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ORIGINAL PAPER

Synthesis and antimicrobial activity of some novel terephthaloyl thiourea cross-linked carboxymethyl chitosan hydrogels

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Abstract Four novel hydrogels composed of carboxymethyl chitosan (CM-chitosan) cross-linked with various contents of terephthaloyl thiourea moieties (TTUCM-chitosan-1, TTUCM-chitosan-2, TTUCM-chitosan-3, TTUCM-chitosan-4) have been successfully synthesized. The hydrogels were characterized by elemental analyses, FTIR, ^{13}C NMR, SEM, XRD, solubility and swell ability in various solvents. The antimicrobial activities of these hydrogels against three crop-threatening pathogenic fungi (*Aspergillus fumigatus*, *Geotrichum candidum* and *Candida albicans*) and against three bacterial species (*Bacillis subtilis*, *Staphylococcus aureus* and *Escherichia coli*) were investigated. The hydrogels showed higher antibacterial activity than the parent CM-chitosan as judged by their higher inhibition zone diameter and lower minimum inhibition concentration. They are more active against Gram-positive bacteria than against Gram-negative bacteria. The results also indicated that the hydrogels have effective antifungal activity as compared with CM-chitosan. The antimicrobial activity of the hydrogels increased with increasing their cross-linking density.

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Keywords Chitosan · Diisothiocyanate · Cross-linking · Hydrogels · Synthesis · Characterization · Antimicrobial activity

Introduction

Polymer hydrogels are a cross-linked network of linear hydrophilic polymers that are capable of imbibing large amounts of water or biological fluids. They are composed of homo polymers or copolymers which are insoluble due to the presence of chemical or physical cross-links such as entanglements or crystallites (Guilherme et al. 2005). Due to their numerous excellent properties, they are extensively applied in many fields such as feminine hygiene, personal care products, horticulture, and incontinence products (Hongmei and Xie 2003; Yoshimura et al. 2005). It is well known that, natural polymers possess better biocompatibility, biodegradability, non-toxicity and easily modified ability than various synthetic polymers. Thus, in response to the environmental concerns, considerable researches have been recently directed toward the use of naturally abundant polymers such as chitosan for production of hydrogels (Ravi Kumar 2000). Chitosan, a linear cationic amino polysaccharide, is obtained by alkaline deacetylation of chitin, the principal exoskeletal component in crustaceans. Because of the low solubility of chitosan in water, its hydrogels do not show high binding water capacities. Carboxymethyl chitosan, an amphoteric

material with high hydrophilic characteristics, can be obtained through the reaction of chitosan with monochloro acetic acid in the presence of sodium hydroxide (Mourya et al. 2010). It is a very important chitosan derivative showing very good water solubility. Recently, hydrogels with high water binding capacity have been successfully produced from carboxymethyl chitosan by its cross-linking in aqueous media, followed by drying of the resultant gel (Zamani et al. 2010; Bidgoli et al. 2010).

Thioureas have strong antifungal activities that are comparable to the activity observed for the common antifungal antibiotic ketoconazole (Phuong et al. 2004; Huehr et al. 1953). Moreover, they have antibacterial and insecticidal properties (Ramadus et al. 1998; Criado et al. 1998). In our previous work, acetyl, chloroacetyl, and benzoyl thiourea derivatives of carboxymethyl chitosan have shown antimicrobial activity much better than that of the parant carboxymethyl chitosan (Mohamed and Abd El-Ghany 2012).

The objective of this work is to prepare some novel hydrogels composed of carboxymethyl chitosan cross-linked by therephthaloyl thiourea moieties. The hydrogels will be characterized by elemental analyses, FTIR, ^{13}C NMR, SEM, XRD, solubility and swell ability in various solvents. The antimicrobial activities of these hydrogels against three crop-threatening pathogenic fungi namely: *Aspergillus fumigatus* (*A. fumigatus,* RCMB 002003), *Geotrichum candidum* (*G. candidum,* RCMB 052006) and *Candida albicans* (*C. albicans,* RCMB 005002) and three bacterial species namely: *Bacillis subtilis* (*B. subtilis,* RCMB 000107), *Staphylococcus aureus (S. aureus,* RCMB 000106) and *Escherichia coli* (*E. coli*, RCMB 000103) are also described. The effect of the cross-linker content on the hydrogels characteristics will be reported.

Experimental section

Materials

Chitosan with a degree of deacetylation of 88 % and molecular weight of 2.0×10^5 was purchased from Acros Organics, NJ, USA. All other chemicals and reagents were of analytical grade, from Aldrich and were used as received. The crop-threatening pathogenic fungi (*A. fumigatus*, *G. candidum* and *C. albicans*) and bacteria (*B. subtilis, S. aureus* and *E. coli*) used for the antimicrobial assay were provided by the Reginol center for Mycology and Biotechnology Culture Collection.

Preparation of carboxymethyl chitosan (CM-chitosan)

Carboxymethyl chitosan was prepared following the method reported previously (Chen and Park 2003) where chitosan (10 g), sodium hydroxide (13.5 g) and solvent isopropanol (100 ml) were suspended in a flask to swell and alkalize at room temperature for 1 h. The monochloro acetic acid (15 g) was dissolved in isopropanol, and added to the reaction mixture dropwise within 30 min and reacted for 4 h at 55 °C. Then the reaction was stopped and isopropanol was discarded. Ethyl alcohol (80 %) was added and solid product was filtered and rinsed with 80–90 % ethyl alcohol to desalt and dewater, and vacuum dried at 50 °C. The degree of substitution of carboxymethyl chitosan was determined by pH-titration (Eyler et al. 1947) and found to be 0.75.

