CHAPTER IV
NOVEL COPPER (II) ALGINATE HYDROGELS AND THEIR POTENTIAL FOR USE AS ANTI-BACTERIAL WOUND DRESSINGS

4.1 Abstract

The incorporation of antimicrobial metals into alginate dressings was an attractive approach to control the inflammatory reaction. In this work, copper (II) cross-linked alginate hydrogels were successfully prepared via a two-step cross-linking procedure with Cu^{2+}. Solid films were created from alginates and copper (II) sulfate solution by the solvent-casting technique. Next, the films were crosslinked on their outer surfaces with copper (II) sulfate solution using a dip coating method in order to improve dimensional stability and to prevent weight loss of the hydrogels. The alginate (2% w/v) containing Cu^{2+} ions in acetate buffer at low pH provided soft films with excellent swelling behavior. In addition, either an increase in Cu^{2+} ions concentration or crosslinking time led to more densely crosslinked networks which limited water absorption of the hydrogels. The hydrogels clearly showed antibacterial activity against *E. coli*, *S. aureus*, Methicillin resistant *S. aureus* (MRSA), *S. epidermidis* and *S. pyogenes* that was proportional to the Cu^{2+} ions concentration. Moreover, copper (II) alginate hydrogels exhibited a tendency to coagulate fibrin, and possibly had an effect on prothrombotic coagulation and platelet activation. The prepared films could be applied as an antibacterial wound dressing providing a moist wound environment, good activity against bacteria and releasing low toxic substance to the skin cells.

4.2 Introduction

The currently accepted concept for the promotion of wound healing is to provide a moist environment to the wound bed [1, 2]. In moist wounds, an environment that is rich in white blood cells, enzymes, cytokines, and growth factors can be easily generated and maintained throughout the healing [3-5]. Much research has focused on the development of materials that provide a moist environment in the wound. One of the most important classes of materials is hydrogels. The first hydrogel was developed

using radiation crosslinking and was observed in further studies to have pain-relieving characteristics along with enhanced healing [6, 7].

Alginate, the biocompatible natural polymer consisting of D-mannuronic acid (M) and L-guluronic acid (G) derived from seaweed, can afford strong, hydrophilic gels leading to high water absorption which limits wound secretions and minimizes bacterial contamination [8]. The gelation of alginate simply occurs in the presence of multivalent cations which have been widely used in pharmaceutical and medical applications including wound dressing materials [9-11].

The incorporation of antimicrobial metals into alginate dressings is an attractive approach to control the inflammatory reaction and prevent/ eliminate infections. Among the various types of multivalent cations, copper (II) has well known as antibacterial activity since ancient times [12]. For example, copper (II) ions from copper oxide (CuO) have been reported as antibacterial agents against *E. coli* [13]. Although the antibacterial activity of copper metal and its derivatives were not significantly different from other metals, but it was not frequently studied because of the instability of copper metal which easily oxidizes in air [14]. However, alginates with incorporated copper (II) were previously fabricated in various forms in order to improve the antibacterial activity [15, 16]. It was reported that copper (II) incorporated with alginate has been used as a wool textile modifier, in order to improve absorption of metals capacity and antibacterial activity. The resulting alginate/copper fabric showed excellent antibacterial properties, evident upon contact with *E.coli* [17].

Generally, an effectual wound dressing should maintain a moist environment upon absorption of the wound exudates as well as provides optimized properties such as flexibility, durability, permeability to water vapor, adequate mechanical properties and protects the wound from secondary infection. It was concluded that in order to accelerate wound healing, a prevention of wound infection was most important. In this work, copper (II) ions incorporated with alginate were interested in order to provide antibacterial activity for wound dressing. The potential antibacterial wound dressing should inhibit the growth of bacteria that was generally found in would infection (e.g. *Staphylococcus aureus*) [18] as well as a various type of bacteria (e.g. the gram positive bacteria, gram negative bacteria and methicillin resistant bacteria).

The flexibility of the crosslinked alginate gel is directly proportional to the properties of the polymer blocks to be bound. For example, crosslinking alginate at G-G blocks which are buckled and stiff allows the M-M blocks and M-G block which are ribbon-like to rotate and increases the flexibility of the matrix. However, Cu^{2+} was reported to be bound with high binding affinity but non-selectively with alginate resulting in copper (II) alginate providing less flexibility and stiffness matrix [19, 20]. In order to overcome this problem, an interesting solution is provided. The preferable environment of guluronic acid (G) for dissolution is one that can decrease of buckled and stiff parts. One of the interested factors is the pH of the environment. It was reported that acid environment can increase the stabilized elastic segments between junction sites leading to increase in swelling.

In this work, the copper (II) crosslinked alginate hydrogels were prepared using guluronic acid (G) preferable solvent (variation of the solvent pH in order to increase solubility of G-G blocks) with the aim to increase the selectivity of the copper (II) crosslinked alginate and to increase gel flexibility. The copper (II) ion crosslinked alginate films were fabricated via solvent casting of alginate followed by immersion in copper (II) sulfate solutions to obtain two simultaneous benefits: an improved dimensional stability and good antibacterial activity. The immersed films were evaluated for their swelling behavior, weight loss, water content, water absorption, and water vapor transmission rate. Moreover, all of the immersed films were evaluated the potential for use as an anti-bacterial wound dressing using disc diffusion method against the several bacteria (gram positive bacteria, gram negative bacteria, methicillin resistant bacteria) as well as bacteria that was found in dermal infections. Also, the films were tested for their ability to accelerate blood coagulation and cytotoxicity.

4.3 Experiment details

4.3.1. Materials

Alginic acid (sodium salt, Brookfield viscosity 20000-40000 cps; hereafter, sodium alginate) and copper (II) sulfate ($CuSO_4.5H_2O$) were purchased from Sigma-Aldrich Corp. (St. Louis, USA). Sodium acetate (anhydrous) was purchased from Fluka (Buchs, Switzerland). Acetic acid (glacial) was purchased from

Mallinckrodt Chemicals, USA. All other chemicals were of analytical reagent grade and used without further purification.

4.3.2 Preparation of Copper (II) Alginate Film

The acetate buffer pH 3.0, 4.0, 5.0 and distilled water were used to be the solvent of alginate at the certain concentrations (0.5 % w/v, 1.0 %w/v and 2.0 %w/v). Copper (II) sulfate solutions (source of copper (II) ions) in various concentrations (0.5 to 2.5 % w/v) at various cross-linking times (5 to 15 min) were added dropwise into a sodium alginate solution to initiate gelation. The volumetric ratio between each of the alginate solution and each of the copper (II) sulfate solution was 10:1 v/v. The mixtures were dried under vacuum for 3-5 days in culture dishes to finally obtain solid films (hereafter, copper (II) alginate films/hydrogels). To improve the dimensional stability of the films, they were re-immersed in the corresponding copper (II) sulfate solutions for 5, 10 and 15 min. All of the immersed films were washed in distilled water for 10 second to eliminate the excess copper and the films were dried at 60 °C for 24 h. After preparation, the hydrogels were cut into discs (14 mm in diameter and 1 mm in thickness) and were further dried to constant weight. The hydrogels were kept in sealed bags prior to further use.

