Preparation and Characterization of Ciprofloxacin-Loaded Alginate/Chitosan Sponge as a Wound Dressing Material

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ABSTRACT: The aim of this study is preparation and characterization of alginate/chitosan sponges including a model antibiotic (i.e., ciprofloxacin) to use in wound and/or burn treatment. Sponges were prepared firstly by the gelation of sodium alginate followed by lyophilization, crosslinking with calcium chloride, and finally coating with chitosan. Sponges were characterized with respect to morphology, water uptake, in vitro drug release behavior, and antimicrobial activity. Investigated and evaluated parameters in all of these studies were selected as the concentration of calcium chloride, alginate viscosity, drug content, and molecular weight of chitosan. Drug release and water uptake were found to be greatly influenced by these parameters. Water uptake and drug release rate were decreased by increasing the crosslinking density, chitosan molecular weight, and alginate viscosity. In the antimicrobial tests, it was obtained that the antimicrobial activity is directly proportional with the release rates and water uptake. Morphological studies showed a highly porous structure with interconnected pores. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 1602–1609, 2006

Key words: alginate sponge; chitosan; wound dressing; ciprofloxacin release; antimicrobial activity

INTRODUCTION

Rapid and proper healing is important in the treatment of wounds such as severe burns, trauma, diabetic, decubitus and venous stasis ulcers, and similar tissue damages. The healing response of tissues involves a complex interaction between cells, extracellular matrix molecules, and soluble mediators.[1] The complexity of this process has been simplified by dividing the healing response into four broad categories that coincide with the temporal sequence of normal healing: homeostasis, inflammation, proliferation, and remodeling.[2]

In cases of severe and large amounts of skin loss, or in the presence of difficult and nonhealing wounds, immediate coverage of the wound surface with a dressing is needed. The dressing achieves the functions of the natural skin by protecting the area from the loss of fluid and proteins, preventing infection through bacterial invasion, and subsequent tissue damage. Numerous skin replacements are either currently available for clinical use or in clinical testing. These include temporary and permanent skin replacements, epidermal and dermal skin replacements, and synthetic/biosynthetic and biologic skin replacements. Biologic components are xenogenic, allogenic, or autogenic.

In this study, ciprofloxacin releasing system was prepared by using alginate and chitosan as skin replacement material to be used in wound healing and/or burn dressing applications. Alginate and chitosan are well known polyionic biopolymers that have been used in many different biomedical applications. Sodium alginate is a particularly attractive biopolymer that has a wide range of applications in the field of biotechnology. Alginate is a linear, unbranched polysaccharide composed of 1,4-linked β-D-mannuronic acid (M-block) and α-L-guluronic acid (G-block), which are found in varying composition and sequence.[3] Gel formation is achieved through the exchange of sodium ions from G-blocks with the divalent cations, such as calcium, and the staking of G-blocks to form an egg-box structure.[4] Alginate is biocompatible, hydrophilic, and biodegradable under normal physiological conditions. On the other hand, unstable mechanical properties due to the ion exchange of crosslinked divalent cations with monovalent cations[5] are the disadvantages of alginate matrix. Chitosan is a polyaminosaccharide, normally obtained by alkaline deacetylation of chitin, which is a polysaccharide that is widely spread among marine and terrestrial invertebrates and lower forms of a plant kingdom.[6,7] Chitosan's availability in a variety of useful

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TABLE I
Alginate/Chitosan Sponge Preparation Formulation Based on Effective Parameters

Sample no.	Chitosan molecular weight (Da)	Chitosan concentration (%)	$CaCl_2$ concentration (%)	Drug/alginate (mg/0.2 g alginate)	Alginate viscosity (cps)	Alginate concentration (%)	Water uptake (%)	Released ciprofloxacin (%)[a]
Effects of chitosan molecular weight								
1	150.000	0.5	10	5	3500	2	821	75
2	450.000	0.5	10	5	3500	2	934	80
3	650.000	0.5	10	5	3500	2	1060	91
Effects of $CaCl_2$ concentration (%)								
4	450.000	0.5	5	5	3500	2	1204	92
2	450.000	0.5	10	5	3500	2	934	80
5	450.000	0.5	20	5	3500	2	742	65
Effects of drug content								
6	450.000	0.5	10	2.5	3500	2	–	55
2	450.000	0.5	10	5	3500	2	–	80
7	450.000	0.5	10	10	3500	2	–	85
Effects of alginate viscosity								
8	450.000	0.5	10	5	250	2	1076	92
2	450.000	0.5	10	5	3500	2	934	80
9	450.000	0.5	10	5	14,000	2	651	53

