Biomaterials based on *N, N, N*-Trimethyl chitosan fibers in wound dressing applications

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Abstract:

In the present work, *N, N, N*-Trimethyl chitosan (TMC) fibers were synthesized successfully and the resulting quaternized materials were characterized by FTIR. The designed TMC fibers with different degree of quaternization achieved high water absorption capability. In antibacterial activity study, TMC fibers showed high antibacterial activity than chitosan fibers against the Gram-negative bacteria *Escherichia coli* (> 63%) and Gram-positive bacteria *Staphylococcus aureus* (> 99%). TMC fibers exhibited no obvious cytotoxicity to mouse embryo fibroblast cells with low extraction concentrations (< 0.05 g/mL). In animal wound healing test, TMC2 fibers could significantly enhance wound re-epithelialization and contraction compared with the control (chitosan fibers). In conclusion, TMC fibers have a potential to be used as wound dressing materials.

Keywords: *N, N, N*-Trimethyl chitosan fibers; Wound dressing; Antibacterial activity; Wound healing

1. Introduction

The skin is the largest organ in the human body and acts as an anatomical barrier from pathogens and any damage, and it also plays a key role in protecting the body against excessive water loss. Skin is often injured by wounding or physical trauma, and damages to skin initiate a series of complicated and orchestrated processes involving the interactions of various cells and matrix components to restore of this functional barrier [1, 2]. Therefore, skin generally needs to be covered with a dressing immediately after it is damaged.

An ideal dressing should maintain a moist environment at the wound interface, allow gaseous exchange, act as a barrier to microorganisms and remove excess exudates [3]. The materials should possess suitable mechanical property, non-toxic, anti-infective property, satisfactory water vapor transmission rate (WVTR) [4] and be removed easily without trauma, among other features.

Current strategies for dealing with skin damage are focused on the acceleration of the wound repair by systematically designed dressing materials [3]. Many wound dressings have been created, such as alginate [5], collagen [6], and chitosan (CS) [7]

with different kinds of forms, e.g., films, foams, fiber or hydrophilic gel. Among these materials, CS fibers have been proposed as safe wound dressing material [8], due to excellent biological properties such as biodegradability, biocompatibility [9]. However, for chitosan fibers, poor liquid absorbing capacity and especially weak antibacterial activity in solid state make it not appropriate to be used in current form when dealt with wounds. Chitosan derivatives achieved by quaternization have shown high bactericidal and liquid absorbance activity [10]. Quaternary ammonium chitosan fibers not only retain the property of chitosan fibers but also enhance the antimicrobial activity [11], which meet the requirement of the wound dressing. In addition, quaternized chitosan fibers could have high liquid absorbance capacity to provide the wound with a moist environment, which prevents excessive eschar formation and chronic clinical problems [12]. In previous reported, quaternized chitosan fibers (QCFs) were prepared and showed high antibacterial activity and nontoxic to the L929 cell [13]. However, it was found that the antimicrobial action was more efficient when the cationic charge is located at the amino group of the chitosan and not on the quaternary substituent [14, 15]. Besides, it was important to evaluate a wound dressing with wound healing experiment.

In the current study, the quaternary ammonium moiety was introduced onto the chitosan backbone directly. *N, N, N*-Trimethyl chitosan (TMC) fibers, the simplest form of quaternary ammonium chitosan derivatives with different degree of quaternization (DQ) were obtained by modifying the surface of CS fibers with iodomethane. The structures of CS fibers and TMC fibers were evaluated by FTIR. Then, the water-absorption ability, antibacterial activity and cytotoxicity of the TMC fibers were also studied. Finally, wound healing capacity of CS fibers and TMC fibers was evaluated using animal wound models. The results indicated that the TMC fiber mats could serve as an ideal wound dressing for wound protection or wound bed preparation.

2. Materials and methods

2.1 Materials

Chitosan fibers (CS fibers, viscosity = 1092 cps, degree of deacetylation = 91.5%,

linear-density = 3.1 cN) were purchased from Qingdao Jifa Group Co., Ltd (Shandong, China). Methyl iodide (CH₃I), Dimethyl Sulfoxide (DMSO) and sodium hydroxide (NaOH) were obtained from Qingdao Yunshan Biotechnology Co., Ltd. Dulbecco's modified Eagle's medium (DMEM), thiazolyl blue tetrazolium bromide (MTT) and D'Hanks Buffer were purchased from Huasheng Chemical. The test strains, *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) were obtained from Microbiology Laboratory of Ocean University of China. Mouse embryo fibroblasts (MEFs) were obtained through primary culture. Other reagents were all analytical grade.

