Research Article

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Chitosan-Alginate Drug Loaded Polymer Network for Accelerated Wound Healing

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ABSTRACT

The biodegradable sponge composed of chitosan and sodium alginate at different ratio (1:3, 2:2, 3:1) was prepared and analyzed for their swelling, *in vitro* drug release and biodegrading ability. Plumbagin a multifunctional agent was incorporated into chitosan and alginate sponge to deter wound infection. The water uptake ability, lysozyme degradation resistance and persistence drug release was found to be more appropriate in the sponge prepared with high ratio of chitosan to alginate. An *in vivo* animal test employed confirms the applicability of the sponge as a wound dressing material without any adverse symptoms. The sponge had better wound healing effect than cotton gauze and by incorporating plumbagin a more accelerated wound healing was observed in animal model.

Key Words: Plumbagin, chitosan, alginate, biodegradable sponge, wound dressing, wound healing

1. INTRODUCTION

Chitin is a biopolymer consisting of poly N-acetyl glucosamine and is widespread in nature as a structural material particularly in marine arthropod shells. Chitin is generally an insoluble material but can be deacetylated by treatment with hot sodium hydroxide to form the soluble polymer chitosan. Chitosan preparations of various molecular weights, degrees of de-acetylation and with further molecular derivatization patterns have attracted much attention because of their potentially beneficial biological properties. These properties include hemostasis, antimicrobial activity stimulation of healing, tissue-engineering scaffolds, and drug delivery [1]. Chitosan is obtained by the hydrolysis of chitin and until now, many researchers have examined chitosan as a promising material for biomedical applications on account of its good biocompatibility, biodegradability, cellular binding capability, antimicrobial activity and wound healing effect [2].

Sodium Alginate is a natural amylose carbohydrate distilled from alga. It is widely applied to food, medicine, textile, printing and dyeing, paper-making and daily chemicals as thickener, emulsifier, stabilizer and binder etc. Sodium Alginate is exploring more in food application, especially since 1980s forward. It is not only a safety food additive, but also a basis material of modeling food or dietary food [3].

Secondary metabolites produced by plants constitute a source of bioactive substances and nowadays the scientific interest has increased due to the search for new drugs from plant origin. *Plumbago* species are reported in the literature for its biological activities such as, anti-parasitic, insect anti-feedant, anti-tumor, wound healing and others, some of them attributed to the presence of special chemical compounds, such as naphthoquinones. Plumbagin is a naphthoquinone well distributed among *Plumbago* species, specially found in their roots [4].

The use of natural polymers as drug carriers has received much attention in the pharmaceutical field due to their safety. In particular, the polysaccharides such as sodium alginate and chitosan have been studied for application in the design of dosage forms for controlled release. The use of natural polymers is valuable based on proven biocompatibility [5].

Wounds can be classified into chronic wounds and acute wounds. Chronic wounds take longer than 8-12 weeks to heal. Examples include diabetic leg ulcers, arterial and venous leg ulcers and pressure sores. Acute wounds can be burn wounds, surgical wound or wounds from trauma. The process of wound healing is a set of coordinated responses to tissue injury that results in tissue contraction, closure, and restoration. The longer it takes for spontaneous wound healing, the worse the outcome usually is, with increasing likelihood of developing hypertrophic scarring and unsightly alterations in pigmentation [6].

The healing process can be thought of as occurring in five phases, namely, inflammation leading to haemostasis and clot formation, fibroplasia and neovascularization, formation of granulation tissue, re-epithelialization, and finally the formation of new extracellular matrix and tissue remodelling. However, topical applications of compounds with anti-oxidative properties using proper materials and antioxidants will be useful against oxidative damage and be helpful to the healing of the wound [7].

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In this research work, chitosan, alginate sponge was prepared at three different concentrations (1:3, 2:2, 3:1) and plumbagin, a multi-functional agent derived from plants of Plumbago species was incorporated to the sponge (CA sponge) to deter wound infection and accelerated wound healing. An *in vivo* animal test was adopted to analyze the sponge for its wound healing effect.