[a] Measured after 4 h.

forms and its unique chemical and biological properties make it a very attractive biomaterial and it is extensively used in many types of applications.[8] Polycations such as chitosan form strong complexes with alginates, which are stable in the presence of Ca^{2+} chelators.[9] Thus, coating alginate sponges with polycations can improve their chemical and mechanical stability. Additionally, the presence of chitosan in a wound dressing is reported to promote fibroblast growth and affect macrophage activity, which accelerates the wound healing process.[10–15]

Alginate/chitosan sponges were prepared first by the gelation of sodium alginate followed by lyophilization, which creates the porous structure, crosslinking with calcium chloride, and finally coating with chitosan to provide mechanical stability and accelerate the wound healing process. During this process, a polyionic complexation occurs between alginate and chitosan because of the polyanionic character of alginate and polycationic character of the chitosan. For the characterization and optimization of polymeric carrier, ciprofloxacin, an antibacterial agent, was loaded into the sponges and some of the effective parameters (i.e., crosslinker concentration, drug content, alginate viscosity, chitosan molecular weight) on the water uptake and drug release rate were evaluated. After this characterization procedure, antimicrobial activity of ciprofloxacin-loaded polymeric carriers was evaluated. Similar effective parameters were used in both water uptake and in vitro ciprofloxacin release studies. *Escherichia coli (E. coli*) was used to determine the antimicrobial activity of released ciprofloxacin from alginate/chitosan sponges as the model bacteria.

EXPERIMENTAL

Preparation of alginate/chitosan sponges

Aqueous sodium alginate solution was prepared at 2% (w/v). Chitosan was dissolved in acetic acid solution of 5.0% (w/v) to prepare solutions of 0.5, 1.0, and 2.0% (w/v). Chitosan was supplied commercially with different molecular weights (i.e., 150.000, 450.000, 650.000 Da) (Fluka, Steinheim, Germany). Alginic acid sodium salts of low, medium, and high viscosity grades (Sigma, St. Louis, MO) were used. Viscosities of 2% solution of low viscosity, medium viscosity, and high viscosity grade sample were respectively 250, 3500, and 14,000 cps at 25°C. First, 1.0 mL of 2% (w/v) alginate solution was poured into the wells of a 24-well plate (well size: 17 mm diameter, 20 mm height), frozen overnight at −80°C, and then lyophilized. The resulting sponge was peeled off from the plate and treated with 5.0, 10.0, and 20.0% (w/v) calcium chloride solution for 10 min. After that, the sponge was treated with chitosan solution for 10 min to interact with chitosan, frozen overnight at −80°C, and lyophilized. Obtained alginate/chitosan sponges were kept in a vacuum dessicator for further analysis. Alginate/chitosan sponge preparation formulations based on effective parameters are shown in Table I.

Characterization of alginate/chitosan sponges

Morphological evaluations

Morphological evaluations of the alginate/chitosan sponges were made by a scanning electron microscope (SEM; JEOL, Japan). In these studies, a small piece of

sponge was put onto the sample holder, coated with gold, and then the SEM micrographs were taken.

Water uptake ability of alginate/chitosan sponges

Water uptake ability of alginate/chitosan sponges was determined by the gravimetric method. In this method, sponge was placed in a buffer solution (pH: 7.4) for a required period of time. The swollen sponges were collected and its weight was determined by first blotting the sponges with filter paper and weighing immediately on an electronic balance (Mettler, Switzerland). The weight of the swollen sponges was recorded at a predetermined time period. The percent water uptake was calculated from the following equation

$$S = \frac{w_t - w_0}{w_0} \times 100$$

where S is the water uptake ability (%), w_t denotes the weight of the sponges at time t, and w_0 is the initial weight of the sponges. The molecular weight of chitosan, alginate viscosity, and calcium chloride concentration were investigated parameters for water uptake ability.

