Kasetsart J. (Nat. Sci.) 49 : 92 - 102 (2015)

Development of Protein-Based Hydrogel Wound Dressing Impregnated With Bioactive Compounds

Wimol Phoudee and Wanida Wattanakaroon*

ABSTRACT

Wound healing is a complex, multi factorial process in which the skin repairs itself after injury. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. However, this process is delayed or impaired in patients with wound-healing abnormalities. These chronic nonhealing wounds are considered a critical problem in clinical practice. This study aimed to synthesize a biocompatible natural protein-based wound dressing that was able to deliver bioactive molecules for the treatment of difficult-to-treat chronic wounds. Bovine serum albumin (BSA) was chosen as a model protein. Dressings were prepared as a hydrogel or gel pad containing 10 $\mu g.mL^{-1}$ ciprofloxacin and 10 μM astaxanthin. The dressing was characterized in terms of morphology, absorption and antibiotic release. Antimicrobial activity, biocompatibility and proliferation were investigated. The results revealed that the hydrogel surface had a porous structure. The absorption capacity of the hydrogel increased with an increase in the immersion time and reached equilibrium within 3 d. The BSA hydrogel had activity against *Pseudomonas aeruginosa* with a log reduction value of 6.19 ± 0.12. At day 1 and 2, 4.24 and 1.31 $\mu g.mL^{-1}$ of ciprofloxacin, respectively, were released from the BSA pad and resulted in 6.32 ± 0.16 and 5.08 ± 0.2 log reduction, respectively. A culture of Vero cells with the dressing material indicated that it had no cytotoxicity. Astaxanthin incorporated into both dressings promoted cell proliferation at the highest rate on day 1. These characteristics suggest both dressings had potential for wound healing.

Keywords: bovine serum albumin, astaxanthin, ciprofloxacin, wound dressing

INTRODUCTION

Wound healing is the natural restorative process of tissue injury which includes three distinct phases—inflammation, tissue formation and tissue modeling. In healthy individuals, the healing response is usually nonproblematic and takes place at an optimal rate. However, it is delayed or impaired in patients with healing abnormalities. As a result, chronic nonhealing ulcers develop (Singer and Clark, 1999). This chronic wound care can be facilitated with the use of dressings, which are designed both to manage the microbial infection and to promote wound healing.

Wound dressings synthesized from biocompatible materials are of interest due to their desired properties such as being nontoxic, non-allergenic, non-adherent and easily removed without trauma (Czaja *et al.*, 2006). This study

Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University, Nakhon Pathom 73000, Thailand.

* Corresponding author, e-mail: wanida@su.ac.th

Received date : 15/08/14 Accepted date : 05/02/15

aimed to synthesize a biocompatible, natural protein-based wound dressing that was able to deliver bioactive molecules for the treatment of difficult-to-treat chronic wounds.

Thus, the focus of the study was to develop wound dressings from the protein-based material that can protect the wound from infection and accelerate tissue repair. Bovine serum albumin (BSA) was chosen as the model protein to demonstrate protein-based dressings. Dressings were prepared as a hydrogel or in the form of a gel pad containing the antibiotic drug and antioxidant with the aim that the latter may be a strategy to improve the wound repair process. Hydrogels are one of the most promising materials for biomedical applications and have great potential for use as wound dressings (Teramoto *et al.*, 2008).

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA, fraction V) and astaxanthin as an antioxidant were purchased from Sigma (St. Louis, MO, USA). Ciprofloxacin was obtained from Fluka (Buchs, Switzerland) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) was purchased from Invitrogen (Eugene, OR, USA). Cell culture reagents were purchased from Gibco BRL (Grand Island, NY, USA). All commercially available solvents and reagents used were of analytical grade and no further purifications were made.

Hydrogel preparation

The 6% (weight per volume, w/v) BSA was dissolved in phosphate buffer with pH 7.4. The suspension was heated at 100 °C for 30 min and rapid-cooled in cold water. An amount of 10 $\mu g.mL^{-1}$ of ciprofloxacin dissolved in 0.133 mM sulfuric acid and 10 µM of astaxanthin dissolved in 0.3% (volume per volume, v/v) dimethyl sulfoxide (DMSO) were added to the hydrogel, followed by 0.25% (weight per volume, w/v) NaCl solution. Subsequently, the suspension was cooled down at 4 °C for 2 hr for complete gelation (Akintayo *et al*., 1999). The gel pad involved heating 8% (w/v) BSA for 20 min. The suspension was then prepared as described above and cast on a 9.8 cm diameter Petri dish followed by air drying under a laminar flow hood for 6 hr. The pad was cut out of a dish into a 2.5 × 2.5 cm square.

