Potential Wound Dressing with Improved Antimicrobial Property

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ABSTRACT: Cultured skin substitutes have provided a new approach for the closure of wounds, but these avascular grafts are subject to microbial destruction that may lead to tissue infection. To minimize wound infection, HN-300 (a new inorganic antimicrobial particle) was incorporated into the collagen–chitosan wound dressing. Antimicrobial, cytotoxicity, release rate, swelling, and mechanical properties of this material were assessed. The antimicrobial property was enhanced with the increasing dosage of HN-300. When the concentration of HN-300 was more than 5%, the films showed sustained and stable antimicrobial property within 144 h. When the concentration of HN-300 was less than 7.5%, the wound dressings were noncytotoxic and compatible for L929 fibroblasts. So 5% HN-300 in antimicrobial films was suitable and the concentration also could improve the mechanical properties of the collagen–chitosan wound dressings. After treatment with 5% glycerol, the film's stability was enhanced and silver ions were protected at the same time. Hence, collagen/chitosan/HN-300 films are of great potential in application as wound dressing in future, because of their good biosafety and physical properties. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 1679–1686, 2007

Key words: biomaterials; biocompatibility; proteins; antimicrobial properties; wound dressing

INTRODUCTION

Cultured skin substitutes have provided a new approach for wound healing. Collagen was one of the most promising materials for its excellent biocompatibility and biodegradability.[1] As the main composition of extracellular matrix, collagen improved the adhesion, proliferation, and differentiation of cells in tissue culture.[2] Chitosan was used in various biomedical fields such as drug delivery carrier, surgical thread, and wound healing materials,[3] and it also could improve the function of collagen films, such as mechanical property, swelling property, and biodegrading rate.[4] Collagen and chitosan could form a polyanion–polycation complex when the concentration of chitosan was more than 10 wt %.[5] So the composites of collagen and chitosan were ideal materials for skin substitutes. To minimize wound infection risk of these avascular grafts, antimicrobial drugs such as broad spectrum antiseptic agents (iodine and silver) and antibiotics should be used. Silver was widely used as one of broad spectrum antiseptic agents, owing to its ability to sterilize varieties of disease—caused by microbes such as aerobe, anaerobe, yeast, fungi, and virus.[6] In addition to the antimicrobial properties, silver also showed anti-inflammatory properties.[7] Silver exerted its antimicrobial effects by interfering with the respiratory chain on the cytochromes and components of the microbial electron transport system. Silver also could bind DNA and inhibit DNA replication.[8] Traditional silver antimicrobials such as silver nitrate and silver sulfadiazine were limited in their stimulation and quick inactivation. In this research, a new type of silver antiseptic agent HN-300 was incorporated into the collagen–chitosan films.

As wound dressing, collagen–chitosan films complexed with antimicrobial agent, which should be crosslinked to keep its structural stability in moist wound environment. Glutaraldehyde and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) were ordinary crosslink agents. Glutaraldehyde could crosslink collagen and chitosan efficiently at the same time, but it was generally thought to result in cytotoxicity.[9,10] Using both EDC and glutaraldehyde needed extensive washing to get rid of residua, but in the process of washing, silver ions would get lost greatly

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from collagen/chitosan/HN-300 films. The antimicrobial films did not need to be deeply crosslinked, since the antimicrobial films would be used on the surface of body and it was convenient to be changed. Glycerol just satisfied the need. Recent studies indicated that the collagen structure was stabilized by direct intramolecular interactions with polyhydric alcohols,[11] and that glycerol could stabilize collagen solvation shell.[12] Therefore, glycerol was used as the post-treatment agent of the antimicrobial films. The antiseptic efficiency, cytotoxic property, release amount, and the swelling capacity of the collagen/chitosan/HN-300 films were investigated in this article.

EXPERIMENTAL

Materials

Chitosan (M_w: 2×10^5, 90.6% deacetylated) was purchased from the Third Institute of Oceanography of the State Oceanic Administration of China (Xiamen City, Fujian Province, China). Collagen type I was separated from fresh bovine tendon according to our reported method.[13] HN-300 was donated by the Jiangsu Hehai Group of the Chinese Academy of Sciences (Taixing City, Jiangsu Province, China). HN-300 containing 3% (w/w) silver was produced by the exchange of silver ions and $NaZr_2(PO_4)_3$ ion exchange carriers. Ag^+ and Ag^{2+} were the main antimicrobial components in HN-300. The diameter of HN-300 particle was about 300 nm.