In vitro ciprofloxacin loading and release

Ciprofloxacin release from the sponges was followed in batch experiments. In these studies, ciprofloxacin-loaded sponges were incubated with 20 mL of phosphate buffer solution (pH: 7.4) within a shaker at constant temperature (37°C). The supernatant were pipetted out periodically (i.e., 1, 2, 4, 12, 24, 48, 72, and 96 h) and the concentration of ciprofloxacin was measured spectrophotometrically (Schimadzu, Mini UV 1240, Japan) at 275 nm. During the release experiments, about 20% of the supernatant was replenished with a fresh buffer solution to obtain warm sink conditions and the removed ciprofloxacin from the release medium was also taken into consideration in the calculations.

Determination of ciprofloxacin activity

E. coli was isolated from urine specimens to use in the determination of bactericidial activity of ciprofloxacin. A bacteria suspension equivalent to the turbidity of a McFarland 0.5 standart, which represents 1×10^8 colonies forming unit (CFU) per milliliter, was prepared by using sterile 0.85% NaCl, and 10 mL of prepared suspension was transferred to sterile test tubes. One of the tubes was chosen as control tube (without sample). Sponges having different formulations were added to these sterile tubes. Each tube was vortexed for 15–20 s and incubation was performed at 37°C overnight. After 24 h of incubation and at various times (i.e., 48, 72, and 96 h. . .) thereafter, the number of bacterial colonies was determined by performing serial dilution, bacterial colony counts on an aliquot from each test vial. For this purpose, tubes were removed from the incubator and vortexed. 0.1 mL from each reaction tube was removed to perform serial dilution bacterial plate counts. Dilutions were achieved by the addition of 0.9 mL aliquots of sterile 0.85% NaCl to 0.1 mL of each sample. Using a 0.001 mL calibrated loop, a purity plate was incubated by subculturing 0.001 mL of the sample onto the surface of blood agar plate (Oxoid, England) and was stroked for isolation. After 24 h, colonies on subculture plates were counted. Taking into account dilution factor, number of colonies in each reaction tube was determined.

RESULTS AND DISCUSSION

Morphological evaluations

Figure 1 shows SEM photograph of the surface of alginate/chitosan sponge and Figure 1(b) shows SEM photograph of the cross section of alginate/chitosan sponge. The sponges exhibited highly porous structures with interconnected pores, as seen in Figure 1(a, b).

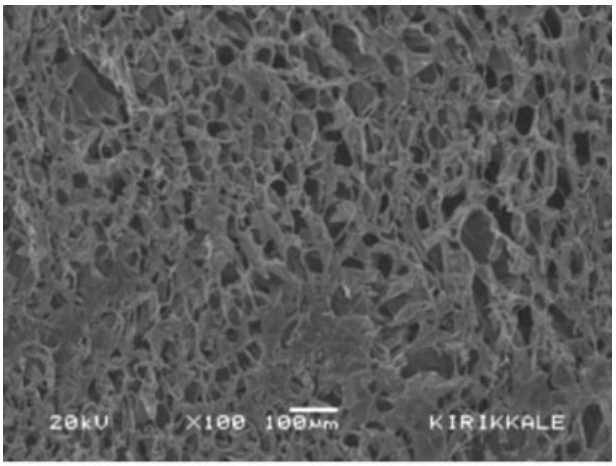
Water uptake ability of alginate/chitosan sponges

The formulations for the preparation of alginate/chitosan sponges with different structures to investigate the effective parameters and the water uptake ability of these sponges were given in Table I. Those effects were discussed individually in the following subsections. Almost all sponges having different formulations reached the maximum water uptake in approximately 8–10 min.

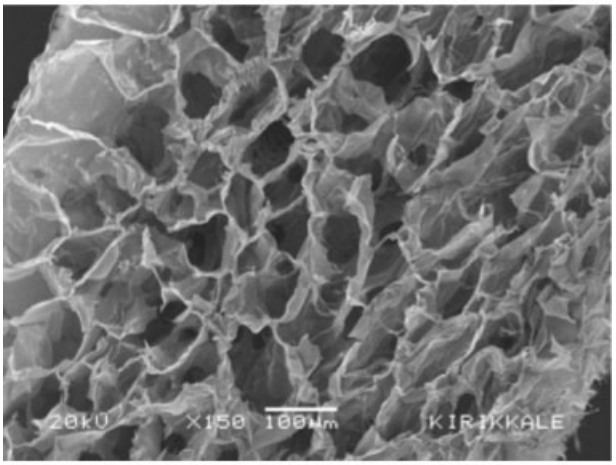
Effects of calcium chloride concentration on water uptake ability of alginate/chitosan sponges

It is well known that the amount of crosslinker directly affects the crosslinking density of the polymer and crosslinking density is increased by increasing the amount of crosslinker.[16] For the confirmation of this information and to optimize the alginate/chitosan sponge formulation for further investigations, sponges were prepared with different amounts of calcium chloride as seen in Table I. These formulations were evaluated for the determination of the water uptake ability for different amounts of crosslinker.