Characterization

The morphological appearance of the BSA gel pad was studied. The freeze-dried sample was fixed on aluminum stubs and coated with gold for observation using scanning electron microscopy (SEM) with a Maxim 2000 scanning electron microscope (CamScan; Cambridge, UK).

Absorption ratio

The absorption-time profile was determined for the gel pad. The pad was weighed and immersed in 5 mL of phosphate buffer at pH 7.4 and 37 °C for predetermined periods. After the surface water was blotted with filter paper, the weight of each sample was recorded and compared with the initial weight of the gel pad. The weight gain was then calculated. The absorption ratio of a gel pad at time *t* was estimated using Equation 1:

$$\text{Absorption ratio} = \frac{W_t - W_0}{W_0} \qquad (1)$$

where W_t is the weight gain at time *t* and W_0 is the initial weight before treatment. The cumulative absorption ratio represents the sum of the current absorption ratio plus the cumulative amount of absorption ratio at time *t*.

Susceptibility

Clinically isolated *P. aeruginosa* TISTR 1467 was used in this study. The strain was grown in nutrient broth (NB) obtained from Lab M (Heywood, UK) at 37 °C. The susceptibility was measured using 9 mL of 16 hr *P. aeruginosa* at 1×10^8 colony forming units per milliliter (cfu.

mL^{-1}) and was mixed with 1 mL of hydrogel. The hydrogel treatments consisted of: hydrogel alone; hydrogel containing 0.133 mM sulfuric acid and 0.3% (v/v) DMSO; hydrogel with either astaxanthin or ciprofloxacin; and hydrogel with ciprofloxacin and astaxanthin. A control broth with bacterial inoculation was also used. Cultures were then incubated at 37 °C for 24 hr. The cell suspensions were serially diluted and viable bacterial numbers were determined by drop-plating on nutrient agar (NA). Plates were incubated overnight at 37 °C, cfu were enumerated, and the density of viable bacteria ($cfu.mL^{-1}$) was calculated. Bacterial killing was reported as a log reduction. The log reduction was calculated relative to the cell count of the control.

***In vitro* antibiotic release**

The ciprofloxacin-loaded BSA pad (2.5 × 2.5 × 0.1 cm) was immersed in 5 mL of phosphate buffer at pH 7.4 and 37 °C to determine the antibiotic drug release from this structure. The initial concentration of ciprofloxacin used in this study was at 125 μg per pad. The phosphate buffer at pH 7.4 was removed completely every day consecutively for 5 d to determine the drug concentration, and fresh phosphate buffer (pH 7.4) was introduced equally to maintain a constant volume. The mixture was centrifuged at 8,000 revolutions per minute at 4 °C for 10 min and the supernatant liquid containing antibiotic was taken. The ciprofloxacin content was measured using spectrophotometry (Biochrom, Cambridge, UK) at a wavelength of 278 nm, and the susceptibility of *P. aeruginosa* to the antibiotic released was performed as described above.

Cytotoxicity

Vero (African green monkey kidney) cells were cultured in a humidified 5% (v/v) CO_2/air environment at 37 °C in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum. The cells were grown at approximately 60–80% confluence of cells.

The toxicity of dressing materials was determined by the MTT reduction assay (Zhang *et al*., 2008). Cells were plated at a density of 2.5×10^4 cells per well (suspension volume 100 μL) in 96-well plates. The dressing materials including the BSA, acid and DMSO, astaxanthin, and ciprofloxacin at the concentrations used in dressings were incubated with the cells for 24 and 48 hr. After each time, the culture medium was removed and the cells were washed with phosphate buffered saline (PBS) at pH 7.4. MTT was added to each well to yield a final MTT concentration of $0.5\ mg.mL^{-1}$. Cells were incubated with the MTT for 4 hr in a CO_2 incubator after which time 100 μL of DMSO was added to dissolve the formed formazan crystals. The results were then read using a microplate reader (Tecan; Männedorf, Switzerland) at 560 nm. Viability was reported relative to the control cells not exposed to the dressing materials.

Cellular proliferation

Cellular proliferation was based on the method reported by Wiegand *et al*. (2009). Cells were plated at an initial density of 4.0×10^3 cells per well (suspension volume 100 μL) in a 96-well plate. The cells were cultured for 1 d and after that time the culture medium was replaced with 200 μL of either MEM medium containing hydrogel at a volume ratio of 1:1 or conditioned MEM medium from the immersion of a gel pad for 1 and 2 d. The hydrogel and pad treatments consisted of: hydrogel and pad alone; hydrogel and pad containing 0.133 mM sulfuric acid and 0.3% (v/v) DMSO; hydrogel and pad with either astaxanthin or ciprofloxacin; and hydrogel and pad with ciprofloxacin and astaxanthin. Cultures grown without dressings were used as controls. The proliferation was assessed consecutively for 5 d of cell culture. After incubation, the wells were washed twice with PBS at pH 7.4 to remove the culture medium treatment. The number of proliferated cells was then measured using the same procedure as in the cytotoxicity test.

