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Development and assessment of hemostasis chitosan dressings

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ABSTRACT

The aim of this study was to prepare and evaluate chitosan dressings treated with sodium hydroxide (NaOH) and/or sodium tripolyphosphate ($Na_5P_3O_{10}$) for haemostatic use. The pure sodium hydroxide-gelled chitosan dressing (CS-B) had water contents (about 95%) and porosities (about 85%) which were similar to those of commercially available chitosan-based products. The CS-B dressing also exhibited homogeneously sized and penetrating pores throughout, whereas the commercially available Clo-Sur PAD showed porous lamellar structures inside and Instant Clot Pad exhibited a heterogeneous pore size distribution. Additionally, the CS-B dressing was flexible and resilient, free of odour and able to recover completely after compression in a hydrated state. Finally, the CS-B sponge absorbed blood quickly, accelerating blood clotting, enhancing red blood cell adhesion and maintaining its original shape after haemostatic testing.

1. Introduction

Chitosan is a biocompatible, antimicrobial material derived from the alkaline N-deacetylation of chitin, a natural biopolymer originating from crustacean shells. This partial deacetylation gives rise to chitosan, a linear polysaccharide with interspersed D-glucosamine and acetyl-D-glucosamine units. For example, chitosan has been found to promote tissue growth and to accelerate wound healing (Brown, Daya, & Worley, 2009; Peter et al., 2010). Moreover, its efficient gel-forming properties and ability to be shaped or incorporated into hydrogels, microspheres and spongy dressings expand its potential applications in biomedicine (Dai, Tegos, Burkatovskaya, Castano, & Hamblin, 2009; Kranokpiraksa et al., 2010; Muzzarelli, 2009, 2010).

There are two commercially available haemostatic dressings in Taiwan: the Clo-Sur PAD (Scion Cardio-Vascular, Inc., FL, U.S.A.) and the Instant Clot Pad (Cosmo Medical Inc., Taiwan). Both are composed of chitosan and are often used to stop trauma-related arterial bleeding, as well as routinely applied post-angioplasty after wound debridement. Clinically, when the chitosan dressing makes contact with a wound, it adheres to and covers the site and attracts red blood cells, forming a seal that prevents further haemorrhaging. The haemostatic mechanism of chitosan involves the agglutination of red blood cells, possibly due to its intrinsic polycationic properties and nonspecific binding to cell membranes (Fischer, Bode, Demcheva, & Vournakis, 2007; Okamoto et al., 2003; Rao & Sharma, 1997). Some reports indicate that chitosan also accelerates coagulation *in vivo* by influencing the activation of platelets (Baldrick, 2010; Chou, Fu, Wu, & Yeh, 2003; Muzzarelli et al., 2007).

These two chitosan products have several shortcomings that may limit their clinical applications, such as having a trace acidulous odour due to the use of acetic acid as a processing solvent, which is potentially allergenic; being too fragile to retain proper shape under compression; and needing a long period of compression to stop bleeding after angioplasty.

We previously reported on chitosan membrane and sponge-like devices that were prepared by thermally induced phase separation, followed by non-toxic sodium hydroxide (NaOH)-based gelation. Finally, sodium tripolyphosphate ($Na_5P_3O_{10}$) was employed as a crosslinking agent to increase mechanical strength. Here, a series of chitosan sponges was fabricated by adjusting the molecular weight of chitosan and the crosslinking conditions. The dressings' basic properties and haemostatic efficacy were then evaluated against the two commercially available products, which served as controls.

2. Materials and methods

2.1. Materials

Chitosan of two different molecular weights, 300 kDa and 70 kDa, were purchased from TCI (Tokyo, Japan). The degree of

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Table 1
Experimental variables used for the preparation of the chitosan dressings. Water content, equilibrium swelling ratio and porosity of the chitosan dressings.

