สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER III

IN VITRO BIOCOMPATIBILITY OF HEXANOYL CHITOSAN FILMS

3.1 Abstract

The present contribution reports for the first time some in vitro biocompatibility evaluations of hexanoyl chitosan (H-chitosan) for possible utilization in biomedical applications. The evaluations comprised the cytotoxicity testing and the attachment, proliferation, and spreading of L929, mouse connective tissue, fibroblast-like cells that were cultured on the surface of H-chitosan film in comparison with those on chitosan film. These films were fabricated by solution-casting technique. Some thermal, physico-chemical, and morphological characteristics of H-chitosan film were also investigated. H-chitosan film exhibited two steps in the loss of its mass at 242 and 299 °C, respectively, while chitosan film exhibited only one at 297 °C. The water contact angle on the surface of H-chitosan film was 76°, while that on the surface of chitosan counterpart was 71°, a result indicating the more hydrophobicity of H-chitosan film in comparison with the chitosan counterpart. Indirect cytotoxicity evaluation of H-chitosan film using L929 revealed non-toxicity of the film to the cells. Lastly, both the attachment and the proliferation of L929 cells on H-chitosan film were inferior to those on tissue-culture polystyrene plate (TCPS). The attachment of the cells on H-chitosan film was better than that on the chitosan counterpart at a short seeding time (i.e., <5 h), while the proliferation of the cells on H-chitosan film was better than that on the chitosan counterpart after 2 and 3 days in culture.

(**Keywords**: Chitosan; Hexanoyl chitosan; Biocompatibility; Fibroblast)

3.2 Introduction

Chitin or poly(N-acetyl-D-glucosamine) is one of the most abundant polysaccharides commonly found in shells of various insects and crustaceans as well as cell walls of various fungi. Chitosan is a partially N-deacetylated derivative of

หอสมุดกลาง สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

chitin. In the food industry, chitosan in its blends with gelatin was developed as edible films (Arvanitoyannis, Nakayama, & Aiba, 1998), while the blends between chitosan and poly(vinyl alcohol) (PVA) was suggested to be suitable materials for food packaging (Arvanitoyannis, 1999). In biology, chitosan is structurally similar to glycosaminoglycans (GAGs), such as condroitin sulfate and hyaluronic acid, in the extracellular matrix (ECM) of connective tissues. As a result, chitosan has been heavily explored as a suitable functional material in biomedical applications (Ma, Wang, He, & Chen, 2001; Muzzarelli et al., 2001; Qi, Xu, Jiang, Hu, & Zou, 2004), due mainly to its biocompatibility, biodegradability, and non-toxicity. Despite the vast applicabilities of this polymer, utilization of chitosan is somewhat limited by its poor solubility in common solvents and its physical properties that are rigid and brittle, a direct result of the strong intra- and inter-molecular hydrogen bonding. Chitosan can be functionalized rather easily through its hydroxyl and/or amine groups (Jayakumar, Prabaharan, Reis, & Mano, 2005). Some chemical modifications such as acylation (Hirano, Ohe, & Ono, 1976; Zong, Kimura, Takahashi, & Yamane, 2000), alkylation (Yalpani & Hall, 1984), and phthaloylation (Kurita, Ikeda, Yoshida, Shimojoh, & Harata, 2002; Nishimura, Kohgo, Kurita, & Kuzuhara, 1991) reactions can be carried out. Most of the organically soluble derivatives of chitosan are used to formulate materials for biomedical applications, such as drug delivery (Tien, Lacroix, Szabo, & Mateescu, 2003) and wound dressing (Pielka et al., 2003). Recently, chitosan derivatives bearing cyclodextrin cavities were developed as novel adsorbent materials (Prabaharan & Mano, 2006). Among such derivatives, acylated chitosans are soluble in various common organic solvents, such as chloroform, benzene, pyridine, and tetrahydrofuran (THF) (Zong et al., 2000). N-acylated chitosan has been fabricated as membranes (Seo, Ohtake, Unishi, & Iijima, 1995), films (Xu, McCarthy, Gross,&Kaplan, 1996), and fibers (Hirano, Usutani, Yoshikawa, & Midorikawa, 1998). Among the various acylated chitosans, N-hexanoyl chitosan (H-chitosan) was found to exhibit the best blood compatibility (Lee, Ha, & Park, 1995). In addition, H-chitosan was found to be anti-thrombogenic and resistant to hydrolysis by lysozyme (Hirano & Noishiki, 1985). Even though H-chitosan is a very interesting derivative of chitosan to be used in biomedical applications, its biological properties with living cells have not yet been available in the open literature. In the

present contribution, H-chitosan film was prepared with solution-casting technique. The main purpose was to investigate the biological properties of H-chitosan by examining for its cytotoxicity and the attachment, proliferation and spreading of L929, mouse connective tissue, fibroblast-like cells that were cultured on H-chitosan film. Biological properties of solution-cast chitosan film were also determined for comparison purposes. In addition, some thermal, physico-chemical, and morphological characteristics of H-chitosan film were also investigated.

