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Control release and biological properties of AgNPs loaded hydroxyethylacryl chitosan/sodium alginate film for potential wound dressing

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Abstract

The aim of this study was to prepare wound dressing from natural hydrogel blend between hydroxyethylacryl chitosan (HC) and sodium alginate (SA). The HC/SA films were prepared by solution casting technique in a proportion of HC:SA of 25:75 w/w with further crosslinked by calcium ion. Silver nanoparticles (AgNPs) were induced into the crosslinked films by soaking the film in silver nitrate solution at various concentrations with later reduced by sodium borohydride before drying to obtain the AgNPs contained HC/SA films. The AgNPs in the film was confirmed by X-Ray Diffractometer (XRD) and X-Ray Fluorescence Spectrometer (XRF). The AgNPs contained HC/SA films evidenced antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Cell viability of the film was also measured using MTT assay. The results showed that all of the films were not cytotoxic for Vero cells. The paracetamol was used as a model drug. The release profiles of paracetamol were studied in phosphate buffer solution (PBS), simulated wound exudate fluid, investigated at 37 °C. Almost the films showed slow release profiles of paracetamol during 3-7 days depending on the formula. The comprehensive results suggest their potential as a wound dressing.

Keywords: control release, hydrogel , hydroxyethylacryl chitosan , silver nanopaticles, sodium alginate

Introduction

Wound dressing has been developed to encourage the wound healing.[1] It can recover the suitable physiological reconstruction of skin and prevent infections and dehydration with a moist environment.[2] For this purpose, kinds of wound dressings have been developed basing hydrogels because they have good efficient absorbing volumes of exudate, and still provide a moist wound healing environment.[3]

Chitosan (CS) is a cationic polymer obtain from the deacetylation of chitin. It is a bio-copolymer consisting of N-acetyl-d-glucosamine and d-glucosamine units. CS has biocompatibility, non-toxicity and biodegradability.[4,5] Moreover, CS has properties of hemostasis, wound healing acceleration, scar prevention and absorption-enhancing properties.[6,7] From these properties, CS has been exclusively applied to wound healing [8,9] and drug delivery.[10,11] However, the dissolution of CS can be achieved in acetic acid or other acids solution, which can cause cytotoxic in the final product.[12] To improve the solubility of CS in acid-free water, CS was modified by introducing hydrophilic groups to its reactive amino groups. Hydroxyethylacryl Chitosan (HC) was one of the interesting derivatives of chitosan which was synthesized by following the Michael addition reaction between chitosan and hydroxyethylacrylate. The obtained HC can dissolve in water without using acidic solution and easily synthesized in high yield.[13]

Sodium alginate (SA), a water-soluble salt of alginic acid, is a naturally occurring polysaccharide found in the cell wall of brown algae. It contains two uronic acids, β-(1-4) linked D-mannuronic acid (M) and α-(1-4) linked L-guluronic acid (G). The structure of SA composes of homopolymeric blocks of M-M, G-G, and alternating sequence of M-G blocks. SA can form hydrogels under mild conditions with multivalent metal cations (e.g.,Ca^{2+}, Zn^{2+}, etc.).[14,15] It is usually used as wound dressing and controlled release due to its nontoxic, biodegradable, biocompatibility and excellent film-forming properties.[16-18] However, the crosslinked film of SA is brittle

which may cause the burst effect in the phosphate buffer saline (PBS), simulated wound exudate fluid, during use in drug delivery application.[19]

It is well known that blending of polymers is an effective method to enhance the performance of polymer. In previous work, the HC/SA blended hydrogel films were prepared using calcium chloride as a nontoxic ionic crosslinker. The hydrogel films have pH-sensitive properties, reducing burst effect of drug releasing in PBS, reasonable mechanical properties, and nontoxicity.[15,20] For these reasons, The HC/SA hydrogel can be used in drug delivery as wound dressing rather than the solely SA. However, HC/SA blended hydrogel film does not show any antibacterial activity, which limited its use in infected wound dressing.