Formulation	Mw of chitosan	Chitosan solution neutralized	Treatment solution (v/v)		Chitosan dressing code name	Water content (%)	ESR (%)	Porosity (%)
			NaOH, 4%	$Na_5P_3O_{10}$, 5%				
A	7×10^4	y	1	0	CS-A	94.72 ± 0.38	24.89	43.85 ± 5.82
B	7×10^4	n	1	0	CS-B	95.12 ± 0.33	36.00	85.57 ± 1.57
C	7×10^4	y	0	1	CS-C	94.34 ± 1.20		
D	7×10^4	n	0	1	CS-D	94.29 ± 0.18		
E	7×10^4	y	3	17	CS-E	92.87 ± 0.55		
F	7×10^4	n	3	17	CS-F	91.01 ± 0.89		
G	3×10^5	y	1	0	CS-G	89.45 ± 2.50	41.83	64.77 ± 3.63
H	3×10^5	n	1	0	CS-H	88.74 ± 0.63	41.94	80.80 ± 5.63
I	3×10^5	y	0	1	CS-I	93.23 ± 0.63		
J	3×10^5	n	0	1	CS-J	92.43 ± 0.67		
K	3×10^5	y	3	17	CS-K	92.16 ± 0.22		
L	3×10^5	n	3	17	CS-L	90.94 ± 0.53		
Clo-Sur PAD	–	–	–	–	Clo-Sur	96.86 ± 0.50	39.87	83.94 ± 6.77
Instant Clot Pad	–	–	–	–	Inst-Clot	96.76 ± 0.22	20.38	84.05 ± 1.53

deacetylation of the chitosan was approximately 83%. Sodium tripolyphosphate ($Na_5P_3O_{10}$, 5%) and sodium hydroxide (NaOH, 1 N) were purchased from SHOWA (Tokyo, Japan) and acetic acid was purchased from Merck-Schuchardt (Hohenbrunn, Germany). All chemicals used in this study were of reagent grade.

2.2. Fabrication of haemostatic chitosan dressing

Chitosan was dissolved in 0.1 N aqueous acetic acid to form a 2% (w/v) chitosan solution. Part of this solution was neutralised to pH 6.0 by adding aqueous 1 N NaOH. Ten millilitres of the acidic and neutralised chitosan solutions were put into 6 cm dish and frozen at −20 °C for 8 h, followed by lyophilisation for at least 24 h. The resultant porous chitosan dressings were further treated with a mixture solution of 1 N aqueous NaOH and 5% $Na_5P_3O_{10}$ at a volume ratio of 3:17 for 3 h to induce gelation and crosslinking (Table 1) (Lin, Chang, Chen, Chou, & Kuo, 2006; Chang, Niu, Kuo, & Chen, 2007). The treated dressings were then washed with distilled water three times, frozen at −20 °C for 4 h and lyophilised again.

2.3. SEM observation of morphology and blood coagulation

Scanning electron microscopy (SEM) was employed to examine the morphology of the chitosan dressings, with emphasis on the porous characteristics resulting from different NaOH/$Na_5P_3O_{10}$ ratios. Prior to SEM, the samples were lyophilised and sputter-coated with gold, followed by observation using a Hitachi S-2700 (Tokyo, Japan) instrument.

A sample of 0.5 mL of human whole blood or platelet-insufficient blood obtained from the TBSF was added to each chitosan dressing. After incubation at 37 °C for a predetermined amount of time, the dressings were fixed, dried and sputter-coated with gold for SEM studies. Blood coagulation on a cover glass was used as a control.

2.4. Water content and equilibrium swelling testing

The water content of the chitosan dressings was determined by swelling the dressing in phosphate buffered saline (PBS) at pH 7.4 at room temperature for 2 h. The wet weight (W_{wet}) of the swollen dressing was measured immediately after gently blotting with filter paper to remove surface liquid, followed by lyophilisation and reweighing (W_{dry}). The water content of the dressing was calculated using the formula:

$$WC = \frac{W_{wet} - W_{dry}}{W_{wet}} \times 100\%$$

The equilibrium swelling ratio (ESR) of the dressing was also calculated, based on the following equation:

$$ESR = \frac{V_w - V_d}{VW} \times 100\%.$$

Here, V_d is the exterior volume of the chitosan dressing (1 cm × 1 cm), measured using a vernier caliper, and V_w is the exte-