3.3 Experimental

3.3.1 Materials

Hexanoyl chitosan (H-chitosan) was synthesized in our laboratory via a heterogeneous acylation reaction of chitosan, prepared from shells of Penaeus merguiensis shrimps [Surapon Foods Public Co. Ltd. (Thailand)], with hexanoyl chloride in a mixture of anhydrous pyridine and chloroform based on the method described by Zong et al. (2000) (see Figure 3.1). The degree of deacetylation (DD) of the feed chitosan was determined based on an infrared spectroscopic method (Sabnis & Block, 1997) to be about 88%, while the viscosity-average molecular weight $\overline{M}_v$ was evaluated from the intrinsic viscosity $[\eta]$ based on the Mark–Houwink equation [i.e., $[\eta] = K\overline{M}_v^{\,a}$, where K and a assume the values 6.59×10^{-3} ml/g and 0.88 (Wang, Bo, Li, & Qin, 1991), respectively] to be about 5.76×10^5 g/mol. The intrinsic viscosity was measured in a mixture of 0.2 M acetic acid and 0.1 M sodium acetate at 30 °C. The as-prepared H-chitosan had a degree of substitution (DS) of the hexanoyl groups on chitosan molecules of about 3.0 (i.e., Calcd.: C 63.23, H 8.96, N 3.04; found: C 63.23, H 9.17, N 3.00). It should be noted that the DS for fully substituted H-chitosan is 4.0 (Zong et al., 2000).

3.3.2 Sample Preparation

H-chitosan film was prepared by casting 1% w/v H-chitosan solution in chloroform on a polytetrafluoroethylene plate and the plate was maintained at room temperature to evaporate as much chloroform from the film as possible. The as-prepared film was further ''dried'' in vacuo for another 24 h. Chitosan film, used

as the reference material, was prepared by casting 2% w/v chitosan solution in 1% acetic acid aqueous solution on a stainless steel plate and let dried at 40 °C for 24 h. The as-prepared chitosan film was further neutralized with 1 M NaOH solution, excessively washed with distilled water, and dried in vacuo at room temperature for 24 h. The obtained H-chitosan and chitosan films were cut into circular disc specimens, of which diameter was about 14 mm.

3.3.3 Physical Characterization

Thermogravimetric analysis (TGA) of H-chitosan and chitosan films was carried out by a Perkin-Elmer Pyris Diamond TG/DTA analyzer at a heating rate of 10 °C min^{-1} in nitrogen atmosphere over a temperature range of 50–400 °C. Samples of approximately 2–3 mg were used. The static contact angle of H-chitosan film was measured using a Tantec Cam-Plus Micro contact angle meter. Approximately 10 µl of deionized water was dropped onto the surface of the film. The result was compared with that measured on the surface of a chitosan film, which was used as the control. The morphology of H-chitosan and chitosan films was examined using a JEOL JSM 5410LV scanning electron microscope (SEM). Each sample was coated with a thin layer of gold using a JEOL JFC-1100E ion sputtering device prior to SEM observation.

3.3.4 Biological Characterization

Mouse connective tissue, fibroblast-like cells (L929: ECACC Cat. No. 85011425) was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, BIOCHROM AG), together with 100 U ml^{-1} penicillin (GIBCO) and 100 µg/ml streptomycin (GIBCO) at 37 °C in a wet atmosphere containing 5% CO_2. When the cells reached 80% confluence, they were trypsinized with 0.25% trypsin containing 1 mM EDTA (GIBCO) and counted by a hemacytometer (Hausser Scientific, USA) prior to further use.

3.3.4.1 Cytotoxicity Test

The cytotoxicity of H-chitosan and chitosan films was evaluated based on a procedure adapted from the ISO10993-5 standard test method. The films were prewashed with 70% ethanol for 30 min and washed 3 times with fresh culture medium prior to further incubation at 37 °C in fresh culture medium for

24 h. The extraction ratio was 10 mg ml^{-1}. After 24 h, the extraction medium was serially diluted to obtain extraction medium samples with concentrations of 1, 0.5, and 0.25 mg ml^{-1}. L929 cells were seeded in wells of a 96-well plate at a density of 10^3 cells per well. After incubation for 48 h, the culture medium was removed and replaced with the as-prepared extraction media and later incubated for another 24 h. The extraction media were then removed and the cells were re-incubated for 24 h in fresh culture medium. The number of living cells was finally quantified with 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazoluim bromide (MTT) assay.

