CHAPTER IV
PREPARATION AND CHARACTERIZATION OF ASIATICOSIDE-LOADED ALGINATE FILMS AND THEIR POTENTIAL FOR USE AS EFFECTUAL WOUND DRESSINGS

4.1 Abstract

A wound dressing material was successfully prepared from alginate, a natural polymer capable of forming into hydrogels, and asiaticoside (PAC), a substance from the plant *Centella asiatica* which has commonly been used in traditional medicine to heal wounds. Various amounts of PAC (i.e., at 2.5, 5 and 10%, based on the weight of alginate) were mixed with alginate in distilled water. The mixtures were later cast into films. The formation into solid films was achieved with the two-step cross-linking procedure with Ca^{2+}. First, a dilute $CaCl_2$ aqueous solution (at 0.05% w/v, 200 mL) was added slowly into an alginate aqueous solution (at 2% w/v, 100 mL). This step imparted the dimensional stability of the obtained "mixed" films. Secondly, the "mixed" films were cross-linked further in either 2.5 or 5% $CaCl_2$ aqueous solution to obtain "immersed" films. This step caused the "immersed" films to be more stable in environment that requires exposure to a high humidity or contact with an aqueous medium. Due to its insolubility in water, PAC existed in the films as discrete entities. The release of PAC from the PAC-loaded alginate "immersed" films was achieved by both the swelling and the erosion of the alginate matrix in the phosphate buffer solution (PBS) that contained methanol at about 10% v/v. The potential for use of both the neat and the PAC-loaded alginate "immersed" films as wound dressings was assessed by indirect cytotoxicity evaluation and direct cell culture, using normal human dermal fibroblasts (NHDF). The results showed that these materials were non-toxic to the skin cells.

Key-words: Alginate; Asiaticoside; Hydrogel; Wound dressing

4.2 Introduction

Wound healing is a specific biological process involving the general phenomenon of tissue regeneration (Boateng, Matthews, Stevens, and Eccleston, 2008). The entire process of wound healing is a complex and ordered cascade of events, which can be divided into four distinct, but overlapping, phases of hemostasis, inflammation, proliferation and maturation (MacKay and Miller, 2003; Enoch and Leaper, 2005; Deng, He, Zhao, and Yang, 2007; Stojadinovic, Carlson, Schultz, Davis, and Elster, 2008). Additionally, wound healing involves a complex series of interactions among different cell types, cytokine mediators and the extracellular matrix (ECM) components (MacKay and Miller, 2003; Enoch and Leaper, 2005; Stojadinovic et al., 2008).

Although wound healing is the natural process of regenerating damaged and/or lost tissues, an appropriate wound dressing should be able to enhance the healing process considerably, by mediating at the right stage of or providing excellent conditions for wound healing (Kokabi, Sirousazar, and Hassan, 2007). Generally, an effectual wound dressing should maintain a moist environment upon absorption of the wound exudates, protect the wound from secondary infection, reduce necrosis of the wound bed, provide adequate gaseous exchange, regulate and/or mediate the release of certain growth factors and cytokines, and also be elastic, biocompatible with tissues and blood, non-toxic and non-antigenic (Purna and Babu, 2000; Lin, Chen, and Run-Chu, 2001; Kokabi et al., 2007; Boateng et al., 2008; Singh and Pal, 2008). Moreover, an effectual wound dressing should promote a rapid healing of the wound and, once healed, the detachment of the dressing should not cause secondary trauma to the neo-tissues (MacKay and Miller, 2003; Boateng et al., 2008).

Based on these requirements, biocompatible polymeric hydrogels are promising materials for uses as wound dressings, since they can be tailor-made with specific needs (Kokabi et al., 2007; Boateng et al., 2008; Singh and Pal, 2008). Such needs, in addition to the general requirements for an effectual wound dressing, include non-irritating and non-adhering properties, immediate pain relief, ease of handling and replacing without compromising patients' comfort, transparency to

allow easy monitoring of the wound bed, and facilitation of the migration and mitosis of epithelial cells (Kokabi et al., 2007; Boateng et al., 2008; Singh and Pal, 2008). By definition, hydrogels are three-dimensional, hydrophilic, water-insoluble polymeric networks (Peppas, Bures, Leobandung, and Ichikawa, 2000; Hoffman, 2002; Liu, Lin, Li, and Liu, 2005; Sokolsky-Papkov, Agashi, Olaye, Shakesheff, and Domb, 2007; Hamidi, Azadi, and Rafiei, 2008; Singh and Pal, 2008). The internal networks may result from physical and/or chemical domains that retain their integrity, either in whole or in part, when being surrounded by a large amount of water molecules. The functions of hydrogels in biomedical applications, including wound dressings, result from their ability to imbibe a large quantity of water (Liu et al., 2005; Singh and Pal, 2008).

Among numerous polymers capable of forming into hydrogels, alginate (see Scheme 4.1), an abundant bio-copolymer of (1→4) glycosidically-linked β-D-mannuronic acid (M) and α-L-glucuronic acid (G) monomers in different covalent sequences or blocks (Augst, Kong, and Mooney, 2006; Dong, Wang, and Du, 2006; Coviello, Matricardi, Marianecci, and Alhaique, 2007; Pielesz and Bak, 2008), is an ideal material for fabrication into wound dressings. It is derived from certain species of algae and certain strains of microbes (Coviello et al., 2007; Pielesz and Bak, 2008). In the presence of multivalent metal cations, e.g., Ca^{2+}, Mg^{2+}, etc., it readily forms into hydrogels, due to the formation of ionic bridges between adjacent G units of the same or different chains into three-dimensional networks (Sartori, Finch, Ralph, and Gilding 1997; Rhim, 2004; Dong et al., 2006; Roger, Talbot, and Bee, 2006; Pongjanyakul and Puttipipatkhachorn, 2007; Zhang et al., 2008). Because of the intrinsic properties of alginate, such as natural abundance, relatively low material and production costs, high water absorbance, ion-exchange capability, biocompatibility, haemostatic property and non-immunogenicity (Groves and Lawrence, 1986; Sartori et al., 1997; Augst et al., 2006; Dong et al., 2006; Coviello et al., 2007; Pongjanyakul and Puttipipatkhachorn, 2007; Hong, Jin, Park, Ahn, and Kim, 2008; Pielesz and Bak, 2008), alginate-based hydrogels have been widely used in pharmaceutical and medical applications, particularly as wound dressing materials (Groves and Lawrence, 1986; Almeida and Almeida, 2004; Augst et al., 2006; Dong

et al., 2006; Coviello et al., 2007; George and Abraham, 2007; Pongjanyakul and Puttipipatkhachorn, 2007).

Nowadays, the development of wound dressings has changed from passive to active types, with an aim of imparting specific functions (Purna and Babu, 2000; Kokabi et al., 2007). The purpose of the present contribution is to develop wound dressings with enhanced wound healing property, through the use of a phyto-chemical. Among various phyto-chemicals, extracts from *Centella asiatica* Linn. Urban or Buabok (in Thai) have traditionally been used to heal wounds, burns and ulcerous abnormalities of the skin (Kartnig, 1988; Cheng and Koo, 2000). The influence of the plant extracts on wound repair [e.g., antinociception, anti-inflammation (Somchit, 2004), antioxidation (Veerendra Kumar and Gupta, 2002; Jayashree, Muraleedhara, Sudarslal, and Jacob, 2003; Gnanapragasam, Ebenezar, Sathish, Govindaraju, and Devaki, 2004; Shinomol and Muralidhara, 2008), immunomodulation (Jayathirtha and Mishra, 2004), antitumor (Huang, Zhang, Zhen, Xu, and Zhen, 2004; Ruan, Lai, and Zhou, 2006) and antikeloid (Widgerow, Chait, Stals, and Stals, 2000)] has been attributed to the presence of four major trisaccharide triterpenoid components in the extracts (i.e., asiatic acid, asiaticoside, madecassic acid and madecassoside) (Inamdar, Yeole, Ghogare, and de Souza, 1996). Among these components, asiaticoside (see Scheme 4.2) is the most active component associated with the healing of wounds (Maquart, Bellon, Gillery, Wegrowski, and Borel, 1990; Shim et al., 1996; Suguna, Sivakumar, and Chandrakasan, 1996; Maquart et al., 1999; Shukla, Rasik, and Dhawan, 1999a; Shukla et al., 1999b). Recently, Sikareepaisan, Suksamrarn, and Supaphol (2008) and Suwantong, Ruktanonchai, and Supaphol (2008) reported the development of electrospun gelatin and cellulose acetate fiber mats as carriers for topical and/or transdermal delivery of asiaticoside from gelatin solutions in 70% acetic acid that contained varying amounts of a methanolic extract of *Centella asiatica* and from cellulose acetate solutions in 2:1 v/v acetone/dimethylacetamide that contained either pure asiaticoside or a crude *Centella asiatica* extract, respectively.

In the present contribution, asiaticoside-containing alginate suspensions were fabricated into thin films by solvent-casting process. The asiaticoside-loaded alginate films were assessed for their potential for use as active wound dressings for

asiaticoside delivery. The as-cast asiaticoside-loaded alginate films were dipped into a calcium chloride ($CaCl_2$) aqueous solution to cross-link the films. These asiaticoside-loaded alginate films were fabricated to meet two requirements, i.e., enhanced wound healing capability and ease of production such that actual utilization could be realized. Various properties (i.e., morphology, mechanical integrity in either dry or wet state, swelling and weight loss behavior, and cytotoxicity) of both of the neat and the asiaticoside-loaded alginate films, as well as the release characteristic of asiaticoside from the asiaticoside-loaded alginate films, were investigated.

4.3 Experimental Details

4.3.1 Materials

Alginate (batch number: 4I311105X; $M_w \approx 1443$ kDa, $M_n = 321$ kDa; powder) was purchased from Carlo Erba (Italy). Calcium chloride ($CaCl_2$; anhydrous) was purchased from Ridel-de-Haën (Germany) and asiaticoside [90% purity; powder; hereafter PAC (see Figure I in Supplementary data for the morphology of the as-received PAC)] was purchased from Shanghai Angoal Chemical Co., Ltd. (China). Disodium hydrogen orthophosphate (anhydrous), sodium dihydrogen orthophosphate and sodium chloride (NaCl) were purchased from Ajax Chemicals (Australia). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (USA). All chemicals were of analytical reagent grade and used without further purification.