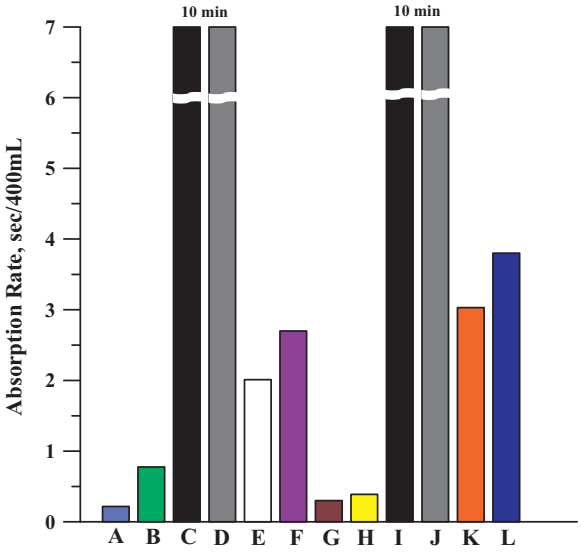

Fig. 1. Absorption rate of 0.4 mL of distilled water on the prepared chitosan dressings.

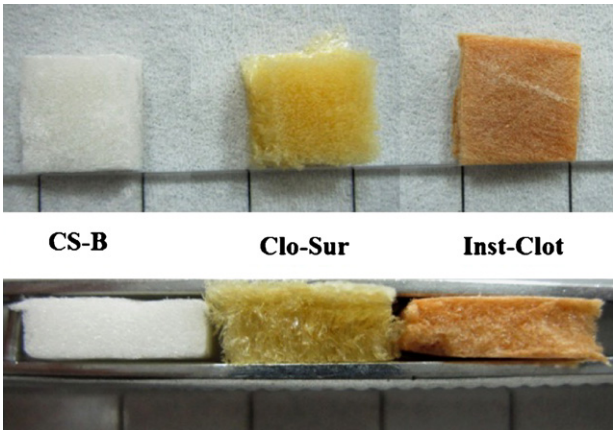

Fig. 2. Photographs of the fabricated chitosan dressing (CS-B) and the commercial chitosan dressings (Clo-Sur and Inst-Clot).

rior volume of the dressing after swelling in distilled water for 1 min.

2.5. Determination of porosity

The porosity of the prepared chitosan dressings was determined using Archimedes' principle. The exterior volume (V_d) of each chitosan dressing (1 cm × 1 cm) was measured using a vernier caliper. The sample was then immersed in a pycnometer containing 99% ethanol solution. The actual volume of (V_a) of the sample was calculated using the formula:

$$V_a = \frac{(W_w - W_o) - (W_t - W_p)}{0.789 \ \ g/cm^3},$$

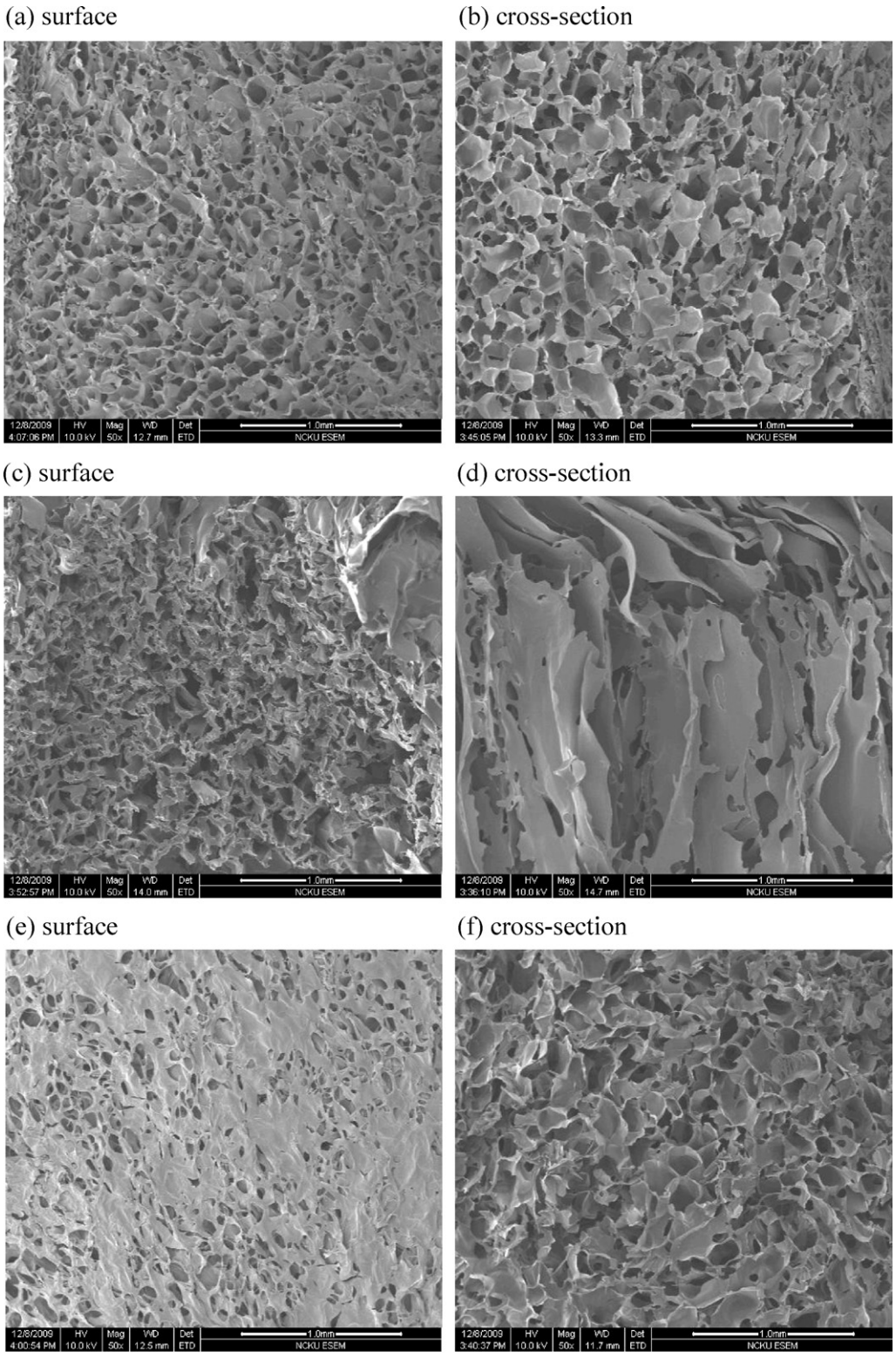

Fig. 3. SEM micrographs of the surface and cross-section of the chitosan dressings: (a, b) CS-B, (c, d) Clo-Sur and (e, f) Inst-Clot dressings.

where W_w is the weight of ethanol and the pycnometer; W_o is the dry weight of the pycnometer; W_t is the combined weight of the ethanol, the pycnometer and the dressing sample; W_p is the combined weight of the dry pycnometer and dry dressing sample; and 0.789 g/cm^3 is the density of 99% ethanol solution. The porosity of the chitosan dressing was then determined using the following formula:

$$\text{Porosity}\ (\%) = \frac{V_d - V_a}{V_d} \times 100\%.$$

Porosity values were expressed as means ± standard deviations ($n = 6$).

2.6. Absorption and blood clotting testing

The absorption rate of the chitosan dressings was determined using distilled water and human whole blood. The latter was obtained from the Taiwan Blood Services Foundation (TBSF, Taipei, Taiwan). Dressings were cut into 1 cm × 1 cm squares and placed into glass bottles. Then, 0.4 mL of distilled water or human whole blood was dispensed onto the dressing. The absorption rate was defined as the time required for the dispensed fluid to be completely absorbed by the dressing.

The blood clotting test was modified from Ong, Wu, Moochhala, Tan, & Lu (2008). Dressings were cut into 1 cm × 1 cm squares and placed into glass bottles. Next, 0.25 mL of human whole blood (containing the anticoagulant citrate dextrose at a 1:6 ratio) was slowly dispensed onto the surface of the dressings. The bottles containing the samples were then incubated at 37 °C. After a predetermined amount of time (30, 60, 90, 120 or 180 s), 20 mL of distilled water were carefully added by dripping water down the inside wall of the bottles, preventing disruption of the clotted blood. Red blood cells that were not entrapped in the clot were haemolysed with distilled water and the absorbance of the resultant haemoglobin solution was measured at 540 nm (UV-VIS spectrophotometer Agilent 8453, Santa Clara, CA, USA). The absorbance of 0.25 mL of whole blood in 20 mL of distilled water was used as a reference value.