3.3.4.2 Cell Attachment and Proliferation

Each of the as-prepared H-chitosan and chitosan films was placed in wells of a 24-well plate and sterilized by 70% ethanol for 5 min. It was washed 2 times with phosphate buffer saline (PBS; pH = 7.2) and then with fresh culture medium. Prior to cell seeding, the film scaffolds were pressed with a metal ring (diameter = 12 mm) and 500 µl of fresh culture medium was pipetted into each well. For cell attachment study, 5.0×10^4 of L929 cells were seeded into each well and allowed to attach to the film scaffolds for 1, 3, 5, and 24 h, after which time the number of the attached cells was determined by MTT assay. Each sample was rinsed with PBS 2 times to remove unattached cells prior to MTT assay. For proliferation study, 3.0×10^4 of the cells were seeded and allowed to attach for 20 h. The cells were later starved with serum-free medium (SFM) (DMEM containing 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin but without FBS) 2 times (viz. SFM were changed twice at 20 and 24 h after the attachment period). L929 proliferation was studied at day 1, 2, and 3. It should be noted that the attachment period after 20 h was referred to day 1. The number of cells was determined by MTT assay. The morphology of the cells attached to the film scaffolds was also observed using scanning electron microscopy (SEM).

3.3.4.3 Cell Morphology and Cell Spreading

To investigate the cell–material interaction, SEM images were taken after L929 cells were allowed to attach and/or proliferate for a certain time interval, stated above. Briefly, the cellular constructs were harvested, washed with PBS, and then fixed with 3% glutaraldehyde. After being rinsed with 0.2 M

phosphate buffer, the samples were dehydrated through a series of graded dimethylsulfoxide (DMSO) and later let dry in air. The samples were then coated with a thin layer of gold using the ion sputtering device and observed in SEM. Cell spreading was analyzed from selected SEM images that were taken after the cells were allowed to attach onto the film scaffolds for 3 and 5 h. The cells that adopted a flattened, polygonal shape, with filopodia- and lamellipodia- like extensions were regarded as spreading cells. On the contrary, the cells that resisted washing and remained tethered to the film surface were regarded as non-spreading cells. The number of cells exhibiting the spreading characteristics was assessed and later calculated as the percentage of the total number of cells that were adhered onto the films.

3.3.4.4 MTT Assay

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of the purple formazan crystals formed is proportional to the number of viable cells. First, each sample was incubated with 0.5 mg ml^{-1} MTT solution in DMEM without phenol red (250 µl per well). After incubation at 37 °C for 1 h, MTT solution was removed. A solution containing 900 µl of dimethylsulfoxide (DMSO) and 125 µl of glycine buffer (pH = 10) was added into each well of a 24-well plate to dissolve the formazan dye. Solutions were then transferred into a cuvette and a Thermospectronic Genesis10 UV–visible spectrophotometer was used to measure the absorbance at 570 nm. The intensity of the absorbance is proportional to the number of living cells.

3.4 Results and Discussion

3.4.1 Thermal Characteristics

Thermal stability of H-chitosan and chitosan films was evaluated by TGA. Figure 3.2 shows TGA results for H-chitosan and chitosan films. According to their corresponding derivative TGA curves (not shown), chitosan film was found to degrade at ca. 297 °C, while H-chitosan film exhibited two steps in the loss of its mass at ca. 242 and 299 °C, respectively, which are in general accordance with our

earlier reported values of 254 and 312 °C (Peesan, Supaphol, & Rujiravanit, 2005), respectively. The first step in the loss of mass should be a result of the loss of the hexanoyl side groups, while the second should correspond to the thermal degradation of the main chain. It should be emphasized that the as-prepared chitosan film contained an appreciable amount of absorbed moisture (i.e., about 12 wt.%), while the as-prepared H-chitosan film did not. The reason is due to the incorporation of the hydrophobic hexanoyl side chains into the structure of H-chitosan and the incorporation of such side groups renders H-chitosan its solubility in some common organic solvents (Peesan et al., 2005; Zong et al., 2000).

3.4.2 Physico-chemistry and Morphology of the Film Surface

The biological response of viable cells on a certain material has been known to be influenced by the physico-chemistry of the surface. A number of studies revealed that such surface properties are important factors determining the type of biological molecules that can be adsorbed on the surface (Boyan, Hummert, Dean, & Schwartz, 1996). Commonly, hydrophilicity or hydrophobicity (Ponsonnet et al., 2003, Ruardy, Schakenraad, van der Mei, & Busscher, 1995; Wang, Wu, & Chen, 2003) and topographical roughness of the surface (Ponsonnet et al., 2003; Zhao, Deng, Chen, & Chen, 2003) are important properties determining biocompatibility of the material. Bearing this in mind, the hydrophilicity or hydrophobicity and morphology of the surfaces of the as-prepared H-chitosan and chitosan films were investigated. The hydrophilicity or hydrophobicity of the surfaces was assessed by means of static contact angle measurement. It was found that the contact angle of a droplet of deionized water on the surface of H-chitosan film was ca. 76°, while that on the surface of chitosan film was ca. 71°. The result indicates that H-chitosan film was more hydrophobic or less hydrophilic than the chitosan counterpart, due obviously to the presence of the hydrophobic side chains. Figure 3.3 shows selected SEM images illustrating the topography of the film surfaces. Clearly, the surface of H-chitosan film appeared to be rougher than that of the chitosan counterpart, possibly a result of the greater evaporation rate of chloroform as the casting solvent for H-chitosan film than that of acetic acid aqueous solution as the casting solvent for chitosan (i.e., due to the difference in the boiling points of the solvents).