It was generally known that silver nanoparticles (AgNPs) were used for numerous antimicrobial applications, such as, in surgical devices, wound dressings, water disinfection, and antibacterial coatings.[21,22] Moreover, AgNPs has shown superior antibacterial properties than its bulk form and highly effective against bacteria, virus and fungi.[23,24] Thus, to improve the effectiveness of antibacterial properties, AgNPs were subjected to incorporate into HC/SA blended hydrogel film. In this work, the hydrogel made from the combination between HC and SA, which containing AgNPs was prepared to establish a new wound dressing and drug release. The drug release profiles from the HC/SA hydrogels in simulated wound exudate fluid or PBS were investigated using paracetamol as a soluble model drug. The effects of AgNPs on the drug release profiles, cytotoxicity and antibacterial activity of hydrogel films were investigated.

Materials and methods

2.1 Materials

Chitosan (degree of deacetylation :85 % MW :270 kDa) was purchased from Eland Co., Ltd. Sodium alginate (MW :1,296 kDa) was purchased from Acros Organics Co., Ltd. Hydroxyethylacrylate was supplied by Thai Mitsui Specialty Chemicals Co , Ltd. Calcium chloride dihydrate was purchased from Merck Millipore Ltd. Silver nitrate was purchased from Fisher Chemical. Sodium borohydride was purchased from Laboratory Chemical. All the chemicals were analytical grade.

2.2 Preparation of hydroxyethylacryl chitosan (HC)

HC was synthesized by Michael addition reaction.[13] Briefly, Chitosan (1 g) was dissolved in 1 %w/v acetic acid (100 ml), and then hydroxyethylacrylate (4 g) was added into the chitosan solution. The solution was stirring at 60 °C for 48 h, and neutralized with 10 %w/v sodium hydroxide before precipitated in acetone. The precipitate was washed with excess acetone and dried in a vacuum oven at 60 °C for 7 days to obtain HC.

2.3 Preparation of HC/SA hydrogel films

HC solution was prepared by dissolving HC (1 g) in distilled water (100 ml) with continuous stirring at 70 °C for 7 days. After cooling the solution to room temperature, SA was added into the HC solution with the weight ratio of HC:SA of 25:75 with further stirring at 70 °C overnight The solution was poured into Petri dish and dried at 60 °C for 24 h to form HC/SA film. The HC/SA film was later immersed in 0.5 M of calcium chloride for 30 min and washed with distilled water for 5 times and then dried at 60 °C overnight to obtain HC/SA hydrogel film.

2.4 Preparation of HC/SA hydrogel films containing silver nanoparticles (AgNPs)

The HC/SA hydrogel films were immersed in 0.5, 1 or 5 mM of silver nitrate for 24 h. The films were washed twice with distilled water and were immersed in 10 mM of sodium borohydride for 30 min. After that, the films were washed twice with distilled water and dried at 60 °C overnight to obtain HC/SA hydrogel films containing AgNPs.

2.5 Characterization of HC/SA film

The XRD patterns of silver were obtained on a X-Ray Diffractometer (XRD, D8 Advance, Bruker BioSpin AG, USA). The quantity of silver was determined using X-Ray Fluorescence Spectrometer (XRF, SRS 3400, Bruker AG, USA). The samples were scanned in the 2Θ range from 5 to 80°.

2.6 MTT assay

Cytotoxicity of the hydrogel films was evaluated by African green monkey kidney fibroblast (Vero) using an MTT assay. The films were soaked into PBS (pH 7.4) at 37 °C for 24 h. Each liquor stock was then received by filtering and subsequently exposed in DMEM (Dulbecco's Modified Eagle Medium). Vero culture was cultured in a medium containing DMEM supplemented with 10 %FBS (Fetal Bovine Serum) and seeded in a 96-well plate at 100 µl/well with later incubated at 37 °C for 24 h. Diluted liquor stock in DMEM (100 µl) was then added to each

plate and incubated at 37 °C for 24 h. Then, 10 µl of MTT solution (5 mg/ml) was added in each well and further incubated at 37 °C for 4 h. After that, the 9:1 mixture of DMSO : 10 % SDS was added in each well plate at a rate of 150 µl/well to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microtiter plate reader. The % cytotoxicity was calculated as follows [25]:

$$\% \text{ cytotoxicity} = \left[\frac{A-B}{A}\right] \times 100 \quad (1)$$

Where A is the absorbance of the control and B is absorbance of the samples.