Synthesis of terephthaloyl thiourea cross-linked CM-chitosan (TTUCM-chitosan) hydrogels

Four different predetermined amounts of terephthaloyl dichloride were dissolved in 20 ml methylene chloride and were added dropwise to solutions of ammonium thiocyanate of appropriate concentrations in 20 ml methylene chloride. The molar ratio of terephthaloyl dichloride to ammonium thiocyanate was 1:2, respectively. Polyethylene glycol-400 (1 ml) was added dropwise to each reaction mixture as a phase transfer catalyst. After stirring for 2 h at room temperature, the white precipitate of the formed ammonium chloride was removed by filtration. Each filtrate (terephthaloyl diisothiocyanate, Scheme 1a) was added to CM-chitosan solution (5.06 g, 20 mmol, in 200 ml of water). The reaction mixture was stirred at 60 °C for 2 h, cooled, and the homogenous cross-linked hydrogels formed (Scheme 1b) were submerged in methanol for 24 h for dewatering to give yellowish white products. The dewatered hydrogels were filtered and dried at 60 °C to constant weights. The ratios of the reactants were varied as shown in Table 1 to give four new hydrogels which were designated as TTUCM-chitosan-1, TTUCM-chitosan-2, TTUCM-chitosan-3,

Scheme 1 Synthesis of TTUCM-chitosan hydrogels

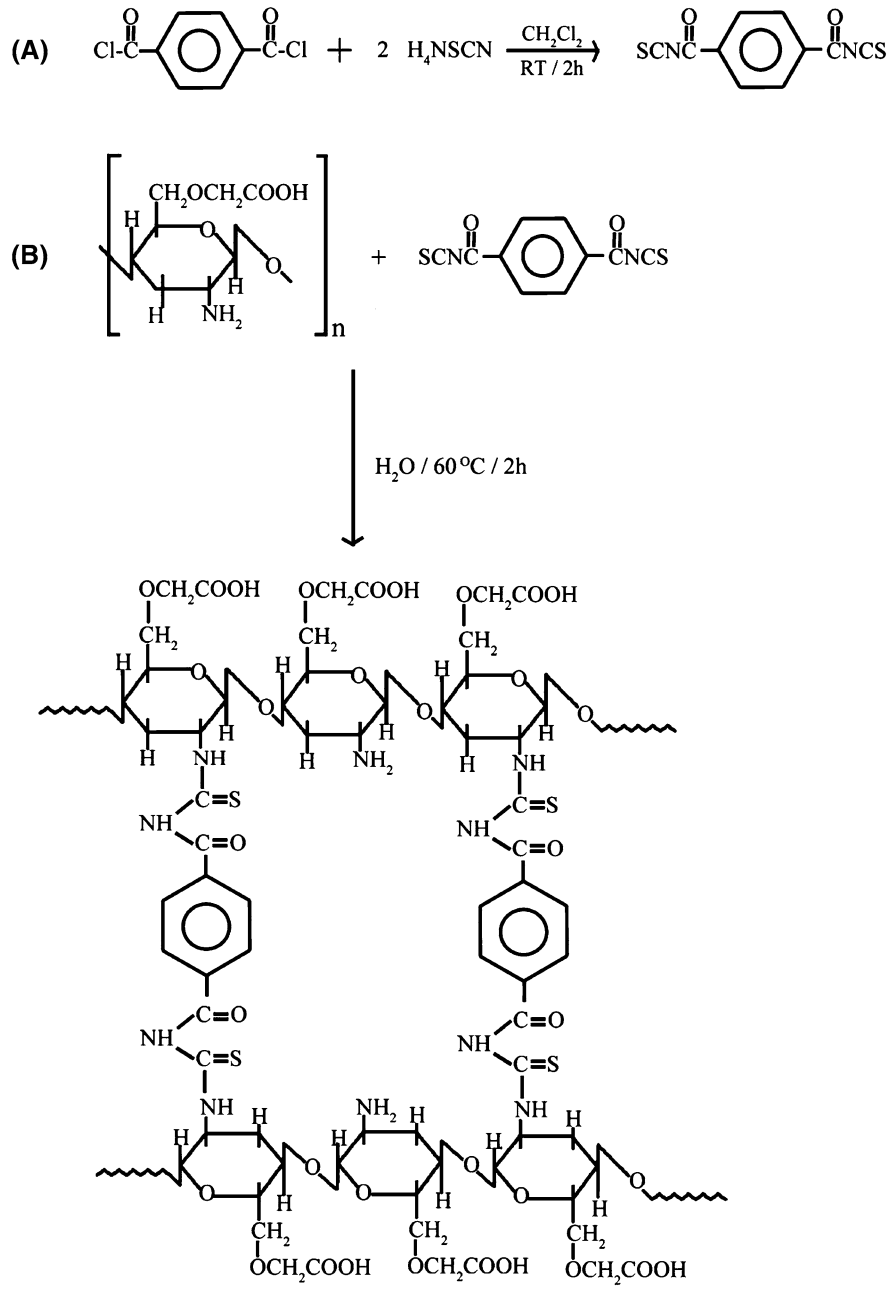

Table 1 Synthesis of TTUCM-chitosan hydrogels having different degree of cross-linking and their elemental analyses

Samples	Terephthaloyl dichloride (mmol)	Amm. thiocyanate (mmol)	CM-chitosan (mmol)	Elemental analyses (%)			
				C	H	N	S
CM-chitosan	–	–	20	43.82	5.63	5.53	–
TTUCM-chitosan-1	1.25	2.50	20	43.90	4.91	5.71	1.83
TTUCM-chitosan-2	2.50	5.00	20	43.98	4.78	5.95	3.27
TTUCM-chitosan-3	5.00	10.00	20	44.49	4.58	6.25	5.42
TTUCM-chitosan-4	10.00	20.00	20	45.37	4.41	7.76	8.58

and TTUCM-chitosan-4 of increasing degree of cross-linking, respectively.

Measurements

Elemental analyses of the hydrogels were done in Perkin-Elmer (Model 2410 series II) C, H, N, S Analyzer (USA) at the Microanalytical Unit, Cairo University (Egypt).

The reaction between CM-chitosan and terephthaloyl diisothiocyanate was confirmed using Tescan Shimadzu FTIR spectrophotometer (Model 8000, Japan). Hydrogel samples were ground well to make KBr pellets under hydraulic pressure of 400 kg/cm^2 and spectra were recorded in the range of 500–4,000 cm^{-1}. In each scan, the amount of hydrogel sample and KBr were kept constant in order to know the changes in the intensities of the characteristic peaks with respect to the amount of terephthaloyl diisothiocyanate.

^{13}C NMR spectra were recorded with Jeol 300 MHz (Japan) in DMSO as a solvent and the chemical shifts were recorded in ppm relative to (TMS) as internal standard.