4.3.2 Preparation of Neat and PAC-loaded Alginate Films

The base alginate solution was first prepared at a fixed concentration of 2% w/v (i.e., 2 g of alginate powder in 100 mL of distilled water). About 200 mL of 0.05% w/v $CaCl_2$ aqueous solution was slowly added to the base alginate solution under constant stirring for 4 h. On the other hand, PAC-loaded alginate suspensions were prepared by first dispersing varying amounts of PAC powder of 2.5, 5 and 10% (based on the weight of the alginate powder) in 100 mL of distilled water. About 2 g of alginate powder was then added to dissolve in each of the PAC aqueous suspensions, followed by the addition of 200 mL of 0.05% w/v $CaCl_2$ aqueous solution under constant stirring for 4 h. The alginate solution and the PAC-loaded

alginate suspensions were poured into 12.5 cm by 22.5 cm polystyrene plates and dried at 50 °C for 4 d. The films obtained after this stage are referred to as "mixed" films. They were cross-linked further by immersing in a $CaCl_2$ aqueous solution (i.e., either 2.5 or 5% w/v) for 10 s. Prior to further investigation, they were washed in distilled water for 10 s and dried in an oven at 50 °C. The films obtained after this stage are referred to as "immersed" films.

4.3.3 Characterization of Neat and PAC-loaded Alginate Films

Morphologies of the neat and the PAC-loaded alginate "mixed" films were observed by a JEOL JSM-5200 scanning electron microscope (SEM) and by an Olympus SZH10 stereo microscope (SM). Each specimen for SEM observation was coated with a thin layer of gold using a JEOL JFC-1100E sputtering device prior to the SEM observation.

Moisture absorption of the neat alginate "mixed" and "immersed" film specimens (circular discs of about 2.8 cm in diameter and about 70 μm in thickness; *a priori* dried at 50 °C for 24 h) was measured at various relative humidity (RH) conditions (i.e., 32.8, 52.9 and 75.0% at 25 °C) on day 7 after incubation. Saturated, aqueous solutions of three different types of inorganic salts, e.g., magnesium chloride ($MgCl_2$), magnesium nitrate ($Mg(NO_3)_2$) and NaCl, were used to generate the specified RH conditions in conditioning jars. The film specimens were placed on wire grids, each of which had been placed above a respective salt solution in a given conditioning jar. The property values were calculated based on the following equation:

$$\text{Moisture absorption (\%)} = \frac{M' - M_i}{M_i} \times 100 , \qquad (1)$$

where M' is the weight of each specimen after incubation in a given conditioning jar for 7 d and M_i is the initial, dry weight of the specimen.

Water absorption and weight loss behavior of the neat alginate "immersed" film specimens (circular discs of about 2.8 cm in diameter and about 80 μm in thickness) were measured in distilled water and simulated body fluid (SBF; procedure for the preparation of SBF is available as Supplementary data) at the

physiological temperature of 37 °C. The property values were measured at various submersion time points and were calculated based on the following equations:

$$\text{Water absorption (\%)} = \frac{M - M_i}{M_i} \times 100\,; \tag{2}$$

$$\text{Weight loss (\%)} = \frac{M_i - M_d}{M_i} \times 100\,, \tag{3}$$

where M is the weight of each specimen after submersion in a respective medium at each submersion time point, M_i is the initial, dry weight of the specimen and M_d is the weight of the specimen in its dry state after submersion in the medium at each time point.

Mechanical integrity, in terms of the tensile strength and the elongation at break, of the neat and the PAC-loaded alginate "immersed" films, either in their dry or wet state, was tested on a Lloyd LRX universal testing machine. Rectangular-shaped (10 mm by 100 mm) specimens of about 90 μm in thickness were cut from the as-prepared films. The gauge length and the crosshead speed were 50 mm and 20 mm·min^{-1}, respectively. To evaluate the mechanical integrity of the films in their wet state, some of the specimens, *a priori* immersed in distilled water for 30 s and blotted with a piece of tissue paper to remove excessive amount of water on their surfaces, were tested.

4.3.4 Release of PAC from PAC-loaded Alginate Films

The actual amounts of PAC in the PAC-loaded alginate "mixed" films were first determined. The specimens (circular discs of about 2.3 cm in diameter and about 70 μm in thickness) were immersed in 20 mL of phosphate buffer solution (PBS, pH = 7.4; procedure for the preparation of PBS is available as Supplementary data) under vigorous stirring for 24 h. This treatment caused the film specimens to disintegrate completely. The actual amounts of PAC were then quantified by high-performance liquid chromatography (HPLC) (see later). The obtained data were back-calculated from the obtained data against a predetermined calibration curve for the as-received PAC.

The release characteristics of PAC from the PAC-loaded alginate "immersed" films were investigated in a medium that was a mixture between PBS

and methanol (at 10% (v/v)), based on the total immersion method (Suwantong et al., 2008). Each specimen (circular discs of about 2.3 cm in diameter and about 70 μm in thickness) was placed in a cellulose dialysis tube (Sigma-Aldrich, USA), filled with 20 mL of the releasing medium at the physiological temperature of 37 °C. At specified immersion time points ranging between 0 to 24 h, 1 mL of the medium (i.e., sample solution) was withdrawn and replaced with an equal amount of the fresh medium at each sampling time point. The amounts of PAC in the sample solutions were determined by HPLC (see later). The obtained data were carefully calculated to determine the cumulative amounts of PAC released from the specimens (reported based on the actual weights of either the as-loaded PAC or the film specimens).

HPLC (Shimadzu LC-10 AD, Japan) was used to quantify the amounts of PAC in the sample solutions. Chromatographic separation of PAC was accomplished using an Inertsil ODS-3 40 C18 column (particle size = 5 μm; column dimension = 4.6 mm by 250 mm) with an Inertsil ODS-3 guard column (particle size = 5 μm; column dimension = 4.0 mm by 10 mm), operating at 1 $mL \cdot min^{-1}$. The HPLC and the guard columns were set at room temperature (25 ± 1 °C). The mobile phase for PAC separation was 26:24:50 (v/v/v) of acetonitrile/methanol/distilled water. The injection volume was 50 μL. A UV detector (Shimadzu SPD-10A, Japan) for PAC was set at 204 nm (i.e., λ_{max}). All of the sample solutions were filtered through a nylon filter (average pore size = 0.45 μm) prior to injection. PAC was separated out over a range of elution periods of 7.4-7.7 min (Suwantong et al., 2008). The calibration curve for PAC was obtained over a concentration range of 0.14-2.95 $mg \cdot mL^{-1}$.

4.3.5 Indirect Cytotoxicity Evaluation

Toxicity of the neat and the PAC-loaded alginate "immersed" films was first evaluated by an indirect method, based on a protocol that was adapted from the ISO 10993-5 standard test method. The evaluation was carried out in 96-well tissue-culture polystyrene plates (TCPS; NunclonTM, Denmark) using normal human dermal fibroblasts (NHDF; 12^{th} passage). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., USA) supplemented by 10% fetal bovine serum (FBS; Invitrogen Corp., USA), 1% L-

glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)]. The specimens (circular discs of about 1.4 cm in diameter) were sterilized by UV radiation for about 1 h on each side. They were then immersed in a serum-free medium (SFM; DMEM containing 1% L-glutamine, 1% lactabumin and 1% antibiotic and antimycotic formulation) for 24 h in an incubator at the extraction ratio of 10 $mg \cdot mL^{-1}$. NHDF were separately cultured in TCPS at 8,000 cells/well in serum-containing DMEM for 24 h to allow cell attachment. After starving the cells with SFM for 24 h, the medium was replaced with an extraction medium and the cells were re-incubated for 24 h. The viability of the cells cultured with each of the extraction media was finally determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see detail of the assay in Supplementary data), The viability of the cells cultured with fresh SFM was used as control.

4.3.6 Morphological Observation of Cultured Cells

Morphologies of NHDF that had been cultured on the surfaces of both the neat and the PAC-loaded alginate "immersed" films were observed by SEM. The specimens (circular discs of about 1.4 mm in diameter) were sterilized by UV radiation for about 1 h on each side. The skin cells (twelfth passage) were then seeded on the surfaces of the specimens at 1×10^4 cells/specimen in a minimum volume of the culture medium and were allowed to attach on the surfaces for 24 h. After removal of the culture medium, the cell-cultured specimens were rinsed with PBS twice. A glutaraldehyde aqueous solution [at 3%, diluted from 50% glutaraldehyde aqueous solution (Electron Microscopy Science, USA) with PBS] was used to fix the cells for 30 min. After the cell fixation, the cell-cultured specimens were rinsed again with PBS. They were dehydrated in ethanol aqueous solutions of varying concentrations (i.e., 30, 50, 70 and 90%) and in pure ethanol for about 2 min at each concentration. They were then dried in 100% hexamethyldisilazane (HMDS; Sigma-Aldrich, USA) for 5 min and later dried in air after the removal of HMDS. Finally, each specimen was mounted on a copper stub and coated with thin layer of gold prior to the SEM observation. It should be noted

that cover glass slides (1 cm in diameter; Menzel, Germany) were used as control substrates on which NHDF were also cultured.

4.3.7 Statistical Analysis

Data were presented as means ± standard errors of means. Statistical analysis was carried out by the one-way analysis of variance (one-way ANOVA) and Scheffe's post hoc test in SPSS 11.5 for Windows software (SPSS). The statistical significance was accepted at $p < 0.05$.