Preparation of collagen/chitosan/HN-300 films

Collagen (CO) or chitosan (CH) was dissolved in 0.05*M* acetic acid to prepare a 0.5% (w/w) solution, respectively. HN-300 was dispersed in the chitosan solution by stirring and ultrasonication. The HN-300 solution was then dropped slowly into the collagen solution with the volume ratio of 1:9 (V_{CH}:V_{CO} = 1:9) and homogenized by stirring. The collagen–chitosan solution was dropped on a polytetrafluorethylene plank and dried for 24 h at 37°C. The same way was used to prepare comparative samples with the amount of silver equal to that of films, with 5% or 10% HN-300.

To improve the stability, these antimicrobial films were immersed in 5% glycerol in water for 1 h and washed with triply distilled water (30 min × 5 times), and then dried for 24 h at 37°C.

Swelling test

The collagen/chitosan/HN-300 films with or without glycerol treatment were placed into distilled water at room temperature, and the wet weight (w) of the film was determined after incubation for 24 h. The swelling ratio of the films was defined as the ratio of weight increase ($w - w_0$) to the initial weight (w_0). Each value was averaged from six parallel measurements.

Silver release assay

Collagen/chitosan/HN-300 films and comparative samples with $AgNO_3$ were immersed in phosphate buffer saline (PBS) (1 cm^2 film/mL PBS), respectively. The whole apparatus was placed in a water bath shaker at 37°C and shaken at 200 rpm. PBS was removed and replaced with fresh medium every 24 h, and the concentration of silver in the extract was examined through atomic absorption spectrometry.

Antibacterial test

Escherichia coli (*E. coli*) DH5α was proliferated in liquid Luria-Bertani (LB) culture medium for 14 h at 37°C, and was shaken at 180 rpm. The optical density of the bacterial suspension was measured with ultraviolet spectroscopy (UV-2550) at 600 nm. Hundred microliters of bacterial suspensions were evenly pipette-loaded on the solid LB culture medium in a dish. Then the LB medium was covered with round pieces of tested films and a filter paper with the same diameter as blank experiment, respectively. The dishes were put in an incubator (37°C, 5% CO_2). Photos were taken after incubation for 12, 36, 50, and 144 h, respectively.

Cell compatibility assay

Five kinds of collagen/chitosan/HN-300 films (the weight percent of HN-300 was 0%, 2.5%, 5%, 7.5%, and 10%, respectively) were immersed in 75% ethanol ultrasonicated for 30 min, washed two times in PBS, and air-dried in a super clean bench. The films (1 cm^2/mL) were then immersed in 1640 culture medium, with 10% bovine serum and incubated for 24 h at 37°C. About 1.96 mL extraction (or 1.96 mL medium as negative control) was added to each well of 24-well polystyrene plates. About 0.04 mL suspension of L929 fibroblasts (5×10^5 cells/mL) was seeded in each well. Plates were cultured in an incubator (37°C, 5% CO_2) for 5 days. On the 1st, 3rd, and 5th day, the culture medium of one plate was removed and 200 μL MTT (0.25 mg/mL) was added to each well, and the plates were incubated for 4 h and then 600 μL of DMSO was added. After incubation for 1 h, absorbance (A) was measured at 490 nm against blank (DMSO). Cell toxicity of pure HN-300 was also checked with the same method.

The cell viability (CV) was calculated according to the following formula:

$$CV = A_{Sample} \times 100/A_{Control}$$

Sterilized film sample (12 mm in diameter) was put in each well of a 24-well plate, and then 2 mL com-

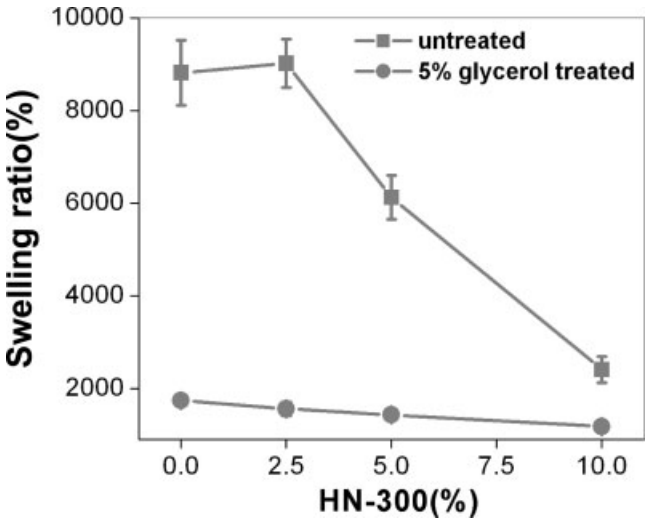

Figure 1 The effect of the concentration of HN-300 on the swelling ratios of collagen–chitosan films treated by 5% glycerol and untreated, respectively. Values are mean ± SD ($n = 4$).

plete medium and 1×10^4 L929 cells were added. The plate was cultured in an incubator (37°C, 5% CO_2) for 4 days. These specimens were fixed in 2.5% glutaraldehyde in 0.1*M* sodium cacodylate buffer (pH = 7.2) at 4°C for 30 min and then dehydrated in a graded series of alcohols. In the end, these specimens were observed using a scanning electron microscope (SEM, LEO1530).