Scanning electron microscopy observations of the hydrogels were carried out as follows: The dry samples spread on a double sided conducting adhesive tape, pasted on a metallic stub, were coated with a gold layer of 100 μm thickness using an ion sputter coating unit (Jeol S150A) for 2 min and observed with a Jeol-JXA-840A Electron Probe Microanalyzer. All the SEM photomicrographs were obtained using an accelerating voltage of 20 kV and at a magnification of 400×.

Powder X-ray diffraction patterns of the hydrogel samples were obtained using Brukur D8 Advance-Germany with a Ni monochromator. The power level was 40 kV/40 mA. The X-ray source was CuKα radiation. The samples were maintained stationary while scattering angles from 4° to 60° were scanned in the reflection mode at a scanning rate of 1° min^{-1}.

Determination of the soluble fraction of the hydrogels: Weighed samples of each hydrogel were stirred overnight in 10 ml of each of the following solvents: acetic acid solution (1 % v/v), DMF, DMSO, THF, NMP, chloroform, methylene chloride, acetone and methanol. The swollen samples were then dried in oven at 60 °C to constant weights. The soluble fraction was calculated according to the following equation: soluble fraction (%) $= [(W_o - W_1)/W_o] \times 100$, where W_o, is the initial weight of the hydrogel and W_1, is the weight of the oven dried hydrogel.

Determination of the swell ability of the hydrogels in various solvents: A known weight of the dry hydrogel sample was immersed in a single solvent (acetic acid solution (1 % v/v), DMF, DMSO, and H_2O) and kept undisturbed at room temperature until equilibrium swelling was reached. The swollen sample was then removed from the immersion media, quickly wiped with filter paper to remove the droplets on its surface and reweighed. The percent swelling was calculated using the following equation: Swelling (%) $= [(W_1 - W_o)/W_o] \times 100$, where W_o, is the weight of the dry hydrogel and W_1, is the weight of the swollen hydrogel. Swelling measurements were made in triplicate, and the error was estimated to be within 1 %.

Antibacterial activities were investigated using agar well diffusion method. The activity of tested samples was studied against the *S. aureus* (RCMB 000106) and *B. subtilis* (RCMB 000107) (as Gram positive bacteria) and *E. coli* (RCMB 000103) (as Gram negative bacteria). Centrifuged pellets of bacteria from a 24 h old culture containing approximately 104–106 CFU (colony forming unit) per ml were spread on the surface of nutrient agar (tryptone 1 %, yeast extract 0.5 %, NaCl 0.5 %, agar 1 %, 1,000 ml of distilled water, PH 7.0) which was autoclaved under 121 °C for at least 20 min. Wells were created in medium with the help of a sterile metallic bores and then cooled down to 45 °C. The activity was determined by measuring the diameter of the inhibition zone (in mm). 100 μl of the tested samples (10 mg/ml) were loaded into the wells of the plates. All compounds were prepared in DMSO, DMSO was loaded as control. The plates were kept for incubation at 37 °C for 24 h and then the plates were examined for the formation of zone of inhibition. Each inhibition zone was measured three times by caliper to get an average value. The test was performed three times for each bacterium culture: penicillin and streptomycin were used as antibacterial standard drugs (Rahman et al. 2001).

Antifungal activities were investigated by screening the tested samples separately in vitro against various fungi (*A. fumigatus*, RCMB 002003; *G. candidum*, RCMB 052006; and *C. albicans*, RCMB 005002), on Sabouraud dextrose agar plates. The culture of fungi was purified by single spore isolation technique. The antifungal activity was by agar well diffusion method (Rathore et al. 2000) as follows:

Sabouraud dextrose agar plates: A homogeneous mixture of glucose-peptone-agar (40:10:15) was sterilized by autoclaving at 121 °C for 20 min. The sterilized

solution (25 ml) was poured in each sterilized petridish in laminar flow and left for 20 min to form the solidified Sabouraud dextrose agar plate. These plates were inverted and kept at 30 °C in incubator to remove the moisture and to check for any contamination.

Antifungal assay: Fungal strain was grown in 5 ml Sabouraud dextrose broth (glucose:peptone; 40:10) for 3–4 days to achieve 105 CFU/ml cells. The fungal culture (0.1 ml) was spread out uniformly on the Sabouraud dextrose agar plates by sterilized triangular folded glass rod. Plates were left for 5–10 min so that culture is properly adsorbed on the surface of Sabouraud dextrose agar plates.

Now small wells of size (4 mm × 2 mm) were cut into the plates with the help of well cutter and bottom of the wells were sealed with 0.8 % soft agar to prevent the flow of test sample at the bottom of the well. 100 µl of the tested samples (10 mg/ml) were loaded into the wells of the plates. All compounds was prepared in DMSO, DMSO was loaded as control. The plates were examined for the formation of zone of inhibition. Each inhibition zone was performed three times for each fungus. Clotrimazole and itraconazole were used as antifungal standard drugs.

To determine the minimum inhibition concentration (MIC) of tested samples, the agar plate method was used; two-fold serial dilutions of each sample were added to nutrient broth for bacteria (beef extract 5 g, peptone 10 g added to 1,000 ml distilled water, pH 7.0) and to Sabouraud dextrose broth for fungi, DMSO was used as the control. Then they were heated in autoclave at 121 °C for 25 min. The culture of each organism was diluted by sterile distilled water to 105–106 CFU/ml, a loop of each suspension was inoculation, the plates were incubated at 37 °C for 24 h for bacteria, and at 30 °C for 3–4 days for fungi. The colonies were counted and the MIC values were obtained. The MIC was considered to be the lowest concentration that completely inhibits against inoculums comparing with the control, disregarding a single colony or a faint haze caused by the inoculums (Damyanova et al. 2000).

Results and discussion

Carboxymethyl chitosan was modified with terephthaloyl diisothiocyanate via reacting of both the isothiocyanate groups with the amino groups of CM-chitosan (Scheme 1b). The amount of terephthaloyl diisothiocyanate (Scheme 1a) with respect to CM-chitosan was varied as described in Table 1 to produce four new terephthaloyl thiourea cross-linked CM-chitosan hydrogels (Scheme 1b) designated as TTUCM-chitosan-1, TTUCM-chitosan-2, TTUCM-chitosan-3, and TTUCM-chitosan-4 of increasing degree of cross-linking, respectively, based on their sulphur contents which were determined by elemental analyses (Table 1).