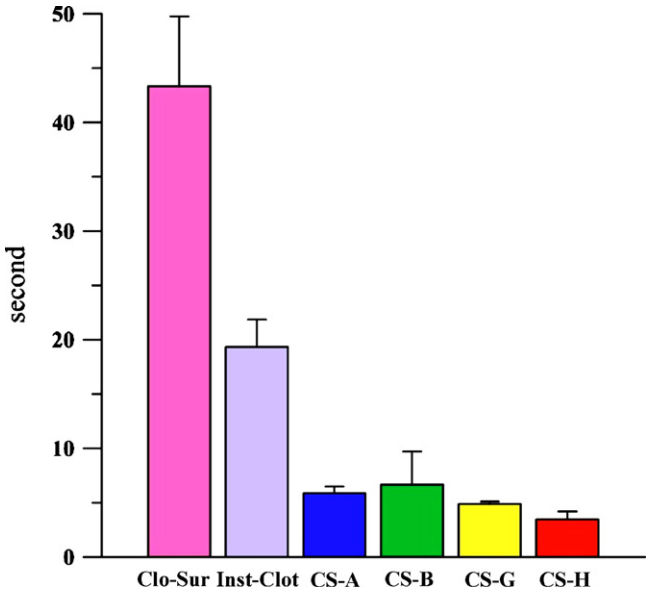

Fig. 4. Absorption rate of 0.4 mL of human whole blood on the chitosan dressings.

2.7. Statistical analysis

All quantitative data were expressed as means ± standard deviations. Differences between means were analysed for statistical significance using the Student's *t* test. *P*-values less than 0.05 were considered statistically significant.

3. Results and discussion

Rapid blood absorption and effective coagulation by the prepared chitosan dressings were our main concerns. We first tested the dressings' absorption of distilled water prior to further examination (Fig. 1). Among the chitosan sponges that we prepared

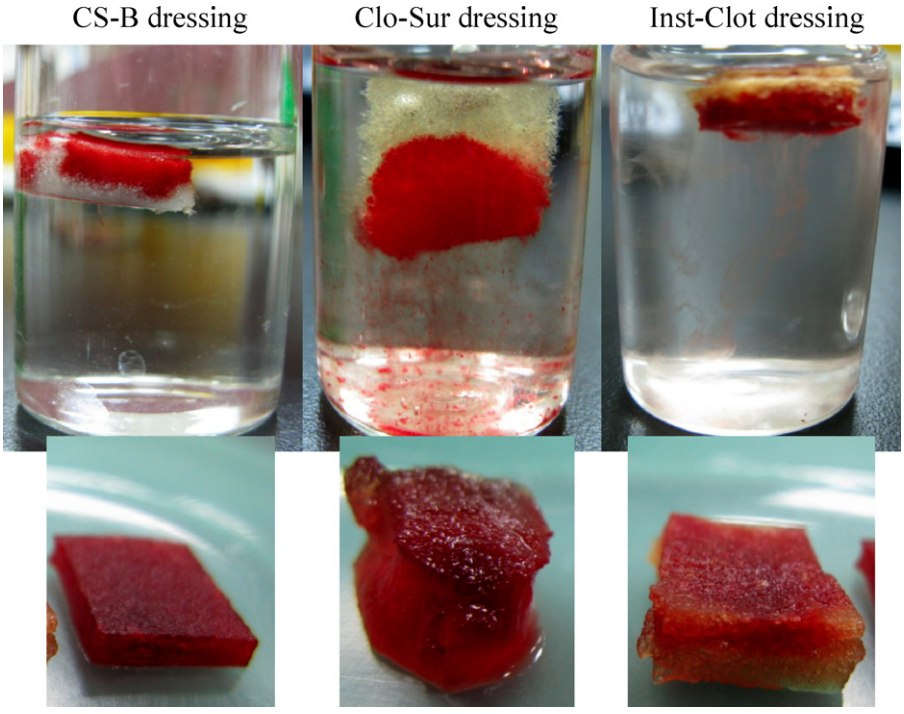

Fig. 5. Photographs showing that more haemoglobin leaked from the Clo-Sur dressing than from the Inst-Clot dressing. The CS-B sponge entrapped red blood cells well and maintained its original shape after absorbing whole blood.