4.4 Results and Discussion

4.4.1 Surface Morphology of Neat and PAC-loaded Alginate Films

The neat and the PAC-loaded alginate films were prepared by the two-step cross-linking procedure with Ca^{2+}. This procedure was improved from the methods utilized by Rhim (2004). In his original account (Rhim, 2004), either the *in situ* or the post-fabrication cross-linking procedure was used. The products obtained from either of these methods were referred to as "mixing" or "immersion" films. In the first method, different amounts of $CaCl_2$ powder (0.04, 0.08 and 0.12 g $CaCl_2$/4 g alginate) were mixed directly into the film-forming alginate solution (i.e., 4 g of alginate and 2 g of glycerol in 200 mL of distilled water) prior to being cast into films. In the second method, *a priori* fabricated films of pure alginate were immersed in $CaCl_2$ aqueous solutions of varying concentrations (i.e., 1, 2, 3 and 5 g $CaCl_2$ in 100 mL of distilled water). Rhim (2004) reported that the quality of the "mixing" films was not good, owing possibly to the inhomogeneous gelation that resulted from the direct mixing of $CaCl_2$ powder in the alginate solution. On the other hand, better film quality was obtained from the direct immersion of the neat alginate films into $CaCl_2$ aqueous solutions (Rhim, 2004).

Here, we combined the benefits of both methods mentioned above (Rhim, 2004) into the two-step cross-linking procedure. In the first step, the neat alginate solution or the alginate/PAC suspensions was/were mixed with a dilute aqueous solution of $CaCl_2$ (0.05% w/v). The treatment with a small amount of Ca^{2+} resulted in partial cross-linking of alginate molecules with the cationic species, which, in turn, facilitated their subsequent fabrication into dimensionally-stable

films. After a proper drying in an oven, the obtained films were referred to as the "mixed" films. The "mixed" films were subsequently immersed in a more concentrated $CaCl_2$ aqueous solution (2.5 or 5% w/v), prior to being washed and dried in an oven. This treatment improved the mechanical integrity of the "mixed" films and the films obtained after this step were referred to as the "immersed" films. The thicknesses of these films ranged between 70 and 100 μm. While the neat alginate "mixed" films were optically clear, the PAC-loaded counterparts were translucent. The opacity of the films was a direct result of the presence of the PAC particles and the degree of the translucence was mainly influenced by the PAC content in a dose-dependent manner.

Figure 4.1 shows representative SEM and OM images of the neat and the PAC-loaded alginate "mixed" films. The surface of the neat alginate "mixed" film was smooth with no noticeable defects. This should be a direct result of the excellent film-forming ability of alginate (Dong et al., 2006) and the slow addition of a quantity of the dilute $CaCl_2$ aqueous solution (i.e., 0.05% w/v) into the alginate solution. The slow addition of the dilute $CaCl_2$ aqueous solution should allow for the gradual and homogeneous formation of the physical cross-links between Ca^{2+} and the alginate molecules. Rhim (2004) showed that, by adding $CaCl_2$ powder directly into an alginate solution, the surface of the obtained "mixing" films was rough. On the contrary to the neat alginate "mixed" film, the surfaces of the PAC-loaded alginate "mixed" films showed evidence of certain PAC entities, which distributed rather homogeneously throughout the mass of the films in a dose-dependent manner. In reference to the morphology of the as-received PAC in Supplementary data, it is quickly recognized that the rod-like features of the as-loaded PAC were retained. However, the size of the as-loaded PAC entities was much smaller than that of the as-received material. This should be a result of the breakage of the originally-loaded PAC entities during mechanical agitation. It is important to emphasize that the presence of the PAC entities throughout the mass of the films is due mainly to the fact that PAC is not water-soluble. Mechanical agitation only facilitates their dispersion and distribution within the base alginate solution, hence within the resulting films.

4.4.2 Moisture Absorption, Water Absorption and Weight Loss Behavior of Neat Alginate Films

Moisture absorption of the neat alginate "mixed" and "immersed" films was first determined to evaluate the potential for uses of them in applications that require an exposure to a high humidity environment. Figure 4.2 shows moisture absorption behavior of the alginate "mixed" films at three different RH conditions, i.e., 32.8, 52.9 and 75.0%. The "mixed" films were able to absorb the greatest amounts of moisture, with the property value increasing with an increase in the RH level (i.e., from 4.3 ± 0.6% at the RH level of 32.8% to 20.6 ± 1.3% at the RH level of 75.0%). These values were clearly lower when the "mixed" films were cross-linked with $CaCl_2$ solutions. Upon cross-linking in the 2.5% $CaCl_2$ solution, the obtained "immersed" films exhibited the moisture contents of 3.7 ± 0.9, 7.4 ± 1.0 and 17.9 ± 1.2% at the RH levels of 32.8, 52.9 and 75.0%, respectively. These values were lower for the "mixed" films that had been cross-linked in the 5% $CaCl_2$ solution (i.e., 0.9 ± 0.6, 4.8 ± 0.6 and 14.5 ± 0.7%, respectively). Previously, Rhim (2004) showed that, at RH levels greater than 52%, the neat alginate film and the alginate films prepared by directly mixing 0.08 g of $CaCl_2$ (per 4 g of alginate) in the casting solution were able to absorb greater amounts of moisture than the films prepared by a direct immersion in a $CaCl_2$ solution (3 g $CaCl_2$ in 100 mL of distilled water). He attributed the phenomenon to the change in the hydrophillicity and/or the decreased segmental mobility of the cross-linked alginate molecules by Ca^{2+} that occurred close to or at the surface of the films (Rhim, 2004). Based on the obtained results, the "immersed" films were chosen for further studies.

Figure 4.3 shows water retention and weight loss behavior of the "immersed" films after immersion in either distilled water or SBF for various time intervals (up to 24 h) at the physiological temperature of 37 °C. These were determined to evaluate the potential for uses of the films in applications that require a contact with an aqueous medium (e.g., wound exudates). The retention of water and the losses in the mass of the "immersed" films occurred more readily when they were submerged in distilled water, with the property values of the films that had been cross-linked in the 2.5% $CaCl_2$ solution being greater than those of the films that had been cross-linked in the 5% solution. In distilled water, greater amounts of water

were able to diffuse into the films due to the greater driving force, which is attributable to the greater chemical potential of water molecules in distilled water in comparison with that of water molecules in SBF. On the other hand, the losses in the mass of the films that occurred more readily in distilled water than in SBF should be due to the differences in the driving forces for diffusion of Ca^{2+} into either medium. In distilled water, no Ca^{2+} are present, thus the driving force for the diffusion of Ca^{2+} from the cross-linked alginate films into distilled water should be greater than that for the diffusion of the ions from the films into SBF, which contains a certain amount of Ca^{2+}. The greater amounts of Ca^{2+} that were diffused from the films into distilled water should be responsible for partial disintegration of the films in the medium. In addition, the presence of another divalent cationic species, i.e., Mg^{2+}, in SBF which would diffuse into the films and take part in the additional cross-linking of the films, despite the loss of certain amounts of Ca^{2+} from the films, could act to prevent the disintegration of the films when they are submerged in the medium.

Specifically, the water retention of the alginate "immersed" films, after having been cross-linked in either 2.5 or 5% $CaCl_2$ solution, increased immediately after 15 min of submersion in distilled water to ca. 3360 and 2280%, respectively. Further increase in the time of submersion in the medium resulted in monotonous increases in the property values of both types of the "immersed" films to reach the final values at 24 h of submersion of 9090 and 5950%, respectively. On the other hand, the property values of both types of the "immersed" films upon their submersion in SBF at the submersion time point of 15 min were much lower at 630 and 540%, respectively. The values increased rather monotonously with further increase in the submersion time to assume the final values at 24 h of submersion of 910 and 820%, respectively. In similar manners, the losses in the masses of both types of the "immersed" films in distilled water at the initial submersion time of 15 min were 18.3 and 7.6%, respectively. These increased to assume the final values at 24 h of submersion of 29.6 and 24.9%, respectively. In SBF, on the other hand, the property values of both types of the "immersed" films at 15 min of submersion were much lower at 0.6 and 0.1%, respectively. At 24 h of submersion in the medium, the property values increased to 6.6 and 1.3%, respectively. Evidently, the lower water retention and weight loss behavior of the 5% $CaCl_2$ cross-linked "immersed" films as

compared to the 2.5% $CaCl_2$ cross-linked counterparts were due obviously to the greater degree of cross-linking as a result of the greater concentration of Ca^{2+}.

4.4.3 Mechanical Properties of Neat and PAC-loaded Alginate Films

The mechanical properties, in terms of the tensile strength and the elongation at break, of both the neat and the PAC-loaded alginate "immersed" film specimens that had been cross-linked with either 2.5% or 5% $CaCl_2$ solution in their dry or wet state were investigated. The results are graphically shown in Figure 4.4. The property values of the "mixed" films in their dry state were also investigated for comparison. The neat "mixed" films in their dry state exhibited the tensile strength and the elongation at break at about 65.4 MPa and 5.2% on average, respectively. Inclusion of the PAC particles within the "mixed" films was responsible for the reduction in the rigidity of the resulting films, i.e., the tensile strength decreasing to about 42.4-44.2 MPa on average and the elongation at break increasing to about 6.3-7.7% on average. Upon cross-linking in either 2.5 or 5% $CaCl_2$ solution, the tensile strength of the neat "immersed" films in their dry state increased slightly to 67.6 and 73.8 MPa on average, respectively. Interestingly, the elongation at break also increased to 7.3 and 7.0% on average, respectively. Marked improvement in the rigidity of the PAC-loaded alginate "mixed" films was observed when they were submerged in the $CaCl_2$ solutions. However, among the PAC-loaded alginate "immersed" films, the values of both tensile strength and elongation at break were not statistically different, as the values appeared to range between 65.3-70.0 MPa on average for tensile strength and 4.3-7.1% and on average for elongation at break.