MATERIALS AND METHODS

Chemicals

Chitosan (MW 300,000 and degree of deacetylation, 92%), sodium alginate (MW 300,000) and plumbagin were purchased from Sigma-Aldrich Co. (USA). All other reagents and solvents used were of analytical grade.

Preparation of Chitosan-Alginate Sponge (CA Sponge)

Chitosan solution was prepared by dissolving chitosan in deionized water containing acetic acid (1.0% by weight). Alginate solution (1.0% by weight) was prepared by dissolving sodium alginate powder in deionized water (Mei Dai, et al., 2009). The chitosan solution was mixed with sodium alginate solution (chitosan-alginate blend ratios by weight were 3:1, 1:1, and 1:3) and blended with a homogenizer at room temperature until an opaque aqueous solution was obtained. The opaque aqueous solution was centrifuged at low speed to remove the trapped air bubbles. The centrifuged solution was then poured into a 6-well plate (well size: 36mm diameter, 20mm height), frozen overnight at -40ºC and then lyophilized at -30ºC for 24 hours in a freeze dryer (Sanyo, Japan).

Preparation of Plumbagin-Loaded CA Sponge

Plumbagin was accurately weighed and dissolved in absolute ethanol. The plumbagin-ethanol solution was mixed with chitosan solution. The prepared solution was then added into alginate solution and blended with a homogenizer, centrifuged, frozen and lyophilized as described earlier (refer section 2.2).

Swelling Ability of Sponges

A known weight of chitosan-alginate sponge was incubated in phosphate buffered saline (PBS, pH-7.4) to determine the swelling ability of CA sponges. The wet weight of the sponge was determined at required period of time by first blotting the sponge with filter paper to remove adsorbed water on the surface and then weighed immediately on electronic balance. The percentage water adsorption of CA sponges in the media was calculated as follows:

$$\text{Water absorption (\%) } E_{SW} = \frac{W_e - W_o}{W_o} X\ 100 \quad \ldots\ldots\ldots\ldots\ldots [1]$$

Where E_{SW} is the percentage water adsorption of chitosan-alginate sponges at equilibrium. W_e denotes the weight of the chitosan-alginate sponges at equilibrium water adsorption, and W_o is the initial weight of the chitosan-alginate sponges. Each experiment was repeated 3 times, and the average value was taken as the percentage water adsorption.

In Vitro Degradation

The chitosan-alginate sponges were incubated at pH7.4 in phosphate-buffered saline (PBS) with 500-1000U/C.C. of lysozyme concentration in 6-well plate and kept at 37ºC. At required period of time, the sponges were taken out, washed with deionized water, frozen and lyophilized. The weights of the sponges were weighed and recorded.

$$\text{Weight loss (\%)} = \frac{W_o - W_f}{W_o} X\ 100 \quad \ldots\ldots\ldots\ldots\ldots [2]$$

Where W_o denote the initial weight of the CA sponges and W_f denotes the final weight of the CA sponges after lysozyme treatment. The experiment was performed in triplicate and the average value was taken as the percentage weight loss.

Drug Loading and Release

Plumbagin incorporated CA sponge was prepared by adding plumbagin solution (final concentration was 1mg/ml in chitosan-alginate solution) to chitosan solution under constant stirring. The mixed solution was then added into alginate solution, blended, centrifuged, frozen and lyophilized as described above.

Drug release was analyzed using 36mm-in-diameter plumbagin-incorporated CA sponges in 6 centrifuge tubes with 10ml phosphate-buffer solution (pH4.0) within a shaker at 37 ºC. All the supernatants were pipetted out periodically and replaced with equivalent volume of fresh phosphate-buffer solution (pH4.0). The concentration of plumbagin was measured using UV spectroscopy at 420 nm.

Persistence test of drug in chitosan alginate sponge

The plumbagin loaded C3A1 sponge and C3A1 sponge were cut into discs under sterile condition. The sponge discs (1.0 cm in diameter) were placed in *Staphylococcus epidermidis* (ATCC 29213) (3hrs grown culture) lawn cultured MH agar plates and incubated at 37 °C for 24 hours. An uncoated sponge disc was used as control to differentiate the efficacy efficiency of the coated plant extract material.