The obtained results were given in Table I. The water uptake ability is decreased by increasing the amount of calcium chloride as expected. This behavior



(a)



(b)

Figure 1 SEM micrographs of alginate/chitosan sponges: (a) surface (b) cross section.

can be explained by the permeation mechanism in hydrogels, meaning that in the hydrogels, the water permeation occurs via a pore mechanism, the reduction in water uptake by the sponge accompanying increased crosslinking density being an important factor. Highly crosslinked sponges tended to show lower water uptake ability, since the highly crosslinked structure could not sustain much water within their network structure.

Effects of chitosan molecular weight on water uptake ability of alginate/chitosan sponges

The molecular weight of the polymer is an effective parameter for water uptake ability of chitosan structures.[16] Therefore, this parameter was also investigated for alginate/chitosan sponges in this study. For this purpose, three different chitosan polymers (i.e., MW: 150.000, 450.000, and 650.000) were used to prepare chitosan sponges. The prepared sponges were evaluated according to the swelling behavior. The obtained water uptake ratios were summarized in Table I.

The water uptake ratios changed approximately between 821 and 1060% according to the molecular weight of chitosan. This is an expected result, because it is well known that the water diffusion is more difficult in the case of higher molecular weight because of the less molecular spaces than lower molecular weights for the same volume of polymer unit.[16]

Effects of alginate viscosity on water uptake ability of alginate/chitosan sponges

The water uptake was decreased by increasing alginate viscosity significantly. The viscosity of alginate depends primarily on the molecular weight of the material[17] and the molecular weight of the polymer is an effective parameter for water uptake ability of the structure. Because of the less water diffusion in the case of high molecular weight alginate, the lowest water uptake was observed for the sponge, which was prepared with high viscosity alginate.

Ciprofloxacin loading and release studies

Similar parameters were selected for ciprofloxacin loading and release studies with water uptake studies (i.e., crosslinker concentration, ciprofloxacin/chitosan ratio, chitosan molecular weight, and alginate viscosity), because in hydrogel type controlled release or drug delivery system, active agent release mechanism is directly proportional with the swelling nature of the hydrogel. According to the obtained release data, very high ciprofloxacin release rate was observed in first 1 h in all formulations as burst release and then very slow and constant release was followed after burst release. The obtained results were given individually in the following subsections and in Table I as the release data that measured after 4 h.

Effects of crosslinker concentration on ciprofloxacin release

Calcium chloride was used as crosslinker with three different concentrations in the preparation of the ciprofloxacin-loaded sponges. The obtained release data were given in Table I. The release rate was directly proportional with the concentration of crosslinker. This is actually related to the crosslinking denstiy, which means that this density is increased with increasing the crosslinker concentration and it is too difficult to release the active agent with the higher crosslinking densities. This behavior was also correlated with the water uptake evaluations based on the crosslinker concentrations as discussed earlier.

Both the water uptake and ciprofloxacin release rate were increased by decreasing the crosslinker concen-

tration. Highest release rate (around 92% total release in 4 h) was obtained with the lowest crosslinker concentration (i.e., 5%).

Effects of ciprofloxacin/alginate ratio on ciprofloxacin release

The active agent/polymer ratio is a well known effective parameter on the active agent release mechanism for many different types of polymer.[18] This can be speculated with the generated holes (or spaces) by releasing the ciprofloxacin molecules, which means that the released ciprofloxacin molecules leave more space after they released in the case of higher ciprofloxacin content. This hole generation is similar to the generation of the holes during the swelling process and of course the amount of the hole numbers will affect the release rate directly. In this study, the amount of ciprofloxacin was fixed and just the amount of chitosan was changed (such as 2.5, 5.0, and 10.0 mg/0.2 g alginate). The obtained release data with those formulations were given in Table I. The release rate was the highest in the case of highest amount of ciprofloxacin used and also the swelling ratios are in so similar trend with release data. In addition, these results supported the expressed hypothesis related with the active agent release and leaving back the molecular holes as mentioned earlier.

