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In vitro gentamicin sustained and controlled release from chitosan cross-linked films

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Abstract A novel wound dressing film was investigated for controlled and sustained delivery of gentamicin, while covering and protecting the wound. Chitosan was cross-linked with Hexamethylene 1,6-bis (aminocarboxysulfonate) to prepare the wound dressing films. Cross-linking reaction was required to control the water retention and release of encapsulated gentamicin. Cross-linked films swell less and became more hydrophilic than chitosan film itself. However, this behavior was inversely proportional to the degree of cross-linking. In vitro gentamicin release from the cross-linked films, at physiological conditions of pH and temperature, was studied for 2 weeks. The effects of gentamicin initial concentration and cross-linking ratio on the kinetics of gentamicin release were evaluated. Results showed that the diffusion rate was governed by initial concentration of gentamicin and degree of cross-linking, since higher gentamicin initial concentration and degree of cross-linking promoted the slower release, while lower gentamicin initial concentration and degree of cross-linking promoted the faster one.

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1 Introduction

Gentamicin is one of the most consumed antibiotics because of its low cost, broad antibacterial spectrum of action, low rate of primarily resistant pathogens, low allergy rate, good stability, and water solubility [1]. Gentamicin binds to components in the bacterial cell and causes the production of abnormal proteins, which is ultimately fatal to the bacteria. Gentamicin can treat many types of bacterial infections, particularly Gram-negative infection; it eliminates bacteria that cause infections in lung, skin, bone, joint, stomach, blood, and the urinary tract infections. However, it is not absorbed from the gut and is consequently only given by injection or infusion.

Gentamicin topical preparations are used to treat the infections of skin. Topical preparations are available in two dosage forms: cream and ointment. According to medication directions, skin wound treatment with topical gentamicin should include three basic steps: (1) washing the affected area with water and soap; (2) application of a small amount of the cream or ointment to the affected area and, finally, (3) covering the treated area with a gauze dressing. This procedure should be repeated at least three times a day for 1 week, or in accordance with a physician's prescription. Thus, traditional gentamicin topical treatment is painstaking, besides it can lead to failure in infection control or delay in wound healing, since the dosage is imprecise and delivered discontinuously. For these reasons, a novel antibiotic-releasing wound dressing was investigated for controlled and sustained release of gentamicin, while covering and protecting the wound.

Drug delivery systems such as poly-methyl-methacrylate beads, poly-acrylic-acid hydrogels, ceramics and inorganic cements loaded with gentamicin have been

developed for osteomyelitis treatment [2–4]. However, they are used as bone implants.

Many researches involving a large number of materials (natural, semi-synthetic or synthetic) have been developed in the skin wound dressing management [5–8]. However gentamicin loaded drug delivery system for this purpose was not developed yet.

Most of synthetic polymers show limited biocompatibility and biodegradability compared to the natural polymers, although some naturally abundant polymeric materials exhibit a limitation in their reactivity, biodegradability, and processability [9, 10]. However, chitosan is recommended as suitable functional material, because of its excellent biocompatibility, biodegradability, non-toxicity, and adsorption properties. In addition, chitosan can accelerate wound healing and has shown hemostatic and bacteriostatic activities [9, 11, 12].

Chitosan is derived from chitin (a natural product found in shrimp and crab shells) by chemical deacetylation. Chitosan have found wide applications such as implants, drug delivery matrix, scaffolds for tissue engineering, skin substitutes, and wound dressings [8, 13, 14]. In bio-delivery applications, chitosan has been used as a vehicle for drug, protein, and gene delivery [15–17].

The retention and the release of a bio-molecule depend on the swelling/deswelling of hydrogel, which may vary by several factors including the amount of water in hydrogel. Chitosan is soluble in acid solutions and can easily form films and membranes by solvent evaporation. However, due to its positive charge in acid medium, caused by amino group protonation, it becomes an extremely hygroscopic hydrogel. To increase the time frame and consistency of kinetics of drug delivery, hydrophilic polymers need to be cross-linked [18]. Several methods are available for the cross-linking of chitosan and the most common is the one using glutaraldehyde as the cross-linker [19]. However, there are concerns over the toxicity of the cross-linking agents used, especially the glutaraldehyde, since the residual retention of which may compromise the biocompatibility of chitosan delivery system.