Statistical analysis

All data were expressed as mean ± standard deviation of *n* independent observations. Statistical analysis of the viability dataset for the control and treatment was performed using Student's *t*-test. Results were considered significant if $P < 0.05$.

RESULTS

Hydrogel structure

BSA, a native and therapeutic protein, has been used as the structural dressing material (Matejtschuk *et al.*, 2000) and physiological carrier for drugs (Tada *et al*., 2005). As reported above, the BSA was formed into a transparent hydrogel. Upon heating at 100 °C, BSA undergoes an irreversible stage due to the disruption of the non-covalent forces, causing partial unfolding and the formation of aggregation states (Lin and Koenig, 1976; Murayama and Tomida, 2004). Gelation occurs when the polymer chains cross-link physically into networks (in this case as a result of temperature) and the rigid structure results from the addition of sodium ions (Bryant and McClements, 2000).

Morphology

The surface and internal morphology of the gel pad was characterized using SEM with the results presented in Figure 1; the pore morphology on the surface of the hydrogel was visible and the BSA gel showed a sponge-like, interconnected, mesoporous structure.

Absorption of hydrogel

Hydrogels are materials that can form a three-dimensional polymeric network capable of retaining water (Peppas *et al.*, 2000). The equilibrium uptake of phosphate buffer at pH 7.4 by the protein gel pad was determined at 37 °C. The cumulative absorption ratio versus time profile (Figure 2) shows that the hydrogel pad underwent rapid swelling. The absorption values increased with an increase in the immersion time and became constant over a period of 3 d. The cumulative absorption ratio after immersion in phosphate buffer at pH 7.4 for 1 d was 1.32, while for 2 and 3 d, the ratios were 2.26 and 2.24, respectively. Apparently, there was partial destruction of the hydrogel structure at days 2 and 3. This may have been due to the fact that the hydrophilic and water-solubilizable albumin substituent (which has a high affinity for water) substantially affected the mechanical properties of the hydrogel.

Susceptibility of *P. aeruginosa* to ciprofloxacin

The incorporation of a broad spectrum antibiotic such as ciprofloxacin in a wound dressing can help prevent bacterial infection. The antimicrobial test indicated that the developed dressing was highly effective against the relevant bacterial strain. The BSA hydrogel-containing antibiotic had activity against *P. aeruginosa* with a log reduction value of 6.19 ± 0.12 (Figure 3). In combination with astaxanthin, ciprofloxacin exhibited a lower effect on the log reduction of *P. aeruginosa*. Chalkley and Koornhof (1985) reported that ciprofloxacin was an effective bactericidal agent against *P. aeruginosa* and *E. coli*. The minimum bactericidal concentration value for *P. aeruginosa* was 0.03 $\mu g.mL^{-1}$.

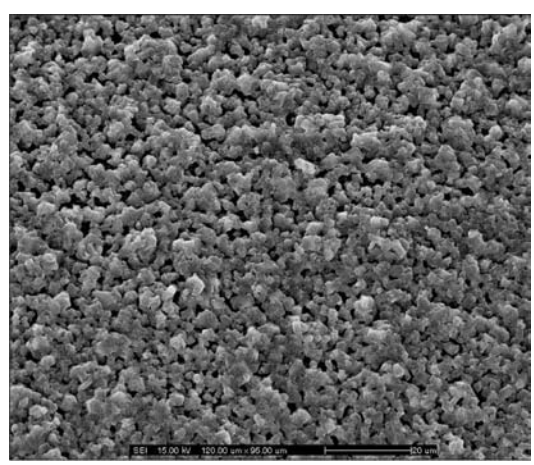

Figure 1 Representative scanning electron micrograph of bovine serum albumin gel pad (Scale bar = 20 μm).