Mechanical property

The mechanical property was measured by a multifunctional electron mechanical analyzer (WDS-5, Shanghai Shenlian Testing Machineries Works) at a crosshead speed of 20 mm/min. The studied samples were cut to 110 ± 1 mm in length and 10 ± 0.5 mm in width for the test.

RESULTS AND DISCUSSION

Swelling test

The holding water ability of a film was an important parameter to evaluate its property for wound dressing. High swelling ratio might prevent the wound from accumulation of unwanted body fluid by the adsorption of exudates. It also assured moist wound environment, which would be helpful for therapy.[14] However, as the swelling ratio was higher than 5000%, the strength of swelled collagen/chitosan/HN-300 films decreased sharply. The films had no strength after 24-h swelling in water, and so a suitable swelling ratio of antimicrobial films was necessary. Figure 1 showed the swelling ratios of these films with different amount of HN-300 in various conditions. The swelling ratio of collagen–chitosan films untreated by glycerol decreased with the increase of HN-300 from 2.5% to 10% obviously. Therefore, when the concentration of HN-300 was more than 2.5%, the swelling ratios were lower than that of the pure collagen–chitosan films. The films with over 5 wt % HN-300 had good strength after 24-h swelling in water. The result indicated that HN-300 could strengthen collagen–chitosan films. To improve the film strength in low concentration of HN-300 (too much HN-300 was harmful to cell, see Cytotoxicity Assay section), the collagen/chitosan/HN-300 films were treated with 5 wt % glycerol solution. The swelling ratio of treated films decreased obviously compared with untreated films, and decreased hardly with the increase in concentration of HN-300. The result suggested that glycerol could decrease the swelling ratio of these films. All films treated by glycerol had good strength after 24-h swelling in water, and then the concentration of HN-300 in antimicrobial films was not restricted by swelling ratio.

Release amount of silver

The *in vitro* release amount of silver from collagen/chitosan/HN-300 films within 7 days was showed in Figure 2. The release amount reached about 45% in films with 2.5% HN-300 on the first day, which showed a typical initial burst effect, while the release amount only reached 12% in films with 10% HN-300. These results indicated that the initial burst drug release was retarded with the increasing amount of HN-300. The release amount of silver in the films with the concentration of HN-300 from 5 to 10% reached

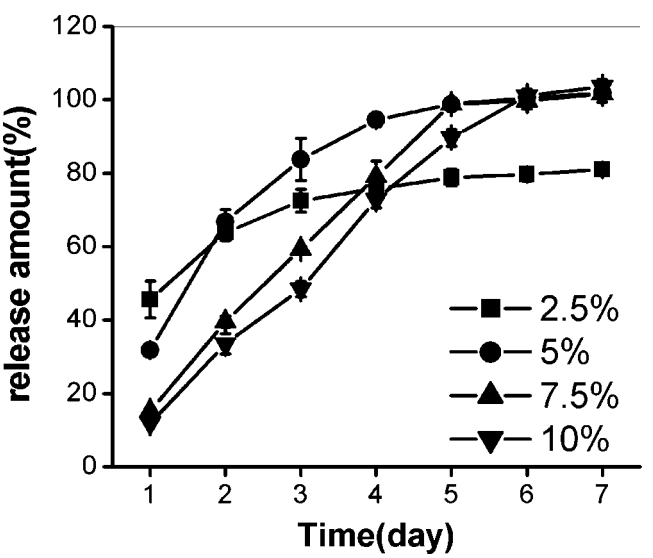

Figure 2 The release profiles of silver from 2.5%, 5%, 7.5%, 10% HN-300-containing collagen–chitosan films. All films were treated with 5% glycerol for 1 h. Values are mean ± SD ($n = 4$).