FTIR characterization of CM-chitosan

FTIR spectroscopy was employed to detect the structural changes of chitosan and CM-chitosan (Fig. 1). The FTIR spectrum of chitosan showed four strong absorption peaks at 1,155, 1,073, 1,030, and 895 cm^{-1} which were characteristic peaks of the saccharide structure. The very strong broad absorption peak around 3,600–3,200 cm^{-1} should be assigned to the stretching vibration of OH, the extension vibration of the NH, and the intermolecular hydrogen bonds of the polysaccharide. Primary amines have two absorption peaks in this region. There were weak absorption peaks at 1,653 and 1,567 cm^{-1} corresponded to amide I and amide II, respectively, which indicated that chitosan had a high deacetylation degree. The FTIR spectrum of CM-chitosan showed, in addition to the above peaks, a strong absorption peak at 1,454 cm^{-1} which could be assigned to the symmetrical stretching vibration of COO^- group. The asymmetrical stretching vibration of COO^- group (around 1,550 cm^{-1}) is overlapped with the deforming vibration of NH_2 at 1,604 cm^{-1} to obtain a very strong peak. The C–O absorption peak became stronger and moved to 1,097 cm^{-1}. The results, which are in accordance with the work of Xie et al. (2002), indicated that substitution occurred at the C6 position.

FTIR characterization of TTUCM-chitosan hydrogels

Figure 2 shows a comparison of the transmission FTIR spectra for four TTUCM-chitosan hydrogels with CM-chitosan. The FTIR spectra of TTUCM-chitosan hydrogels are concerned, first; the broad band between 3,600 and 3,200 cm^{-1} due to the OH and NH group stretching vibration is observed. In addition, the characteristic absorbance of NH_2 at 1,604 cm^{-1} disappeared; these results show that NH_2 group had reacted with terephthaloyl diisothiocyanate. This is also well illustrated by the disappearance of the doublet peak at 3,435 and

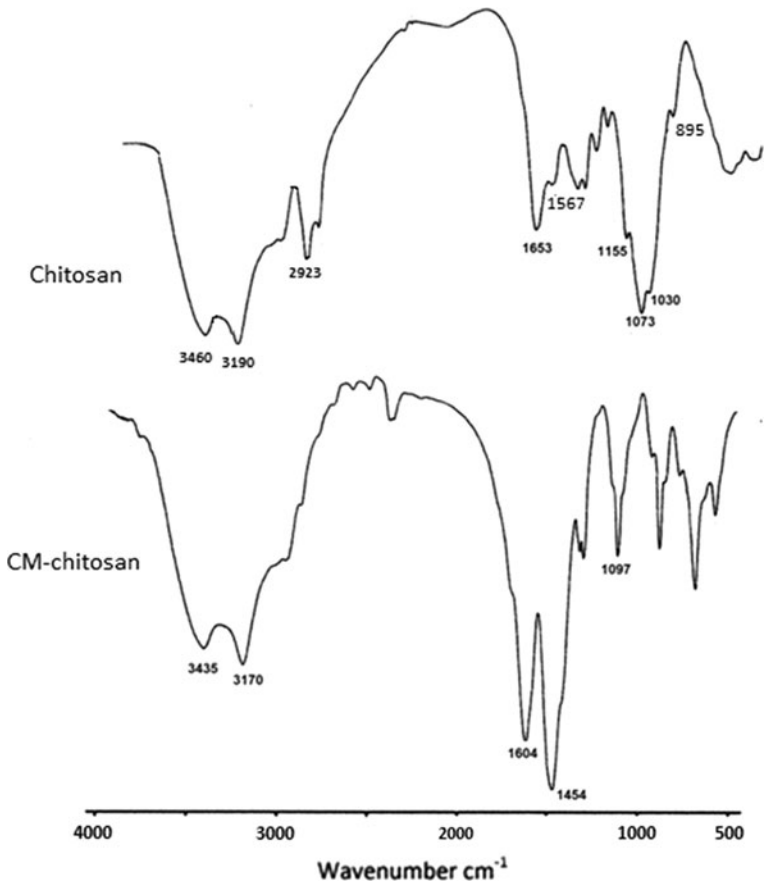

Fig. 1 FTIR spectra of chitosan and CM-chitosan

3,170 cm^{-1} corresponding to the $–NH_2$ group and the appearance of a single peak around 3,436 cm^{-1} for NH group. Second; the characteristic broad absorption band around 1,652 cm^{-1} assigned the overlap of C=O, NH (secondary amide) and C=C (phenyl), the intensity of this band increased with increasing the cross-linking density of the hydrogels, i.e., from TTUCM-chitosan-1 to TTUCM-chitosan-4. Third; the strong broad absorption band around 1,060 cm^{-1} corresponded to C=S group overlapped with C–O stretching vibration. The bending vibration around 1,420 cm^{-1} and around 575 cm^{-1} indicate the absorption of N–C–S group. Again intensity of these bands increased with increasing the cross-linking density of the hydrogels. These evidences confirm the structure of the TTUCM-chitosan hydrogels.

^{13}C NMR characterization of TTUCM-chitosan hydrogels

^{13}C NMR spectrum of TTUCM-chitosan-4 hydrogel was shown in Fig. 3. The signals can be identified as follows: $\delta = 63.65$ ppm attributed to C2; $\delta = 65.24$ ppm represented carbon C6; $\delta = 65.55$ ppm attributed to C3 and C5 (superimposed); $\delta = 69.71$ ppm represented C1 and C4 (superimposed); $\delta = 72$ ppm attributed to C7; $\delta = 127.01$, 128.57, 131.41 and 131.63 ppm attributed to aromatic carbons; $\delta = 166.86$ ppm and $\delta = 196.3$ ppm attributed to C=O and C=S respectively. These signals confirm the structure of the TTUCM-chitosan-4 hydrogel.