Upon submersion of these "immersed" films in distilled water for 30 s, they became much softer and more pliable, as the tensile strength decreased significantly to range between 0.74-2.03 MPa on average and the elongation at break increased immensely to range between 49-96% on average. This should be a direct result of the plasticizing effect of water molecules that were absorbed by the films during the submersion. Interestingly, the inclusion of PAC powder in the films caused the PAC-loaded films in their wet state to be stronger (i.e., for the 2.5% $CaCl_2$ cross-linked films, they were about 74-98% stronger and, for the 5% $CaCl_2$ cross-linked counterparts, they were about 36-70% stronger), yet more pliable (i.e., for the 2.5% $CaCl_2$ cross-linked films, they were about 27-54% more pliable and, for the 5%

$CaCl_2$ cross-linked counterparts, they were about 67-78% more pliable), than the neat films. The "immersed" films that had been cross-linked in 5% $CaCl_2$ solution also appeared to be stronger than those cross-linked in 2.5% $CaCl_2$ solution (i.e., 62% stronger for the neat films and 27-39% stronger for the PAC-loaded films). Finally, the increase in the content of PAC powder in the films caused the films to be increasing stronger and less pliable. Pavlath, Gossett, Camirand, and Robertson (1999), Rhim (2004) and Roger et al. (2006) reported that the tensile strength of alginate films was increased, while the elongation at break was decreased by $CaCl_2$ treatment and it has been found to depend on the $CaCl_2$ concentration and the treatment time. Apparently, the obtained results agreed well with these previous reports.

4.4.4. Release Characteristics of PAC from PAC-loaded Alginate "Immersed" Films

The alginate "immersed" films that had been prepared from alginate solutions containing PAC at 5 and 10% w/w of the alginate powder were chosen for further investigation of the release characteristics of PAC therefrom. However, prior to the investigation, the actual amounts of PAC in these films needed to be determined. For this purpose, the "mixed" films that had been prepared from the same alginate solutions as *a priori* mentioned were used. The results showed that the actual amounts of PAC in the "mixed" films were (4.6 ± 0.2) and (8.6 ± 0.4)%, respectively, which correspond to the loading efficiency values of about (91.8 ± 3.1) and (87.3 ± 3.9)% on average, respectively (n = 3). These values were used as the bases to calculate the cumulative amounts of PAC released from the PAC-loaded alginate "immersed" films.

The release characteristics of PAC from the alginate "immersed" films that had been prepared from alginate solutions containing PAC at 5 and 10% w/w of the alginate powder and subsequently cross-linked in either 2.5 or 5% $CaCl_2$ solution were investigated by the total immersion method in a medium comprising PBS and 10% v/v of methanol (hereafter, the medium). The addition of methanol was to facilitate the dissolution of PAC in the medium, as PAC is a water-insoluble substance. Previously, Suwantong, Opanasopit, Ruktanonchai, and Supaphol (2007) and Suwantong et al. (2008) included methanol in the dissolution media to facilitate

the dissolution of curcumin, PAC and a crude *C. asiatica* extract during the release experiments. As shown in Figure 4.5, the release of PAC from the PAC-loaded alginate "immersed" films are reported in two different manners, i.e., as the percentages of the weights of PAC released divided by the actual weights of PAC in the specimens and as the percentages of the weights of PAC released divided by the actual weights of the specimens.

Based on the results shown in Figure 4.5, the shape of the release profiles for all of the investigated materials was essentially similar and it can be divided into four stages, regardless of the ways in which the results were reported. Let us take the profiles of the 2.5% $CaCl_2$ solution cross-linked PAC-loaded alginate "immersed" films, that were reported as the percentages of the weights of PAC released divided by the actual weights of PAC in the specimens in Figure 4.5a, as examples. The first stage correlated to a delayed release of PAC from the PAC-loaded materials through the dialysis membrane (i.e., 0 to about 11% of the PAC released within the first 60 min for the 5% PAC-loaded alginate "immersed" films and 0 to about 8% within the first 30 min for the 10% PAC-loaded counterparts). The second stage corresponded to the burst release of PAC from these materials, which was characterized by an abrupt increase in the release rates of PAC from the materials (i.e., from about 11% of the PAC released at an immersion time point of 1 h to about 30% at 2 h for the 5% PAC-loaded alginate "immersed" films and from about 8% at an immersion time point of 30 min to about 35% at 1 h for the 10% PAC-loaded counterparts). The third stage referred to the sustained release of PAC from the materials (i.e., from about 30% of the PAC released at an immersion time point of 2 h to about 90% at 12 h for the 5% PAC-loaded alginate "immersed" films and from about 35% at an immersion time point of 1 h to about 91% at 12 h for the 10% PAC-loaded counterparts). The last stage signified the gradual decrease in the release rates of PAC from the materials to reach plateau values (i.e., from about 90 to 97% over an immersion time period of 12 to 24 h for both types of the materials). Clearly, at any given immersion time point, the amount of PAC released from the 10% PAC-loaded alginate "immersed" films was greater than that from the 5% PAC-loaded counterparts. This is due obviously to the greater PAC concentration in the films, hence greater driving force for diffusion. The four different stages

characterizing the release of PAC from the 2.5% $CaCl_2$ solution cross-linked PAC-loaded alginate "immersed" films were also observed in the 5% $CaCl_2$ solution cross-linked PAC-loaded counterparts, with the value, for a particular PAC loading at any given time point, of the 5% $CaCl_2$ solution cross-linked films being slightly lower than that of the 2.5% $CaCl_2$ solution cross-linked counterparts. This is obviously a result of the increase in the degree of cross-linking when the films had been cross-linked in the 5% $CaCl_2$ solution (Dong et al., 2006).

Based on the results shown in Figure 4.5a, it is evident that the maximum amounts of PAC released from the alginate films were about 97% for both 5 and 10% PAC-loaded alginate films that had been cross-linked in 2.5% $CaCl_2$ solution, about 96% for 5% PAC-loaded alginate films that had been cross-linked in 5% $CaCl_2$ solution and about 92% for 10% PAC-loaded alginate films that had been cross-linked in 5% $CaCl_2$ solution. It is clear that, within 24 h of immersion in the medium, almost all of the as-loaded PAC was able to be released into the medium. Though not shown, we observed that both the neat and the PAC-loaded alginate "immersed" films would be broken up into small pieces over a period of time upon submersion in PBS and, hence, the releasing medium. This is based on the fact that Na^+ is the only cationic species present in PBS and, upon submersion of the alginate films into this medium, ionic exchange between Ca^{2+} and Na^+ can occur, leading to the gradual disintegration of the films. Based on this, it is expected that the observed high release efficiencies of the PAC-loaded alginate "immersed" films should be due mainly to the swelling and the surface erosion mechanisms (Pongjanyakul and Puttipipatkhachorn, 2007). By reporting the released amounts of PAC based on the actual weights of the specimens as in Figure 4.5b, slightly different information can be obtained. Clearly, the maximum amounts of PAC released from the alginate films were about 4.3 and 4.2% for the 5% PAC-loaded alginate films that had been cross-linked in 2.5 and 5% $CaCl_2$ solutions, respectively, and about 7.7 and 7.3% for the 10% PAC-loaded alginate films that had been cross-linked in 2.5 and 5% $CaCl_2$ solutions, respectively.

The release kinetics of a drug from a carrier can be characterized according to the following equations (Peppas and Khare, 1993; Ritger and Peppas, 1987):

$$\frac{M_t}{M_\infty} = kt^n, \text{ for } \frac{M_t}{M_\infty} < 0.6, \quad (4)$$

where M_t is the cumulative amount of the drug released at an arbitrary time t, M_∞ is the cumulative amount of the drug released at an infinite time, n is an exponent characterizing the mechanism with which the release kinetics can be described, and k is the release rate of the drug that incorporate the physical character of the releasing system.

The case for normal Fickian diffusion is characterized by the value of n being 0.5 and Case II diffusion is by the value of n being 1.0, while the case for non-Fickian or anomalous diffusion is characterized by the value of n being between 0.5 and 1.0 (Ritger and Peppas, 1987; Peppas and Khare, 1993; Verreck et al., 2003). In case of the Fickian diffusion (i.e., n = 0.5), a straight line is expected when the fractional cumulative amount of drug released (i.e., M_t/M_∞) is plotted as a function of $t^{0.5}$ (Figure 4.6a). The results from the analyses (i.e., values of parameters k and the r^2, which signifies the goodness of the fits) are summarized in Table 4.1. Based on the Fickian diffusion mechanism, the values of the rate parameter 'k' for all of the PAC-loaded alginate "immersed" films were essential similar as they were found to be between 4.06×10^{-3}-4.32×10^{-3} $s^{-0.5}$. In case of the non-Fickian diffusion, the diffusion exponent 'n' and the rate parameter 'k' can be obtained from the values of the slope and the intercept of the plot of $\ln(M_t/M_\infty)$ version $\ln(t)$ (Figure 4.6b) and the results of such analyses are also summarized in table 4.1. Based on the non-Fickian diffusion mechanism, the values of the diffusion exponent 'n' and the rate parameter 'k' for the 5% PAC-loaded alginate films that had been cross-linked in 2.5% $CaCl_2$ solution were 0.84 and 0.13×10^{-3} $s^{-0.84}$, respectively. For the 5% PAC-loaded alginate films that had been cross-linked in 5% $CaCl_2$ solution, they were 0.76 and 0.11×10^{-3} $s^{-0.76}$, respectively. For the 10% PAC-loaded alginate films that had been cross-linked in 2.5% $CaCl_2$ solution, they were 0.64 and 1.13×10^{-3} $s^{-0.64}$, respectively. Finally, for the 10% PAC-loaded alginate films that had been cross-linked in 5% $CaCl_2$ solution, they were 0.74 and 0.39×10^{-3} $s^{-0.74}$, respectively.

4.4.5 Cytotoxicity Evaluation of Neat and PAC-loaded Alginate "Immersed" Films

The potential for use of the PAC-loaded alginate "immersed" films as wound dressings was assessed by investigating the cytotoxicity of these materials, using normal human dermal fibroblasts (NHDF) as reference. First, the cytotoxicity was evaluated indirectly by culturing NHDF with extraction media (prepared at the extraction ratio of 10 $mg \cdot mL^{-1}$) of the film specimens in comparison with those that had been cultured with the fresh culture medium. The results are shown in Figure 4.7 for a given amount of $CaCl_2$ solution used to cross-link the films; the viabilities of NHDF for all of the PAC-loaded alginate "immersed" film specimens were lower than that for the neat materials. The greater amount of $CaCl_2$ solution used to cross-link the films decreased slightly the viabilities of the cells for all of the film specimens. Notwithstanding, the viabilities of the cells for all of the film specimens were greater than 90% with respect to that of the cells that had been cultured with the fresh culture medium, indicating that none of the "immersed" film specimens released substances in the levels that were harmful to the skin cells. Recently, we also showed that the viabilities of NHDF that had been cultured with the extraction media (prepared at three different extraction ratios of 0.5, 5, and 10 $mg \cdot mL^{-1}$) of both solvent-cast films and electrospun fiber mats that were prepared from CA solution containing PAC at the amount of 40% w/w were greater than or equal to 90% with respect to the cells that had been cultured with the fresh culture medium, indicating also that the materials were not harmful to the skin cells (Suwantong et al., 2008).