After zone formation, both plumbagin loaded and control discs were transferred aseptically to another *S. epidermidis* (ATCC 29213) lawn cultured MH agar plate and incubated as the previous day. This procedure was followed till there was no visual zone formation of all the two disc samples against the used pathogen [8].

Wound Healing Test

The albino rats (140 – 200 g, 6 to 7 weeks old) were used in this wound healing test after getting ethical clearance from the ethical committee. After anaesthetizing with chloral hydrate, the dorsal hair of the rats was removed. Full-thickness wound of 1.5X1.5 cm^2 was excised from the back of the rats. Each wound was covered with an equal size of drug loaded CA sponge, or blank CA sponge or cotton gauze for comparison. Treated rats were placed in individual cages, and the healing wounds were observed on the 0^{th}, 5^{th} and 15^{th} day using a digital camera (16.1 MP Nikon, China).

RESULTS AND DISCUSSION

Preparation of wound healing sponges

The chitosan-alginate sponges were successfully prepared in this

work and were shown in Figure.1. In Figure.2, the flexibility of the sponge was shown by bending it with a forceps.

Figure.1 Chitosan-alginate sponge

(a) The C3A1sponge of chitosan-alginate mixture
(b) The C3A1 sponge with plumbagin

Figure.2 Flexibility of chitosan-alginate sponge

(a) C3A1 sponge bended using a foil covered forceps
(b) Freeze dried sponge showing the liability

Swelling ability of chitosan alginate sponges

The swelling ability of the prepared three different sponges was analyzed using sterile distilled water for its water up taking ability in a period of time. The weight of the sponges before and after water treatment was tabulated in Table.1. The recorded values were used to analyze the percentage water absorption of chitosan alginate sponges using the equation (1).

Table.1 Swelling ability of chitosan alginate sponges

S. No.	Time of water uptake (min)	C3A1 Initial weight (g)	Final weight (g)	C2A2 Initial weight (g)	Final weight (g)	C1A3 Initial weight (g)	Final weight (g)
1.	5	0.0245	0.0327	0.0245	0.0307	0.0245	0.0289
2.	10	0.0327	0.0355	0.0307	0.0335	0.0289	0.0315
3.	15	0.0355	0.0384	0.0335	0.0364	0.0315	0.0334
4.	20	0.0404	0.0404	0.0364	0.0374	0.0344	0.0354
5.	25	0.0424	0.0424	0.0374	0.0374	0.0354	0.0364

The water uptake ability depends on the porous structure of the inner matrix of sponge which is attributed to the water retained within the pores. As the ratio of alginate was increased to chitosan, the water up taking ability of the chitosan alginate sponges gets decreased [9]. Each pore surrounded by fibrillar wall in the structure of C3A1 sponge can hold water and hence the water uptake ability reaches 73.1 %. The C2A2 sponge showed decreased values of 52.7 % because the fibrous network of the sponge attenuate the ability of the water retained within pores. The C1A3 sponge gave even lesser value of 44.5 % in which the pores are surrounded by thin and fragile walls. According to previous researchers this might be due to the fibrillar walls formed by a part of the un-reacted alginate, when the sponge was dipped into the water [9]. Due to the swelling and dissolving of this un-reacted alginate, the pore size gets increased and as a result water upholding ability of the C1A3 sponge gets decreased. There might be other reasons like prevalence of sealed-in cells in sponges that can decrease the ability of water absorption as water cannot get into the sealed-in cells of sponges.

***In vitro* degradation of chitosan alginate sponges**

The *in vitro* degradation of the prepared three different sponges by lysozyme was analyzed in a period of time and the values were tabulated in Table.2. The recorded values were used to analyze the percentage of chitosan alginate sponge enzymatic degradation using the equation (2).