3.4.3 Cytotoxicity Test

The indirect evaluation of cytotoxicity of H-chitosan film was conducted using L929, mouse connective tissue, and fibroblast-like cells. The number of living cells (reported as the percentage of the controls) after the cells were cultured in extraction medium solutions for 24 h is shown in Figure 3.4. Apparently, the number of living cells was found to be about 85%, 88%, and 85% at the extraction medium concentrations of 0.25, 0.5, and 1 mg ml^{-1}, respectively. Recently, we reported an indirect cytotoxicity evaluation of microwave-treated carboxymethyl chitin (CM-chitin) and microwave-treated carboxymethyl chitosan (CM-chitosan) films using L929 and found that the number of living cells after the cells were cultured in the extraction media from microwave-treated CM-chitin films was about 89% on average, while that after the cells were cultured in the extraction media from microwave-treated CM-chitosan films was constant at about 95% up to the medium concentration of 0.5 mg ml^{-1} and decreased to about 80% at the medium concentration of 1 mg ml^{-1} (Wongpanit et al., 2005). In reference to our previous results, H-chitosan film appeared to be less toxic than microwave-treated CM-chitosan film and it may be concluded that H-chitosan film was non-toxic when the extraction medium concentration was less than or equal to 1 mg ml^{-1}.

3.4.4 Cell Attachment, Proliferation, and Spreading

Attachment of cells on the surface of a material is one of the prerequisites for evaluating its biological compatibility for possible utilization in biomedical applications. The number of cells attached on a substrate relates directly to the absorbance determined by MTT assay. In this work, H-chitosan and chitosan films were made by solution-casting technique. Tissue-culture polystyrene plate (TCPS) was used as a positive control. Figure 3.5 shows the absorbance intensity after L929 cells were seeded on the film scaffolds and TCPS for 1, 3, 5, and 24 h. The result shows that the attachment of L929 cells on TCPS was much better than that on both types of the film scaffolds. At short seeding times (i.e., 1 and 3 h), the attachment of L929 cells on H-chitosan film was better than that on the chitosan counterpart, while, at longer seeding times (i.e., 5 and 24 h), the trend was reversed. On any type of surfaces, the attachment of the cells was found to increase monotonously with increasing the seeding time. Proliferation of L929 cells after they

were cultured on the film scaffolds was studied at day 1, 2, and 3. The cells were allowed to attach on the scaffold and TCPS surfaces for 20 h (viz. the number of cells after 20 h in culture was taken as day 1). Figure 3.6 shows the absorbance intensity obtained after 1, 2, and 3 day(s) in culture. Evidently, the proliferation of L929 cells on TCPS was better than that on both types of the film scaffolds. After 1 day in culture, the proliferation of the cells on H-chitosan film was comparable to that on the chitosan film. On the contrary, after 2 and 3 days in culture, the proliferation of the cells on H-chitosan film was slightly better than that on the chitosan film. With increasing the time in culture, the proliferation of L929 cells on any type of surfaces increased assumingly linearly. Both the attachment and the proliferation results suggest the applicability of H-chitosan as a wound dressing or a tissue scaffolding biomaterial. The morphology of L929 cells after they were cultured on H-chitosan and chitosan films for a period of 1, 2, and 3 day(s) was examined by SEM. Figure 3.7 shows selected SEM images of L929 cells that were cultured on the film scaffolds. These SEM images reveal the morphology of L929 cells when they were in contact with the materials. Clearly, for both types of the film scaffolds, the number of cells per unit area was found to increase with increasing culture time. For a given time in culture, cells were found to spread rather evenly over the film surfaces. A close-up SEM image (see Figure 3.7d) reveals that the cells adhered to H-chitosan surface by the formation of filopodia, while the cells bridged to one another probably with the proteins they synthesized. The evidence of cell-to-cell interaction is indicative of non-cytotoxic response of the cells to the substrate. Furthermore, the cells exhibited different cell shapes on both types of the film scaffolds. The different cell morphologies could be due to the different stages of the cells while being cultured on the film surfaces. For cell spreading, the SEM images that were taken for the cell attachment assay were analyzed. The cells that adopted a flattened, polygonal shape, with filopodia- and lamellipodia-like extensions were regarded as spreading cells. In contrast, the cells that resisted washing and remained tethered to the film surfaces were regarded as non-spreading cells. The number of cells with the spreading morphology was counted and calculated as the percentage of the total number of cells that were adhered on the films. Figure 3.8 shows the results of such analyses. After 3 h in culture, about 43% of the cells attached on H-chitosan

film began to spread, while cells attached on the chitosan counterpart were still in round shape. After 5 h in culture, the amount of the spreading cells increased to about 60% for both types of the film scaffolds.