The materials were further evaluated by the direct culture of NHDF on their surfaces. Only the neat and the 10% PAC-loaded alginate "immersed" films were evaluated and the cells that had been cultured on glass substrates were used as control. The results as shown in Figure 4.8 illustrate the morphologies of the cells in three magnifications. After having been cultured for 24 h, all of the cells on the control substrates appeared in their characteristic spindle-like morphology. Similar behavior was also observed for the cells that had been cultured on the neat alginate "immersed" film substrates. Interestingly, the number of cells on the films that had been cross-linked in 5% $CaCl_2$ aqueous solution was slightly lower than that on the

ones that had been cross-linked in 2.5% $CaCl_2$ aqueous solution. As for the PAC-loaded materials, much less number of NHDF were able to attach on these substrates and the morphologies of the majority of the cultured cells were still round, suggesting that the substrates exhibited a non-adherent property towards the cells, due perhaps to the presence of the PAC particles. Recently, we showed that the morphologies of NHDF that had been cultured on the surfaces of both of the solvent-cast films and the electrospun fiber mats that were prepared from CA solution containing PAC at the amount of 40% w/w assumed the normal spindle-like character, despite the much greater viability of the cells attached on the fiber mat surface (Suwantong, Ruktanonchai, and Supaphol, 2010). The inferiority of the PAC-loaded alginate "immersed" films in supporting the attachment of NHDF could be due to the existence of PAC as a heterogeneous entity throughout the mass of the films [in contrast to our previous report on PAC-loaded CA films and fiber mats within which PAC was homogeneously dispersed (Suwantong et al., 2010)].

Even though the cell culture studies seem to suggest that the PAC-loaded alginate "immersed" films showed non-adherent property towards the skin cells, they did not pose any threat to them as the extraction media prepared from these materials contained none of the substances in the levels that were harmful to the skin cells. As the materials with intended use as wound dressings, the non-adherent property of a dressing should be beneficial to a healing wound as it does not cause secondary trauma to the wound during the detachment of a dressing. In addition, the presence of PAC should help promote the production of collagen of migratory or proliferated fibroblasts in the wound area (Lu et al., 2004; Suwantong et al., 2010)

4.5 Conclusion

Alginate films containing pure asiaticoside (PAC, at 2.5, 5 and 10%, based on the weight of alginate) were successfully prepared as wound dressings by solution casting using the two-step cross-linking procedure with Ca^{2+}. The addition of an amount of dilute $CaCl_2$ aqueous solution into the film-forming alginate solution helped to stabilize the shape of the obtained films (i.e., "mixed" films). Further cross-

linking by immersing the mixed films in either 2.5 or 5% $CaCl_2$ aqueous solution (i.e., "immersed" films) improved the stability of the films in a humid environment or upon their contact with an aqueous medium. In an aqueous medium that contained appreciable amounts of divalent cations, e.g., simulated body fluid (SBF), the stability of the films was enhanced, due to additional cross-linking by the cations in the medium. Due to the insolubility of PAC in water, PAC existed within the films as heterogeneous entities. This caused the PAC-loaded films to be translucent. The amounts of the as-loaded PAC were close (i.e., greater than or equal to 87% on average) to those originally loaded into the film-forming PAC-loaded alginate mixtures. Almost all of the as-loaded PAC could be released from the PAC-loaded alginate "immersed" films (i.e., greater than or equal to 92% on average) into the releasing medium of phosphate buffer solution (PBS) and 10% v/v of methanol within 24 h. The mechanism with which PAC was released from these films should be a combination of swelling and surface erosion of the films upon submersion in the Na^+-rich, aqueous medium. Finally, both the neat and the PAC-loaded alginate "immersed" films appeared to be non-toxic to the normal human dermal fibroblasts, despite the fact that the cells did not seem to attach on the PAC-loaded materials as well as they did on the neat alginate "immersed" films.

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4.7 Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.09.048.

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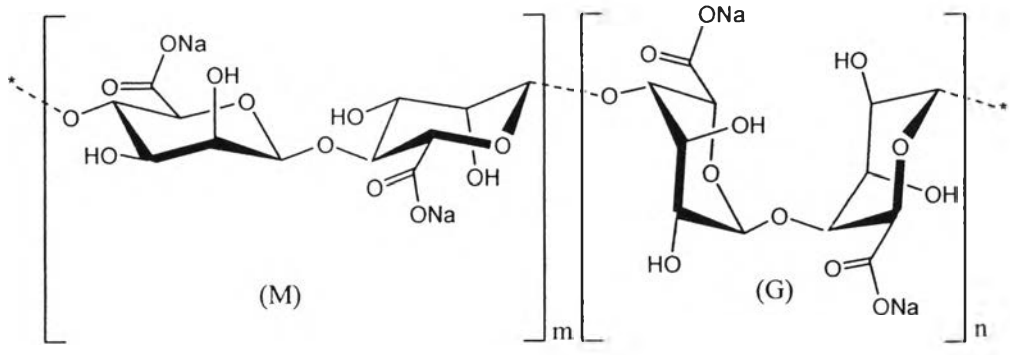
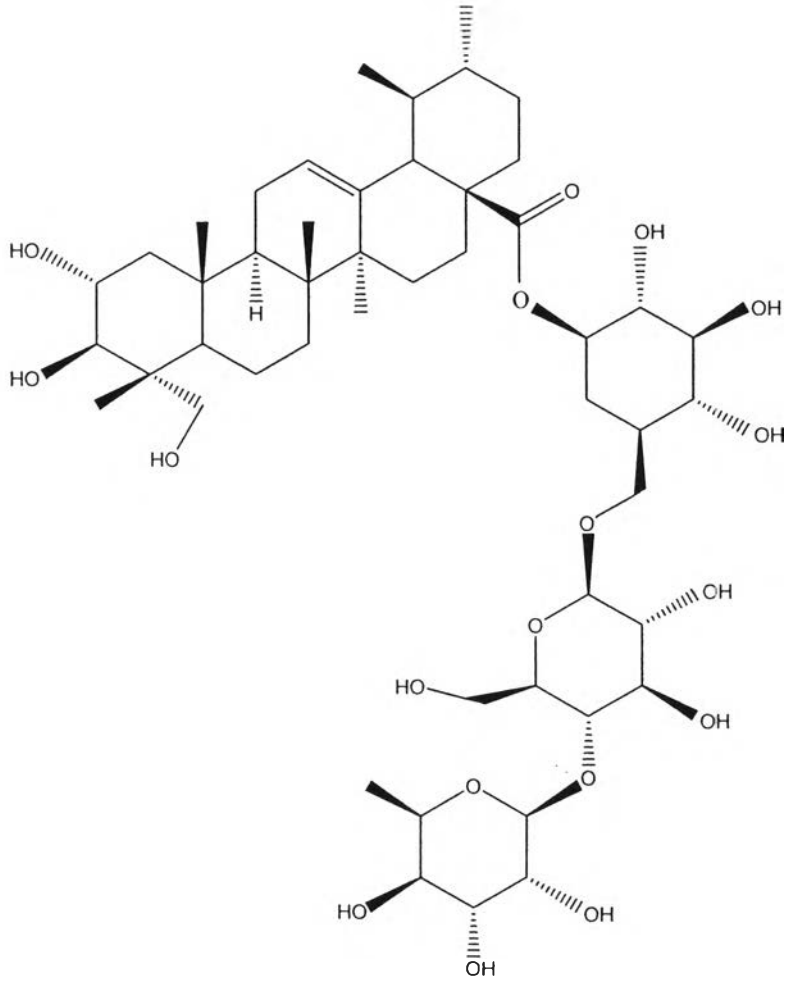


Scheme 4.1 Chemical structure of sodium alginate: (M) stands for β-D-mannuronic acid and (G) for α-L-glucuronic acid building blocks.



Scheme 4.2 Chemical structure of asiaticoside.