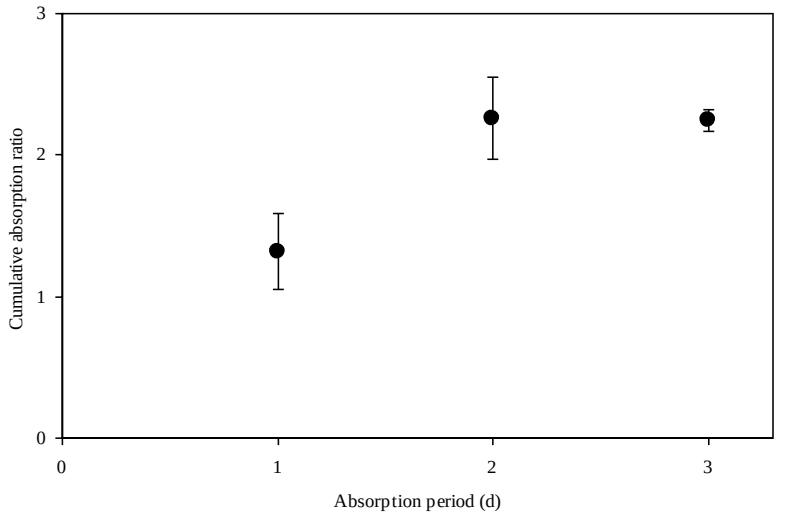

Figure 2 Cumulative absorption ratio of the bovine serum albumin gel pad. Experiment was conducted at 37 °C in phosphate buffer at pH 7.4. Results are reported as the mean ± SD of 3–6 measurements.

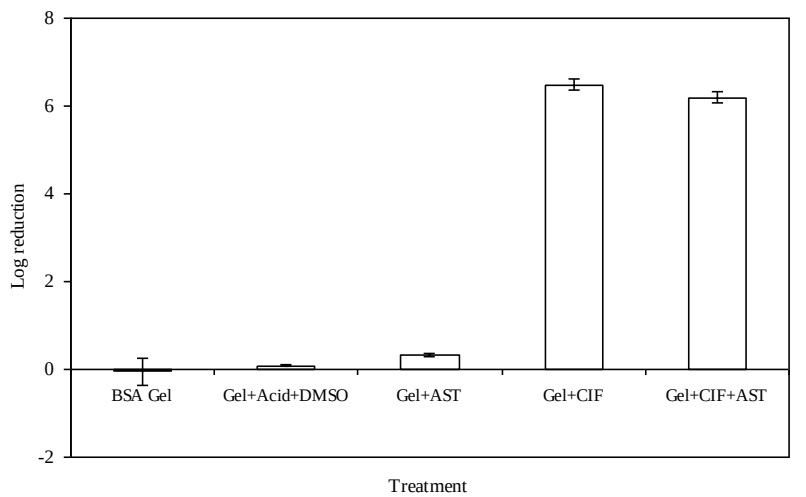

Figure 3 Effectiveness of different treatments at killing *P. aeruginosa* after 24 hr exposure. (BSA Gel = Untreated BSA hydrogel; Gel+Acid+DMSO = Hydrogel containing 0.133 mM sulfuric acid and 0.3% (volume per volume) dimethyl sulfoxide; Gel+AST = Hydrogel with 10 μM astaxanthin; Gel+CIF = Hydrogel with 10 $\mu g.mL^{-1}$ ciprofloxacin; and Gel+CIF+AST = Hydrogel with 10 $\mu g.mL^{-1}$ ciprofloxacin and 10 μM astaxanthin). Results are reported as the mean ± SD of 3–10 measurements.

***In vitro* ciprofloxacin release**

The release of ciprofloxacin from the BSA pad and its effect on bacterial inhibition were investigated. The release profile from the dressing demonstrated a high burst release of antibiotic drug over the first 1–2 d, followed by a gradual release over time. The drug release was almost complete within 3 d, with 4.24 and 1.31 μg.mL^{-1} of ciprofloxacin being released at days 1 and 2 (Figure 4a), respectively, resulting in 6.32 ± 0.16 and 5.08 ± 0.2 log reduction, respectively (Figure 4b).