Effects of chitosan molecular weight on ciprofloxacin release

In the release studies, chitosan molecular weight was selected as another effective parameter on release mechanism according to the previous studies made with chitosan polymers.[18] Ciprofloxacin-loaded sponges were prepared with different molecular weight chitosan polymers (i.e., MW: 150.000, 450.000, and 650.000) and the obtained release data were shown in Table I. The ciprofloxacin release was decreased by increasing the chitosan molecular weight. This behavior depends on the higher tightness of the polymeric chains in the case of chitosan sponges prepared with higher molecular weight chitosan polymers. In this case, water uptake was also decreased as expressed earlier.

Effects of alginate viscosity on ciprofloxacin release

In the release studies, three different viscosities were studied and the obtained data were compared. The obtained release data were given in Table I. The sponge prepared with the lowest alginate viscosity showed the highest release rate as expected and the highest water uptake discussed earlier.

Ciprofloxacin activities

In this part of the study, ciprofloxacin was selected as a model antibiotic to use in the preparation of alginate/chitosan sponges for wound healing studies. Ciprofloxacin, like other quinolones, appears to inhibit synthesis and/other conformation by inhibiting the activity of DNA gyrase, an enzyme responsible for ATP-dependent negative supercoiling of bacterial DNA.[19]

In the studies of antibacterial (or antimicrobial) activities of the ciprofloxacin-loaded chitosan sponges, similar effective parameters were used in both swelling and in vitro ciprofloxacin release studies (i.e., crosslinker concentration, ciprofloxacin/alginate ratio, chitosan molecular weight, and alginate viscosity). *E. coli* was used to determine the antimicrobial activity of released ciprofloxacin from sponges as the model bacteria. In those studies, initially, the same number of bacterial colonies (i.e., 10^8 CFU/mL) was added into the medium contained ciprofloxacin-loaded sponges. Afterwards, the viable cells were counted and reported periodically (i.e., each day up to the death of all cells). During these studies, microscopic observations were collected and evaluated. Sponge preparation formulations are shown in Table I and the obtained results related with the antimicrobial activities were given and discussed individually in the following subsections.

Effects of calcium chloride concentration on antimicrobial activity

Different amounts of calcium chloride were used to prepare sponges for the determination of calcium chloride concentration effects on antimicrobial activities. And the obtained results were given in Figure 2.

The most effective calcium chloride concentration were 5% over the antimicrobial activity. Because in this case, the release rate was the highest as discussed earlier and the amount of *E. coli* was dropped in a very short time.

Effects of ciprofloxacin/alginate ratio on antimicrobial activity

The highest antimicrobial activity (or shortest time for death of bacteria) was achieved with the formulation of the highest ciprofloxacin/alginate ratio as shown in Figure 3. This is an expected result, because in vitro Ciprofloxacin release rate showed that the fastest release rate occurred in that case.

Effects of chitosan molecular weight on antimicrobial activity

The chitosan molecular weight was studied as an effective parameter on both water uptake and in vitro