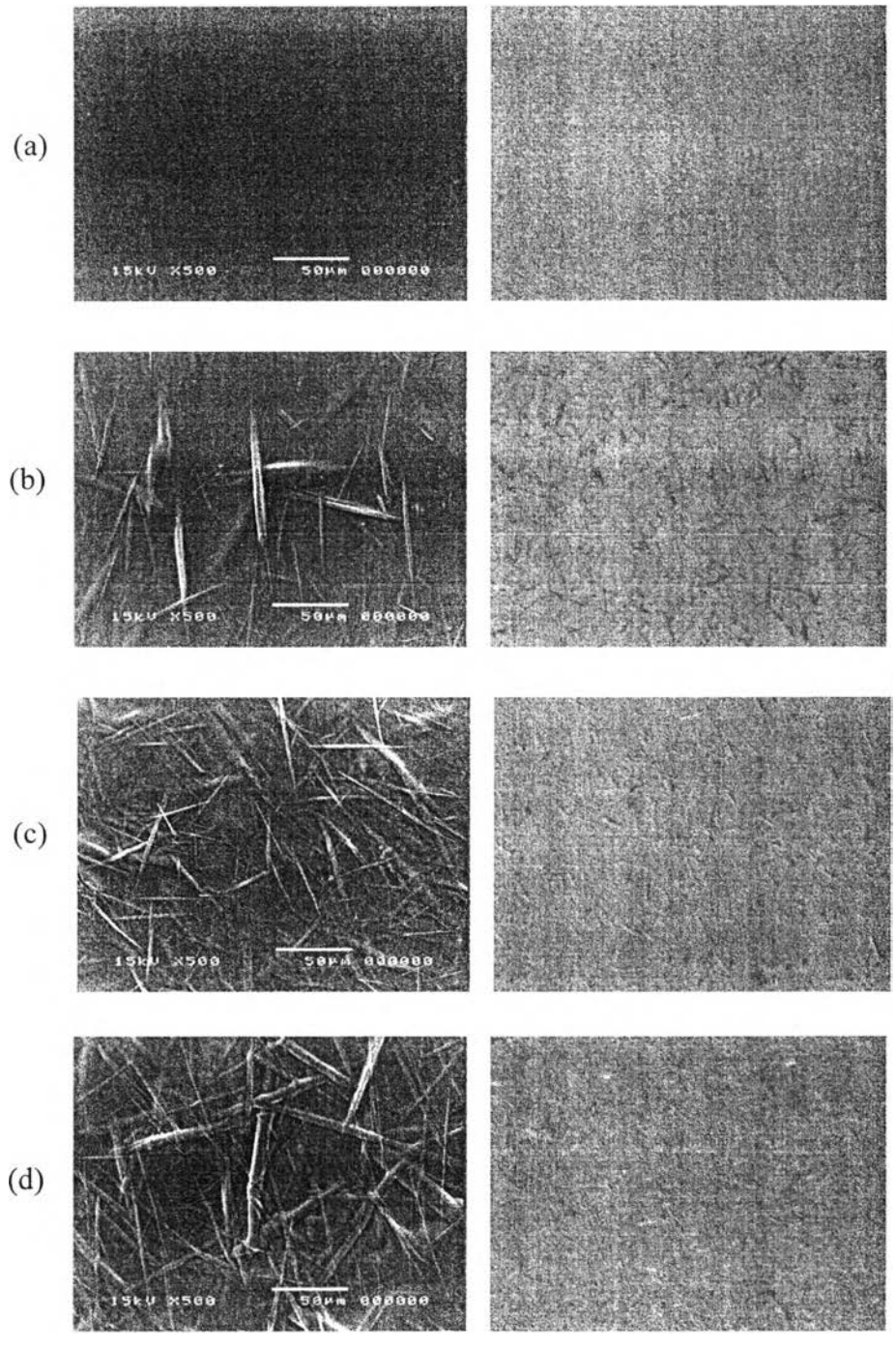


Figure 4.1 Representative scanning electron microscopic (SEM) and corresponding optical microscopic (OM) images of (a) neat alginate "mixed" films, (b) 2.5% PAC-loaded alginate "mixed" films, (c) 5% PAC-loaded alginate "mixed" films, and (d) 10% PAC-loaded alginate "mixed" films.

Remark: PAC = pure asiaticoside

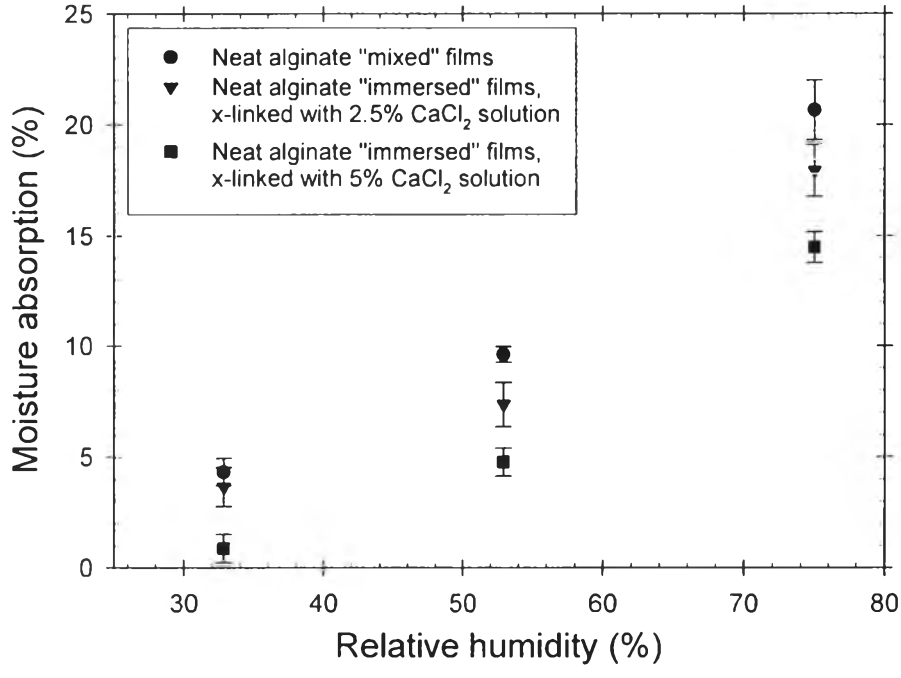
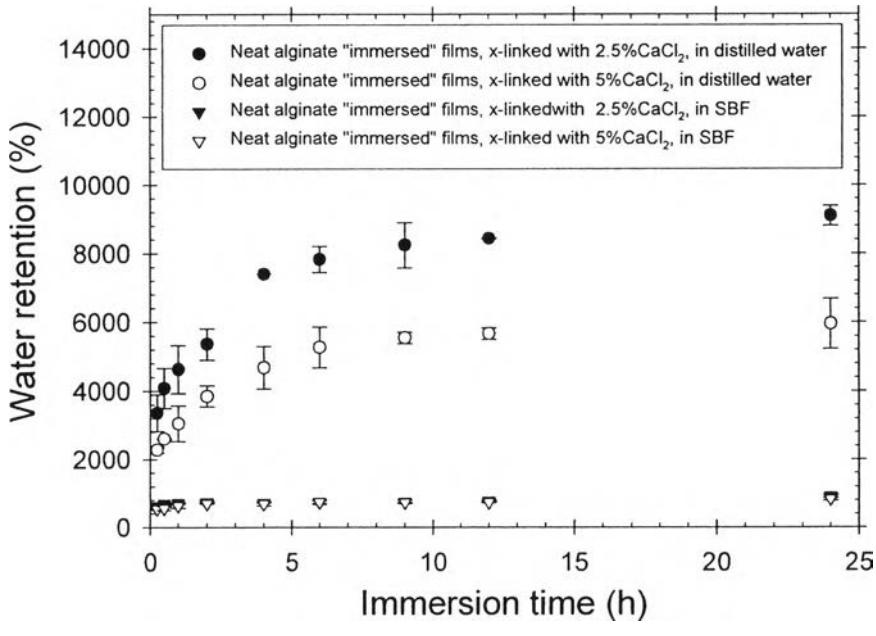
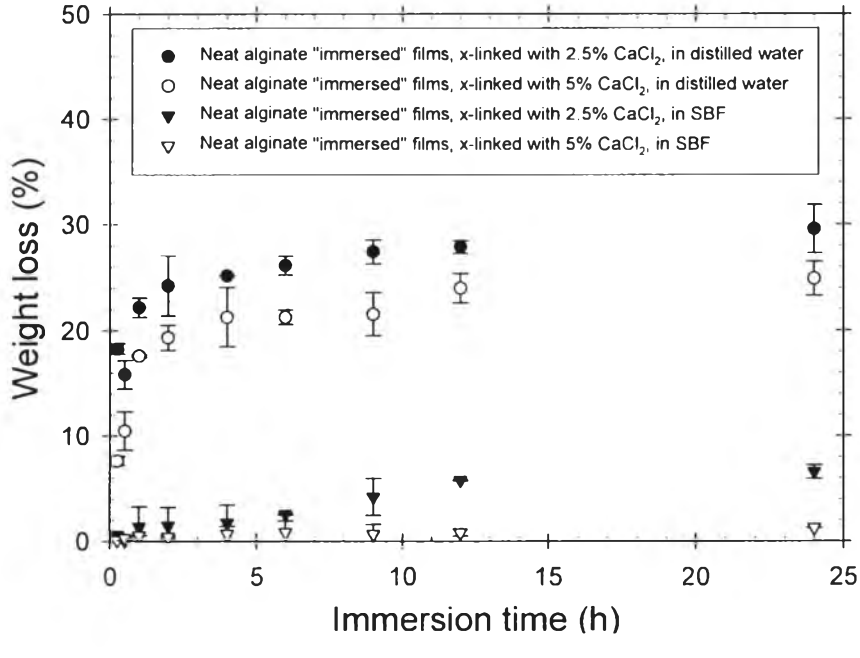


Figure 4.2 Moisture absorption of neat alginate "mixed" films and the corresponding "immersed" films after having been cross-linked with 2.5 or 5% $CaCl_2$ aqueous solution at three relative humidity (RH) levels of 32.8, 52.9 and 75.0% (@ 25 °C) on day 7 after incubation (n = 10).



(a)



(b)

Figure 4.3 (a) Water retention and (b) weight loss behavior of neat alginate "immersed" films after having been cross-linked with 2.5 or 5% $CaCl_2$ aqueous solution upon their submersion in distilled water (pH 6.9) or simulated body fluid (SBF, pH 7.4) for various time intervals at the physiological temperature of 37 °C ($n = 3$).