Recently, a novel water-soluble, bisulfite blocked diisocyanate has been prepared and used as a cross-linking agent for the network formation with chitosan gel [20, 21]. In fact, the bisulfite-blocked diisocyanate is the analogous bis-(aminocarboxysulfonate), which must be devoid of any toxic effects of the relative diisocyanate. Also, the bisulfite derivatization of a diisocyanate group makes the blocked cross-linker soluble in water to allow for easy processability of cross-linked chitosan gel formations. Thus, 1,6- Hexamethylene diisocyanate (HMDI), a well studied diisocyanate was selected as cross-linking agent and reacted with sodium bisulfite to prepare the analogous blocked diisocyanate: Hexamethylene 1,6-bis-(AminoCarboxy-Sulfonate)–HBACS (Fig. 1).

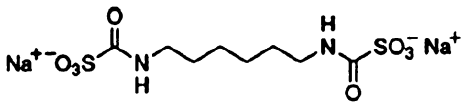

Fig. 1 Structure of hexamethylene 1,6-bis(aminocarboxysulfonate)

Therefore, this research designs a chitosan film; cross-linked through the aforesaid water-soluble blocked diisocyanate cross-linker, and loaded with gentamicin for sustained and consistent delivery of the antibiotic. The resultant biomaterial was characterized for cross-linkage by FTIR, for hydrophilicity by Contact Angle and for swelling behavior by Gravimetric method. Moreover, the in vitro gentamicin controlled release was studied over an extended period of 2 weeks and the effects of gentamicin initial concentration and cross-linking ratio on the kinetics of gentamicin release were also evaluated.

2 Experimental

2.1 Materials and instrumentation

High molecular weight chitosan (Mw $\sim$ 100,000 and >75% deacetylated), acetone, acetic acid, sodium bisulfite, ninhydrin, sodium hydroxide, sodium carbonate, and hexamethylene diisocyanate (HMDI) were purchased from Aldrich Chemical Co., Milwauki, Wisconsin, USA. Gentamicin sulfate and phosphate buffered saline (PBS) were purchased from Sigma Chemical Co. St. Louis, MO, USA.

FTIR analyses were performed on a MIDAC—M series FTIR instrument from MIDAC Corporation (Costa Mesa, CA, USA); and Contact angle analyses were performed in a Contact Angle Instrument (Model: VCA-2000) obtained from AST Products, Billerica, MA, USA.

2.2 Synthesis of hexamethylene 1,6-bis (aminocarboxysulfonate)

In a 100 ml round-bottom flask containing a magnetic stir bar, 6.73 g (40 mmol) of HMDI was added to 8.36 g $Na_2S_2O_5$ (44 mmol) dissolved in 15.53 ml H_2O and was stirred for 20 h. The product was precipitated with acetone and dried in vacuum. Insoluble polymeric byproducts were removed by dissolving the product in water (30 ml) followed by filtration. Product was isolated from the filtrate by precipitation in acetone and dried in vacuum, resulting in a white powder, as described earlier [21].

2.3 Cross-linking reaction

Cross-linking was performed by a modification in the procedure described [21]. Briefly, in a 250 ml flask, 1.5 g of chitosan were dissolved in 1.0% aqueous acetic acid with vigorous stirring. After total dissolution, the desired amount of Hexamethylene 1,6-bis (aminocarboxysulfonate) was added and the solution was heated to 40°C under stirring for 24 h for completely reaction.

Two cross-linking ratios were tested: 30% and 50% (w/w), based on the NH_2 availability on chitosan.

2.4 Preparation of gentamicin loaded films

After cross linking reaction, the desired amount of gentamicin sulfate was added to the solution and stirred for 24 h for completely homogenization. One ml of the resultant solution was placed in each of the circular plastic molds of 16 mm diameter. The contents in the molds were allowed to dry at room temperature to form the circular films.

Two initial gentamicin concentrations were tested: 1% and 10% (w/w), based on chitosan concentration.