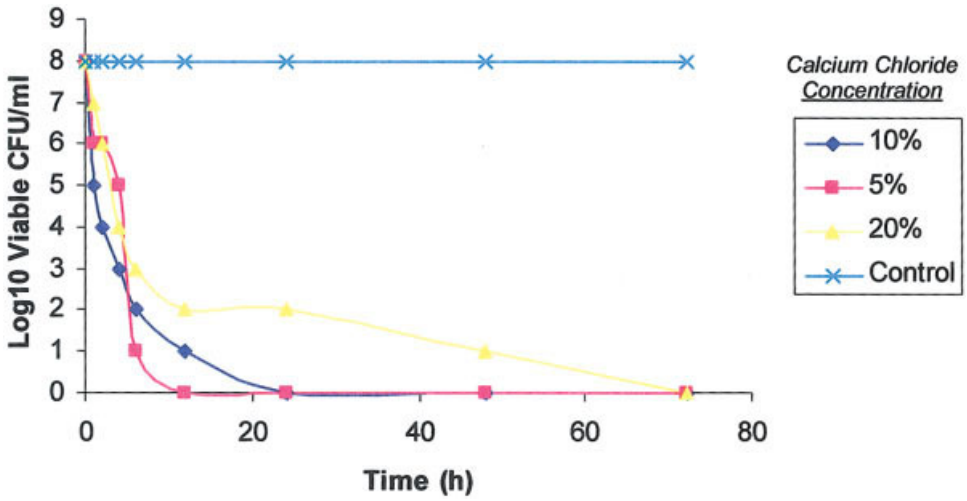


Figure 2 Effects of calcium chloride concentration on antimicrobial activity (chitosan molecular weight: 450.000 Da; chitosan concentration: 0.5%; drug/alginate: 5 mg drug/0.2 g alginate; alginate viscosity: 3500 cps; alginate concentration: 2%). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ciprofloxacin release rate because of the tightness of the molecular chains for different molecular weights of chitosan polymers. Therefore, this parameter was also suggested as an effective parameter on antimicrobial activity. Three different types of chitosan with different molecular weights (i.e., 150.000, 450.000, and 650.000) were used to prepare ciprofloxacin-loaded alginate/chitosan sponges and they are used to investigate the effect of chitosan molecular weight on antimicrobial activity. The obtained results were given in Figure 4. The highest antimicrobial activity (or shortest time for death of bacteria) was achieved with the formulation of high molecular weight chitosan (i.e., MW: 650.000). This is an expected result, because in vitro ciprofloxacin release rate showed that the fastest release rate was occurred with this formulation.

Effects of alginate viscosity on antimicrobial activity

The highest antibacterial activity (or shortest time for death of bacteria) was achieved with the formulation of low viscosity alginate. This is an expected result, because in sponge prepared with low viscosity alginate the water uptake and ciprofloxacin release rate is the highest (as discussed earlier) and this causes death of bacteria quickly and in a shorter time period than the other formulations (Fig. 5).

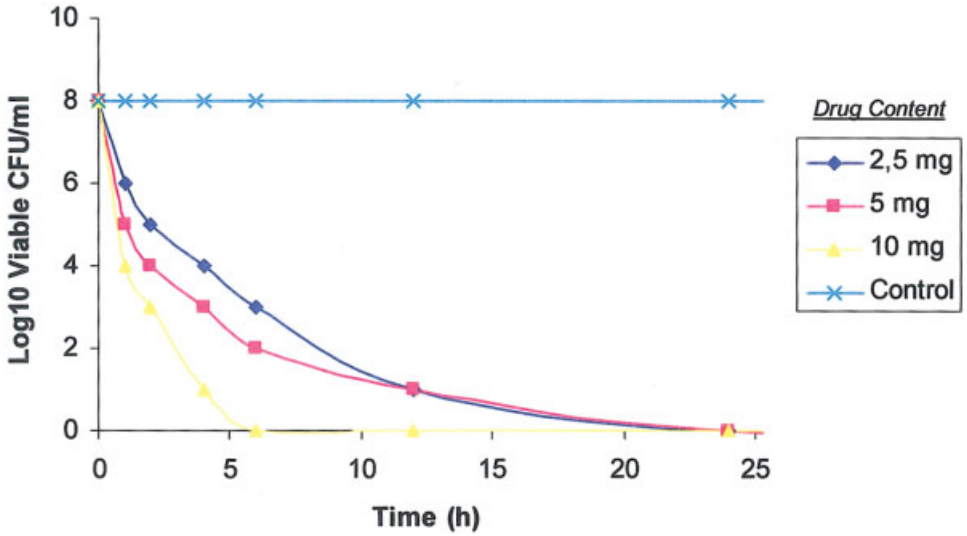


Figure 3 Effects of drug content on antimicrobial activity (chitosan molecular weight: 450.000 Da; chitosan concentration: 0.5%; $CaCl_2$ concentration: 10%; alginate viscosity: 3500 cps; alginate concentration: 2%). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