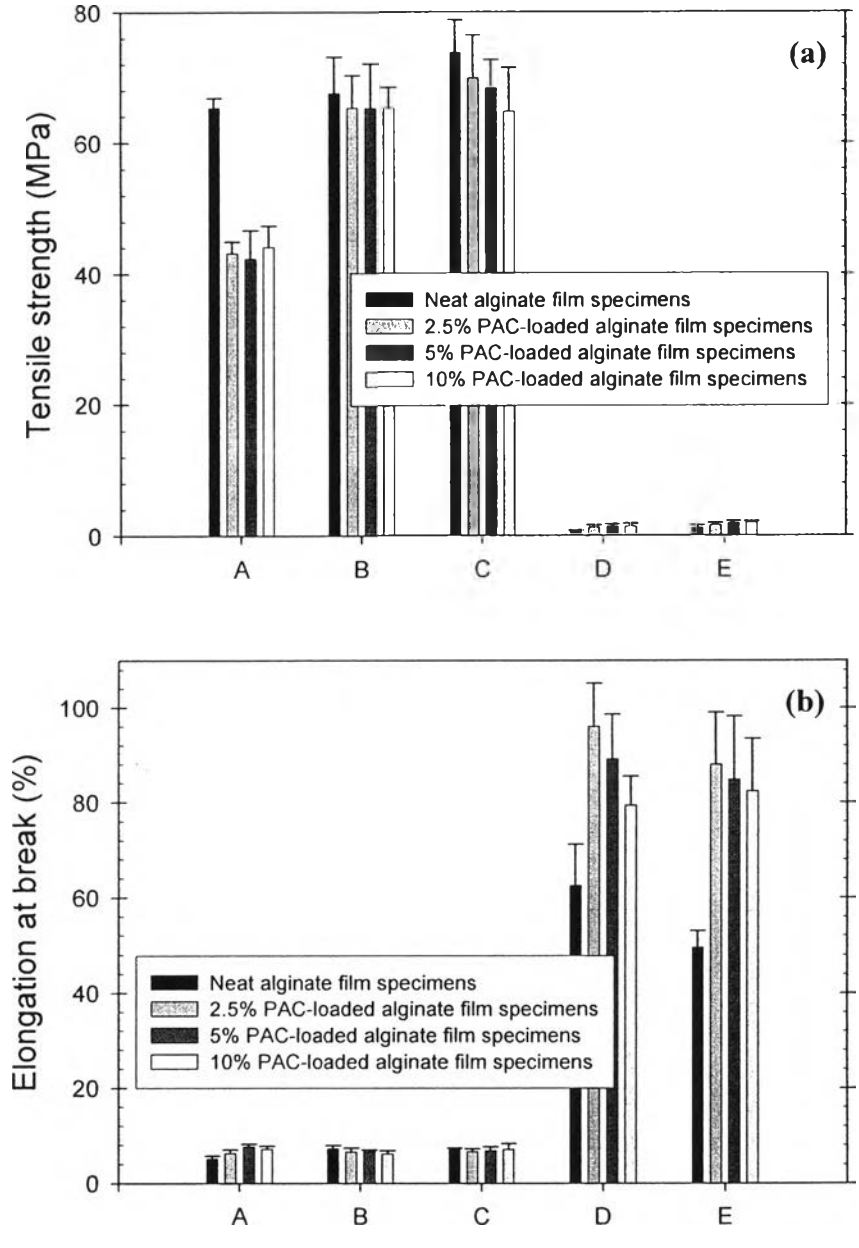


Figure 4.4 Mechanical properties in terms of (a) tensile strength (MPa) and (b) elongation at break (%) of neat and asiaticoside (PAC)-loaded alginate films in dry or wet state (n = 5). A: "mixed" film specimens in dry state, B: "immersed" film specimens cross-linked with 2.5% $CaCl_2$ aqueous solution in dry state, C: "immersed" film specimens cross-linked with 5% $CaCl_2$ aqueous solution in dry state, D: "immersed" film specimens cross-linked with 2.5% $CaCl_2$ aqueous solution in wet state, and E: "immersed" film specimens cross-linked with 5% $CaCl_2$ aqueous solution in wet state.

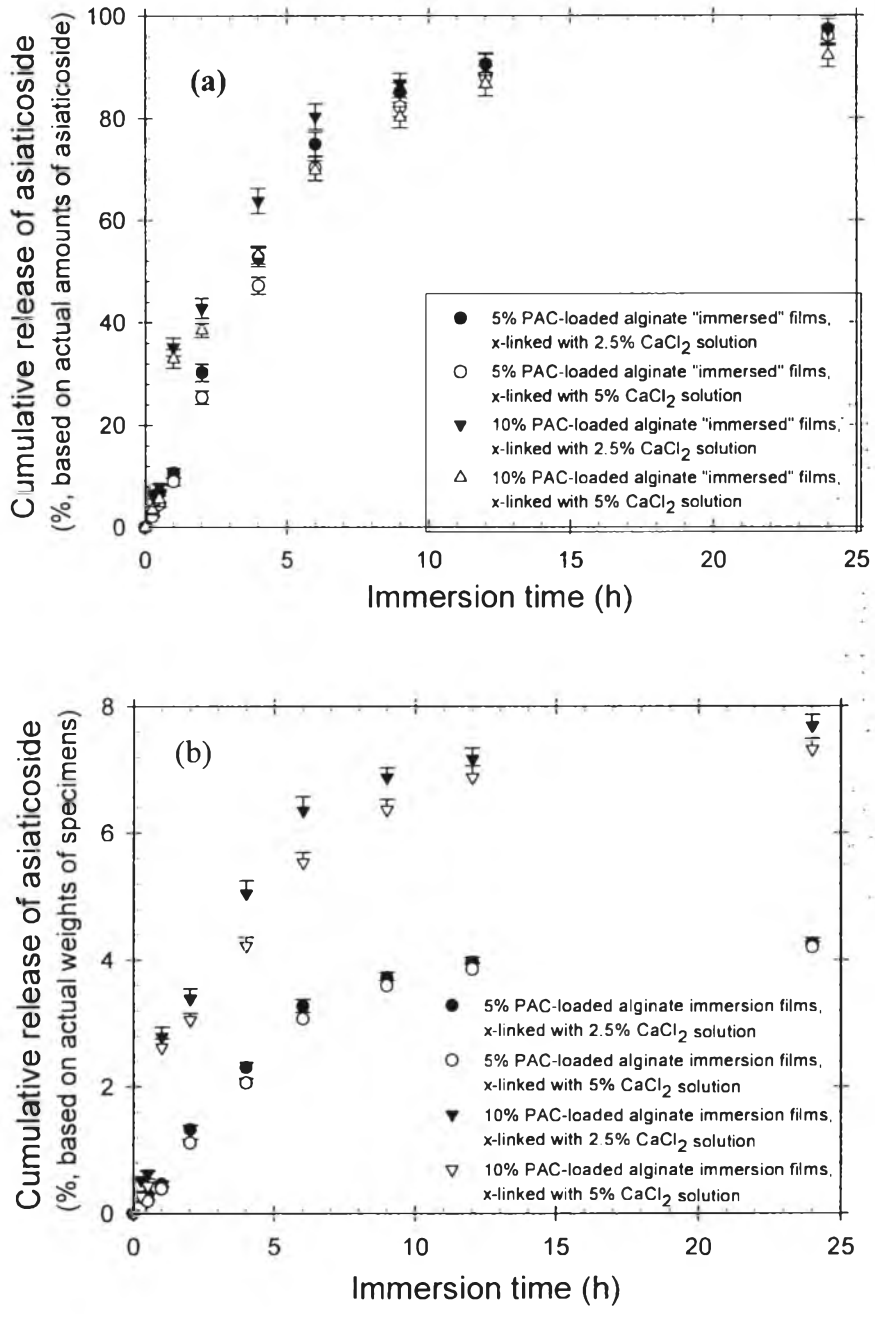


Figure 4.5 Cumulative release profiles of PAC from 5% or 10% PAC-loaded alginate "immersed" films after having been cross-linked with 2.5% or 5% $CaCl_2$ aqueous solution, reported as (a) the percentages of the released weights of PAC divided by the actual weights of PAC in the specimens and (b) the percentages of the released weights of PAC divided by the actual weights of the specimens, in the releasing medium containing 90:10 v/v phosphate buffer solution (PBS)/methanol at the physiological temperature of 37 °C ($n = 3$).

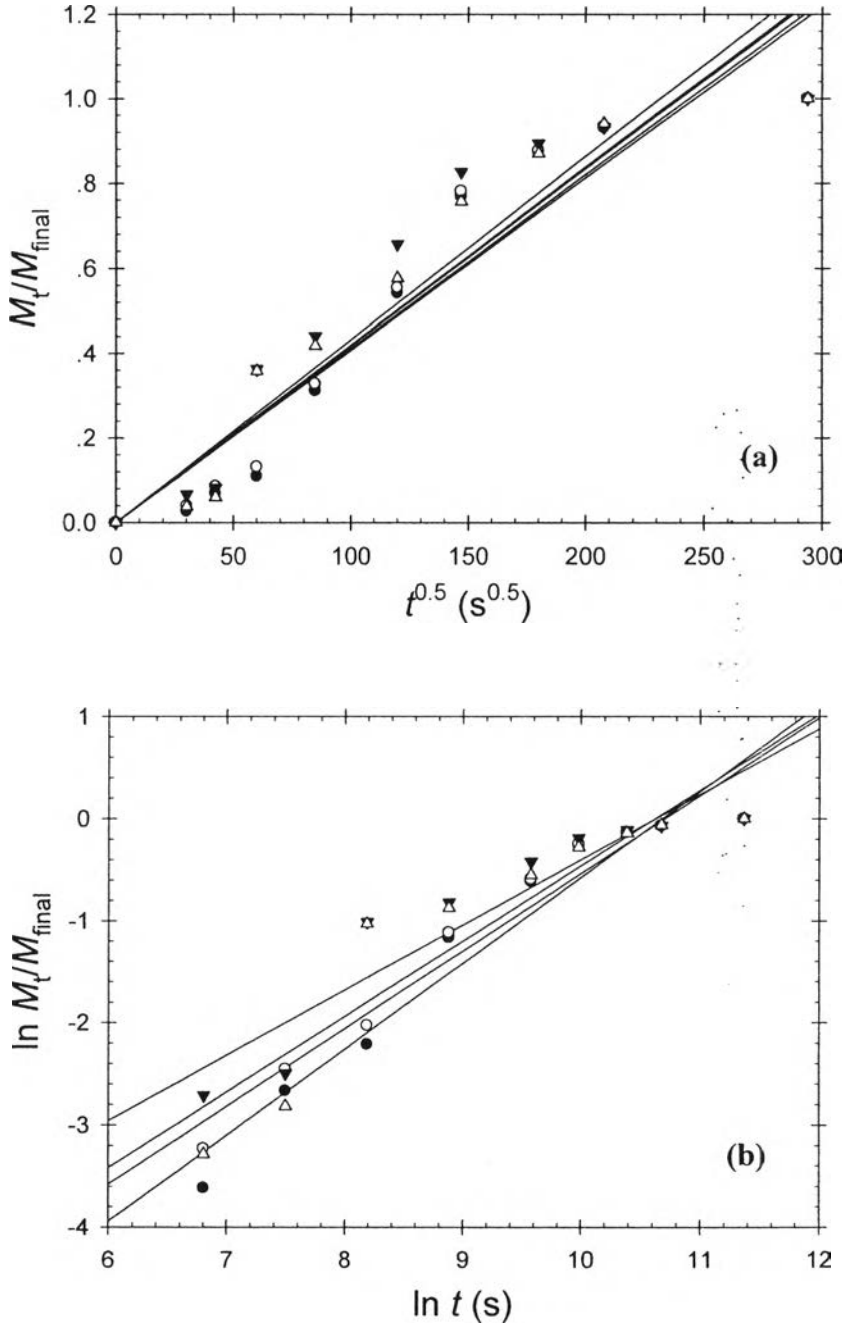


Figure 4.6 Fitting curves of the release kinetics of PAC from 5% or 10% PAC-loaded alginate "immersed" films after having been cross-linked with 2.5% or 5% $CaCl_2$ aqueous solution based on (a) Fickian diffusion type of release mechanism and (b) non-Fickian diffusion type of release mechanism. Keys: 5% PAC-loaded alginate "immersed" films, cross-linked with (●) 2.5 or (○) 5% $CaCl_2$ solution and 10% PAC-loaded alginate "immersed" films, cross-linked with (▼) 2.5 or (△) 5% $CaCl_2$ solution.