(Table 1), the ones treated with pure 4% NaOH solution absorbed 0.4 mL of distilled water in less than 1 s (CS-A, CS-B, CS-G and CS-H dressings). In contrast, dressings treated with pure 5% $Na_5P_3O_{10}$ solution or with a mixture of NaOH and $Na_5P_3O_{10}$ at a volume ratio of 3/17 exhibited a slower absorption rate. Specifically, the sponges treated with pure $Na_5P_3O_{10}$ needed over 10 min to absorb 0.4 mL of distilled water (CS-C, CS-D, CH-I and CS-J dressings).

It was observed that chitosan dressing treated with 4% NaOH was flexible and resilient, as assessed macroscopically. Measurement of resistance to compression (hardness) indicated that the chitosan dressings treated with 5% $Na_5P_3O_{10}$ were slightly tougher. In general, these dressings had a high degree of recovery to original shape upon immersion in distilled water or whole blood than other sponges.

All chitosan dressings prepared in this study exhibited a high water content of about 89–95%, whereas the commercially available chitosan-based products had a slightly higher water content of 96% (Table 1). The porosity of the prepared dressings was also determined, falling in the range of 44.4–85.6%. Variation among the porosities of the chitosan dressings was observed, related to whether the chitosan solutions were titrated to pH 7.0 (Table 1). The two titrated chitosan samples, with molecular weights of 300 kDa and 70 kDa, respectively, exhibited lower porosity than the non-treated ones due to the pre-gelation reaction of the NaOH solution with the soluble chitosan molecules. SEM observation also demonstrated fewer pores and more lamellar structures in the pH-titrated CS-A and CS-G samples (data not shown). The CS-A as well as CS-B dressings exhibited lower swelling ratios than the commercially available chitosan-based products, Clo-Sur dressings and Inst-Clot dressings. Based on our preliminary results regarding the water content, absorption rate, porosity, macroscopic characteristics and ease of preparation of various chitosan dressings, we chose those treated with pure NaOH for further examination and used the Clo-Sur and Inst-Clot dressings for comparison.

Photographs of the CS-B dressing and commercially available chitosan-based products are shown in Fig. 2. The CS-B sponge had a white appearance, while the Clo-Sur and Inst-Clot dressings were more yellow and brown in colour and had an acidulous odour. Scanning electron micrographs of these dressings are presented in Fig. 3, indicating a porous structure. The CS-B dressing had a more homogenous pore-size distribution and displayed penetrating pores both on the surface and in the cross-sectional view. On the other hand, the Clo-Sur dressing exhibited lamellar sheet structures on the inside, while a heterogeneous pore distribution was observed on the surface and inside of the Inst-Clot dressing.

3.1. Blood absorption

The NaOH-treated chitosan dressings presented faster absorption rates, absorbing 0.4 mL of human whole blood in less than 5 s. Meanwhile, the Clo-Sur dressing required more time (about 45 s) to completely absorb the same amount of blood (Fig. 4). The distinctly rapid absorption rate of the CS-B dressing may be attributed to its homogeneous and penetrating porous structure, while the Clo-Sur dressing contained lamellar sheets that may have inhibited percolation of the blood.

To assess blood coagulation, the dressings were placed in 0.9% saline solution. As seen in Fig. 5, the Clo-Sur dressing swelled significantly in saline and blood leakage from the dressing was observed. Similar phenomena were also observed for the Inst-Clot dressing, although less blood leached out of the sponge. In contrast, blood was well entrapped inside the CS-B dressing, despite slight swelling.