Scanning electron microscopy observations of TTUCM-chitosan hydrogels

Microstructures of the hydrogels surface were investigated by scanning electron microscopy as presented in Fig. 4. It could be seen that the hydrogels have a channel like surface with an extremely porous structure. While the hydrogels have a similar surface appearance, the distribution and the size of their pores are different. The distribution of porosity became more uniform and

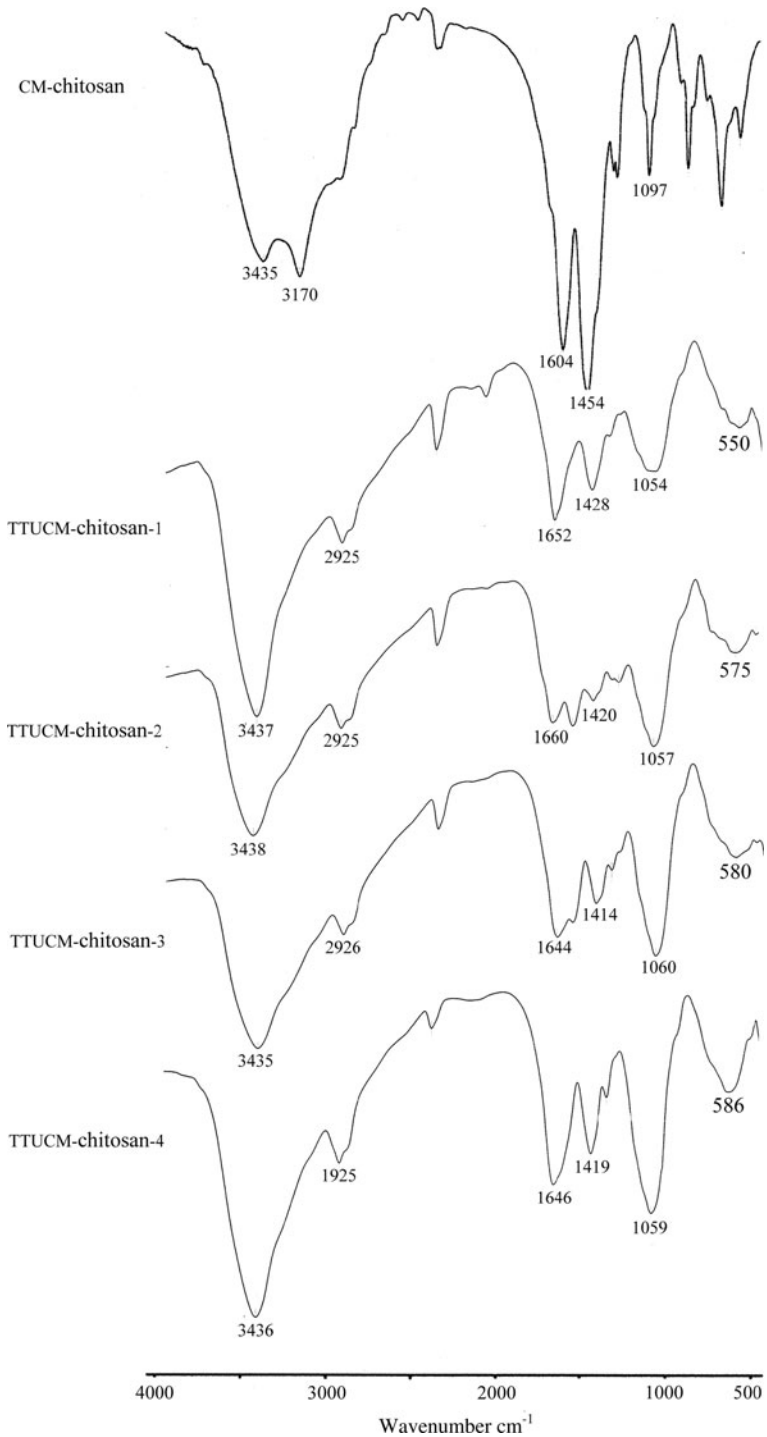

Fig. 2 FTIR spectra of CM-chitosan and TTUCM-chitosan hydrogels

dense with increasing concentration of terephthaloyl thiourea cross-linking moieties incorporated into the gel (TTUCM-chitosan-4 hydrogel). When terephthaloyl thiourea content rose, from TTUCM-chitosan-1 to TTUCM-chitosan -4, it would be expected that the equilibrium water content of the hydrogels increased, which led to more pores in hydrogels. The microporous surface structure of the hydrogels could lead to high surface areas with low diffusion resistance in the matrix. This expectation may be supported by the pore

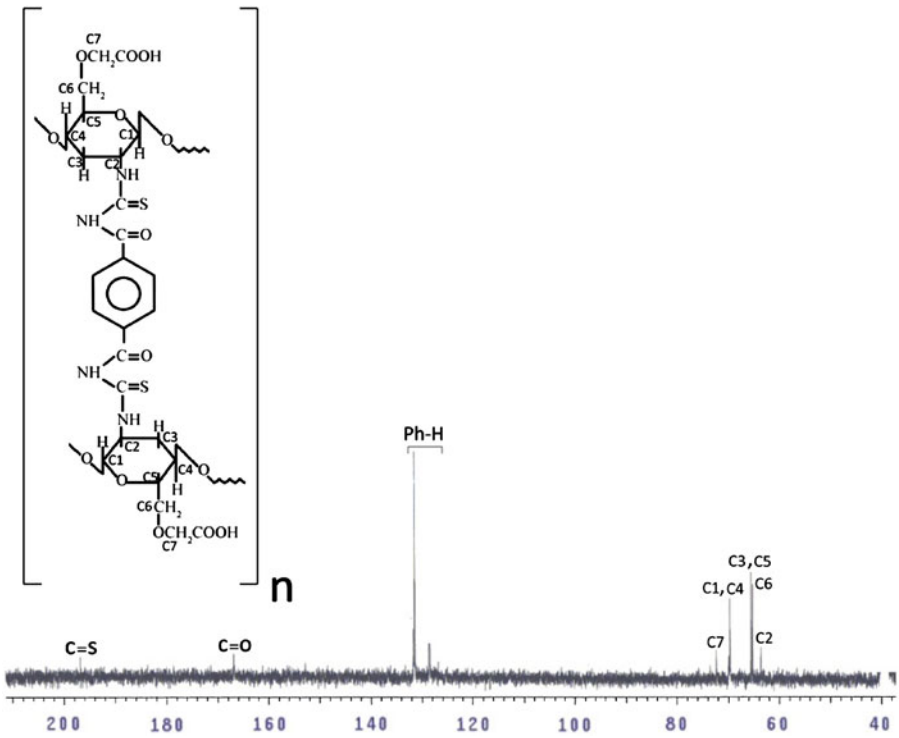

Fig. 3 ^{13}C NMR spectrum of TTUCM-chitosan-4 hydrogel

size of the hydrogels which decreased with increasing the cross-linking density of the hydrogels, e.g., from TTUCM-chitosan-1 to TTUCM-chitosan-4 hydrogels.