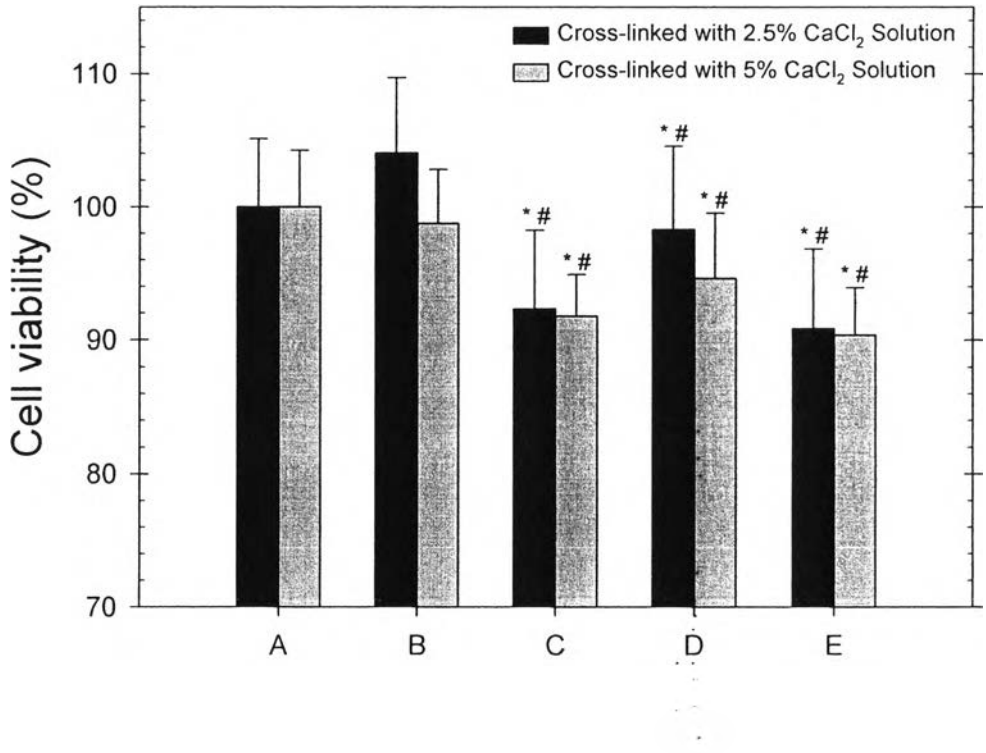


Figure 4.7 Viabilities of normal human dermal fibroblasts (NHDF) that were cultured for 24 h with extraction media from neat and PAC-loaded alginate "immersed" films after having been cross-linked with 2.5% or 5% CaCl2 aqueous solution in comparison with viability of the cells that were cultured with fresh culture medium ($n = 3$). A: fresh culture medium, B: neat alginate "immersed" films, C: 2.5% PAC-loaded alginate "immersed" films, D: 5% PAC-loaded alginate "immersed" films, and E: 10% PAC-loaded alginate "immersed" films.

Remark: *$p < 0.05$ compared with control at a given cross-linking condition and #$p < 0.05$ compared with the neat alginate "immersed" films at a given cross-linking condition.

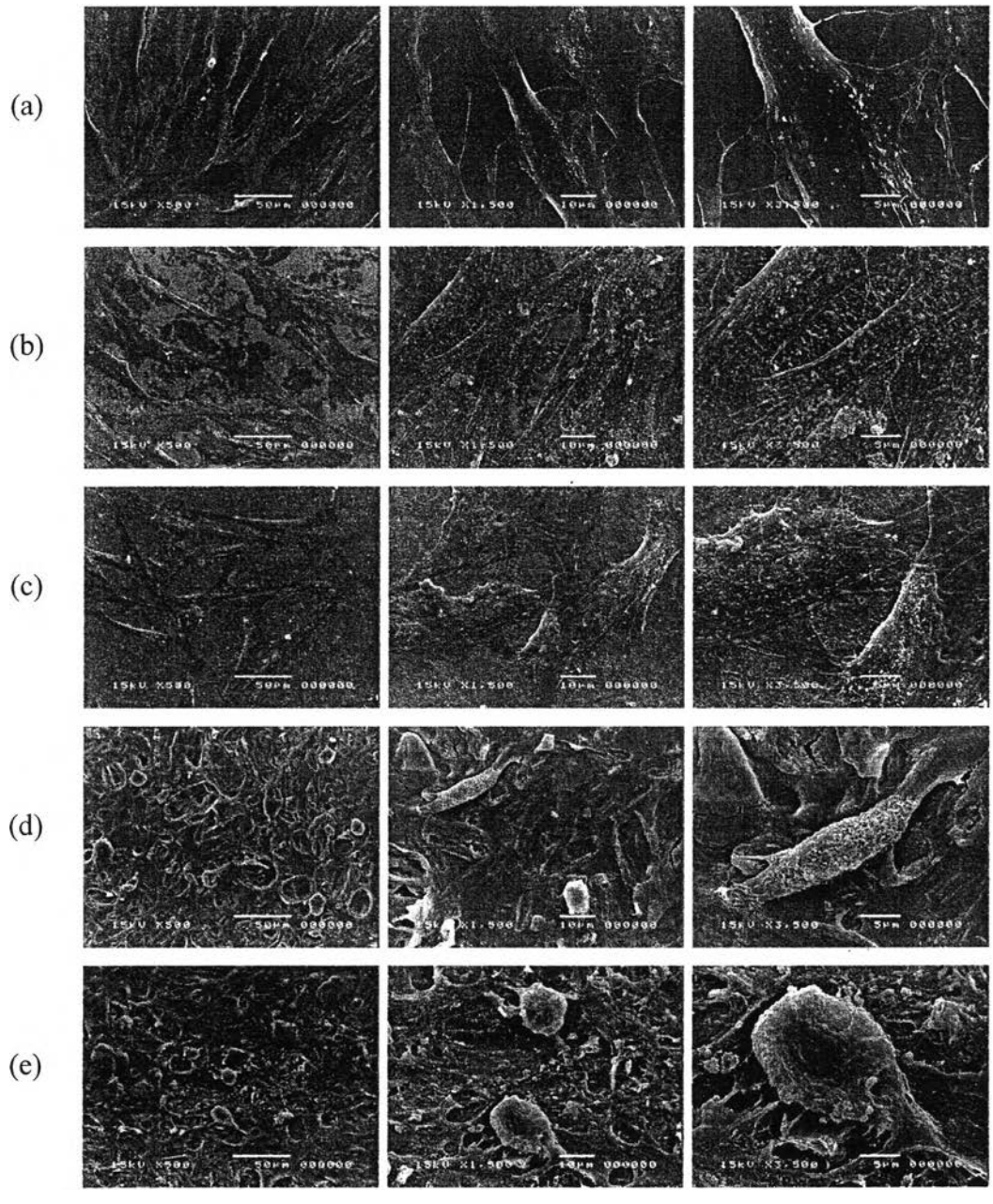


Figure 4.8 Representative SEM images illustrating morphologies of normal human dermal fibroblasts (NHDF) that were cultured for 24 h on (a) glass slide (control), (b) neat alginate "immersed" films cross-linked with 2.5% $CaCl_2$ solution, (c) neat alginate "immersed" films cross-linked with 5% $CaCl_2$ solution, (d) 10% PAC-loaded alginate "immersed" films cross-linked with 2.5% $CaCl_2$ solution, and (e) 10% PAC-loaded alginate "immersed" films cross-linked with 5% $CaCl_2$ solution. The magnification of 500x, 1500x and 3500x were showed in the first, the second and the third column respectively.

Table 4.1 Analyses on the release kinetics of PAC from PAC-loaded alginate "immersed" film specimens ($n = 3$)

Type of sample	Based on the Fickian diffusion type of release mechanism			Based on the non-Fickian diffusion type of release mechanism		
	k ($s^{-0.5}$)	n	r^2	k (s^{-n})	n	r^2
5% PAC-loaded alginate "immersed" films cross-linked with 2.5% $CaCl_2$ aqueous solution	4.06×10^{-3}	0.5	0.91	0.13×10^{-3}	0.84	0.94
5% PAC-loaded alginate "immersed" films cross-linked with 5% $CaCl_2$ aqueous solution	4.10×10^{-3}	0.5	0.91	0.11×10^{-3}	0.76	0.95
10% PAC-loaded alginate "immersed" films cross-linked with 2.5% $CaCl_2$ aqueous solution	4.32×10^{-3}	0.5	0.87	1.13×10^{-3}	0.64	0.88
10% PAC-loaded alginate "immersed" films cross-linked with 5% $CaCl_2$ aqueous solution	4.19×10^{-3}	0.5	0.90	0.39×10^{-3}	0.74	0.86