2.2. Synthesis and characterization of TMC fibers

2.2.1. Synthesis

TMC fibers were synthesized by methylation of CS fibers with iodomethane under alkaline conditions. In detail, 1 g of CS fibers was added to a mixture solution (40 mL of DMSO and 6 mL of 15% (w/v) NaOH solution). After 10 min, methyl iodide was added and the reaction mixture was refluxed at 45 °C for 15 min. The dosage of methyl iodide was changed to obtain three kinds of TMC fibers with different DQ. The reaction was stopped by dropping in a 50 mL ethanol. The obtained TMC fibers were washed in a mixture of 10% (w/v) NaCl solution and ethanol (25:75) for ion-exchange. The DQ of the TMC fibers was measured by titrating the amount of Cl⁻ ions on the TMC with aq AgNO₃ solution [16].

2.2.2. The Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectra (FTIR) of CS fibers and TMC fibers recorded by method of KBr pellets on a NEXUE470 spectrometer (Nicolet, Madison, USA).

2.3 Swelling behaviors

The swelling study of the samples was determined by a gravimetric method [17]. Initially, samples were prepared and the dry weights were accurately measured. Then each sample was immersed in certain amount of deionized water at room temperature for 1 h. After that, excess water on the surface of swollen hydrogels was blotted out with filter paper and the weight of specimens was determined. The water absorptivity of the materials is defined using the following equation:

$$\text{Water absorption (\%)} = (w - w_0)/w_0 \times 100\%$$

Where w_0 and w represent the weight of the sample before and after immersion in water, respectively.

2.4. Antimicrobial activity

Antibacterial activity test was performed by recording the number of growing bacteria colonies on the Petri dish. One loopful of the bacteria was inoculated in nutrient broth (NB), which shook in a shaker at 37 °C for 18 h. The cultures of *E. coli* and *S. aureus* containing $\sim 10^6$ CFU/mL were prepared by appropriately diluting with NB and used for the antibacterial test. CS fibers and TMC fibers of 100 mg were placed into solution which contained 1mL of the bacterium suspensions and 9 mL 0.1 mol/L PBS solution, followed by incubation in a shaker at 37 °C for 18 h. Control experiment (without the addition of CS fibers or TMC fibers) was also conducted. At last, 100 μ L of the bacterial broth was spread onto agar plates. The numbers of colonies were counted after incubation for 18 h at 37 °C and the ratio of inhibited the growth of bacteria (IRG) was calculated which is defined as:

$$\text{IRG (\%)} = (N_a - N_b)/N_a \times 100\%$$

Where N_a and N_b are the average values of colonies of the control group and the experimental groups, respectively.

2.5. Cytotoxicity assays

In vitro cell viability in the presence of TMC fibers was investigated by the MTT assay using MEFs. MEFs were cultured in supplemented with 10% fetal bovine serum at 37 °C in 5% CO₂. The cells were seeded on 96-well plates at a density of 1×10^4 cells per well and cultured for 12 h. Subsequently, the medium was replaced with fresh medium with a different concentration of the extract of the fibers (ranging from 0.0125 g/mL to 0.1 g/mL). The culture medium was used as blank control. The plates were incubated at 37 °C, 5% CO₂ and 95% relative humidity for 24 h and 48 h. At the end of the incubating, MTT (10 μ L) was added and the plates were incubated for an additional 4 h. Supernatants were removed and the formazan salts were dissolved

followed by addition of 150 μ L DMSO on a shaking water bath at 37 $^{\circ}$ C for 10 min. The absorption values were measured at 490nm. The relative growth rate (RGR) was expressed by the following equation:

$$\text{RGR (\%)} = D_t / D_{nc} \times 100\%$$

Where D_t and D_{nc} are the absorbances of the tested sample and the blank control.