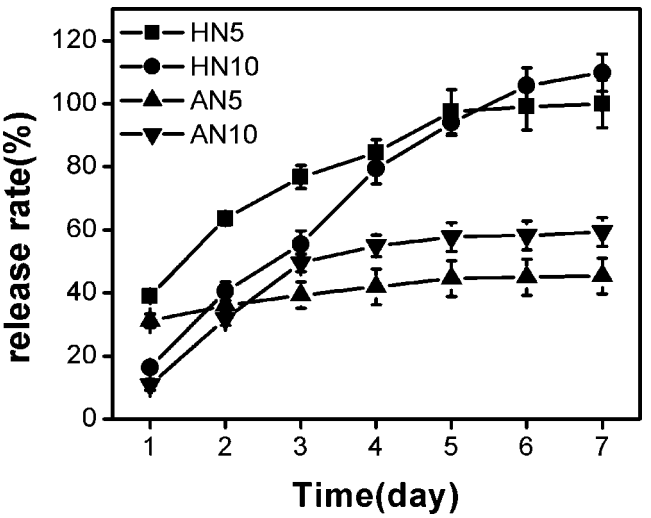

Figure 3 The release profiles of silver from HN-300 containing collagen–chitosan films and $AgNO_3$ containing collagen–chitosan films. HN5: 5 wt % HN-300, HN10: 10 wt % HN-300, AN5 and AN10: the amount of silver in collagen/chitosan/$AgNO_3$ films was equal to the amount in the collagen/chitosan/HN-300 films with 5 and 10% HN-300, respectively. All films were treated with 5% glycerol for 1 h. Values are mean ±SD ($n = 4$).

above 98% after 7 days. The release amount of silver in the films with 2.5% HN-300 reached about 80% after 7 days.

Comparative samples (collagen/chitosan/$AgNO_3$ films) were used in this experiment. The release profiles of the comparative samples were shown in Figure 3. On the first day, the release amount of comparative samples was just a little lower than that of the experimental samples (collagen/chitosan/HN-300 films) with equivalent silver. Over the next few days, the release amount of silver from comparative samples fell off rapidly, and the release stopped after the fifth day. From the beginning to the fifth day, the release amount of silver from the films with the concentration of silver equivalent to 5 and 10% HN-300 were 44.6% and 57.7%, respectively. The values were less than that of the experimental samples (98%). What does this show? Here a phenomenon should be mentioned. In the process of preparing $AgNO_3$/chitosan solution through stirring and ultrasonication, the color of the solution changed from transparent to gray as shown in Figure 4. Four kinds of solution prepared with 0.05*M* acetic acid were shown in Figure 4. They were (a) the solution with 0.025% $AgNO_3$, (b) the solution with 0.5% chitosan, (c) the solution with 0.5% chitosan and 0.025% $AgNO_3$, and (d) the solution with 0.5% chitosan and 0.5% HN-300. The comparison of (a), (b), and (c) showed that the change of solution color happened only in chitosan solution containing $AgNO_3$. Being combined with silver ions, oxygen in the air may make silver ions oxidized and form silver oxides on the condition of heating or exposure to light. In the experiment, 0.025% $AgNO_3$ solution (a) did not change in color obviously. It meant the energy, including the heat produced by ultrasonication or exposure to light, was not enough to form silver oxides. Why gray silver oxides could form in the solution c? According to the research of Yoshizuka,[15] chitosan could be used to adsorb various metal ions in solution including silver ions. In the experiment, the silver–chitosan composed of a catalytic activity system, and it accelerated the formation of silver oxides. Figure 3 showed that about half of silver in comparative samples could not release effectively to exploit its antimicrobial

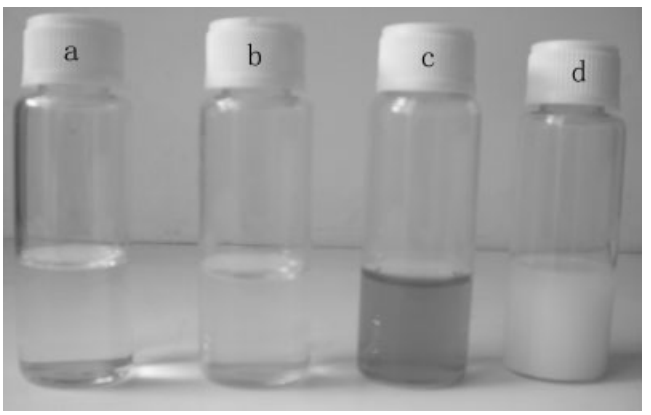

Figure 4 The image of the solutions prepared with 0.05*M* acetic acid. (a) 0.025% (w/w) $AgNO_3$, (b) 0.5% (w/w) chitosan, (c,d) 0.5% (w/w) chitosan solution containing 0.025% $AgNO_3$ and 0.5% HN-300, respectively.

