariente, Kim, & Atala, 2001). The results are shown in Fig. 6A, and are expressed as percentage of viable cells in the presence of the test the modified chitosan (p-CHI$_{100}$) versus positive control without the material. It was shown that the tested material diluted 1:5 and 1:10,which was corresponding to the concentration 0.033 g/ml and 0.018 g/ml, respectively inhibited cell growth only in 20% compared to the control (cells without material). In turn, the cell viability reached the level of 50% following treatment with modified chitosan at concentration 0.1 g/ml (dilution 1:1).

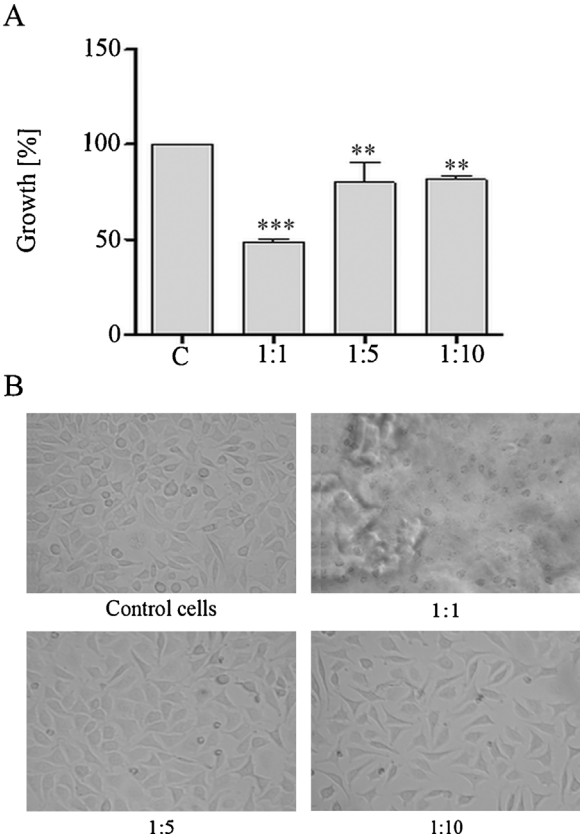

Fig. 6. A) MTT viability assay of composite chitosan (dilution of p-CHI$_{100}$ 1:1, 1:5, 1:10) following 24 h of incubation with L929 cells. **p < 0.01, ***p < 0.001 vs. control (untreated) cells. The mean ± SD values from three independent experiments. B) Morphological examination of L929 cells (control) and p-CHI$_{100}$ (dilution 1:1, 1:5, 1:10) under light microscope (magnification ×125).

The morphological examination of L929 cells treated with modified chitosan confirmed cytotoxicity data. Fig. 6B shows that morphology of fibroblasts cultured with modified chitosan diluted 1:5 and 1:10 presented unchanged morphology in relation to the control. On the contrary, sample diluted 1:10 affected the morphology of L929 cells (cells were shrunken, detached from the culture plate). These results clearly showed that L929 cells were able to proliferate and maintain their fibroblast morphology in the presence of the tested modified polymers, depending on their concentration. In order to evaluate the tested chitosan composites for wound healing materials use, certain parameters like solubility and absorbing properties have to be considered. An average mass of a single sponge of 20 mm in diameter and 5 mm tall is approximately 0.05 g. According to our research, the sponges were able to absorb up to 30 times their weight in wound exudate, while their solubility did not exceed 11%. Thus, the concentration of modified chitosan in contact with the wound would be at 0.004 g/ml. It can be assumed that the amount of modified chitosan in the composites would not affect their wound healing ability negatively.

4. Conclusion

Regenerative medicine is currently one of the most promising and rapidly growing fields of life sciences. There has been a recent increase of interest in the design, development and advancement of the biomaterials based scaffolds. However, there are certain functional requirements that have to be met for these materials to be used in regenerative medicine. The research results presented in this paper successfully prove the potential use of chitosan modified with propylphosphonic anhydride in water-soluble composites. These composites have high porosity, antioxidant and antimicrobial properties, are highly absorbent and maintain excellent biocompatibility. The presented composites have high potential for use in wound dressing and can provide a controlled, moist healing environment upon contact with the wound exudates, necessary for skin regeneration.

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