4.3.3 Physical and Chemical Characterization

After the initial addition of copper (II) sulfate solution to a sodium alginate solution, the amount of free copper (II) ions in the clear solution that was left from the crosslinking of the alginate chains was quantified by a Shimadzu UV-1800 spectrophotometer. Chemical interaction between the copper (II) ions and certain chemical functional groups of alginate was examined on a copper (II) alginate film using a Nicolet Fourier transform infrared (FT-IR) 360 spectrophotometer. FT-IR spectra of sodium alginate were also recorded for comparison using the KBr pellet method. Morphologies of the copper (II) alginate films both before and after subsequent immersion in the corresponding copper (II) sulfate solutions were observed with a JEOL JSM-5200 scanning electron microscope (SEM).

4.3.4 Determination of Equilibrium Water Content and Water Absorption

The films were immersed in the simulated body fluid pH 7.4 (SBF) at 37 °C which was imitated with physiological conditions of human body, allowed to swell until of constant weight for 24 h. The obtained hydrogels were removed from the droplets of liquid (the excess of SBF on the hydrogel surface) by filter paper prior to being weighed. The equilibrium water content and water absorption can be calculated as follows:

$$\text{Equilibrium water content (\%)} = \frac{W_s - Wd}{W_s} \times 100, \quad (1)$$

where W_s is the weights of the hydrogel at the equilibrium swollen state. It should be noted that W_d was obtained after the swollen hydrogels were dried in an oven at 60 °C for 24 h.

$$\text{Water absorption (\%)} = \frac{W_s - W_i}{W_i} \times 100; \quad (2)$$

$$\text{Weight loss (\%)} = \frac{W_i - Wd}{W_i} \times 100, \quad (3)$$

where W_i are the initial dry weights of the films (before being immersed in SBF).

4.3.5 Water Vapor Transmission Rate (WVTR)

The water vapor transmission rate (WVTR) was measured following the monograph of the European Pharmacopeia. The experiment measured the rate of water transport from a glass bottle containing 25 mL of water, which used a disc of hydrogel (14 mm in diameter) as the cap for the bottle. The glass bottle with the hydrogel cap was kept in an oven at 35 °C for 24 h. WVTR was calculated by using following equation

$$\text{WVTR} = \frac{(W_i - W_t)}{A \times 24} \times 10^6 \quad \text{g/(m}^2\text{.h)} \quad (4)$$

where WVTR is expressed in g/(m^2.h), A is the area of the diameter of the bottle (mm^2), W_i and W_t are the weight of bottle before and after placed in an oven, respectively.

4.3.6 Swelling Behavior

Studies were carried out based on the kinetics of water sorption dynamics method utilized by Balakrishnan and Lee [21, 22]. Briefly, the films were weighed for initial dry weight before being immersed in distilled water at 37 °C. After a specified immersion time, the films were taken out and the water droplets on the surface of the films were removed with filter paper sheets prior to being weighed. The swelling ratio (Q_m) was calculated by following expression:

$$Q_m = \frac{W_s - W_i}{W_i}, \quad (5)$$

where W_s and W_i are the weights of the film hydrogels in their swollen state and initial dry state, respectively. The crosslink density (ν_e, mol/cm^3) of the copper (II) cross-linked hydrogels was calculated based on the Flory-Rehner equation:

$$\nu_e = -\left[\ln(1-\nu_2) + \nu_2 + \chi_1 \nu_2^2\right]\left[V_1\left(\nu_2^{1/3} - \frac{2\nu_2}{f}\right)\right]^{-1} \quad (6)$$

where χ_1 is the interaction parameter, f is the cross-linking functionality, V_1 is the molar volume of water (18.062 cm^3/mol) and ν_2 is the volume fraction of polymer in the hydrogels at the equilibrium swollen state. According to Balakrishnan et al. (2005) and Lee et al. (2000), χ_1 and f were assumed to be 0.35 and 2, respectively, for alginate-water systems. The value of polymer volume fraction (ν_2) was calculated by the following equation:

$$\nu_2 = \left(1/\rho_p\right)\left[\left(Q_m/\rho_s\right) + \left(1/\rho_p\right)\right]^{-1} \quad (7)$$

where ρ_p is the polymer density (0.8755 g/cm^3), ρ_s is the water density (0.9933 g/cm^3 at 37 °C) and Q_m is the swelling ratio.

4.3.7 Cumulative Copper (II) Ions Release

The copper (II) crosslinked alginate hydrogels were cut to discs (9 mm in diameter, 1 mm in thickness, 102.02 ± 14.0 mg) and each individual disc was immersed in a bottle containing 10 ml of SBF solution at 37°C under agitation at 60 rpm. At various time intervals, the immersion solution was collected for investigation

of the cumulative release of copper species. A replacement of 10 ml of SBF solution into each bottle was performed at each time interval. The concentration of copper species was measured using a Varian SpectrAA300. The initial content of copper (II) ions in copper (II) cross-linked alginate hydrogel can be calculated corresponding to amounts of added copper (II) ions.

4.3.8 Antibacterial Evaluation

Specimens of hydrogel were cut into discs (9 mm in diameter), which were further studied via the disc diffusion method [The US clinical and laboratory standards institute (CLSI) disc diffusion method]. Both gram-negative bacteria, *E. coli*, ATCC 25922 and gram-positive bacteria, *S. aureus*, ATCC 25923 were used to test for antibacterial activity of copper (II) alginate hydrogels. Methicillin resistant *S. aureus* (MRSA), DMST 20654, *S. epidermidis*, ATCC 12228 and *S. pyogenes*, DMST 17020 which caused dermal infection, were also selected to evaluate their antibacterial activity. All of the bacteria was diluted until the colonies equal to 10^8 CFU (colony forming unit)/ mL and then 200 µL bacteria solution were transferred on Difco™ Mueller Hinton agar dishes. The hydrogel specimens were placed on the agar culture dishes and incubated at 37 °C for 24 h. An inhibition zone was clearly seen around each specimen whenever there was antibacterial activity.

4.3.9 Indirect Cytotoxicity Evaluation

The ISO 10993-5 standard test method was used to evaluate toxicity of the copper (II) alginate hydrogel, which was used in both L929 cells (mouse fibroblast) and NHDF (Normal human dermal fibroblast). The cells were cultured in Dulbecco's modified Eagle's medium, DMEM [composition of 10% fetal bovine serum (FBS; Invitrogen Corp., USA), 1% l-glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation which was contained penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)]. The disc specimens (14 mm in diameter) were sterilized with 70%v/v ethanol for 30 min. The extraction medium was prepared by immersing each disc specimen (weighed in mg of hydrogel) in serum free media (SFM; DMEM containing 1% l-glutamine, 1% lactalbumin and 1% antibiotic and antimycotic formulation) for 1, 2 and 3 days with

extraction ratios of 15, 25 and 75 mg/mL, respectively. The cells were then separately cultured using SFM in 24-well tissue-culture polystyrene plates (TCPS; NunclonTM, Denmark) which were incubated at 37 °C for 24 h. Since L 929 had higher growth rate compared to NHDF, it was seeded with lower cell number than NHDF and the cell density was optimized in each well (5000 cells per well for L929 and 10,000 cells per well for NHDF). The obtained extraction media was used as the media for the cells to grow. The relative cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

4.3.10 Blood Clot Formation Assay

Blood clot formation initiated by the hydrogels was demonstrated via fibrin formation on the surface of the hydrogels. First step, human blood from Chulalongkorn hospital (200 μL) was dropped on the surface of each sample including 6 mm in diameter of the obtained hydrogels (i.e. the copper (II) alginate films after cross-linking the surface by 1.0% $CuSO_4$ solution and 2.5% $CuSO_4$ solution, respectively) and the glass slides as controls. The samples were immediately covered with glass cover slides and then incubated for 5 min at room temperature. Afterwards, the samples were gently washed to remove excess blood using PBS buffer (pH 7.2). In order to observe fibrin formation, all of the samples were fixed with 3 %v/v of glutaraldehyde in PBS buffer for 30 min at room temperature, and rinsed with PBS buffer. Finally, the samples were dehydrated with a series of ethanol solutions (30, 50, 70, 90, and 100%) for 2 minutes each and air dried. The morphology of fibrin formation was observed by SEM.