2.5 Experimental design for in vitro gentamicin release from cross-linked chitosan

The effects of cross-linking ratio (0, 30, and 50%) and initial antibiotic concentration (0, 1, and 10%) on gentamicin in vitro release were investigated in a 2 × 2 factorial design (Table 1).

2.6 FTIR analyses

FTIR analyses on above thin film samples were performed on a MIDAC—M series FTIR instrument. The samples were scanned in the range of 500–2,000 cm^{-1}.

2.7 Contact angle

The experiments for contact angle measurements were performed in order to evaluate water affinity (hydrophilicity), an important parameter for wound dressings. The water contact angle with chitosan films was obtained by carefully placing a drop of water on the film surface and then, after 30 s, both right and left angles of contact were measured by the contact angle instrument, using a reported method [22].

Table 1 Experimental design for in vitro gentamicin release

Sample	Cross-linking ratio (%)	Gentamicin initial concentration (μg/ml)
1	30	150
2	30	1,500
3	50	150
4	50	1,500

2.8 Swelling behavior

Swelling behavior of the films was assessed by the gravimetric method. Samples films were kept in a vacuum desiccator for 24 h before determining dry mass (m_D) by weighing to ±0.0001 g places on an electronic balance. Then, the samples were immersed for 24 h in 0.1 M PBS maintained at pH 7.4 at 37°C. The soaked samples were then blotted with filter paper to remove non-absorbed surface water and then weighed again to determine wet mass (m_W). Swelling ratio (S) was calculated, using the Eq. 1:

$$S(\%) = [(m_W - m_D)/m_W] \times 100 \quad (1)$$

Reported values are averages of three samples.

2.9 In vitro release

Experiments on in vitro release of gentamicin from the chitosan films (Table 1) were performed at 37°C in 0.1 M PBS, pH 7.4. The release medium was collected after each 24 h and replaced with fresh buffer each time. This procedure was repeated for 14 days and the experiment was carried out in triplicate.

A colorimetric procedure, previously established [23], was used for gentamicin quantification. The method is based on the ninhydrin reaction with primary and secondary amines present in gentamicin. This reaction produces a purple color and the absorption of the gentamicin–ninhydrin mixtures at 400 nm has a linear relationship with the gentamicin concentration.

The collected medium was previously reacted with ninhydrin for 15 min at 95°C, followed by determination of visible absorbance at 400 nm. Gentamicin concentration was taken from the standard curve.

3 Results and discussion

Topical formulations of chitosan for wound healing and related applications and their effects on wound related components are being intensely investigated [14]. The use of chitosan for the controlled delivery of bio-molecules is on rise recently [16, 17]. However, chitosan forms extremely hygroscopic hydrogels and the controlled retention and release of a bio-molecule from chitosan could be achieved by an appropriate biocompatible cross-linker that has physicochemical compatibility with chitosan. Thus,

this work evaluates such a cross-linked chitosan based controlled release device to be later used for wound dressing film for sustained gentamicin release.

3.1 Cross-linker and cross-linking

To attain the cross-linking, first HBACS was synthesized as a cross-linker by a known method; it was then incorporated in required amounts (Table 1) in acidic solution of chitosan, by the formation of urea linkage between HBACS and preferably the amino group of chitosan. Apparently, cross-linking must affect the hydrophilic behavior of the cross-linked chitosan film; therefore, the water-contact angles and swelling behavior of the films were measured. However, confirmation of chitosan cross-linking with HBACS was done by FTIR. The examination of the Hexamethylene 1,6-Diurea cross-linked chitosan films through various parameters used in this work is described as follows.

3.1.1 FTIR

The infrared spectra of chitosan and chitosan-HBACS cross-linked films are presented in Fig. 2. The development of cross-linking was shown by a well-resolved peak between 1,565 and 1,475 cm^{-1}, characteristic of NH deformation in secondary amides (Amide II band). In addition, a peak at about 1,600 cm^{-1}, representing $C = 0$ stretch in secondary amides (Amide I band), and another around 1,700 cm^{-1}, which suggest the presence of carbonyl groups, were only observed for cross-linked films, and its resolution was greater for the 50% cross-linked samples [20].