X-ray diffraction of TTUCM-chitosan hydrogels

X-ray diffraction was employed to study the effect of cross-linking on the hydrogel morphology and the patterns are presented in Fig. 5. It could be noted that the hydrogels are less crystalline than the parent CM-chitosan. The crystallinty of the hydrogels decreases with increasing their degree of cross-linking. This is because of a significant change in the functional groups of CM-chitosan occurred after cross-linking. It is suggested that a significant level of hydrogen bonding in the CM-chitosan powder was reduced after cross-linking via NH_2 groups, thus forming a smaller fraction of crystalline phase and a larger fraction of amourphous phase.

Solubility of TTUCM-chitosan hydrogels in various solvents

It is well known that the high hydrophilicity of CM-chitosan is due to the primary amine and the carboxylic groups that make CM-chitosan readily soluble in water and in dilute acid and alkali solutions. Hence the solubility of the new hydrogels was studied in different solvents at room temperature. The results show that the hydrogels are insoluble in acetic acid solution (1 % v/v), DMF, DMSO, THF, NMP, chloroform, methylene chloride, acetone and methanol since no soluble fraction of all the hydrogels were obtained. This indicates successful formation of cross-linked network in these hydrogels.

Swell ability of TTUCM-chitosan hydrogels in various solvents

The results of swelling of TTUCM-chitosan hydrogels in various solvents have been summarized in Table 2. These results represent the average of three comparable experiments for each sample. The data showed that all the hydrogels are greatly swelled in all the investigated solvents, as they are much hydrophilic in nature due to their highly polar thiourea moieties which can form much more hydrogen bonds with the investigated solvents. The highest degree of swelling for the hydrogels is observed with acetic acid solution (1 % v/v) as compared with the aprotic solvents which may be attributed to the basicity of the thiourea moieties of the cross-linker. The highest swelling degree for the aprotic solvent is observed in DMSO due to its higher polarity. On increasing the cross-linking density, the swell ability increases. This is well illustrated from the

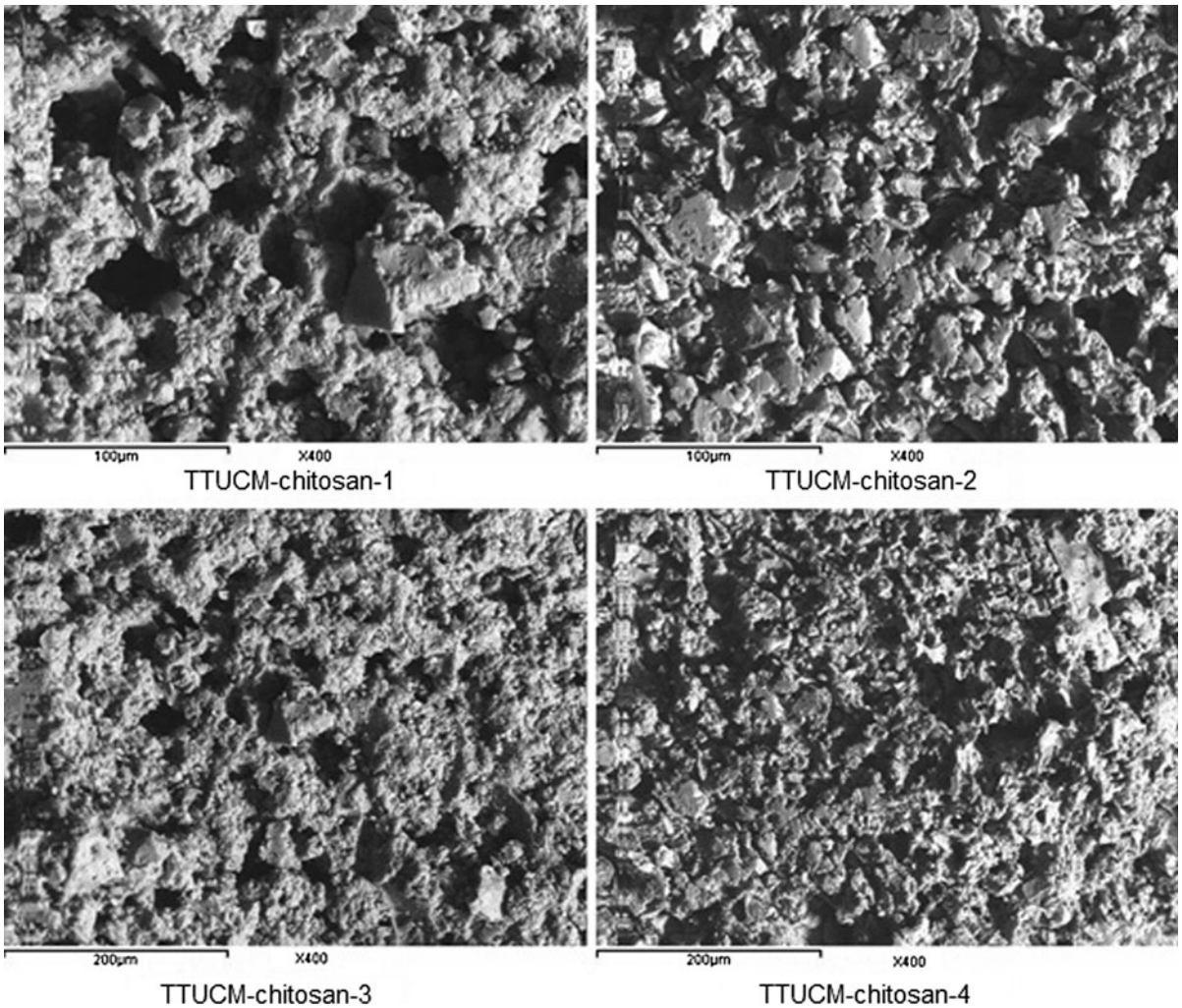

Fig. 4 SEM of TTUCM-chitosan hydrogels at a magnification of ×400

data reported for swell ability in case of TTUCM-chitosan-4 as compared with that of the TTUCM-chitosan-1 hydrogel irrespective to the nature of the investigated solvents. This experimental finding may be attributed to the fact that increasing the cross-linking density lowered the total attraction forces between the neighboring chains of CM-chitosan, thus permitting more diffusion of the solvents between the chains and consequently increased its swell ability. Also, the hydrophilicity of the hydrogels increases with increasing the cross-linking density.