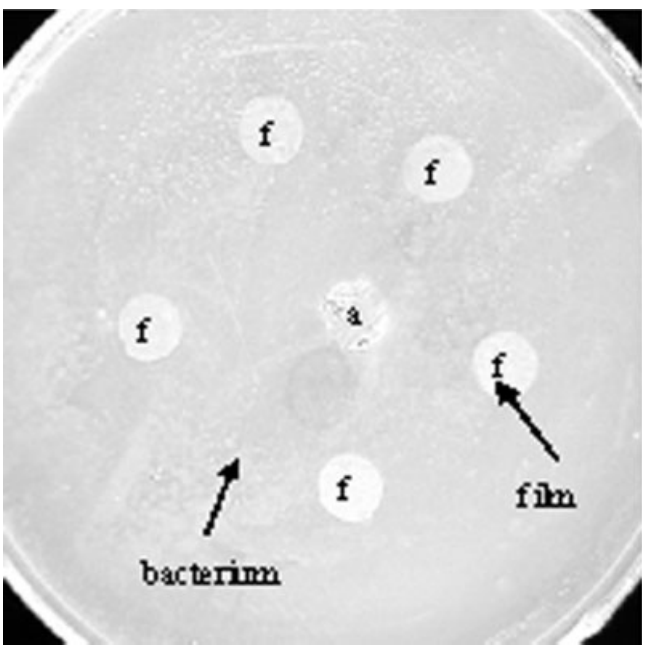

Figure 5 The image of the inhibition zones around HN-300-free collagen–chitosan films after incubation for 12 h. a, filter paper; f, collagen–chitosan films (collagen:chitosan = 9 : 1 (wt)).

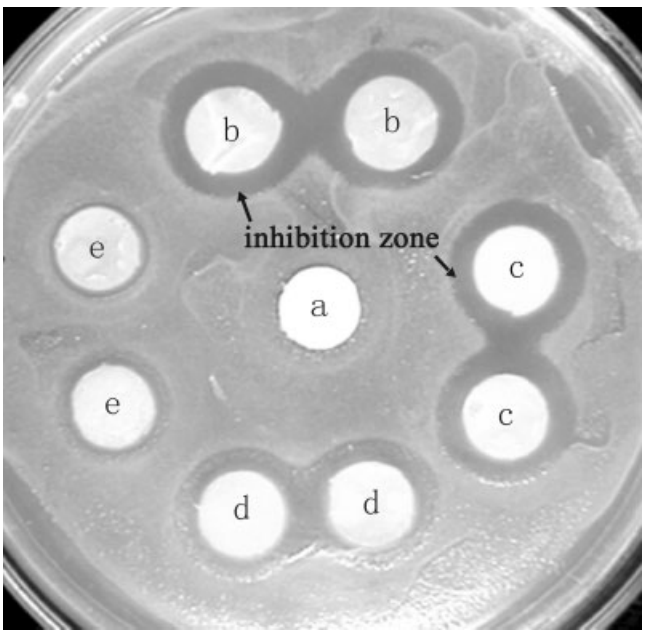

Figure 6 The image of the inhibition zone of HN-300-containing collagen–chitosan films with different ratios (wt) after incubation for 12 h. a, filter paper; b, 10%, c, 7.5%, d, 5%; e, 2.5%.

ability. In the same condition, HN-300 as new inorganic antimicrobial particle defended oxidization and protected silver effectively in the process of preparing experimental samples. It explained the reason for using HN-300.

To test the release form of silver from collagen/chitosan/HN-300 films, the extract of these experiment samples was centrifuged at 20,000 rpm, and then the concentration of silver in the supernatant layer was also examined through atomic absorption spectrometry. No difference in concentration was found between the extraction and its supernatant layer. The result indicated the silver ions were released by ion exchange, and not by HN-300 nanoparticles or local material degradation.