Table.2 *In vitro* degradation of chitosan alginate sponges

S. No.	Time of degradation (min)	C3A1 Initial weight (g)	Final weight (g)	C2A2 Initial weight (g)	Final weight (g)	C1A3 Initial weight (g)	Final weight (g)
1.	30	0.0700	0.0685	0.0700	0.0612	0.0700	0.0602
2.	30	0.0685	0.0634	0.0612	0.0534	0.0602	0.0514
3.	30	0.0634	0.0595	0.0534	0.0515	0.0514	0.0484
4.	30	0.0595	0.0566	0.0515	0.0506	0.0484	0.436
5.	30	0.0566	0.0528	0.0506	0.0487	0.0436	0.0387

The property to be considered for the prepared sponges was stability as they have a physical contact with wounds for few days. The results in Table.2 represent the percentage of weight loss of cross-linked sponges as a function of degradation by lysozyme over time. The weight loss of chitosan sponge ranges 25 %, 30.4 % and 45 % for C3A1, C2A2 and C1A3 respectively. The sponges prepared with the mixing ratio of chitosan to alginate of 1:3 showed 1.5 – 3 times higher weight loss than that of 3:1 and 2:2. The order of the weight loss was C1A3>C2A2>C3A1. Probably because of the higher cross-linking degree of C3A1 sponges it was considered stronger and so can withstand the lysozyme degrading action better than C1A3 and C2A2 sponges.

Drug loading and *in vitro* release

The release of plumbagin from sponge was examined to evaluate its sustaining ability and was shown in Table.3.

Table [3] *In vitro* release of plumbagin from the chitosan alignate sponges

S.No	Incubation time of sponge in PBS (hours)	OD value (420 nm) C3A1	C2A2	C1A3
1	1	0.022	0.026	0.030
2	2	0.025	0.030	0.036
3	3	0.031	0.034	0.039
4	4	0.033	0.037	Not identifiable

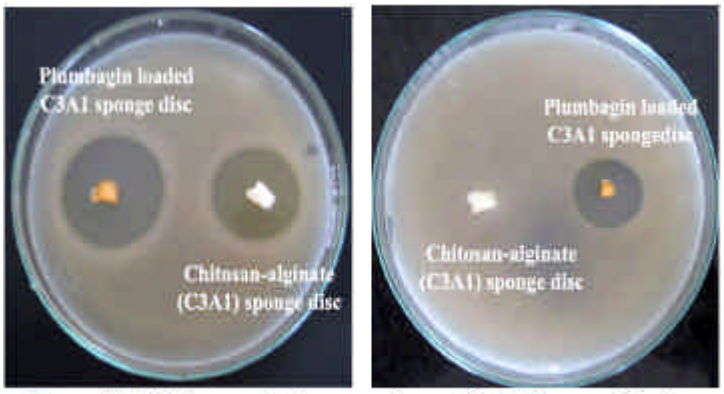
The drug release behavior of chitosan-alginate sponges loaded with plumbagin, a hydrophobic drug was investigated. In 4 days of observation, about 50 % of drug (approx. 0.05 mg) released from C3A1 sponge and 70 % from C2A2 and 80 % of the drug released from C1A3 sponge had been evaluated in 3 days. These indications revealed better sustained release property of drug from C3A1 sponge than C2A2 and C1A3 sponges. This property of C3A1 sponge may be due to the interaction of amino group on chitosan with carboxyl groups on alginate.

Persistence test of drug loaded chitosan sponge

The C3A1 sponge coated with the drug plumbagin and the uncoated C3A1 sponge were used to evaluate their bioefficacy against the *Staphylococcus aureus* (ATCC 29213).

The drug coated sponge disc exhibited 36 mm of inhibition zone against *S. epidermidis* (ATCC 29213) while the uncoated sponge sample showed 15 mm inhibition zone for the same pathogen in the first day of incubation in persistence test (Figure.3).

Figure.3 Persistence tests of sponges against *S. epidermidis* (ATCC 29213)



Zone of inhibition on 1st day Zone of inhibiton on 5th day

For 4 consecutive days the two discs were transferred aseptically to fresh bacterial pathogen inoculated Muller-Hinton agar plates to evaluate the persistence of antimicrobial activity. The coated sample exhibited good bioefficacy in the entire number of days analyzed, while the uncoated sponges showed little less bioefficacy comparatively (Table 4).