The antibacterial activity of TTUCM-chitosan hydrogels

Table 3 shows the antibacterial activity of the CM-chitosan and TTUCM-chitosan hydrogels using inhibition zone method. Compared with CM-chitosan, all the hydrogels have a higher antibacterial activity. Several mechanisms elucidating the antimicrobial activity of chitosan have been postulated. The most acceptable mechanism is the interaction between positively charged chitosan molecules and negatively charged microbial cell membrane. The interaction is mediated by the electrostatic forces between the protonated NH_3^+ groups of chitosan and the electronegative charges on the microbial cell surfaces. This electrostatic interaction results in twofold interference: (1) by promoting changes in the properties of membrane wall permeability, thus provoke internal osmotic imbalances and consequently inhibit the growth of the microorganisms, and (2) by the hydrolysis of the peptidoglycans in the microorganism wall,

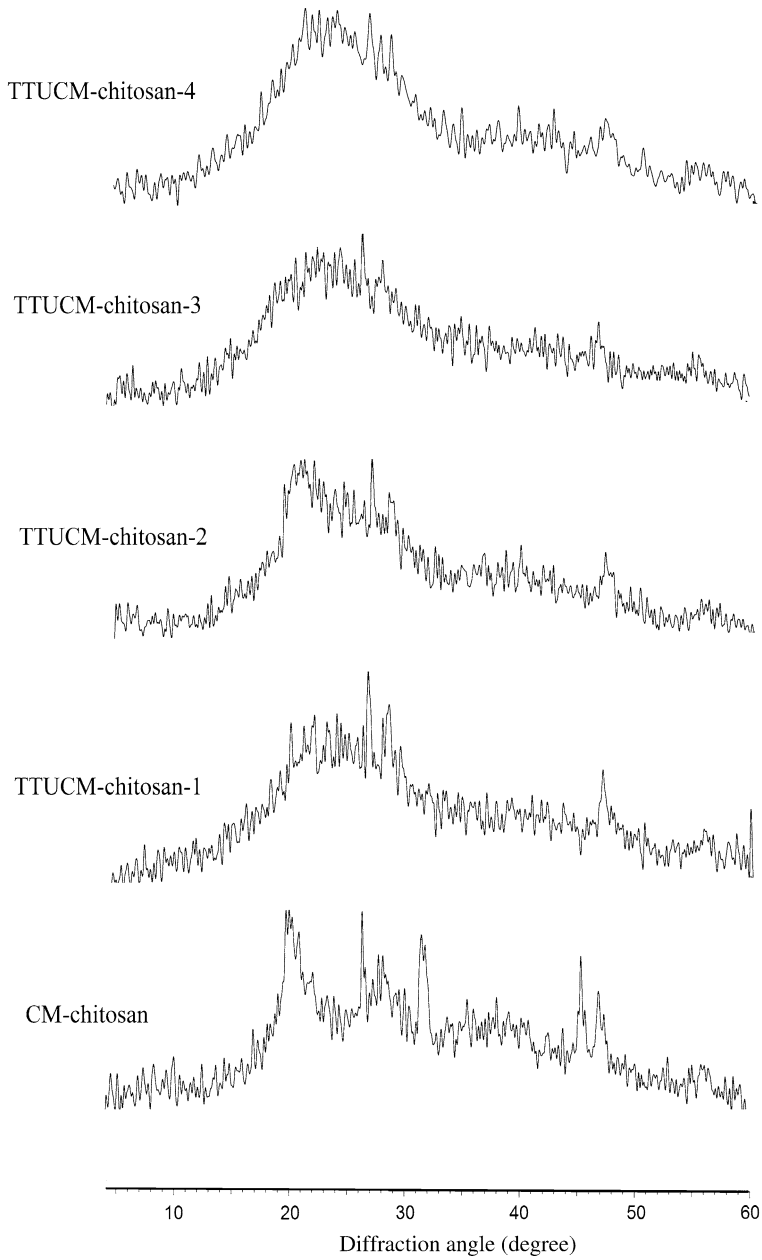

Fig. 5 Powder X-ray diffraction of CM-chitosan and TTUCM-chitosan

Table 2 Swell ability of TTUCM-chitosan hydrogels in various solvents

Solvent	Swell ability (%)			
	TTUCM-chitosan-1	TTUCM-chitosan-2	TTUCM-chitosan-3	TTUCM-chitosan-4
H_2O	1,385	1,542	1,575	2,172
DMF	1,193	1,841	2,171	2,392
DMSO	1,505	2,089	2,458	2,686
Acetic acid	2,324	2,400	2,681	2,784

Table 3 Inhibition indices of TTUCM-chitosan hydrogels against *B. subtilis*, *S. aureus* and *E. coli*

Samples	Inhibition zone (mm)		
	Tested microorganisms		
	B. subtilis (RCMB 000107)	*S. aureus* (RCMB 000106)	*E. coli* (RCMB 000103)
CM-chitosan	11.3 ± 0.08	10.1 ± 0.20	0
TTUCM-chitosan-1	16.2 ± 0.15	14.1 ± 0.19	9.7 ± 0.22
TTUCM-chitosan-2	19.3 ± 0.28	16.2 ± 0.16	11.6 ± 0.27
TTUCM-chitosan-3	20.3 ± 0.19	17.2 ± 0.22	13.3 ± 0.39
TTUCM-chitosan-4	21.3 ± 0.28	20.2 ± 0.23	16.7 ± 0.15