4.3.11 Statistical Analysis

Standard deviations (SD) from triplicate end point data were plotted as error bars on all graphs. Differences between control and samples were determined using one-way analysis of variance (ANOVA) and the Scheffe's post hoc test with SPSS 11.5 for Windows software (SPSS). Statistically significant differences were set at $p < 0.05$.

4.4 Results and discussion

4.4.1 Physical and Chemical Characterization

A UV-vis spectrophotometer was used to measure the free copper (II) ions in the solution before and after addition of alginate solution. The absorbance spectra clearly showed the disappearance of the absorbance pattern of cupric ions from copper (II) sulfate after adding either sodium alginate concentrations of 0.1% w/v or 0.5 % w/v (Figure 4.1b & 4.1c). As shown in Figure 4.1a, the absorbance spectrum of cupric ions occurred in the IR range of approximately 808 nm [23]. The result indicated that there was no copper (II) ion left in the system, it can be assumed that copper (II) ions were bound by alginate chains.

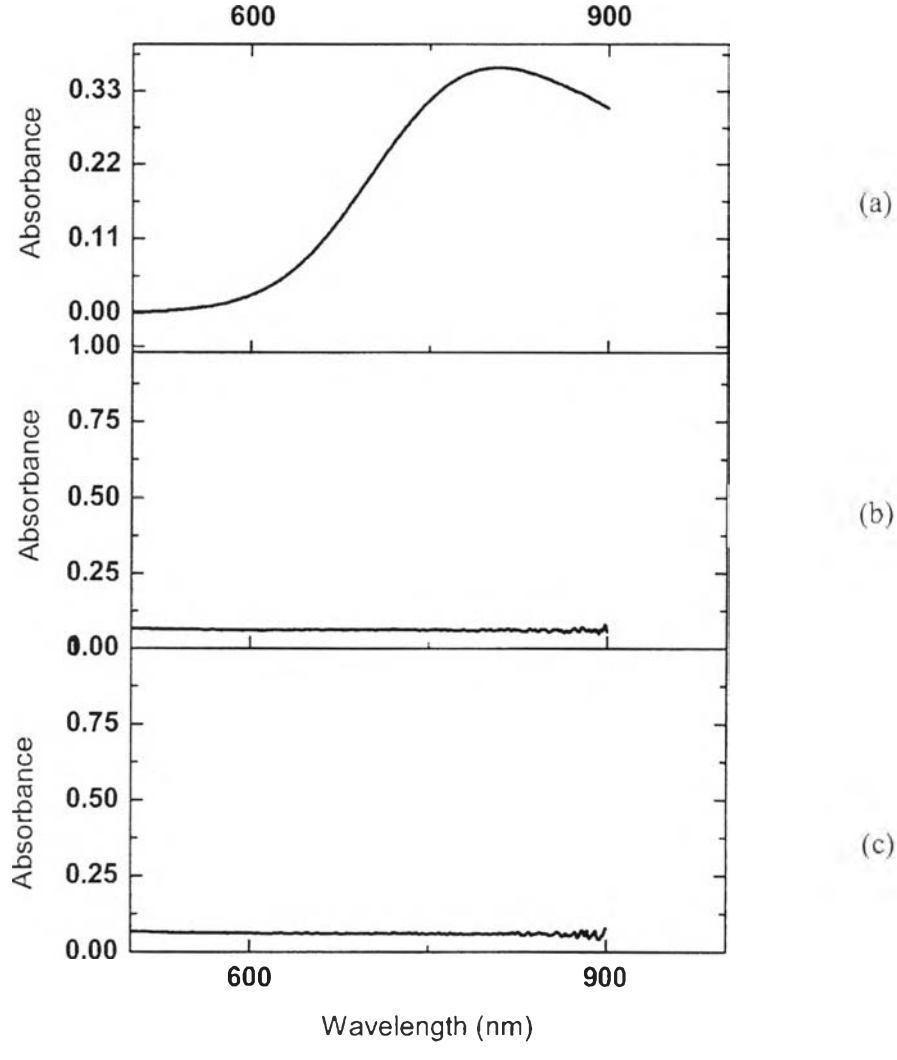


Figure 4.1 (a) The absorbance spectrum of 2.5 % w/v $CuSO_4$ Solution, (b) copper (II) alginate film with 0.1 % $CuSO_4$ solution and (c) copper (II) alginate film with 0.5 % $CuSO_4$ solution.

The FT-IR spectrum (Figure 4.2) of the sodium alginate powder clearly showed a carbonyl group, hydroxyl group and carboxyl and carboxylate group around wave numbers 1,766 cm^{-1} , 3,440 cm^{-1} and 1,000 to 1,400 cm^{-1}, respectively [including β-d-mannuronic acid (m blocks) and α-L-glururonic acid (g blocks)]. The crosslinking between sodium alginate and copper (II) ions directly led to the interaction between unlike charges (i.e. negatively charged carboxylate groups of alginate and positively charged copper (II) ions) [24]. This was supported by FT-IR spectrum of copper (II) alginate film (2.5% $CuSO_4$ solution at pH 4, 15 minutes) which showed the reduction in wave number of carbonyl and hydroxyl peaks to 1,635 cm^{-1} and 3,382 cm^{-1}, respectively.