3.1.2 Contact angle measurement

The results obtained for the non-cross linked chitosan film were Right: 85.7° and Left: 85.2°. However, for both cross-linked films (30% and 50% cross-linked), the contact angles were 0°, since the water drop spread on the films. Thus, cross-linking increased the hydrophilic character of chitosan films.

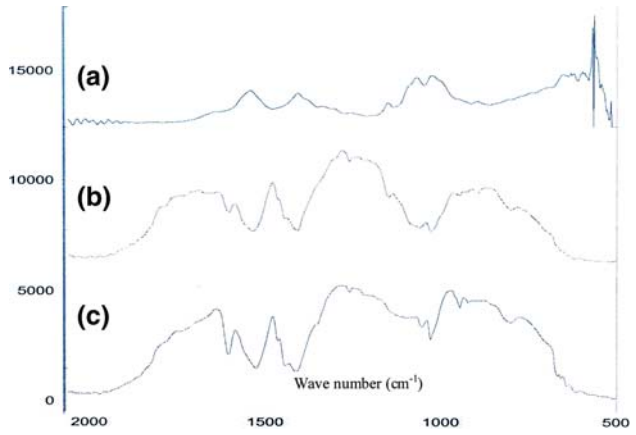

Fig. 2 Infrared spectra of chitosan and chitosan–HBACS cross-linked films. (a) Chitosan film, (b) 30% Chitosan–HBACS cross-linked film, and (c) 50% Chitosan–HBACS cross-linked film

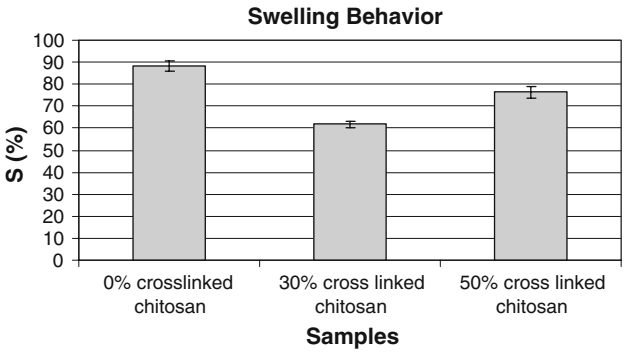

Fig. 3 Swelling behavior of Chitosan and HBACS cross-linked samples

3.1.3 Swelling behavior

The swelling behavior of chitosan films is presented in Fig. 3. As expected, cross-linking suppressed swelling. However, this effect was inversely proportional to the degree of cross-linking, which can be attributed to the hydrophilic character of the cross linking agent.

3.2 In vitro gentamicin release study

Results of in vitro release of gentamicin are presented in Fig. 4.

As expected, higher degree of cross-linking produced slower release, since cross-linking created a polymeric network that might have acted as a barrier to fast diffusion of gentamicin.

Comparing sample pairs with the same degree of cross-linking but different gentamicin initial concentrations (1 and 2; 3 and 4), it was observed that higher gentamicin initial concentration promoted slower percent release and

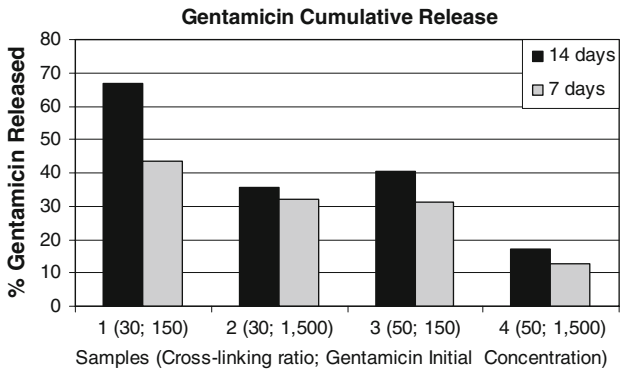

Fig. 4 Gentamicin cumulative release

the lower gentamicin initial concentration showed higher percent release. Therefore, the mechanism of gentamicin diffusion was mainly governed by its initial concentration. Indeed, the diffusion rate was inversely proportional to gentamicin initial concentration.