leading to the leakage of intracellular electrolytes such as potassium ions, and other low molecular weight proteinaceous constituents (e. g. protein, nucleic acid, glucose, and lactate dehydrogenase) (Feng et al. 2000). Since such mechanism is based on electrostatic interaction, it suggests that the greater the number of cationized amines, the higher will be the antimicrobial activity. Carboxymethylation of chitosan allowed the synthesis of CM-chitosan with higher hydrophilicity, with better solubility in aqueous media and with greater positive charge density; where in CM-chitosan the –COOH groups may react with the NH_2 groups intra or intermoleculary and changed these NH_2 groups into NH_3^+ groups leading to increased polycationic character (non-pH dependent positive charges on CM-chitosan). Further, the introduction of acyl thiourea moieties onto CM-chitosan increases its solubility both in organic and aqueous media (Aranaz et al. 2010) and also increases its cationic centers; thus their C=O, NH, and C=S groups can be protonated and consequently the net positive charge was strengthened, leading to a better antibacterial activity. Another proposed mechanism is the binding of chitosan with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis via penetration of chitosan into the nuclei of the microorganisms (Hadwiger et al. 1986). Terephthaloyl thiourea moieties incorporated onto hydrophilic CM-chitosan apart the CM-chitosan chains away from each other, decrease their intermolecular hydrogen bonds, and increase their solubility; the reason for the easy of penetration of the TTUCM-chitosan hydrogels into the cells of microorganisms and prevent the growth of the cell by preventing the transformation of DNA to RNA to obtain a higher antibacterial activity. The third mechanism is the chelation of metals, suppression of spore elements and binding to essential nutrients to microbial growth (Cuero et al. 1991). It is well established that the carboxylic and thiourea moieties have excellent metal-binding capacity (Arslan et al. 2009; Emara et al. 2011). This explains the observed higher antibacterial activity of TTUCM-chitosan hydrogels relative to the parent CM-chitosan.

Moreover, the TTUCM-chitosan hydrogels were more active against the Gram-positive bacteria than against the Gram-negative bacteria (Table 3). As the strongest TTUCM-chitosan-4 hydrogel caused inhibition zone diameter *of B. subtilis* and *S. aureus* of 21.3 ± 0.28 and 20.2 ± 0.23 mm, respectively, corresponded to 16.7 ± 0.15 mm of *E. coli*. This may be attributed to their different cell wall. The cell wall of Gram-positive bacteria is fully composed of peptide polyglycogen. The peptidoglycan layer is composed of net works with plenty of pores, which allow foreign molecules to come into the cell without difficulty and allow more rapid absorption of ions into the cell. But the cell wall of the Gram-negative bacteria is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipids. Because of the complicated bilayer cell structure, the outer membrane is a potential barrier against foreign molecules with high molecular weight. Therefore, the hydrogels have different effects on the two kinds of bacteria. An additional evidence for the greater activity of the TTUCM-chitosan hydrogels against Gram-positive bacteria than that against Gram-negative bacteria comes from their minimum inhibitory concentration (MIC) values. MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Since the MIC values of the strongest TTUCM-chitosan-4 hydrogel against *B. subtilis* and against *S. aureus* were 0.98 and 3.9 μg/ml, the MIC value against *E. coli* was 31.3 μg/ml.

Table 4 Inhibition indices of TTUCM-chitosan hydrogels against *A. fumigatus*, *G. candidum* and *C. albicans*

Samples	Inhibition zone (mm)		
	Tested microorganisms		
	A. fumigatus (RCMB 002003)	G. *candidum* (RCMB 052006)	*C. albicans* (RCMB 005002)
TTUCM-chitosan	12.1 ± 0.10	9.3 ± 0.09	5.4 ± 0.07
TTUCM-chitosan-1	15.1 ± 0.34	12.4 ± 0.26	6.4 ± 0.13
TTUCM-chitosan-2	17.2 ± 0.18	15.4 ± 0.50	7.9 ± 0.12
TTUCM-chitosan-3	19.1 ± 0.23	17.2 ± 0.15	12.6 ± 0.19
TTUCM-chitosan-4	21.5 ± 0.28	20.9 ± 0.19	16.7 ± 0.14

From Table 3, it is interesting to note that the TTUCM-chitosan-1 hydrogel showed the lowest antibacterial activity, relative to the other hydrogels. On the other hand, the TTUCM-chitosan-4 hydrogel exhibited the highest antibacterial activity. The activity of the other hydrogels lies in between these two cases. The different inhibitory effect may be attributed to thiourea moieties content incorporated into the hydrogels. Thus, the inhibitory effect increased with increasing the cross-linking density.

The antifungal activity of TTUCM-chitosan hydrogels

The antifungal activities of TTUCM-chitosan hydrogels against *A. fumigatus*, *G. candidum and C. albicans* are shown in Table 4. The results show that all the hydrogels had effective activities against the tested fungi, compared with the parent CM-chitosan, with inhibitory indices ranging from 6.4 ± 0.13 to 21.5 ± 0.28 mm inhibition zone (Table 4). Moreover, the MIC values of the strongest TTUCM-chitosan-4 hydrogel against *A. fumigatus*, *G. candidum and C. albicans* were 0.98, 1.95, and 31.3 μg/ml, respectively. Generally chitosan has been reported as being very effective in inhibiting spore germination, germ tube elongation and radial growth (El-Ghaouth et al. 1992). The antifungic mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly with fungal growth, similarly to the effects observed in bacteria cells (El-Ghaouth et al. 1992). The microscopic observation reported that chitosan molecules diffuse inside hyphae interfering on the enzymes activity responsible for the fungus growth (Eweis et al. 2006). The high swell ability of TTUCM-chitosan hydrogels is expected to enhance the diffusion of the active ingredient inside the pathogens, which may lead to a disturbance of the enzyme activities responsible for the growth criteria, instead of the adsorption of the insoluble compounds on the fungal hyphae surface. The results also showed that the highest antifungal activity was observed for TTUCM-chitosan-4. This may be due to its higher thiourea moieties content. Pentachloronitrobenzene and chlorothalonil are usually used as fungicides, however, the chloro–groups in these fungicides constitute a big problem in the environment due to their toxicity (Nester et al. 2003; Avadi et al. 2004). TTUCM-chitosan hydrogels may induce lower pollution to the environment.

Conclusion

Four novel terephthaloyl thiourea carboxymethyl chitosan (TTUCM-chitosan) hydrogels have been successfully synthesized via cross-linking different concentrations of terephthaloyl diisothiocyanate onto CM-chitosan chains. These hydrogels were characterized by much better antimicrobial activity than the parent CM-chitosan. The results also indicated that the TTUCM-chitosan hydrogels have stronger activity against Gram-positive bacteria than Gram-negative bacteria. The degree of cross-linking of the hydrogels was related to antimicrobial activities; higher cross-linking resulted in stronger antimicrobial activity.

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