2.7 Antibacterial Activity Test

Antibacterial activity of the HC/SA films against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) were assessed by using an inhibition zone method. Firstly, 100 µl of 108 CFU/ml bacteria suspension was spread on LB agar plate, then the HC/SA films with a cut in 1x1 cm^2 were placed onto the surface of the agar. After 24 h incubation at 35 °C, the diameter of the inhibition zone was measured.

2.8. Swelling behavior

The degree of swelling was carried out in distilled water for 24 h. Dried hydrogels with appropriate size were weighed, and then immersed in 50 mL of distilled water at 37 °C. The swollen samples were taken from the fluid at time interval. After wiping with tissue paper, the weights were measured. The degree of swelling was calculated as follows:

$$\text{Degree of swelling (DS)} = \left[\frac{M_s-M_i}{M_i}\right] \times 100\ \% \quad (2)$$

where M_s and M_i are the weights of the swollen wet hydrogel and the initial dry hydrogel, respectively.

2.9. Stability test

Dried hydrogels of appropriate size were weighed and soaked in distilled water at 37 °C. After 24 h, the samples were dried and weighed. The gel content was calculated as follows:

$$\text{Gel content} = \left[\frac{M_d}{M_i}\right] \times 100\ \% \quad (3)$$

where M_i is the initial dry weight of hydrogel and M_d is the dry weight of hydrogel after immersing.

2.10 In vitro drug release studies

The HC/SA film was adhered to plastic board at 3 sides to form a 3-side sealed bag. Paracetamol as a model drug (0.15 g) was filled into the 2x2 cm^2 gap of the bag and the entrance was then sealed. After that, the HC/SA side of the sealed bag was covered by cellulose acetate (CA, filter size 0.2 µm). The drug-loaded bag was then floated onto the 100 ml of PBS solution by facing down the CA side to the surface of the medium. The system was shaken in the water bath at 37 °C. At selected time intervals, 0.2 mL of the medium was examined and replaced by fresh medium. The amount of released paracetamol from the drug-loaded bag was determined the absorbance at 242 nm using a UV–Vis spectrophotometer. The percentage of released paracetamol was evaluated from standard calibration curves.

Results and discussion

3.1 Characteristics of HC/SA film

Table 1 Result of XRF analysis of the hydrogel films.

sample	Chemical elements presence (%)			
	Ag	CaO	Na_2O	Cl
HC25SA75Ca0.5	-	99.8	-	0.16
HC25SA75Ca0.5Ag0.5	0.59	95.5	3.81	-
HC25SA75Ca0.5Ag1	1.48	93.9	4.09	0.21
HC25SA75Ca0.5Ag5	10.2	85.5	3.47	0.57

The chemical compositions of the hydrogel films were measured by XRF and summarized in Table 1. The results confirmed the presence of calcium ion and silver in the hydrogel films. For calcium ion content, it was seen that calcium ion was detected in all samples of hydrogel films since it was applied as crosslinking agent in all films. For silver measurement, silver could not be detected in HC25SA75Ca0.5 film as it was not applied in this film. On the other hands, silver could be detected in the silver-loaded hydrogel films and its contents were increased as the relatively high concentration of $AgNO_3$ was applied into the films (HC25SA75Ca0.5Ag0.5 (0.59%), HC25SA75Ca0.5Ag1 (1.48%) and HC25SA75Ca0.5Ag5 (10.2%)). However, it is important to highlight that this technique only can detect elements with AMU over 22, which means that elements like C, O or H cannot be detected with this technique.[26]

To further confirm the crystal structure of the AgNPs in HC/SA films, XRD measurements were employed as displayed in Fig. 1. The diffraction pattern of the HC25SA75Ca0.5Ag5 film could show sharp peaks at 2θ = 38.474(111), 44.387(200), 64.660(220), and 77.639(311). They represented, respectively, diffraction peaks of the crystal of pure AgNPs.[27] Furthermore, no peaks of other impurity crystalline phases were detected. However, no peaks have been detected on the diffraction patterns of HC25SA75Ca0.5Ag0.5, HC25SA75Ca0.5Ag1 films as relatively minimal amount of $AgNO_3$ was applied. From XRF and XRD results, it could be concluded that the HC25SA75Ca0.5Ag0.5, HC25SA75Ca0.5Ag1 and HC25SA75Ca0.5Ag5 films contain AgNPs in the structure of films.