Comparing couples of sample pairs with the same gentamicin initial concentrations, but different degrees of cross-linking (1 and 3; 2 and 4), higher cross-linking ratio produced slower release. This was of course expected as the higher cross-link density increases the barrier for gentamicin diffusion. Thus, the diffusion rate was also inversely proportional to the degree of cross-linking.

Figure 5 shows the gentamicin concentration in the releasing medium during the 14 days of study. According to this graphic, all samples showed an intensive release of gentamicin in the first 3 days. This behavior can be attributed to the gentamicin incorporated to the film surface that is easily delivered. However, as soon as the superficial gentamicin was released, the release rate has been gradually decreasing, since the polymeric network act as a barrier for gentamicin diffusion. For this reason, all samples released more than half of the gentamicin released in 14 days in the first week (Fig. 4).

The aim of this present study was to develop a novel antibiotic-releasing wound dressing for controlled and sustained release of gentamicin, while covering and protecting the wound. Thus, the gentamicin concentration in the releasing medium should reach the minimal inhibition concentration for wound pathogens. *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* are the most common pathogens found in skin infected wounds. Minimal inhibition concentration of gentamicin for *P. aeruginosa* and *E. coli* varies between 0.06 through 8 μg/ml and for *S. aureus*, it is between 0.12–1.0 μg/ml [24].

Therefore, the initial concentration of gentamicin and cross-linking ratio should be selected according to pathogen in target and also to the period of treatment. For *S. aureus* infection, for example, all the samples could be employed, since the minimal inhibition concentration of gentamicin for this pathogen was reached during the 14 days.

Both samples 2 and 4 (gentamicin initial concentration = 1,500 μg/ml) would be employed for treatment of *P. aeruginosa* and *E. coli* infections, since they maintained gentamicin concentration higher than 8 μg/ml in the first week.

Prolonged treatments require slow diffusion and hence higher gentamicin initial concentration and cross-linking ratio, while for shorter treatments; both gentamicin initial concentrations and cross-linking ratio could be set according to the gentamicin minimal inhibition concentration for the target pathogen.

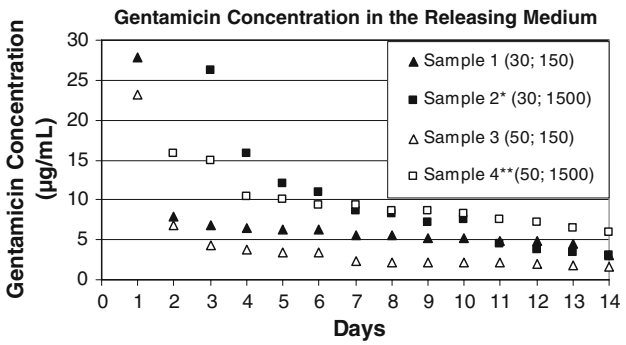

Fig. 5 Gentamicin concentration in the releasing medium. *The concentration of gentamicin for this sample was 324.5 and 95.2 μg/ml in the first and second days, respectively. **Gentamicin concentration for this sample was 135.2 μg/ml in the first day

4 Conclusions

Chitosan–HBACS cross-linking reaction was efficient and confirmed by FTIR and hydrophilic behavior analyses. Cross-linked films swell less than chitosan film itself; however, this effect was inversely proportional to cross linker concentration, due to the hydrophilic character of the cross linking agent.

Moreover, according to contact angle analysis, cross-linked films became more hydrophilic than the chitosan film itself.

In vitro gentamicin controlled release was carried out at 37°C and in pH 7.4, in order to simulate the body conditions. Results showed that the diffusion rate was governed by initial concentration of gentamicin and degree of cross-linking. Higher gentamicin initial concentration and degree of cross-linking promoted the slower release: after 14 days of release, less than 20% of gentamicin initial concentration had been delivered (sample 4). On the other hand, lower gentamicin initial concentration and degree of cross-linking promoted the faster release, with almost 70% of the gentamicin initial concentration released after 14 days (sample 1).

Additional experiments on wound healing were carried out with selected samples and will be published in communication later.

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