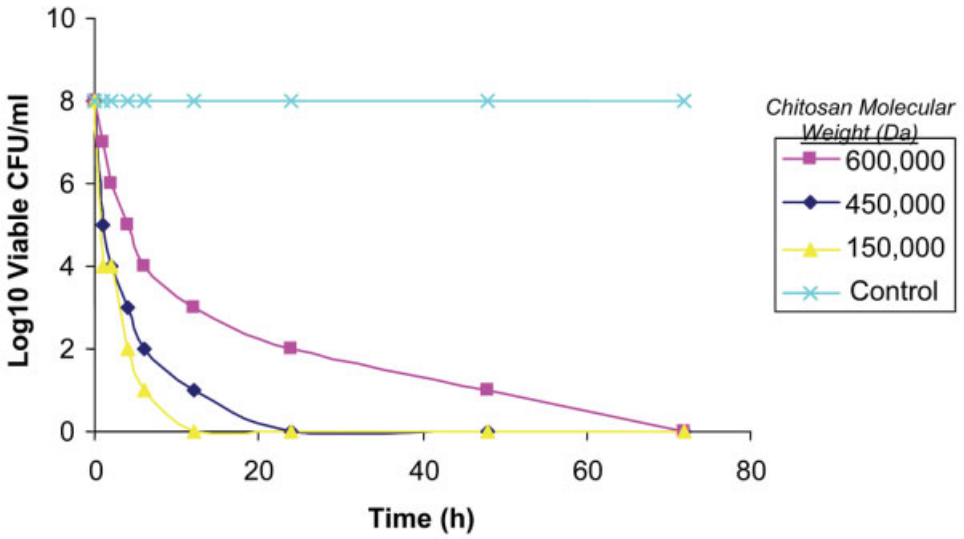


Figure 4 Effects of chitosan molecular weight on antimicrobial activity (chitosan concentration: 0.5%; $CaCl_2$ concentration: 10%; drug/alginate: 5 mg drug/0.2 g alginate; alginate viscosity: 3500 cps; alginate concentration: 2%). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CONCLUSIONS

A highly porous sponge composed of alginate and chitosan was fabricated through the simple freeze-drying technique to use as a wound dressing. This structure will achieve the role of natural skin by protecting the area from the loss of fluid and proteins. The sponge can also protect the wound from the infections by releasing the loaded antibiotic with desired (or required) release rate. The obtained data showed that the water uptake ability and drug release rate was affected significantly by the crosslinking density, alginate viscosity, and chitosan molecular weight. In the antimicrobial activity determination studies, some expected results were obtained, such as the antimicrobial activity was increased with increasing ciprofloxacin release rate. In all effective parameter investigations, the death rate of the *E. coli* colonies was increased by increasing released ciprofloxacin from the sponges. This system could be used as a very promising alternative wound dressing to use in wound/burn dressing applications and wound healing studies.

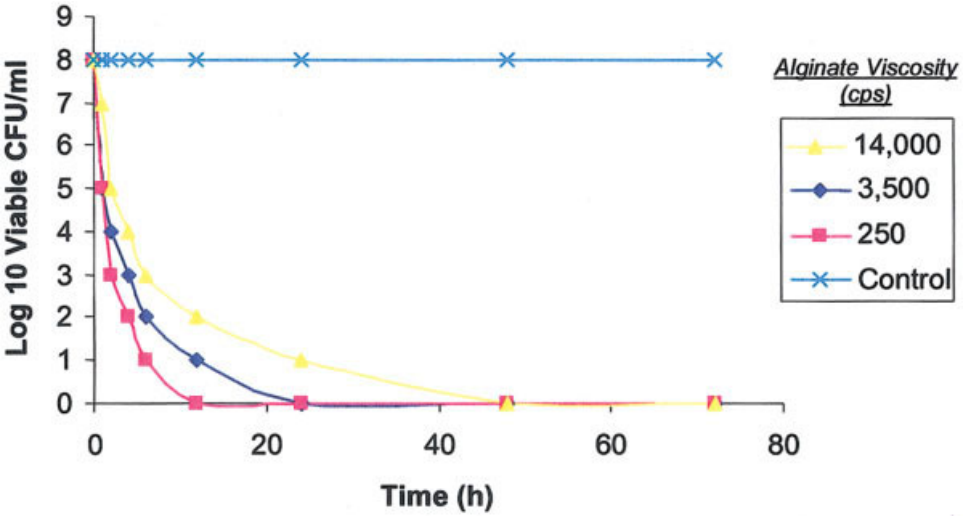


Figure 5 Effects of alginate viscosity on antimicrobial activity (chitosan molecular weight: 450.000 Da; chitosan concentration: 0.5%; $CaCl_2$ concentration: 10%; drug/alginate: 5 mg drug/0.2 g alginate; alginate concentration: 2%). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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