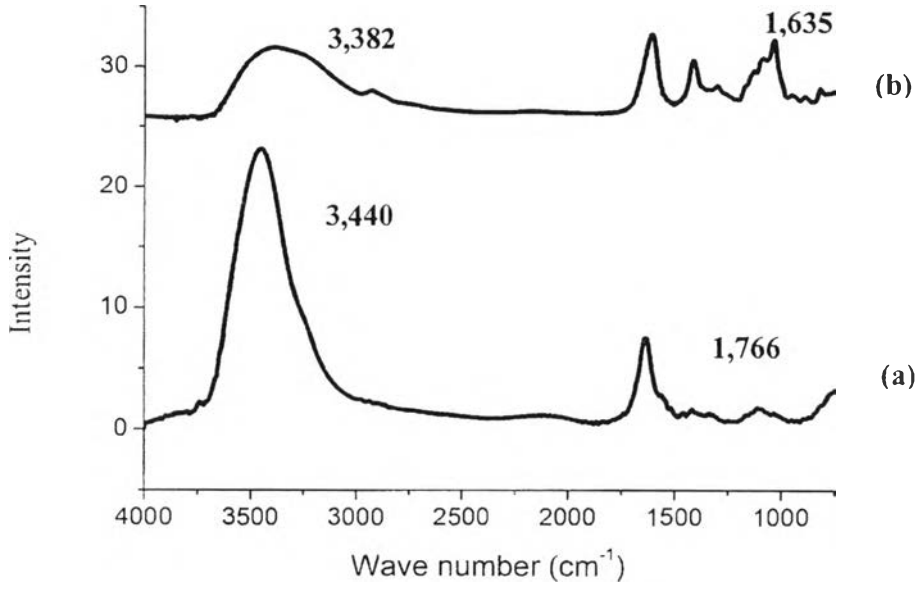
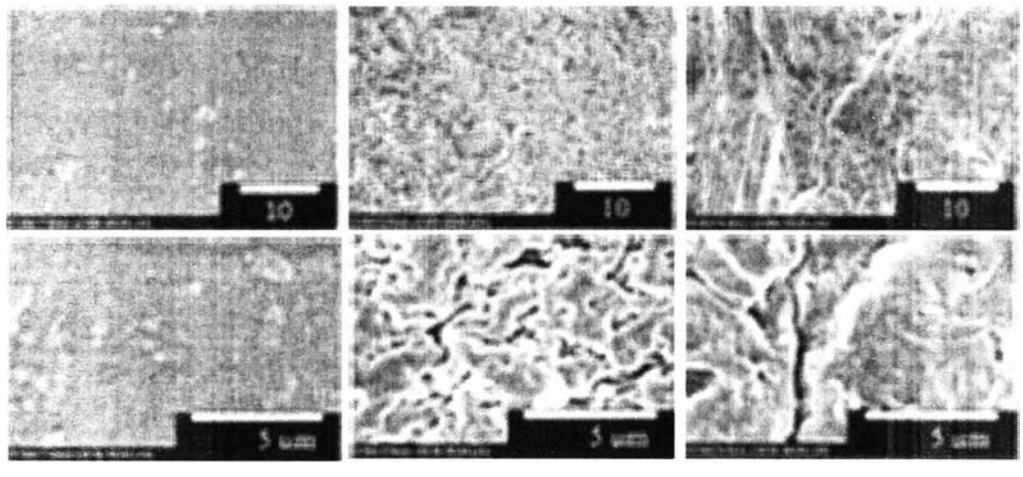


Figure 4.2 FT-IR spectrum of (a) sodium alginate powder and (b) copper (II) alginate film was cross-linked at 2.5% $CuSO_4$ solution, 15 minutes.

The surface observation of the copper (II) crosslinked alginate film was carried out via SEM which focused on the surface of the film both before and after immersing in copper (II) sulfate concentrations of 1.5% w/v and 2.5% w/v. Figure 4.3a & 4.3b showed significant differences between a surface that was crosslinked together between nearby carboxylate groups and copper (II) ions outside the surface. With increasing copper (II) sulfate concentration, Figure 3b and Figure 3c showed the decreasing in void volume of the film resulting from increasing crosslinking points. All of these results were directly related to the improvement of the dimensional stability of the copper (II) alginate film.



a b c

Figure 4.3 Representative scanning electron microscopic images (a) Copper (II) cross-linked alginate film before cross-linking the surface, (b) Copper (II) cross-linked alginate film after cross-linking the surface with 1.5% $CuSO_4$ solution and (c) Copper (II) cross-linked alginate film after cross-linking the surface with 2.5% $CuSO_4$ solution.

The interaction of alginate and divalent metal ions can occur in a variety of ways including bridging in G-G blocks, G-M blocks and M-M blocks, respectively. The stiffness of the individual chain is different which increased in the following order: G-G <M-G <M-M blocks [25].

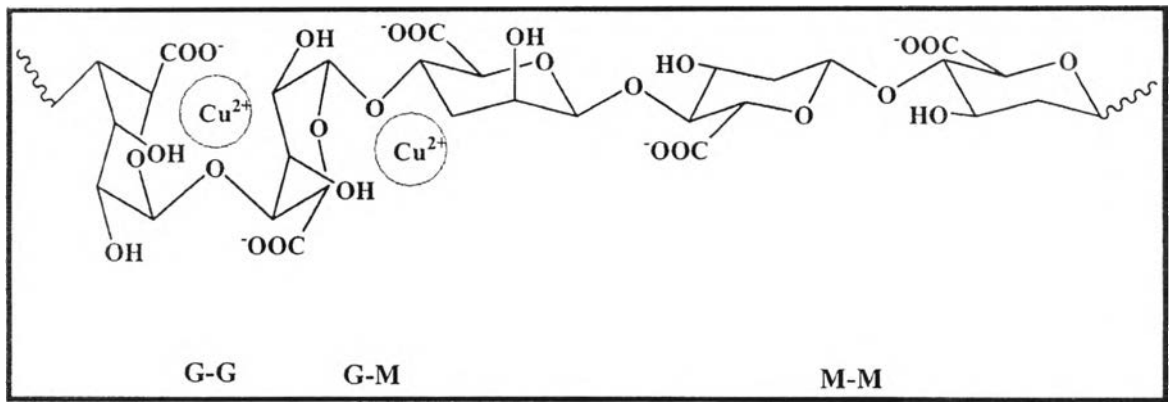


Figure 4.4 The expected interactions of the copper (II) alginate which were occurred in acid solution. The interaction mainly occurred at the G-G blocks which were bound between copper ions and nearby carboxylate and hydroxyl groups. Another possibility was

In order to obtain the flexible matrix, the metal ions should be bound in G-G blocks of the alginate chain which allows other blocks to rotate freely in the matrix. From the FT-IR results, the interaction of Cu^{2+} in the cross-linked alginate occurred at both the carboxylate groups and hydroxyl groups which can be assumed that Cu^{2+} bound mainly with G-G blocks and some part of G-M blocks of the chain. (Figure 4) Moreover, Cu^{2+} may penetrate into inner core of alginate due to their small ionic radii (87×10^{-3} nm) which expected to be crosslinked alginate with high metal content.

4.4.2 Equilibrium Water Content and Water Absorption and Water Vapor Transmission Rate (WVTR)

As shown in Table 4.1 and Table 4.2, when equilibrium water content increased then the water absorption decreased. The results clearly showed the inversely proportional between equilibrium water content and water absorption because if the water content within sample structure is high leading to reduce the sample ability to absorb more water.

The equilibrium water content and water absorption of the all immersed films were in a range of 73 % to 85 % and 220 % to 600 %, respectively. The results indicated the excellent absorption ability of the copper (II) alginate hydrogel to absorb liquid into its structure together with maintaining its original shape.

Table 4.1 Representative water content, water absorption and water vapor transmission rate (WVTR) of copper (II) cross-linked alginate using different pH ranges (n = 3).

pH range	Water content (%)	Water absorption (%) in 24 h immersion time	WVTR ($g/m^2/h$)
3.00	76.66 ± 0.12	292.52 ± 0.23	156.24 ± 0.26
4.00	73.55 ± 0.06	306.45± 0.19	160.24 ± 0.37
5.00	79.32 ± 0.15	254.35 ± 0.22	121.62 ± 0.59
Distilled water	83.74 ± 0.08	181.34 ± 0.18	89.95 ± 0.21

Table 4.2 Representative water content, water absorption and water vapor transmission rate (WVTR) of copper (II) cross-linked alginate using different polymer concentrations (using acetate buffer pH 4) (n = 3).