Antimicrobial character

The aim of the present study was to develop an antimicrobial collagen–chitosan wound dressing. Chitosan, an alkaline mucopolysaccharide, exerts its antimicrobial effects by two mechanism[16]: (1) NH_3^+ groups of chitosan prevent nutrients from entering the cell to interfere bacterial metabolism by adsorbing bacterial cell membrane with negative charge and form a polymer membrane, (2) NH_3^+ groups of chitosan flocculate cells by adsorbing the cellular electronegative substance, disturb the physiological activities of the bacteria, and kill them via pervasion.[17,18]

In the present study, the antimicrobial property of the films with 10% chitosan was examined. After putting collagen/chitosan films into dishes for bacterial culture (*E. coli.* DH5α), the dishes were incubated for 12 h at 37°C, and no inhibition zone was observed around these specimens (Fig. 5). This result seemed to conflict with the antimicrobial property of chitosan that was reported in acid solution.[16–18] NH_3^+ groups of chitosan exist only in the acid environment; thus, almost no NH_3^+ groups appear in the neutral culture environment of bacteria (pH 7.4) in the experiment. That was why chitosan-containing films did not inhibit proliferation of bacteria around the sample as shown in Figure 5. In addition, collagen may be helpful for bacteria adherence and shield the immunology reaction of the host from bacteria.[19] To increase its antimicrobial property, it was thus necessary to develop a new type of antimicrobial wound dressing by adding antibacterial.

The antimicrobial capacity of metallic ion descends in the following order: Ag > Hg > Cu > Cd > Cr > Ni > Pb > Co > Zn > Fe.[20] Silver was chosen in our study to provide the collagen–chitosan wound dressing with antimicrobial property. Traditional silver antimicrobials, such as silver nitrate and silver sulfadiazine, were limited in clinic use for their quick inactivation; moreover, silver sulfadiazine was commonly applied twice per day and silver nitrate up to 12 times per day, and such frequent dressing changes would greatly afflict burn patients.[8] HN-300 was an ideal antimicrobial for its good stability. As shown in Figure 6, the inhibition zone around the films, whose diameters reflected the antimicrobial efficiency, enlarged with the increasing concentration of HN-300. It was because silver ions were the main antimicrobial components in HN-300. The number of silver ions was mainly influenced by the dosage of HN-300. When antimicrobial films contacted body fluid, ion exchange

TABLE I
Antimicrobial Activity of Collagen/Chitosan/HN-300 Films Illuminated by the Inhibition Zone Diameters (mm)

	Concentration of HN-300 (wt %)				
Incubation time	Blank	2.5%	5%	7.5%	10%
12 h	9.0 ± 0.1	10.3 ± 0.2	14.2 ± 0.5	14.4 ± 0.1	14.8 ± 0.2
36 h	9.1 ± 0.1	10.1 ± 0.2	10.8 ± 0. 4	11.4 ± 0.4	12.3 ± 0.3
60 h	9.1 ± 0.1	9.5 ± 0.4	9.9 ± 0.6	11.4 ± 0.6	11.7 ± 0.6
144 h	9.0 ± 0.3	9.1 ± 0.2	9.6 ± 0.2	10.2 ± 0.1	10.4 ± 0.2

Values are mean ± SD ($n = 4$).

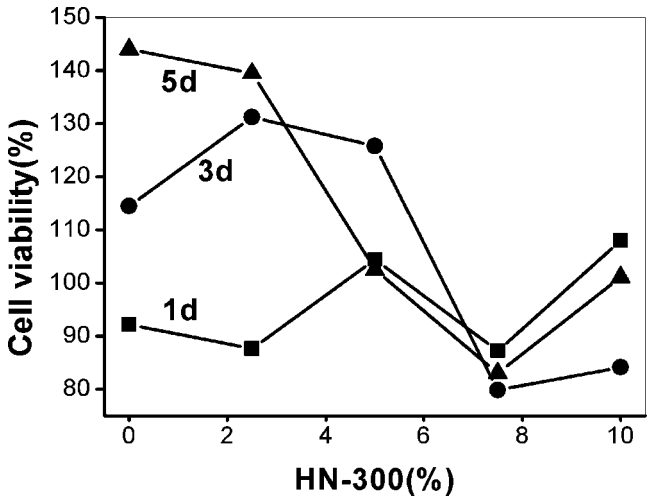

Figure 7 The effect of the concentration of HN-300 on the cell viability of collagen–chitosan films, determined by the MTT assay. Values are mean ($n = 5$).

happened between cations in body fluid and silver ions in collagen/chitosan/HN-300 films. As shown in Table I, the inhibition zone appeared after 12-h incubation, and then decreased with time. These results indicated that some bacteria resumed growth near the outer edge of the inhibition zone. The inhibition zone of the films with the concentration of HN-300 ranged from 2.5% to 10% and were significantly different from that of the controlled filter paper in 36 h, while the inhibition zone of the films with 2.5% HN-300 were similar to that of the controlled filter paper after 36 h. Only the films with the concentration of HN-300 ranging from 5 to 10% showed sustained and stable antimicrobial property within 144 h.