2.6. Wound healing test

A full-thickness excision wound model was used to monitor wound closure. Six male rats (Qingdao Coastal Institute for Drug Control, China) weighting 180 - 220 g were used for this study. The animals were randomized into two groups for two time points (6 and 12 days). After hair removal and anesthesia, four 1.5 cm $\times$ 1.5 cm full-thickness wounds were created on the dorsum of each rat. The wounds of three rats were covered with TMC2 fibers equal size to wounds size , and in the control group, the wounds were sterilely covered by chitosan fibers. On top of the wounds, a piece of Tegaderm was applied to prevent the rats from removing the treatments. The dressings were changed every three day. On day 1, 3, 6, 9 and 12 post-surgery, the wounds were photographed. The wound size was determined by IPP 6.0 software based on the pictures. The amount of wound healing was calculated using the following formula:

$$\text{Amount of wound healing (\%)} = (A_0 - A_t) / A_0 \times 100\%$$

Where A_0 represents the area of the initial wound and A_t is the open area of wound at the time of biopsy on day 3, 6, 9 and 12 accordingly.

2.7. Histological examination

On day 6 and 12, the animals were scarified under anesthesia, and wounds were removed from animals for histological examination. Skin specimens were fixed in 10% buffered formalin, processed, embedded in paraffin. Histology analysis was assessed from the wound sections stained with hematoxylin and eosin (H&E). All sections were analyzed using light microscopy.

3. Results and discussion

3.1. Preparation and characterization of TMC fibers

TMC fibers were typically synthesized by direct quaternization of the amino groups of CS fibers using alkyl halides under alkaline conditions. Alkyl groups were easily introduced into the amine of CS fibers. The degree of quaternization can be accurately tailored by varying the constant of CH_3I while keeping reaction temperature and reaction time (Table 1), which were 19%, 25% and 32%, respectively.

The FTIR spectrum of CS fibers and TMC fibers showed peaks assigned to the saccharide structure at 898 cm^{-1} and 1154 cm^{-1} seen in Fig. 1 [18]. The sharp band at 2879 cm^{-1} was due to the stretching vibration of backbone aliphatic C-H [12], and the band at 2353.7 cm^{-1} was the stretching of CO_2 in the environment. The band due to angular deformation of N-H (1597.8 cm^{-1}) was quite reduced for TMC fibers, which was caused by trimethylation of the primary amine. The new band at 1453 cm^{-1} in TMC fibers, was correspond to an asymmetrical stretching of C-H in the methyl groups. These indicated introduction of quaternary ammonium groups onto chitosan chain.

Fig. 1. FTIR spectra of CS fibers and TMC fibers

3.2. Swelling study

The swelling study of the samples was determined by a gravimetric method. The samples were almost saturated after being immersed in deionized water for 1 h. With the increase of DQ, the water absorption capacity of TMC fibers was 100%, 190% and 285% (Table 1), obviously higher than that of CS fibers which had the lowest value of 20%. TMC fibers had more positive charges with a higher DQ value and were more flexible to absorb water, this may be ascribed to the intermolecular

interactions such as van derWaals forces increased [19]. The ability of improved swelling ratio could also reduce wound maceration [20] and prevent eschar development. Hence, the TMC fibers with high water absorption capacity could be a good candidate for wound dressing applications.

Table 1.

Reaction conditions for the synthesis *N*-quaternary compounds and water absorption ability

Sample	CH ₃ I (g)	DQ (%)	Water absorption (%)
CS	–	–	20 ± 3
TMC1	0.3	19	100 ± 6
TMC2	0.6	25	190 ± 5
TMC3	1	32	285 ± 4

3.3. Antibacterial activities

The antibacterial activities of CS fibers and TMC fibers against Gram-negative bacteria *E. coli* and the Gram-positive *S. aureus*, were tested by using the viable cell-counting method. Three kinds of TMC fibers were used to determine the effect of the alkyl substituent on the antibacterial activities. The antibacterial activity was noted that there were an 85% and 92% reduction in viable *E. coli* after treated with TMC2 fibers and TMC3 fibers compared with the control (Fig. 2a). However, the CS fibers and TMC1 fibers showed low antibacterial action (52% and 63%) against *E. coli*. In the case of *S. aureus*, all three types of TMC fibers exhibited a significantly higher antibacterial ratio (> 99%) than the CS fibers (24%) (Fig. 2b).