Concentration of alginate(%w/v)	Water content (%)	Water absorption (%) in 24 h immersion time	WVTR ($g/m^2/h$)
0.50	84.47 ± 0.22	228.68 ± 0.24	113.45 ± 0.44
1.00	73.55 ± 0.06	306.45 ± 0.19	160.24 ± 0.28
2.00	69.54 ± 0.38	599.87 ± 0.27	168.69 ± 0.31

Many different parameter influenced gel formation. One of the major effects for the gel formation was the variation of the pH range. In an acid environment, the carboxyl ions, $-COO^-$, became protonated to carboxylic groups, –COOH. This decrease in the negative charges not only lowered repulsive forces between alginate molecules, but also lowered the electrostatic interaction between alginate and water molecules forcing alginate molecules to come closer to each other, leading to gel formation. Otherwise, dealing with strong alkalis led to a gradual breakdown of the polysaccharide chains [26].

Therefore, acetate buffer (pH 3.0-5.0) and distilled water were used to compare properties of the obtained copper (II) alginate in this study. Table 1 showed the relationship between pH and water absorption, which provided higher absorption ability when using acid solvents but trends to be reduced in neutral pH of distilled water. The results shown no significant difference of water absorption between pH 3.0 and 4.0 but a slight decrease at pH 5.0 because of decreasing acidic condition and lower solubility of G-G blocks and M-G blocks (Manuronic acid: pKa 3.38, Guluronic acid: pKa 3.65). It was reported that the solubility of alginate in acetate buffer (pH 4) led to increase the stabilized elastic segments between junction sites leading to allow further swelling [27].

The absorption ability can be related with the flexibility of the chains, the higher absorption ability being caused by higher elasticity segments of the chains moving apart from each other. The results showed the absorption ability using acid solvent over neutral pH which meant to be more M-G blocks and M-M blocks moving around and thus assumed that copper (II) ions were possibly fixed in the G-G blocks of the chain.

As shown in Table 4.2, increasing the polymer concentration led to a water absorption increase. It is understandable that increasing the polymer concentration leads to increased gel formation because of higher amount of entangled chains. Gel formation is directly proportional to polymer concentration but the polymer concentration should not be too high because a viscous liquid will be obtained leading to difficulty to form into particular shapes.

An example of the serious issues in the area of burn wounds is the rapid evaporation and exudation of the body fluid leading to a decrease in body temperature and increased rate of metabolism (drying out wound area) [28]. Therefore, wound dressing materials should maintain a moist environment in the wound area. WVTR is the experiment which provided water transmission and absorption ability of the materials. The value of WVTR in this research was in the range of 89 to 169 $g/m^2/h$ which is attractive compared to some commercial wound dressings (Table 4.1 and Table 4.2) [29]. In general, there is no standard value for WVTR, as it depends on the water loss in each type of wound. For example, if the WVTR is high, it will cause a dry condition around the wound surface. When the WVTR is low, it will allow agglomeration of exudates which can cause a bacterial infection.

4.4.3 Swelling Behavior and Crosslink Density of Copper (II) Alginate Films

All of the copper (II) alginate films including various concentrations of copper (II) sulfate solution (1.0 % w/v, 1.5 % w/v, 2.0 % w/v and 2.5 % w/v) and crosslinking time (5 min, 10 min and 15 min) showed the same pattern of swelling behavior. Thus, only the results of copper (II) alginate using a crosslinking time 15 min (Figure 4.5) are reported. The swelling behavior of the hydrogels increased rather rapidly during first 8 h of the immersion time. Further increasing in the immersion time resulted in a gradual increase in the water absorption to eventually assume a plateau value at 24 h of the immersion time (the hydrogels at their equilibrium swollen state). Various concentrations of copper (II) sulfate solution and crosslinking time had an effect on crosslink density, which was inversely proportional to the swelling ratio. The increase in copper (II) sulfate solution decreased the swelling of the immersed film due to increase in crosslinking points. Likewise, increase in crosslinking time provided less swelling. Furthermore, the swelling ratio information can be calculated to crosslink density of entanglement chains following the Flory-Rehner equation (Table 4.3). Equation (6) and (7) indicate that swelling ratio and crosslink density depend on each other. The crosslink density of copper (II) alginate hydrogels were shown to strongly increase with crosslinking time increased but slightly increase with concentration of copper (II) ions increased.

This result indicated that important factors to crosslink alginate with divalent metal ions were not only crosslinking concentration but also the interval time to be allowed them to form crosslinked structures.

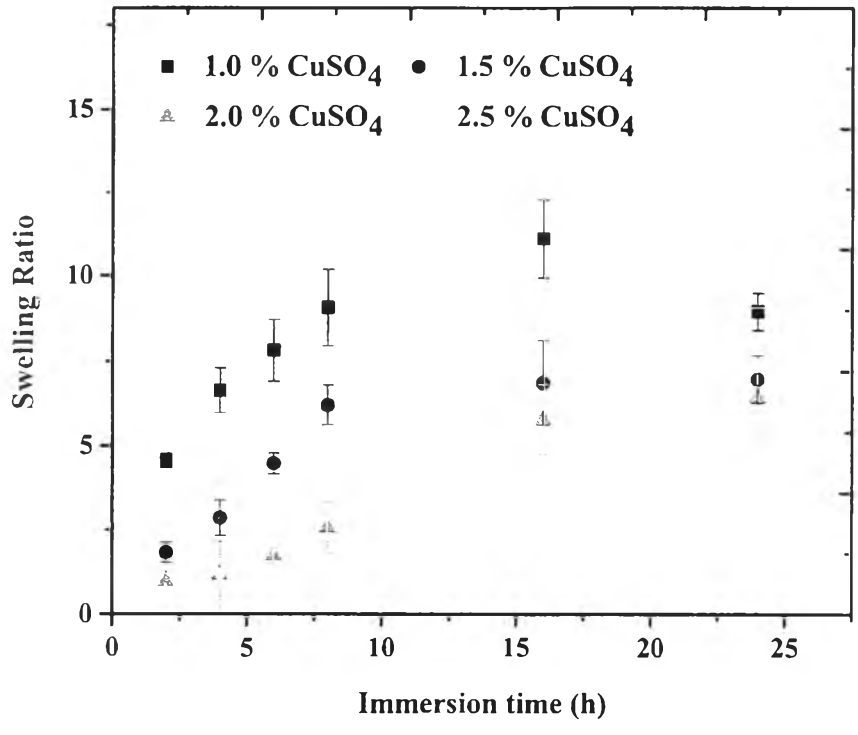


Figure 4.5 Representative swelling behavior of copper (II) alginate immersed film cross-linked with 1.0% w/v $CuSO_4$, 1.5% w/v $CuSO_4$, 2.0% w/v $CuSO_4$ and2.5% w/v $CuSO_4$ solution using cross-linked time interval of 15 minutes (n = 3).

Table 4.3 the cross-linking density of copper (II) alginate immersed film calculated using the Flory-Rehner equation (n = 3).