Cytotoxicity assay

Cytotoxicity of antimicrobial films' extraction was a very important parameter to evaluate biomaterials. In the present study, the extraction of collagen/chitosan/HN-300 films was used to examine the CV of our samples. Figure 7 was the CV of films with 0–10 wt % HN-300 on the 1st, 3rd, and 5th day. On the 1st day, all samples had no obvious negative effect on cells, when compared with films without HN-300. It indicated that the extraction of films with 0–10 wt % HN-300 did not affect the adhesion of cells. The extraction

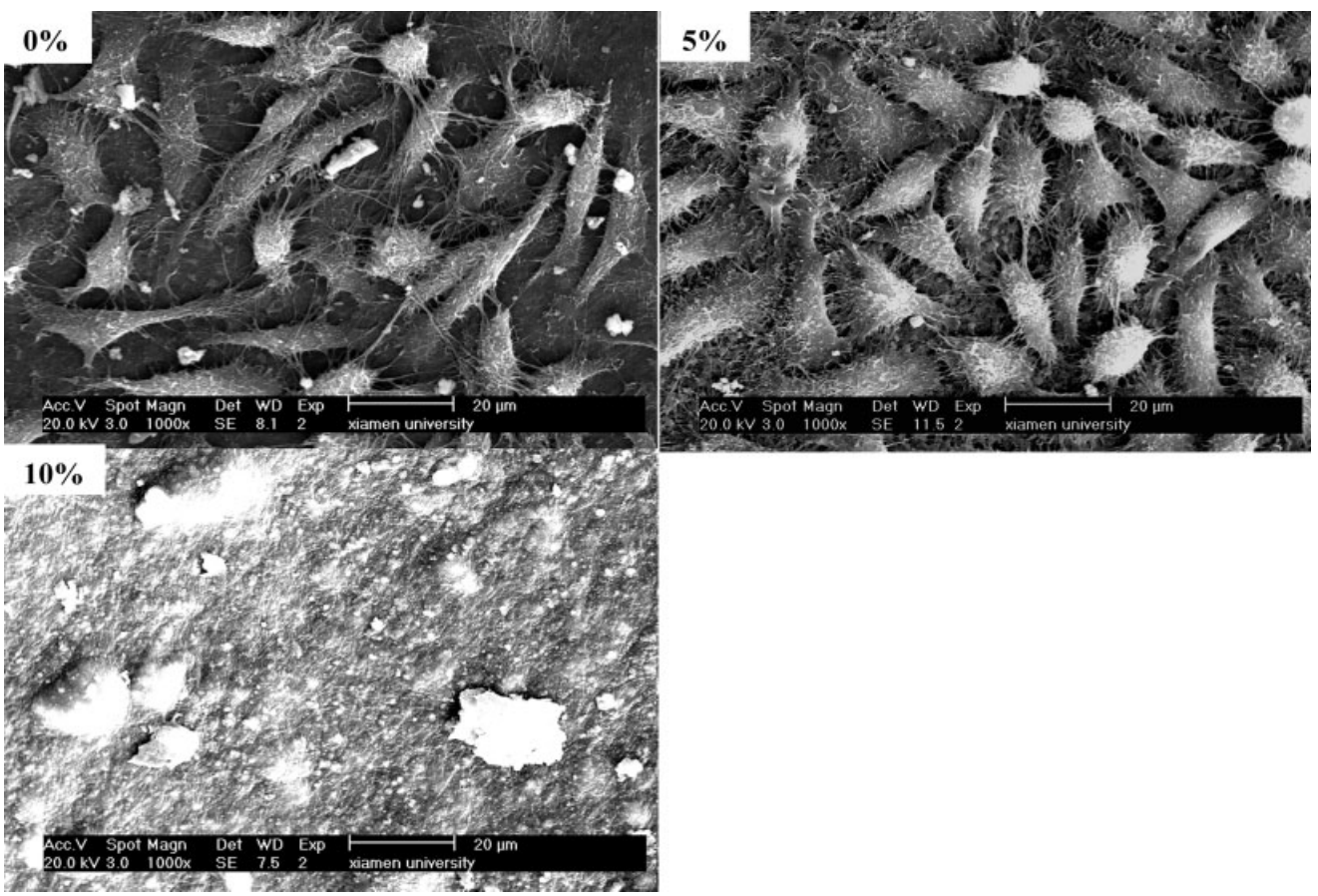

Figure 8 The SEM images of L929 growing on 0%, 5%, 10% HN-300-containing collagen–chitosan films after culture for 4 days.