The results indicated that high DQ of the TMC fibers showed higher antibacterial activity. This outcome was in line with previous report [14]. The quaternary ammonium groups with permanent positive charges can enhance the antibacterial activity of chitosan [21]. The role of the cationic charge in the antimicrobial effect is believed to be associated with the binding of the polymer to the bacterial cell wall [22]. Compared Fig. 2a with 2b, the TMC fibers showed different antimicrobial activities against *E. coli* and *S. aureus*. On the one hand, the presence of negatively

charged teichoic acid (TA) within the surface of *S. aureus* should make them more attractive to the positively charged [23]. Electrostatic interaction occurs between the positive charges on the polymer and the negatively charged anionic components of the bacterial surface, which leads to an increase of bacterial membrane permeability and cell growth inhibition [14]. On the other hand, Gram-negative bacteria possess an outer membrane (OM) composed of lipopolysaccharide (LPS), which provides the bacterium with a hydrophilic surface [24]. Overcoming the OM is a prerequisite for any materials to exert bactericidal activity towards Gram-negative bacteria. Therefore, TMC fibers had stronger antibacterial ability against *S. aureus* than *E. coli*. As *S. aureus* is the most common pathogen responsible for secondary infections in wounds [25], TMC fibers have a good potential use as anti-infective materials in wound healing.

Fig. 2. Inhibition of CS fibers and TMC fibers against *E. coli* (a) and *S. aureus* (b). Bar graphs show * $p < 0.05$ vs CS fiber.

3.4. Cytotoxicity studies

In order to investigate the biocompatibility cytotoxicity of TMC fibers, the cytotoxicity of the materials was evaluated by MTT assay with MEFs. The cytocompatibility results indicated that TMC fibers were not obviously toxic to MEFs with low extraction concentrations (< 0.05 g/mL). The cytotoxicity was also found to be related to the DQ of the derivatives (Fig. 3). Compounds having a higher DQ value (TMC3) were found to exhibit higher cytotoxicity, which owing to high cationic charge densities. Polycations are considered to be cytotoxic because cationic macromolecules could interact with cell membranes [26, 27]. Thus, the results indicated that the TMC fibers were cytocompatible with a low degree of quaternization, which made it good candidate to be used as wound dressing.

Fig. 3. Cell viability measured by MTT assay for CS fibers and TMC fibers extract of different concentrations after 24 h (a) and 48 h (b) of incubation. Bar graphs show * $p < 0.05$ vs CS fibers.

3.5. Wound healing and histology

3.5.1. Wound healing test

In the wound healing test, the full-thickness wounds were created on the dorsum of each rat, and the TMC2 fibers were chosen in this test, which had the high antibacterial activity and low cytotoxicity among three kinds of TMC fibers. The representative images of the wound healings showed that the wound contractions with TMC2 fibers were faster than that of the CS fibers (Fig. 4a). Wound closure was analyzed in each group as a percentage of the reduction in wounded area at day 3, 6, 9 and 12 (Fig. 4b). On the 6th day, the wound areas decreased gradually and achieved approximately 58% when treated with TMC2 fibers, whereas the wound size reduction in CS fibers was 30%. In addition, the wounds were little sign of inflammation or infection formation and seemed to be neat and moist in TMC2 fibers group. However, a scab formed over the surface of wounds in CS fibers group. The results were in line with previous report that the wounds maintained in either a moist or a wet environment were equally effective in promoting healing, when compared with a dry environment [28]. After absorbing wound exudates, a moist environment can enable an easy and painless dressing change without damaging to the newly formed skin, and protect wounds against bacterial invasion [29].

Fig. 4. (a) Micrographs showing wounds on day 0, 3, 6, 9 and 12 after treated with CS fibers and TMC2 fibers. (b) The amounts of wound healing at different times. Bar graphs show * $p < 0.05$ vs CS fibers.

3.5.2. Histological observations

The histological results of the wounds treated with CS fibers and TMC2 fibers were performed on postoperative day 6 and 12 (Fig. 6). Compared with CS fibers group, the length of the newly formed epithelium was significantly increased and the red blood cells in neovascularization were also found in TMC2 fibers group on day 6. The glands and granulation tissue comprised abundant fibroblasts appeared in TMC2 fibers group on day 12. Moreover, a number of inflammatory cells infiltrations were detected after treated with CS fibers. The results indicated TMC2 fibers exhibited higher ability of re-epithelialization and well-organized granulation tissue, compared with CS fibers group.

Fig. 5. Histology of wounds at 6 days and 12 days post-surgery. I: Inflammatory corpuscle, G: Glands, N: Neovascularization.

4. Conclusions

TMC fibers with various DQ were synthesized by modified the surface of CS fibers under different reaction conditions. The study showed TMC fibers had higher absorption capacity, higher antibacterial property compared with CS fibers, and were no toxic to MEFs with low extraction concentrations. *In vivo*, TMC2 fibers exhibited excellent wound healing activity in full-thickness excision wound model in rats. The results indicated that TMC fibers were potential wound dressing materials for wound healing.

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380 Figure captions

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