Cross-linking time	Concentration (% w/v)	Volume (mL)	v_e (mol/cm^3) x 10^5
5 min	1.00%	50	4.23 ± 0.10
	1.50%	50	4.62 ± 0.06
	2.00%	50	4.88 ± 0.18
	2.50%	50	6.54 ± 0.09
10 min	1.00%	50	6.21 ± 0.18
	1.50%	50	7.26 ± 0.20
	2.00%	50	7.47 ± 0.04
	2.50%	50	8.04 ± 0.02
15 min	1.00%	50	7.13 ± 0.08
	1.50%	50	7.49 ± 0.11
	2.00%	50	7.95 ± 0.05
	2.50%	50	8.23 ± 0.15

4.4.4 Weight Loss Behavior of Copper (II) Alginate Film

The weight loss behavior of copper (II) alginate film was shown in Figure 6 which clearly showed the relationship between weight loss and the copper (II) sulfate concentration. The increasing in copper (II) sulfate concentration led to more crosslinking which provided stability to the immersed film resulting in less weight loss during the immersion time increased. As shown in Figure 4.7, the crosslinking time also has an effect on stability of the immersed film (indicated by weight loss) due to generation of more crosslinks between surfaces of the immersed film as crosslinking time increased. It was concluded that crosslinking time, 15 min showed the highest film stability due to less weight loss.

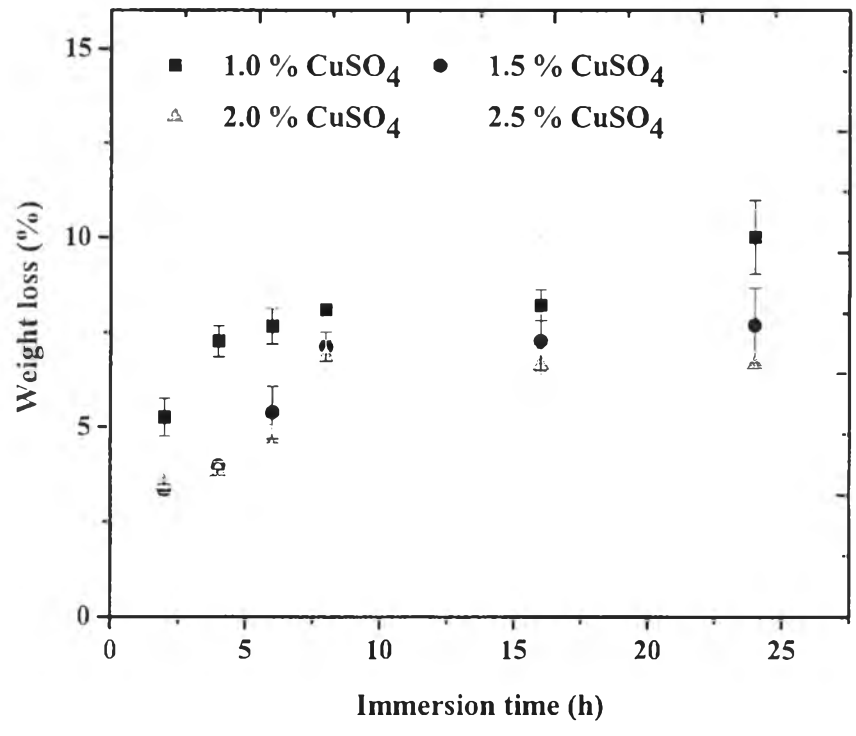


Figure 4.6 Representative weight loss (%) of copper (II) alginate immersed film cross-linked with 1.0% w/v $CuSO_4$, 1.5% w/v $CuSO_4$, 2.0% w/v $CuSO_4$ and 2.5% w/v $CuSO_4$ solution using cross-linked time intervals of 15 minutes (n = 3).

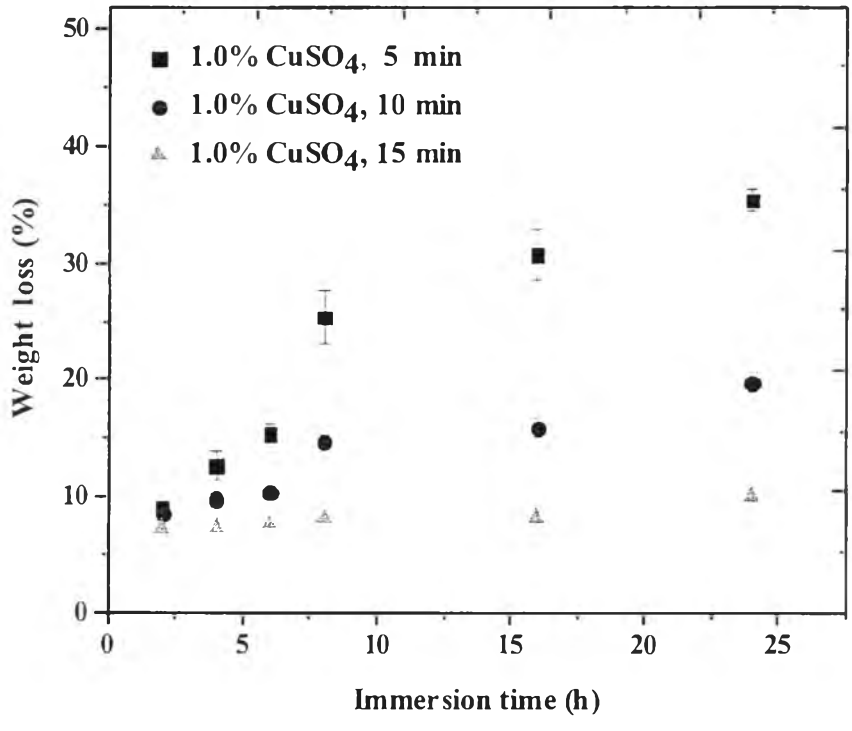


Figure 4.7 Representative weight loss (%) of copper (II) alginate immersed film cross-linked with 1.0% w/v $CuSO_4$, using difference cross-linking time (5, 10 and 15 minutes) (n = 3).

4.4.5 Cumulative Copper (II) Ions Release

The initial contents of copper (II) in the copper (II) cross-linked alginate hydrogel were 10.01 ± 0.89, 14.84 ± 0.33, 20.76 ± 0.21, and 25.86 ± 0.15 mg for a 1.0 g of alginate hydrogel cross-linked with 1.0% w/v $CuSO_4$, 1.5% w/v $CuSO_4$, 2.0% w/v $CuSO_4$, and 2.5% w/v $CuSO_4$ solution, respectively. After a 72 h immersion in SBF, the released contents of copper (II) ions within the hydrogels were 4.93 ± 0.15, 6.91 ± 0.10, 9.36 ± 0.10 and 12.56 ± 0.19 mg for alginate hydrogel cross-linked with 1.0% w/v $CuSO_4$, 1.5% w/v $CuSO_4$, 2.0% w/v $CuSO_4$, and 2.5% w/v $CuSO_4$ solution, respectively. These values corresponded to 49 ± 2, 47 ± 3, 45 ± 2, and 48 ± 3 cumulative release (%) based on the initial amount of copper loaded in the polymer solutions.