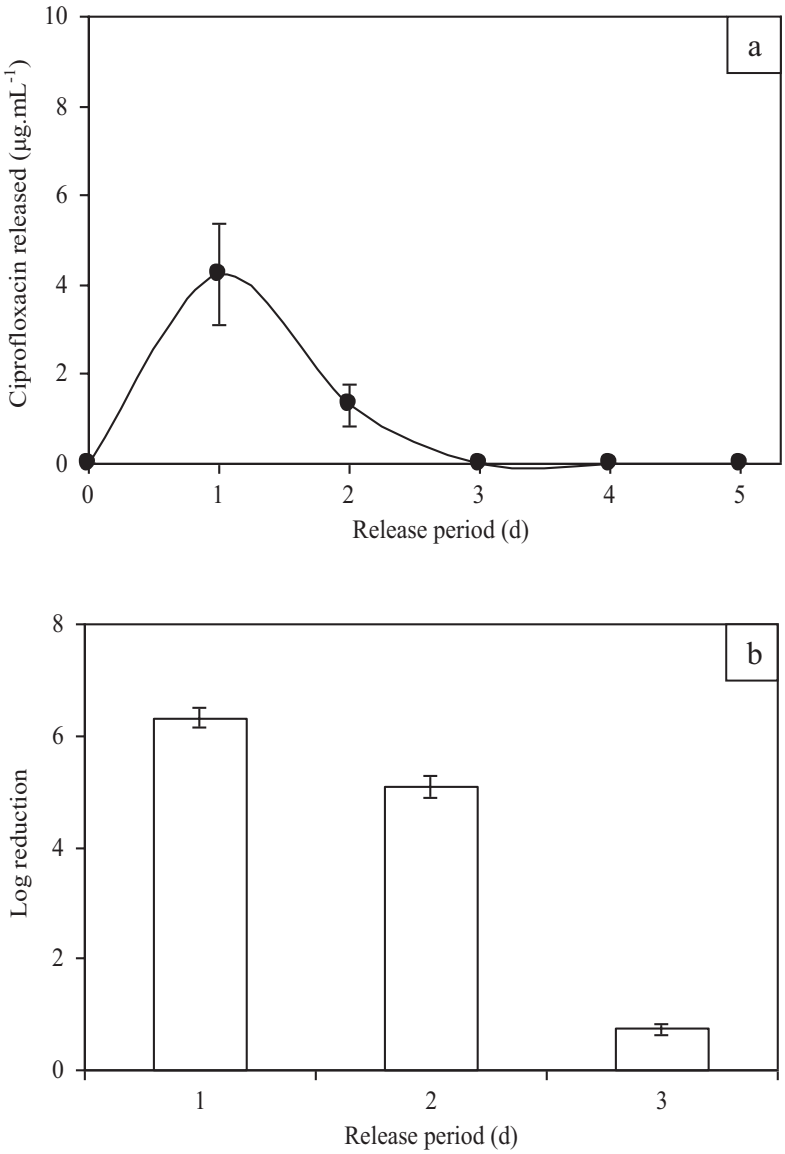

Figure 4 Release of ciprofloxacin from the bovine serum albumin gel pad: (a) Ciprofloxacin content released; (b) Effectiveness at killing *P. aeruginosa* over time. Results are reported as the mean ± SD of 3–6 measurements.

Cytotoxicity

To explore whether the dressing was toxic, the cell viability was examined after exposure to materials of dressing for 24 and 48 hr as measured by the MTT reduction. As illustrated in Figure 5, a culture of Vero cells with the dressing materials indicated that they had no toxic effect. Specifically, cells were hardly affected by the BSA, ciprofloxacin and astaxanthin used. However, cells were sensitive ($P < 0.05$) to the sulfuric acid and DMSO at a high concentration of 1% (v/v).

Cellular proliferation

Cellular behavior was clarified by testing proliferation on the hydrogel. The proliferation of Vero cells was investigated for 5 d. The data in Figures 6 and 7 show that the number of proliferating cells greatly increased in the presence of astaxanthin. Astaxanthin incorporated into both dressings promoted cell proliferation at the highest rate on day 1 ($P < 0.05$). However, ciprofloxacin used in dressings lowered the antioxidant potency. The astaxanthin contents in the hydrogel and gel pad were measured as 7.61 and 7.44 μM, respectively. The astaxanthin content and antioxidant activity released from the gel pad was the highest on the first day and began to decrease over days 2–5 (data not shown).

DISCUSSION

Novel wound dressings containing antibiotic and antioxidant based on a BSA protein were developed. The investigation emphasized the antimicrobial activity, morphology and absorption ratio of the hydrogel pad, the antibiotic release profile and its effect on bacterial inhibition. Cell cytotoxicity and proliferation of dressings were studied using an anchorage-dependent cell line.

The surface electrical properties of proteins have an important characteristic of ionic interactions with other biomacromolecules. Zhao *et al*. (2009) reported that heat-induced physical denaturation of BSA demonstrates more positive