3.5 Conclusions

The biocompatibility of a chitosan derivative, hexanoyl chitosan (H-chitosan) was assessed by evaluating its cytotoxicity and the attachment, proliferation, and spreading of L929, mouse connective tissue, fibroblast-like cells that were cultured on the surface of H-chitosan film. The results were compared with those observed on chitosan film. Both H-chitosan and chitosan films were fabricated by solution-casting technique. Some thermal, physico-chemical, and morphological characteristics of H-chitosan film were also investigated. H-chitosan film exhibited two steps in the loss of its mass, while chitosan film exhibited only one. The addition of the hydrophobic hexanoyl side chains rendered the hydrophobicity to the surface of H-chitosan film in comparison with that of the chitosan film, as suggested by the contact angles of a droplet of deionized water on the film surfaces. The surface of H-chitosan film also appeared to be rougher than that of the chitosan film, due possibly to the difference in the volatility of the casting solvents used. The indirect cytotoxicity test revealed that H-chitosan film was nontoxic to L929 cells. The attachment of L929 cells on H-chitosan film was inferior to that on tissue-culture polystyrene plate (TCPS), but was superior to that on the chitosan counterpart at a short seeding time (i.e., <5 h). On the other hand, the proliferation of L929 cells on H-chitosan film was inferior to that on TCPS, but was slightly better than that on the chitosan counterpart after 2 and 3 days in culture.

3.6 Acknowledgements

The authors acknowledge partial support received from (1) the National Research Council of Thailand (through a research program ''Multidisciplinary Research Series in Tissue Engineering of Skin''), (2) the Petroleum and Petrochemical Technology Consortium [through a Thai governmental loan from the

Asian Development Bank (ADB)], and (3) the Petroleum and Petrochemical College (PPC), Chulalongkorn University. A special thank is due to Waradda Mattanavee for her kind assistance with the contact angle measurement.

3.7 References

Arvanitoyannis, I. S. (1999). Journal of Macromolecular Science – Review in Macromolecular Chemistry and Physics, C39, 205.

Arvanitoyannis, I. S., Nakayama, A., & Aiba, S. (1998). Carbohydrate Polymers, 37, 371.

Boyan, B. D., Hummert, T. W., Dean, D. D., & Schwartz, Z. (1996). Biomaterials, 17, 137.

Hirano, S., & Noishiki, Y. (1985). Journal of Biomedical Materials Research, 19, 413.

Hirano, S., Ohe, Y., & Ono, H. (1976). Carbohydrate Research, 47, 315.

Hirano, S., Usutani, A., Yoshikawa, M., & Midorikawa, T. (1998). Carbohydrate Polymers, 37, 311.

Jayakumar, R., Prabaharan, M., Reis, R. L., & Mano, J. F. (2005). Carbohydrate Polymers, 62, 142.

Kurita, K., Ikeda, H., Yoshida, Y., Shimojoh, M., & Harata, M. (2002). Biomacromolecules, 3, 1.

Lee, K. Y., Ha, W. S., & Park, W. H. (1995). Biomaterials, 16, 1211.

Ma, J., Wang, H., He, B., & Chen, J. (2001). Biomaterials, 22, 331.

Muzzarelli, R. A. A., Biagini, G., DeBenedittis, A., Mengucci, P., Majni, G., & Tosi, G. (2001). Carbohydrate Polymers, 45, 35.

Nishimura, S. I., Kohgo, O., Kurita, K., & Kuzuhara, H. (1991). Macromolecules, 24, 4745.

Peesan, M., Supaphol, P., & Rujiravanit, R. (2005). Carbohydrate Polymers, 60, 343.

Pielka, S., Paluch, D., Kus, J. S., Zywicka, B., Solski, L., Szosland, L., et al. (2003). Fibres & Textiles in Eastern Europe, 11, 79.

Ponsonnet, L., Reybier, K., Jaffrezic, N., Comte, V., Lagneau, C., & Lissac, M. et al. (2003). Materials Science and Engineering, C23, 551.

Prabaharan, M., & Mano, J. K. (2006). Carbohydrate Polymers, 63, 153.

Qi, L., Xu, Z., Jiang, X., Hu, C., & Zou, X. (2004). Carbohydrate Research, 339, 2693.

Ruardy, T. G., Schakenraad, J. M., van der Mei, H. C., & Busscher, H. J. (1995). Journal of Biomedical Material Research, 29, 1415.

Sabnis, S., & Block, L. H. (1997). Polymer Bulletin, 39, 67.

Seo, Y., Ohtake, H., Unishi, T., & Iijima, T. (1995). Journal of Applied Polymer Science, 58, 633.

Tien, C. L., Lacroix, M., Szabo, P. I., & Mateescu, M. A. (2003). Journal of Controlled Release, 93, 1.

Wang, W., Bo, S., Li, S., & Qin, W. (1991). International Journal of Biological Macromolecules, 13, 281.

Wang, Y. W., Wu, Q., & Chen, G. Q. (2003). Biomaterials, 24, 4621.

Wongpanit, P., Sanchavanakit, N., Pavasant, P., Supaphol, P., Tokura, S., & Rujiravanit, R. (2005). Macromolecular Bioscience, 5, 1001.

Xu, J., McCarthy, S. P., Gross, R. A., & Kaplan, D. L. (1996). Macromolecules, 29, 3436.