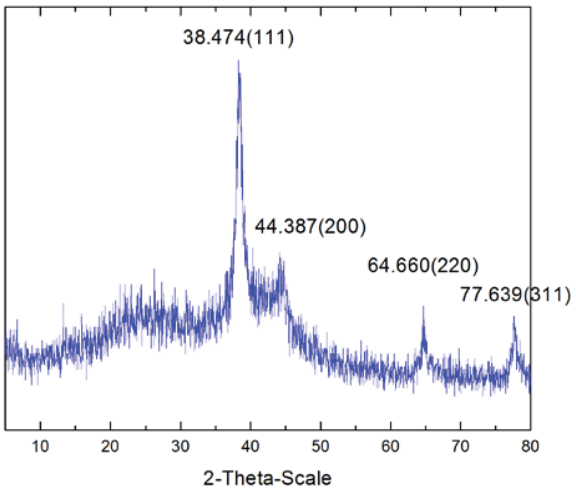

Figure 1 The XRD pattern of HC25SA75Ca0.5Ag5

3.2 Cytotoxicity

Table 2 % Cell viability of the hydrogel films.

sample	% cell viability
Control	100
HC25SA75Ca0.5	90.96 ±2.44
HC25SA75Ca0.5Ag0.5	88.08 ±0.58
HC25SA75Ca0.5Ag1	91.89 ±2.71
HC25SA75Ca0.5Ag5	96.77 ±2.65

Cell viabilities of the HC/SA films on Vero cells are shown in Table 2. After 24 h incubation, the cell viability treated with all HC/SA film formula showed approximately 90% cell viability (% cell viability more than 50% [29]). These results indicate the HC/SA films were not toxic to human cells.

3.3Antibacterial Activity

The antibacterial activities of the HC/SA films are shown in Table 3. The HC25SA75Ca0.5 does not show antibacterial activity against *E. coli* and *S. aureus* while the AgNPs-loaded HC25SA75Ca0.5 does. However, the effect of the contents of silver in the hydrogel film on antibacterial activity was not significantly different. Thus, the minimum concentration of $AgNO_3$ (0.5 mM) for the preparation of the hydrogel film was selected for further study on the swelling behavior and in vitro drug releasing behavior.

Table 3 Antibacterial activity against *E. coli* and *S. aureus* of the hydrogel films.

Sample	Zone diameter (mm)		Activity
	E. coli	*S. aureus*	
HC25SA75Ca0.5	0	0	Inactive
HC25SA75Ca0.5Ag0.5	13±2	14.5±2.4	active
HC25SA75 Ca0.5Ag1	12±0.45	16.5±1.63	active
HC25SA75 Ca0.5Ag5	13.4±1	15.3±1.7	active

3.4 Swelling behavior

The swelling behavior of the hydrogel films in distilled water at ° 37C was investigated. For calcium crosslinked films, the results (Fig. 2a) showed the influence of concentration of crosslinking reagent on the swelling behavior. The degree of swelling decreased with increasing the concentration of $CaCl_2$. It was because high concentration of $CaCl_2$ brought about high crosslinking density of the film. Fig. 2b showed the influence of the loading of silver into the film on the swelling behavior. The results obviously showed no significant difference comparing with their hydrogel films without silver at the same crosslinked condition suggesting that the silver depositing inside the film structure as AgNPs pattern rather than Ag^+ ion corresponding to XRD results.