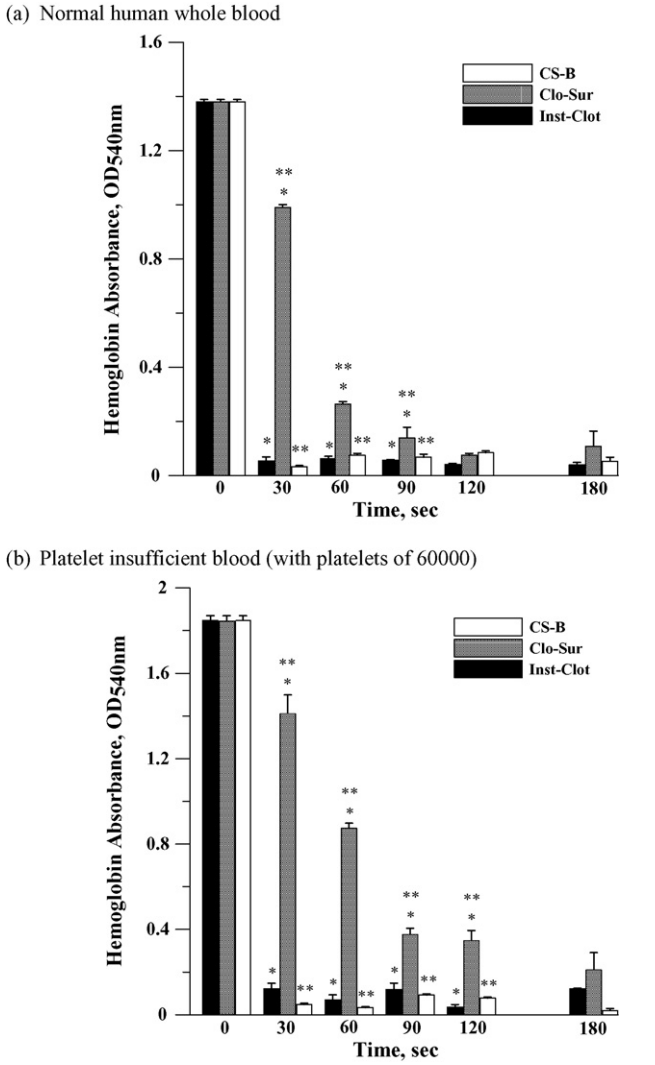

Fig. 6. Blood clotting rates of the CS-B, Clo-Sur and Inst-Clot dressings for (a) human whole blood and (b) platelet-insufficient blood, determined based on the absorbance of haemoglobin from lysed, non-coagulated red blood cells. The * indicates a significant difference between the Inst-Clot and Clo-Sur dressings at each time-point, while the ** indicates a significant difference between the Clo-Sur and CS-B dressings at each time-point ($p < 0.05$, Student's t test, $n = 3$).

3.2. In vitro whole blood clotting test

To evaluate whether the CS-B dressing could increase the rate of blood clotting, human whole blood containing a normal (about 250,000 platelets/ml) or decreased number (60,000 platelets/ml) of platelets was dripped on dressings for 30–180 s before haemolysis of the RBCs that were not entrapped in the resultant clot. The absorbance of the resultant haemoglobin-containing solution was measured, with a high absorbance value indicating a slower clotting rate. As shown in Fig. 6, the CS-B dressing and Inst-Clot yielded significantly lower absorbance values than the Clo-Sur dressing after 30 s of incubation with human whole blood ($p < 0.05$). The absorbance value for the Clo-Sur dressing was similar to that of the other two dressings after 120 s. The platelet-insufficient blood could also form clots on the CS-B dressing after 30 s. The Inst-Clot dressing exhibited a slightly higher absorbance value, and thus slower clotting than the CS-B dressing, while the Clo-Sur sponge could not form clots completely even after 180 s.