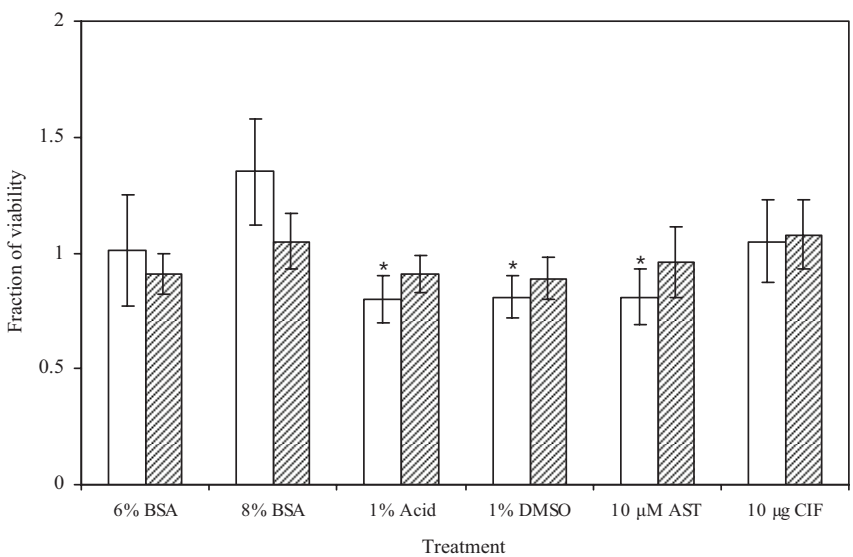

Figure 5 Cytotoxicity of dressing materials for 24 hr (clear bar) and 48 hr (diagonally striped bar) relative to the viability of control cells unexposed to dressing materials. Dressing material treatments: BSA = Bovine serum albumin; Acid = Sulfuric acid; DMSO = Dimethyl sulfoxide; AST = Astaxanthin; and CIF = Ciprofloxacin. The mean ± SD of 4–20 determinations are presented. * indicates measurement is significantly ($P < 0.05$) different from control cells.

zeta potential values than from the native form BSA. This might promote the electrostatic binding with oppositely charged macromolecules.

The hydrogel pad was characterized by the pore area and pore size distribution on the surface, indicating that this BSA hydrogel system could be advantageous for loading and allowing the sustained release of active ingredients. Furthermore, the mesoporous structure is related to the gel swelling level and may confer a high liquid permeability of the reactants through the gel matrix.

Fluid absorption of the hydrogel increased with an increase in the immersion time and reached equilibrium within 3 d. This was due to the hydrophilic nature of albumin. This hydrogel is expected to absorb wound fluids gradually, allowing for good healing of impaired wounds.

The results indicated the efficacy of the antibiotic-incorporated wound dressing. It is likely that bacterial survival is dose-dependent. It should be noted that drug quantities higher than the minimum inhibitory concentration value should be released in order to eradicate all bacteria within a few days and prevent infection.

Wound dressings, apart from absorbing wound exudates during the healing process, may also work as reservoirs for suitable drugs for localized delivery or to prevent bacterial infection. A release study was performed to simulate a situation in which a wound is created and therefore the skin in this area cannot function as a barrier against bacteria. After the onset of an infection, it is crucial to respond immediately to the presence of large numbers of bacteria. The dressing described in the current study provides a burst-release mechanism of the antibiotic which ensures delivery of a therapeutic dose to the wound during the acute period following injury.

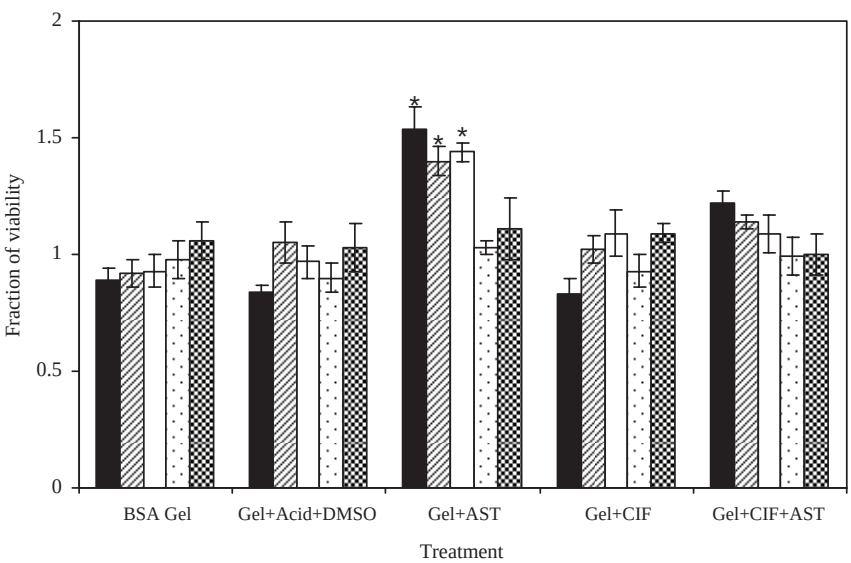

Figure 6 Cell viability in BSA hydrogel with incubation period of 1 d (solid bar), 2 d (diagonally striped bar), 3 d (clear bar), 4 d (dotted bar), and 5 d (cross-hatched bar) relative to the viability of control cells. Dressing material treatments: BSA = Bovine serum albumin; Acid = Sulfuric acid; DMSO = Dimethyl sulfoxide; Gel+AST = Gel + 10 μM astaxanthin; Gel+CIF = Gel + 10 $\mu g.mL^{-1}$ ciprofloxacin; Gel +CIF+AST = Gel + 10 μM astaxanthin + 10 $\mu g.mL^{-1}$ ciprofloxacin. The mean ± SD of 3–5 determinations are presented. * indicates measurement is significantly ($P < 0.05$) different than for control cells.