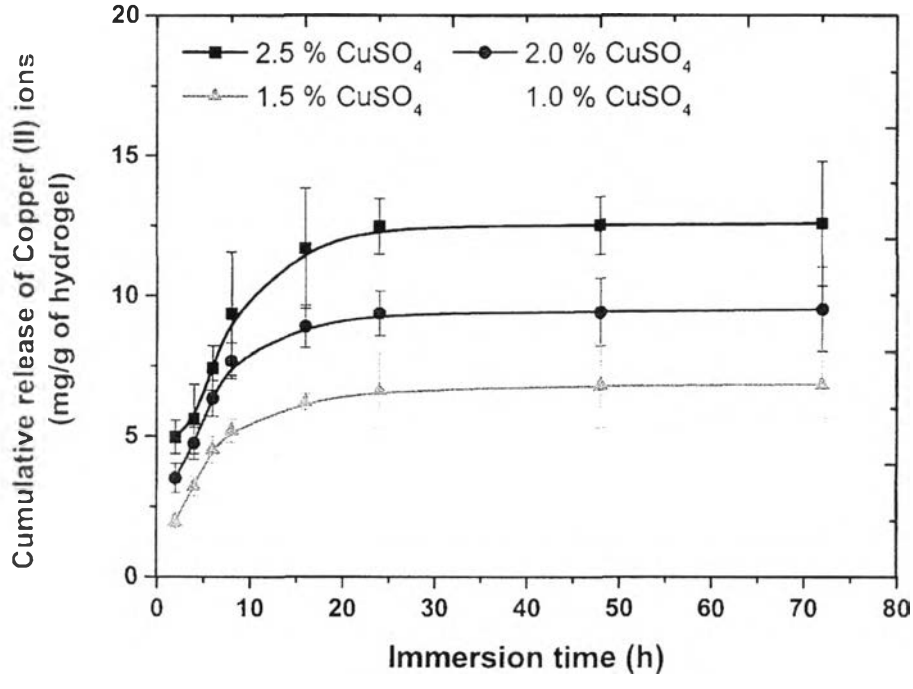


Figure 4.8 Cumulative release of Copper (II) ions (mg / g of hydrogel) from copper (II) crosslinked alginate hydrogel with 1.0, 1.5, 2.0 and 2.5% w/v $CuSO_4$ solutions in different submersion time in SBF, at 37 °C (n = 3).

The cumulative release of copper (II) ion from all hydrogels in a function of the submersion time was shown in Figure 4.8. The cumulative amount of the released copper (II) ions increased rapidly during the first 8 h of the submersion time. A slight but continuously released up to 24 h of the submersion time was observed. An initial fast release is possibly because the copper particles dispersing close to the surface of polymer hydrogels and absorbing near the surface could diffuse to the solution rapidly in initial time. The slow release rate might be described by the assumption that the ions were encapsulated in the inner core of the hydrogel network and a long distance to diffuse through the network leaded to a longer releasing time.

4.4.6 Antibacterial Activity Evaluation

Disc specimens of copper (II) cross-linked alginate films were tested via the disc diffusion method against *E. coli*, *S. aureus*, Methicillin resistant *S. aureus* (MRSA), *S. epidermidis* and *S. pyogenes*. The activity increased with increasing in copper (II) sulfate concentration which can be observed through the zone of inhibition. Figure 4.9 shows the activities of hydrogels using different concentrations of copper (II) sulfate solution. The activity was determined by the circular area around each sample which correlated with ability to release antibacterial substances to inhibit the bacterial growth. The hydrogels using 0.5 to 0.8 % w/v of copper (II) sulfate solution are active toward *S.aureus* but if the concentration increased from 1.0 to 2.5 % w/v, this led to improved activity toward *E.coli*, Methicillin resistant *S. aureus* (MRSA), *S. epidermidis* and *S. pyogenes*.

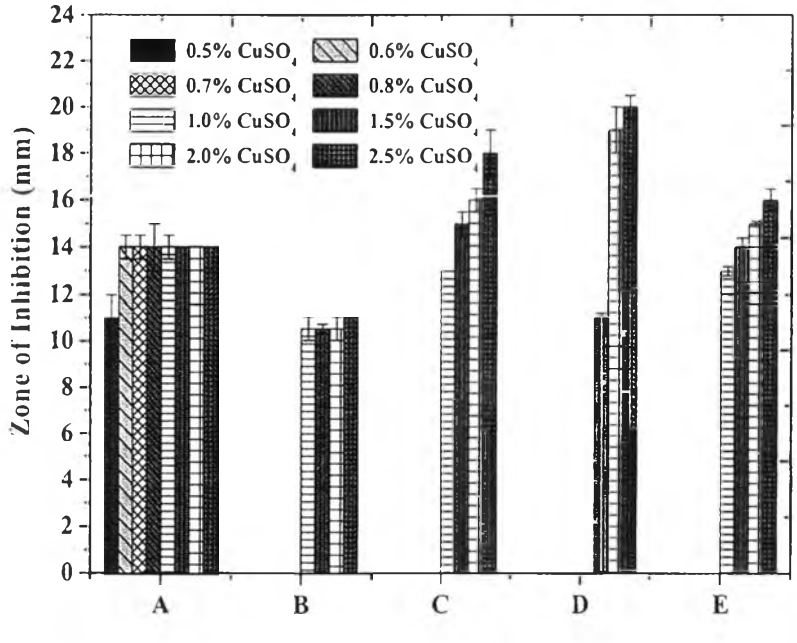


Figure 4.9 Representative the inhibition zone of antibacterial activity of copper(II) alginate immersed film using different concentration of $CuSO_4$ solution(0.5 – 2.5 %w/v) with against *S. aureus* ATCC (A), *E. coli* ATCC (B), *S. aureus* DMST (MRSA) (C), *S. epidermidis* ATCC (D) and *S. pyogenes* DMST (E),The diameter of sample is 9 mm (n = 3)

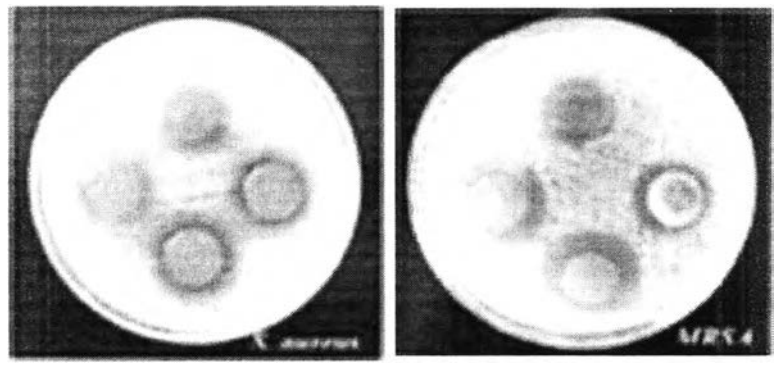


Figure 4.10 Selected images of Disc diffusion method for the assessment of the antibacterial activity of copper (II) alginate hydrogels with against *S. aureus* and MRSA