Yalpani, M., & Hall, L. D. (1984). Macromolecules, 17, 272.

Zhao, K., Deng, Y., Chen, C. J., & Chen, G. Q. (2003). Biomaterials, 24, 1041.

Zong, Z., Kimura, Y., Takahashi, M., & Yamane, H. (2000). Polymer, 41, 899.

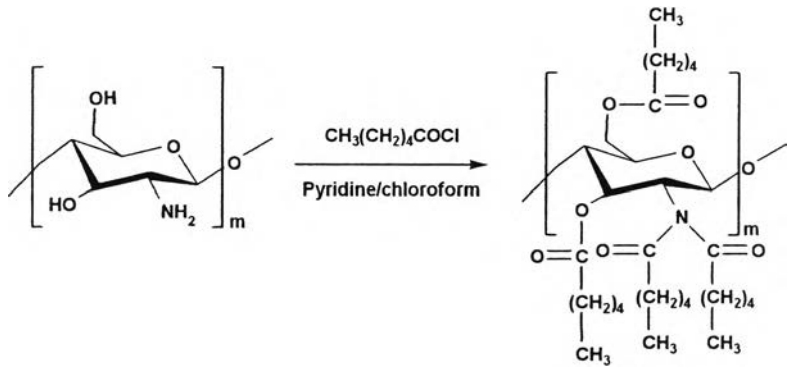


Figure 3.1 A synthesis route for perfect H- chitosan.

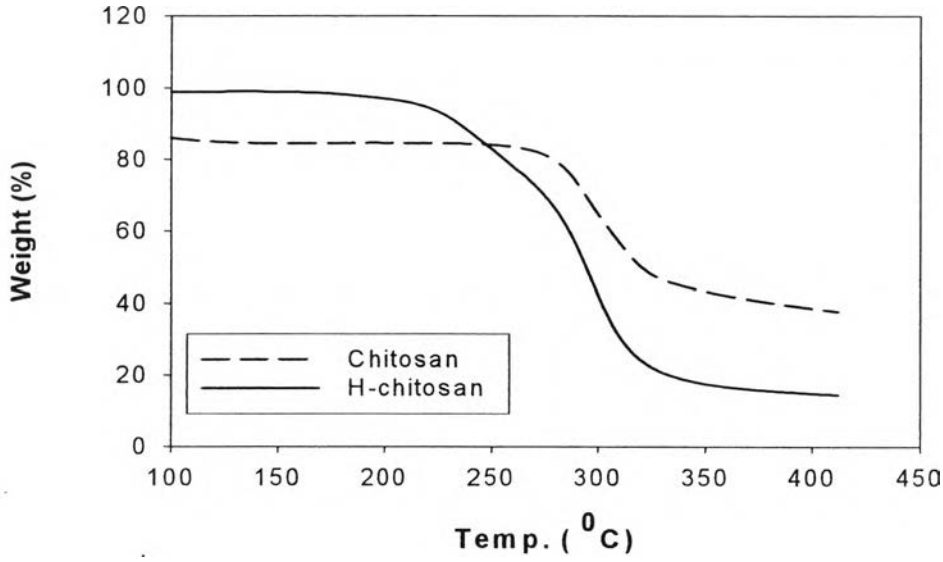
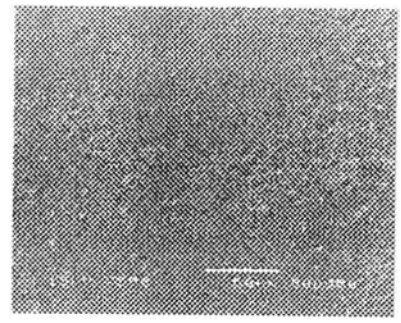
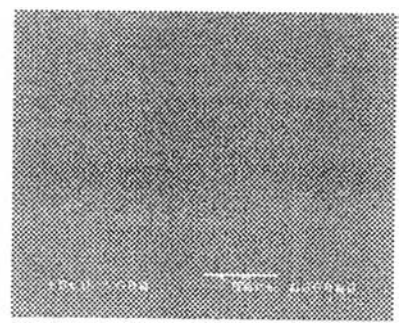


Figure 3.2 TGA curves for chitosan and H-chitosan films. The heating rate was $10°Cmin^{-1}$.



(a)



(b)

Figure 3.3 SEM micrographs of films made of (a) H-chitosan. Mag = 500x; (b) chitosan. Mag = 500x.