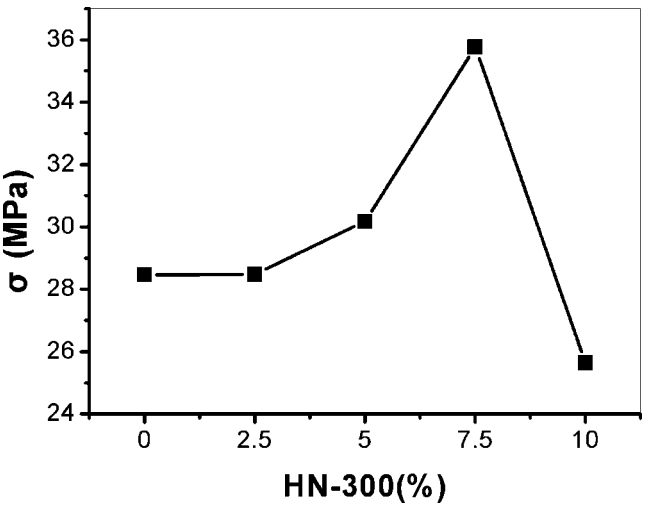

Figure 9 The effects of the concentration of HN-300 on the fracture stress of the collagen–chitosan films. Values are mean ($n = 4$).

of films with 0–2.5 wt % HN-300 accelerated the growth of cells during the experiment. On the 3rd and 5th day, the extraction of films with 5 wt % HN-300 did not affect cell growth, while the extraction of films containing 7.5–10% HN-300 would inhibit cell growth in a certain degree (Fig. 7). HN-300 particle alone was cytotoxic even when its weight was equal to 0.5 wt % of collagen–chitosan films. The CV of HN-300 solution on the 1st, 3rd, and 5th day was 79.6%, 55.6%, 39.2%, respectively. It indicated that cytotoxicity of HN-300 could be reduced by slowly releasing it from collagen–chitosan film. Collagen/chitosan/HN-300 films just acted as sustained release system. HN-300 would improve the antimicrobial function of collagen–chitosan films, and the films could reduce the cytotoxicity of HN-300.

Cell compatibility assay

After L929 fibroblasts were cultured for 4 days on the collagen/chitosan/HN-300 films, SEM was used to observe the cells on the films as shown in Figure 8. The fibroblasts grew well on the films containing 0, 2.5, 5, and 7.5 wt % HN-300, but no cell growth was observed on the films containing 10 wt % HN-300. From Figure 8, it also could be observed that most of the cells were polygon or spindle-shaped. They had plenty of microvilli and pseudopods around them, by which they contacted with each other and coupled tightly on the films. This phenomenon indicated that cells grew well. At the same time, the contact inhibition also could be observed in the place with a mass of cells. The inhibition led these cells to form spherical shape. No cell was found on the films with 10 wt % HN-300. The SEM results showed that the weight percent of HN-300 in the antimicrobial films should be less than 10%, to ensure its preferable biocompatibility.

Mechanical property

The mechanical properties of collagen/chitosan/HN-300 films were shown in Figure 9. The fracture stresses of all samples were larger than 20 MPa. It was also revealed from Figure 9 that the fracture stress of the wound dressing increased from 28.5 MPa to 35.8 MPa as the concentration of HN-300 was less than 7.5%, whereas it decreased to 25.7 MPa as the concentration of HN-300 was 10%. Thus, HN-300 might act as reinforcing agent in the wound dressing to enhance the fracture stress.

CONCLUSIONS

Inorganic antimicrobial particles HN-300 were incorporated into the collagen–chitosan wound dressing. Comparing with $AgNO_3$, HN-300 could protect silver from oxidation and inactivation, effectively, in the process of preparing antimicrobial films. The antimicrobial property was enhanced with the increase in the concentration of HN-300. When the concentration of HN-300 was more than 5%, the films showed sustained and stable antimicrobial property within 144 h. When the concentration of HN-300 was less than 7.5%, the wound dressings were noncytotoxic and compatible for L929 fibroblasts. So 5% HN-300 in antimicrobial films was suitable, and the concentration also could improve the mechanical properties of the collagen–chitosan wound dressings. The collagen/chitosan/HN-300 films could sustain the release of silver ions, and the cytotoxicity test indicated that the sustained release reduced the cytotoxicity of HN-300. Thus, HN-300 improved the antimicrobial function of collagen–chitosan films, and the films reduced its cytotoxicity. After treatment with 5% glycerol, the film's stability was enhanced, and silver ions were protected at the same time. Hence, collagen/chitosan/HN-300 films were of great potential in application as a antimicrobial wound dressing.

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