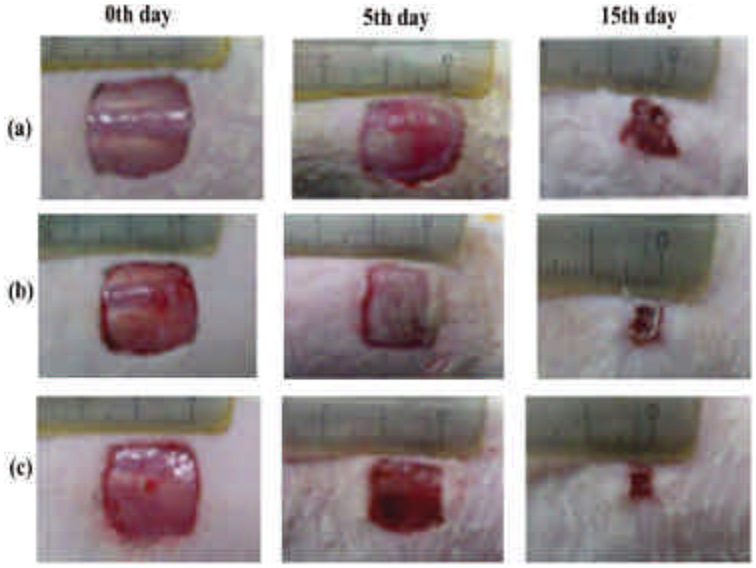
Table.4 Persistence test of sponges against *S. epidermidis* (ATCC 29213)

S.No	Type of chitosan sponge	Incubation time (days)	Zone of inhibition (mm)
1.	C3A1 sponge	1	15
		2	9
		3	7
		4	3
		5	No clear zone
2.	C3A1 plumbagin sponge	1	36
		2	28
		3	22
		4	18
		5	14

Wound Healing Test

Ten percent extract of Plumbago plant was prepared in saline for topical application in previous references by researchers for wound healing [10]. Wound healing activity was studied using excision and incision wound models in rats following topical application. Based on the examined properties, C3A1 sponge was used as a wound dressing material in wound healing test. Full thickness wounds were made on the back of each rat (Figure.4), dressed with normal sterile cotton gauze (as control), C3A1 and C3A1-plumbagin sponges for their wound healing behavior analysis. As a control the wound was covered with cotton gauze. On the 15th postoperative day the dressing material adhered to wound surface was removed and the loss of tissue formation at the wound site was noticed. When the cotton gauze was peeled, slight bleeding and inflammation can be noticed due to the dryness of the gauze. While in the case of C3A1-plumagin sponge, the underlying granulation tissue was formed and in C3A1 sponge there was no bleeding as it can hold moisture condition but the formation of tissue is very meager.

Figure.4 Wound healing test.



(a) cotton gauze as control - at 0th, 5th, and 15th day
(b) C3A1 sponge - at 0th, 5th, and 15th day
(c) C3A1 – plumbagin sponge - at 0th, 5th, and 15th day

The wound on the 15th day was more contractive than the wound observed on the 5th post-operative day; the healing of the C3A1-plumbagin sponge treated wound was obviously faster than the C3A1 sponge and cotton gauze treated wound.

CONCLUSION

A biodegradable sponge, composed of chitosan (CS) and sodium alginate (SA), incorporated with plumbagin was successfully obtained in this work. The swelling ability, *in vitro* drug release and degradation behaviours, and an *in vivo* animal test were employed to confirm the applicability of this sponge as a wound dressing material. The release of plumbagin from the sponges could be controlled by the crosslinking degree of the polymers and released from the sponges in an extended period for up to 4 days for accelerated wound healing. An *in vivo* animal test using albino rats showed that sponge had better effect than cotton gauze, and adding plumbagin into the sponge enhanced the therapeutic healing effect.

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