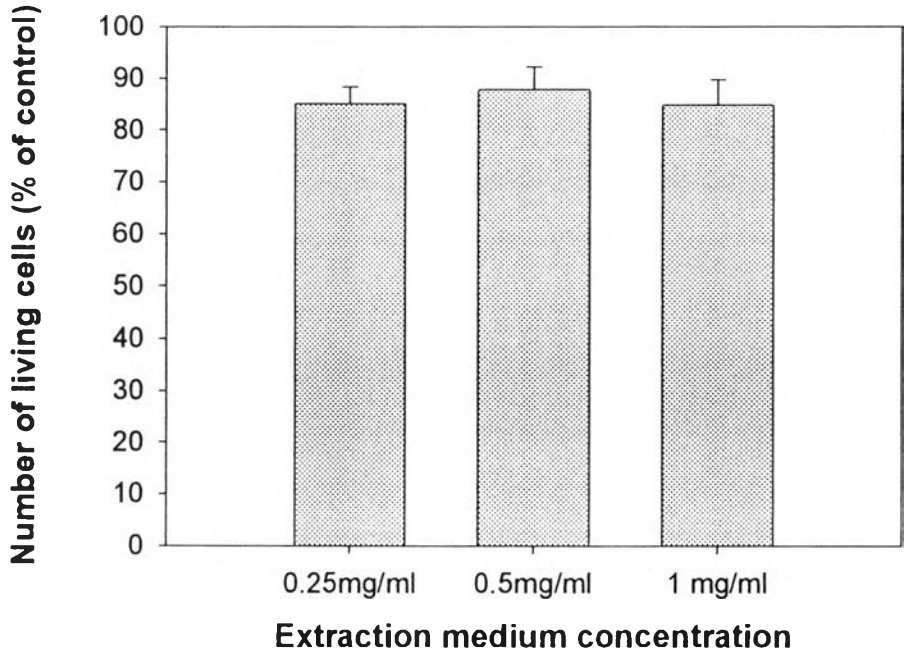


Figure 3.4 Number of living cells after cultured with extraction medium for a period of 24 hours for H-chitosan film.

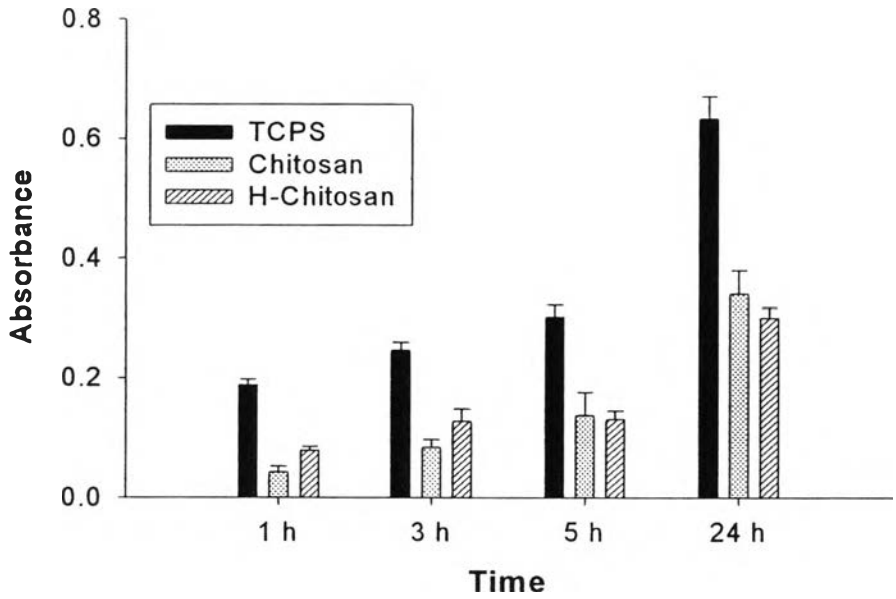


Figure 3.5 Attachment of L 929 cells on TCPS, chitosan and H-chitosan films after cell culture for 1, 3, 5, and 24 hours.

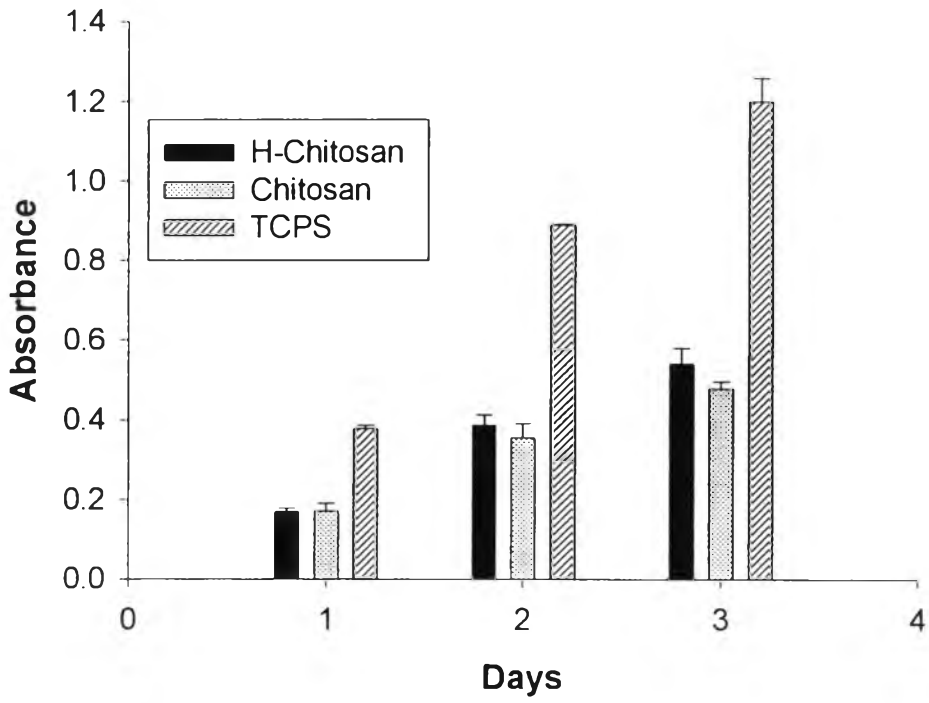


Figure 3.6 L929 cells proliferation on TCPS, chitosan film, and H-chitosan film.