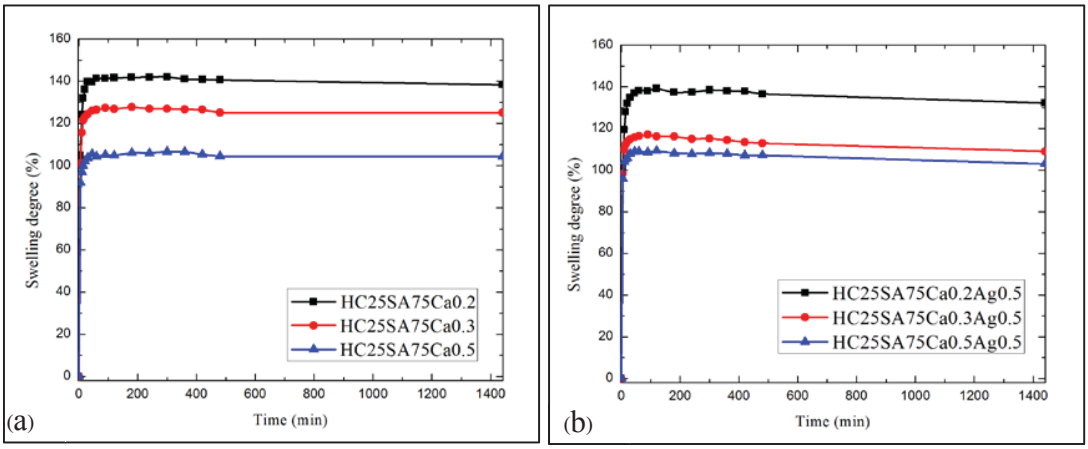

Figure 2 Degree of swelling of HC/SA hydrogel films in distilled water at 37 °C. (a) HC25SA75 with varying concentrations of $CaCl_2$ as crosslinking agent; (b) HC25SA75 with varying concentrations of $CaCl_2$ and containing AgNPs.

3.5 Gel content

Gel contents of the hydrogel films crosslinked by $CaCl_2$ at various concentrations and their AgNPs-loaded films are displayed in Table 4. The values of all samples are almost 100%. Considering both the concentration of crosslinking agent (0.2–0.5 M of $CaCl_2$) and the adding of AgNPs, less effect on gel content was observed. The results suggested their potential to be used in applications requiring permanent contact with water.

Table 4 Gel content of HC/SA hydrogel in distilled water at 37 °C

Sample	**Gel content** (%)
HC25SA75Ca0.5	99.2±0.19
HC25SA75Ca0.2	98.6±0.31
HC25SA75Ca0.3	98.3±0.80
HC25SA75Ca0.2Ag0.5	97.8±0.23
HC25SA75Ca0.3 Ag0.5	97.3±0.49
HC25SA75Ca0.5Ag0.5	97.3±0.95

3.6 In vitro drug release studies

Paracetamol was used as a soluble model drug to study drug release behavior of the HC/SA hydrogels. Fig. 3 showed the release profiles of paracetamol at 37 °C in PBS. Almost the paracetamol was released from the films and all the films demonstrated the linearity of drug release profile. It could be seen in Fig. 3a that the HC25SA75Ca0.5 could completely release all of paracetamol within 7 days, while the HC25SA75Ca0.5Ag0.5 spent 8 days to gain 60% releasing of paracetamol. It could be stated that AgNPs was affected to lower the drug releasing rate of paracetamol. It was mentioned that AgNPs can sustain release of free silver ions from the nanoparticles.[28] This silver ion can then crosslink with COO^- group of SA and also generate coordination interaction with loan pair electron of N of chitosan bringing about the extra crosslinking point within the HC/SA films. This phenomenon could impede the release of paracetamol from the HC/SA films. The effects of varying concentrations of $CaCl_2$ on drug releasing rate were also investigated as the results shown in Fig. 3b. The 60% of paracetamol was released from HC25SA75Ca0.5Ag0.5 within 8 days, while the paracetamol was completely released from the HC25SA75Ca0.2Ag0.5 and HC25SA75Ca0.3Ag0.5 within 3 and 5 days, respectively. It is

clearly seen that the higher degree of crosslinking resulting in the trap of paracetamol into the structure of HC/SA films, thus, the lower drug release rate was obtained.