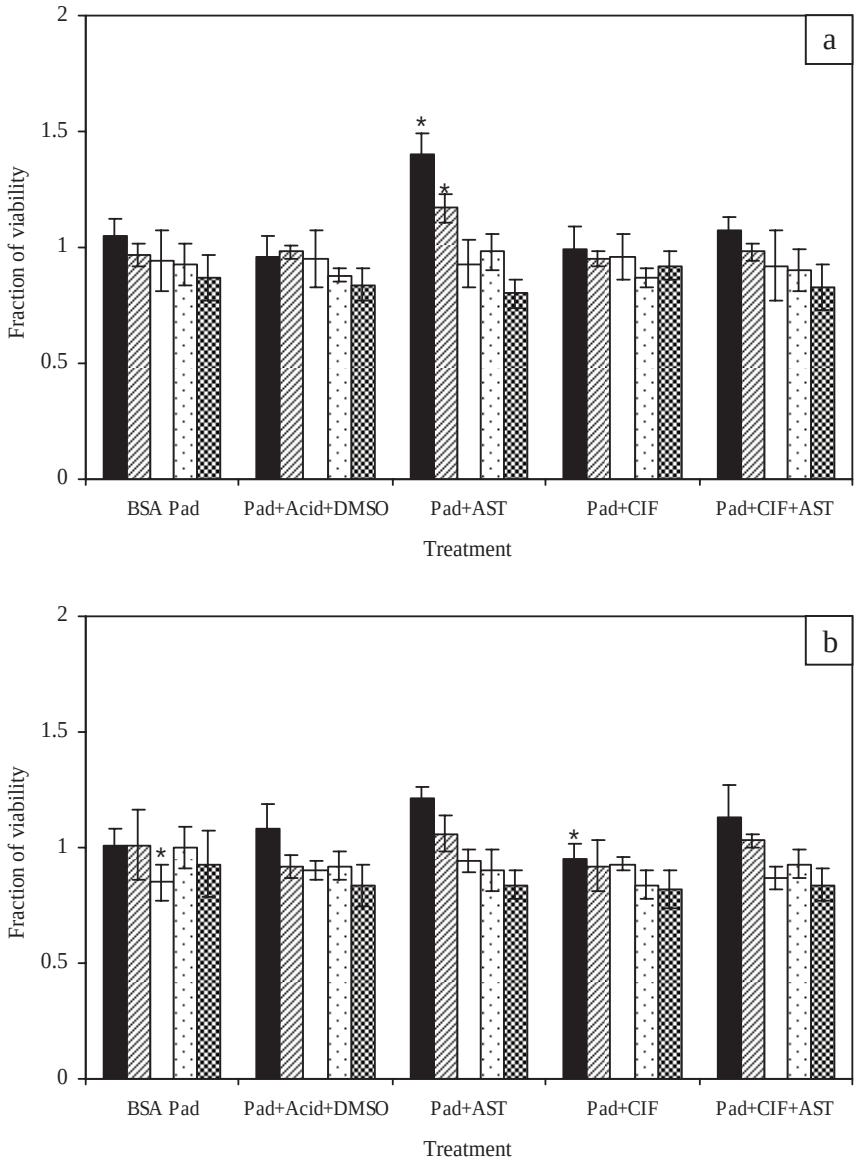

Figure 7 Cell viability in BSA gel pad with incubation period of 1 d (solid bar), 2 d (diagonally striped bar), 3 d (clear bar), 4 d (dotted bar), and 5 d (cross-hatched bar) relative to the viability of control cells for immersion period of: (a) 1 d; (b) 2 d. The data are reported as the fraction of the viability of the cells incubated with conditioned MEM medium from the immersion of a gel pad. Pad treatments: BSA Pad = Untreated BSA pad; Pad+Acid+DMSO = Pad containing 0.133 mM sulfuric acid and 0.3% (volume per volume) dimethyl sulphoxide; Pad+AST = Pad with astaxanthin; Pad+CIF = Pad with ciprofloxacin; Pad+AST+CIF = Pad with ciprofloxacin and astaxanthin. The mean ± SD of 3–5 determinations are presented. * indicates measurement is significantly ($P < 0.05$) different than for control cells.

In the studies examining cytotoxicity, the dressing material was found to be biocompatible. This meets the essential requirements of ideal wound dressings.