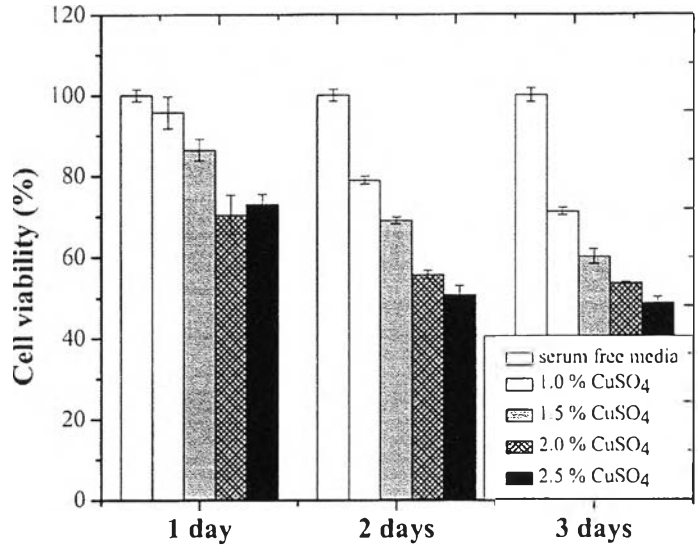
From the antibacterial results were corresponding to release characteristic of copper (II) ions from alginate. The higher amount copper (II) ion release showed the higher antibacterial activity. The released copper (II) ions from each copper (II) alginate specimen can be estimated to a 0.49-1.26 mg of copper (II) ions (9 mm in diameter, 1 mm in thickness, 102.02 ± 14.0 mg which were satisfied amount to be an inhibitor of the microbial activity at relatively low copper (II) concentration [30]. The ability to inhibit the growth of bacteria of the copper alginate hydrogel was higher with gram positive bacteria (i.e. *S. aureus,* Methicillin resistant *S. aureus* (MRSA), *S. epidermidis* and *S. pyogenes*) than gram negative bacteria (*E. coli*). This can be explained in term of the antisense targeting of the genes, as the gene encoding bacterial is highly similar in sequence among Staphyloccus species (85-100% gene similarity in S. aureus, Methicillin resistant *S. aureus* (MRSA), *S. epidermidis* and *S. pyogenes*) and different sequences in *E.coli* species [31]. Therefore, Cu^{2+} possibly preferred to be bound with the antisense target of gram positive more than gram negative bacteria. Although the cell wall of gram positive bacteria is thicker than that of gram negative bacteria, the small hydrated radius of Cu^{2+} facilitates penetration into the intracellular matrix of the bacteria as well as gram positive bacteria containing teichoic acid, which has negatively charges, leading to attraction with positively charge substances (e.g., Cu^{2+}).

Copper (II) ions can be classified as an 'intermediate metal', which may interact in variety of ways. The first possibility is that binding to phosphates can cause destabilizing of the DNA double helix [32]. Copper (II) can also form chelates with carboxylate and amino groups resulting in decreasing stability of the helix [33].

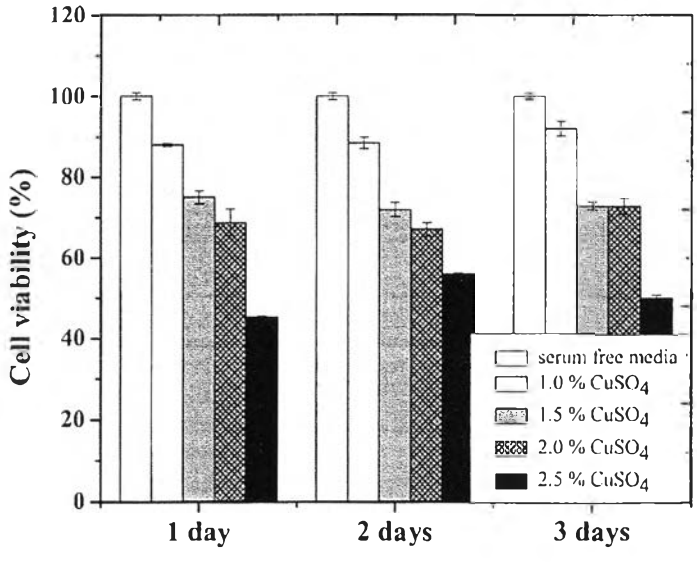
Figure 4.10 shows the inhibition zone of copper alginate hydrogels in the presence of *S.aureus* and MRSA, the clear zone showed larger areas in MRSA than that in *S. aureus*. MRSA was developed from *S. aureus* to be resistant to many kind of penicillin and is classified as a harmful human pathogenic. This result indicates that even though both bacteria are in the same species, there were some parts of different genes which can interact with active Cu^{2+}. The copper (II) alginate hydrogel this has the potential to be an active surface to inhibit various types of bacteria, including MRSA.

4.4.7 Indirect Cytotoxicity Evaluation

The copper (II) crosslinked alginate films were tested for their toxicity using L929 cells (mouse fibroblast) and NHDF cells (normal human dermal fibroblast). The variation of the extraction ratio (extraction media from copper (II) alginate films after having been cross-linked with 1.0%, 1.5%, 2.0% and 2.5% $CuSO_4$ (mg) per fresh culture medium (mL)) was performed to optimize concentration of copper (II) ions in the culture media . It was found that 15 mg/mL of extraction ratio provided relative cell viability of over 75 % at each extraction interval: 1, 2 and 3 days which can be concluded that 15 mg/mL of extraction ratio released the least toxic substance. Likewise, as shown in Figure 4.11a and Figure 4.11b the relative cell viability is acceptable when concentration of copper (II) sulfate was increased to 2.0 % w/v, but tended to reduce to 50 % cell viability when corresponding copper (II) sulfate concentration increased to 2.5 % w/v.



(a)



(b)

Figure 4.11 Viabilities of L929 cells (a) and normal human dermal fibroblasts (NHDF) (b) that were cultured with extraction media from copper (II) alginate after having been cross-linked with 1.0%, 1.5%, 2.0% and 2.5% $CuSO_4$ solution (n =3)

4.4.8 Blood Clot Formation Assay

a b c

Figure 12 Representative scanning electron microscopic images (a) Fibrin formation on the control glass, (b) Fibrin formation on the copper (II) cross-linked alginate film after cross-linked the surface by 1.0% $CuSO_4$ solution and (c) Fibrin formation on the copper (II) cross-linked alginate film after cross-linked the surface by 2.5% $CuSO_4$ solution.

Alginates have been used as hemostats in wound dressings. They act as metal ion donors as they contain mannuronic (M) or guluronic (G) groups with a high metal content [34]. There have been reports that alginate materials activate coagulation more than non-alginate materials. The extent of coagulation activation is affected differently by the alginate M or G group composition. Activation of the coagulation system can occur upon contact with a copper (II) crosslinked alginate hydrogel which was observed via scanning electron microscopy. The results show significant differences in the structures of fibrin formation between glass controls and hydrogel surfaces (Figure 4.12). Figure 4.12a shows a regular fibrin structure on the

glass control slides. A dense and compact structure of fibrin was obtained when contact to the surface of the hydrogel. When using a high concentration of divalent metal ions such as copper (II) ions, the distribution of fibrin formation and coagulation of the fibrin will improve as shown in Figure 4.12c, possibly leading to form the clot on the wound. It was demonstrated that alginate-containing copper (II) ions possibly has a potentiating effect on prothrombotic coagulation and platelet activation.

4.5 Conclusions

An alginate dressing, incorporating copper was successfully prepared using low pH and 2.0 % w/v of alginate which provided dimensional stability and antibacterial activity. The prepared hydrogels/films provided excellent liquid absorption properties. WVTR information showed the ability to allow water vapor to pass through the film and prevention of agglomeration of exudates that promoted accelerated wound healing. Copper (II) ions can be released from the incorporated film to be an active substance to disinfect bacteria. The antibacterial activity of the prepared films caused bacterial disinfection when using just 1.0 % w/v of copper (II) sulfate. Moreover, the blood clot assay was demonstrated that alginates containing copper (II) ions possibly had the potentiating effect on prothrombotic coagulation and platelet activation. From the results it can be concluded that this material can possibly be applied for use as an antibacterial wound dressing base on their low-toxicity for skin cells (fibroblasts) and excellent anti- bacterial activity.

4.6 ACKNOWLEDGEMENTS

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