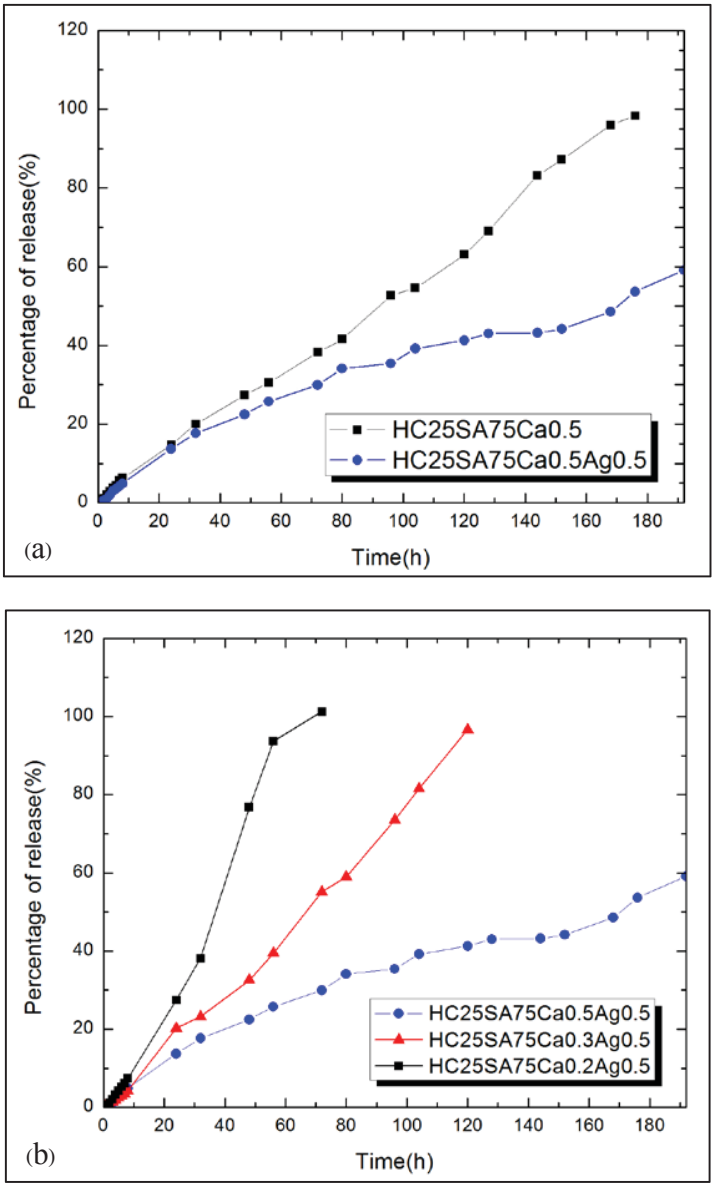

Figure 3 Drug release profiles of HC/SA hydrogel films in PBS at 37 °C (a) the effect of AgNPs in HC25SA75Ca0.5; (b) HC25SA75 with varying concentrations of $CaCl_2$ as crosslinking agent.

Conclusion

In this study, the properties of hydrogel films based on the combination of hydroxyethylacryl chitosan (HC) and sodium alginate (SA) using calcium chloride as a crosslinker with containing AgNPs were investigated for their potentiality in a wound dressing and control drug release. The XRF and XRD results confirmed AgNPs in the structure of HC/SA hydrogel film. The swelling behavior in distilled water at 37 °C showed the decrease in degree of swelling with increasing the concentration of $CaCl_2$. The values of gel content in all films were mostly 100%. In vitro drug release profiles demonstrated the linearity and showed mostly release of paracetamol within 3-7 days depending on the types of HC/SA films. Moreover, all of the prepared hydrogel films have been proven to

be not toxic to our living cells and the AgNPs-loaded HC25SA75Ca0.5 showed antibacterial activity against *E. coli* and *S. aureus*. The comprehensive results of this study suggested their potential as a wound dressing and control drug release.

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