SEM evaluation of blood clot formation on the three chitosan dressings revealed that the red blood cells formed larger aggregates

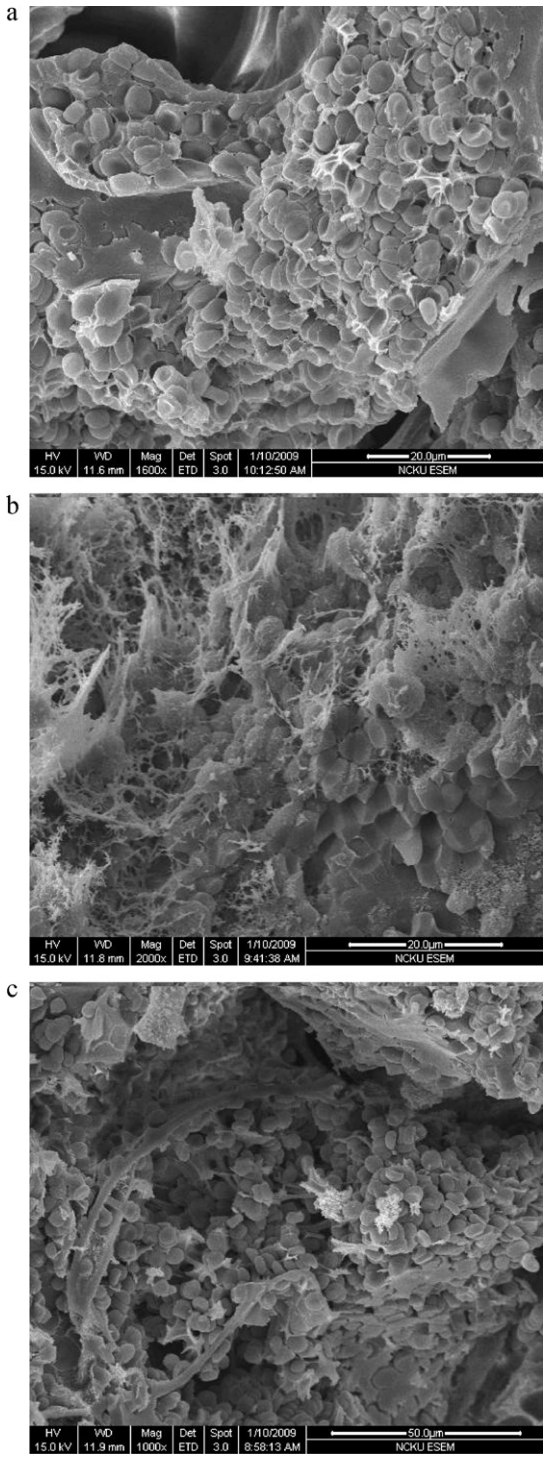

Fig. 7. SEM micrographs of human whole blood on the (a) CS-B, (b) Clo-Sur, and (c) Inst-Clot dressings after 30 s of absorption.

(Fig. 7a and b). More specifically, the red blood cells seemed to have coalesced into an erythrocyte clot or plug on the CS-B and Inst-Clot dressings. Conversely, fewer aggregates were observed on the Clo-Sur dressing while more extensive fibrin was noted on the surface, perhaps caused by attached platelets (Fig. 7c).

We have checked the pH changes of the solution resulting from immersion of the CS-B, Clo-Sur and Inst-Clot dressings in distilled water for 120 min, using latex and polypropylene film as control groups. The pH values decreased significantly from pH 6.8 to pH 4.7 and 5.0, respectively, for the Clo-Sur and Inst-Clot dressings after 120-min immersion. On the other hand, the CS-B dressing, latex and polypropylene film did not cause any obvious pH changes in the surrounding solution during testing. The decreased pH noted for some of the dressings indicated their inherent acidity, which was achieved or retained during preparation.

4. Conclusion

In the present study, we established a modified procedure for preparing chitosan dressings. After freezing and lyophilisation to produce a porous chitosan matrix, the matrix was treated with NaOH or a mixture of NaOH and $Na_5P_3O_{10}$. The matrices were then washed and lyophilised again to produce dressings. By modifying the gelation technique, the dressings' flexibility, texture, appearance, odour and blood absorption and coagulation could be improved, especially in comparison with those of commercially available chitosan-based products. Taken together, the results of the physical examination of the dressings and the haemostatic assays demonstrated that the NaOH-treated chitosan dressing was optimal for enhancing haemostasis. This preparation led to faster and more clotting and retained its original shape and flexibility after contact with human blood.

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