Previous studies provided strong evidence for a role of oxidative stress from excessive production of reactive oxygen species in the pathogenesis of non-healing ulcers and suggested that astaxanthin has anti-ulcer properties (Kamath *et al*., 2008; Schäfer and Werner, 2008). Similarly, the current study showed that astaxanthin significantly enhances cellular proliferation, and as a result, it may accelerate tissue repair. During the wound healing process, dressings are used for the regeneration and repair of dermal and epidermal tissues. This highlights the important role of antioxidant in the wound-healing activity.

Further refinement and development of protein-based biomaterials such as the proteins of legumes as wound dressings are likely for future study.

CONCLUSION

A protein-based hydrogel composed of an antimicrobial drug and antioxidant was prepared and tested. The results suggested that this dressing may possess many advantages for the treatment of wound-healing disorders. Therefore, protein-based material is promising as a wound dressing.

ACKNOWLEDGEMENTS

This work was supported by Thailand Research Fund (TRF) Master Research Grants (TRF-MAG) and by Silpakorn University, Nakhon Pathom, Thailand under contract number MRG-WII535E027. The authors are indebted to Associate Professor Kalyanee Jirasripongpun and Dr. Nuananong Jirakanjanakit for providing Vero cells. The authors would like to convey special appreciation to the academic committee of the 25th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2013) for providing the opportunity for this work to be published in this journal.

LITERATURE CITED

Akintayo, E.T., A.A. Oshodi and K.O. Esuoso. 1999. Effects of NaCl, ionic strength and pH on the foaming and gelation of pigeon pea (*Cajanus cajan*) protein concentrates. **Food Chem.** 66: 51–56.

Bryant, C.M. and D.J. McClements. 2000. Influence of NaCl and $CaCl_2$ on cold-set gelation of heat-denatured whey protein. **J. Food Sci.** 65: 801–804.

Chalkley, L.J. and H.J. Koornhof. 1985. Antimicrobial activity of ciprofloxacin against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* determined by the killing curve method: Antibiotic comparisons and synergistic interactions. **Antimicrob. Agents Chemother.** 28: 331–342.

Czaja, W., A. Krystynowicz, S. Bielecki and R.M. Brown Jr. 2006. Microbial cellulose- the natural power to heal wounds. **Biomaterials** 27: 145–151.

Kamath, B.S., B.M. Srikanta, S.M. Dharmesh, R. Sarada and G.A. Ravishankar. 2008. Ulcer preventive and antioxidative properties of astaxanthin from *Haematococcus pluvialis*. **Eur. J. Pharmacol.** 590: 387–395.

Lin, V.J.C. and J.L. Koenig. 1976. Raman studies of bovine serum albumin. **Biopolymers** 15: 203–218.

Matejtschuk, P., C.H. Dash and E.W. Gascoigne. 2000. Production of human albumin solution: A continually developing colloid. **Br. J. Anaesth.** 85: 887–895.

Murayama, K. and M. Tomida. 2004. Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy. **Biochem.** 43: 11526–11532.

Peppas, N.A., Y. Huang, M. Torres-Lugo, J.H. Ward and J. Zhang. 2000. Physicochemical

foundations and structural design of hydrogels in medicine and biology. **Annu. Rev. Biomed. Eng.** 2: 9–29.

Schäfer, M. and S. Werner. 2008. Oxidative stress in normal and impaired wound repair. **Pharmacol. Res.** 58: 165–171.

Singer, A.J. and R.A.F. Clark. 1999. Cutaneous wound healing. **New Engl. J. Med.** 341: 738–746.

Tada, D., T. Tanabe, A. Tachibana and K. Yamauchi. 2005. Drug release from hydrogel containing albumin as crosslinker. **J. Biosci. Bioeng.** 100: 551–555.

Teramoto, H., T. Kameda and Y. Tamada. 2008. Preparation of gel film from *Bombyx mori* silk sericin and its characterization as a wound dressing. **Biosci. Biotechnol. Biochem.** 72: 3189–3196.

Wiegand, C., T. Heinze and U.C. Hipler. 2009. Comparative *in vitro* study on cytotoxicity, antimicrobial activity and binding capacity for pathophysiological factors in chronic wounds of alginate and silver-containing alginate. **Wound Rep. Reg.** 17: 511–521.

Zhang, X., F. Yang, C. Xu, W. Liu, S. Wen and Y. Xu. 2008. Cytotoxicity evaluation of three pairs of hexabromocyclododecane (HBCD) enantiomers on HepG2 cell. **Toxicol. In Vitro.** 22: 1520–1527.

Zhao, Y., F. Li, M.T. Carvajal and M.T. Harris. 2009. Interactions between bovine serum albumin and alginate: An evaluation of alginate as protein carrier. **J. Colloid Interf. Sci.** 332: 345–353.