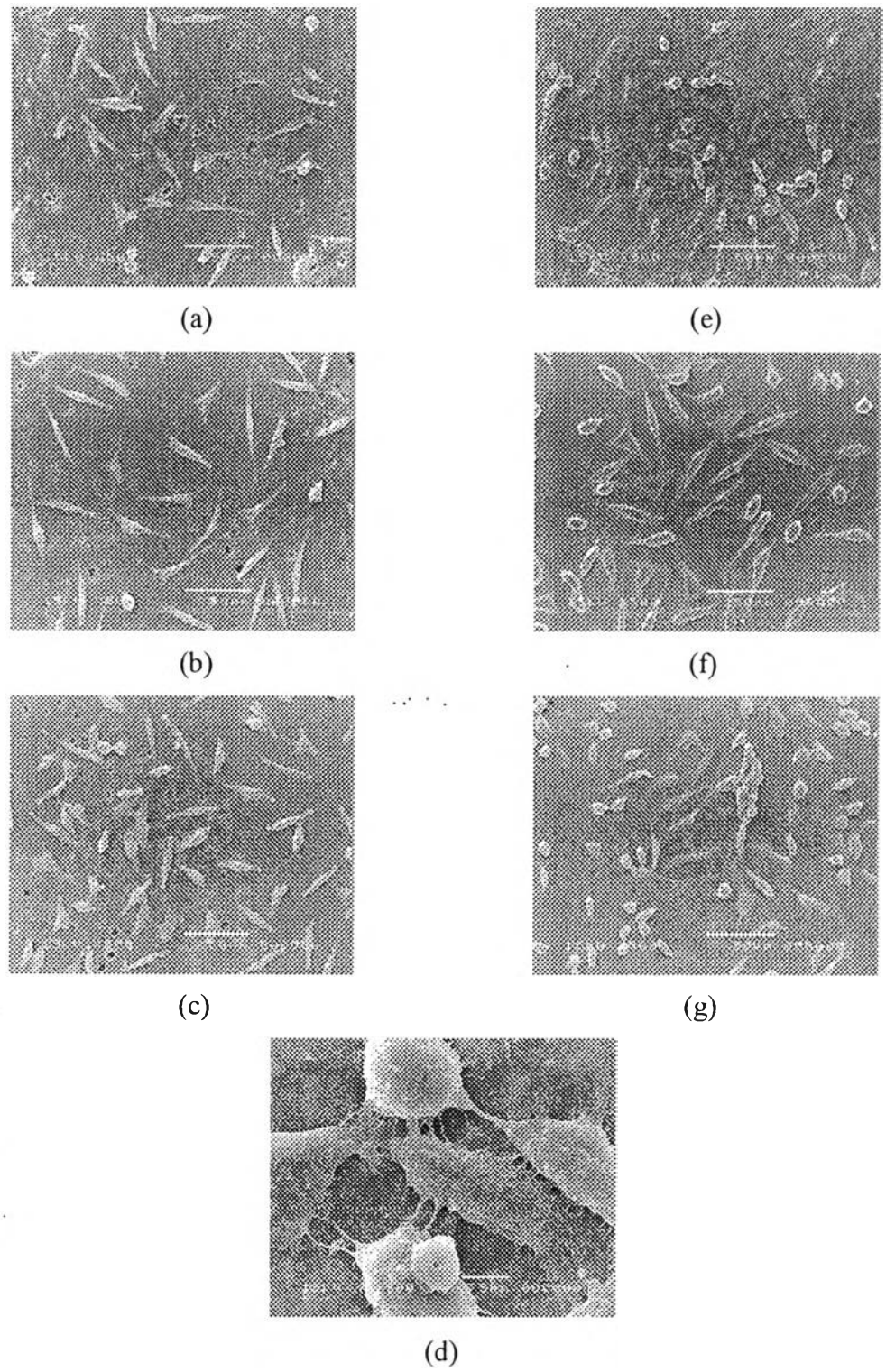


Figure 3.7 SEM micrographs of L929 cells proliferated on H-chitosan film (a) at day 1, Mag = 500x; (b) at day 2, Mag = 500x; (c) at day 3, Mag = 500x; (d) at day 3, Mag = 3,500x; and on chitosan film (e) at day 1, Mag = 500x; (f) at day 2, Mag = 500x; (g) at day 3, Mag = 500x.

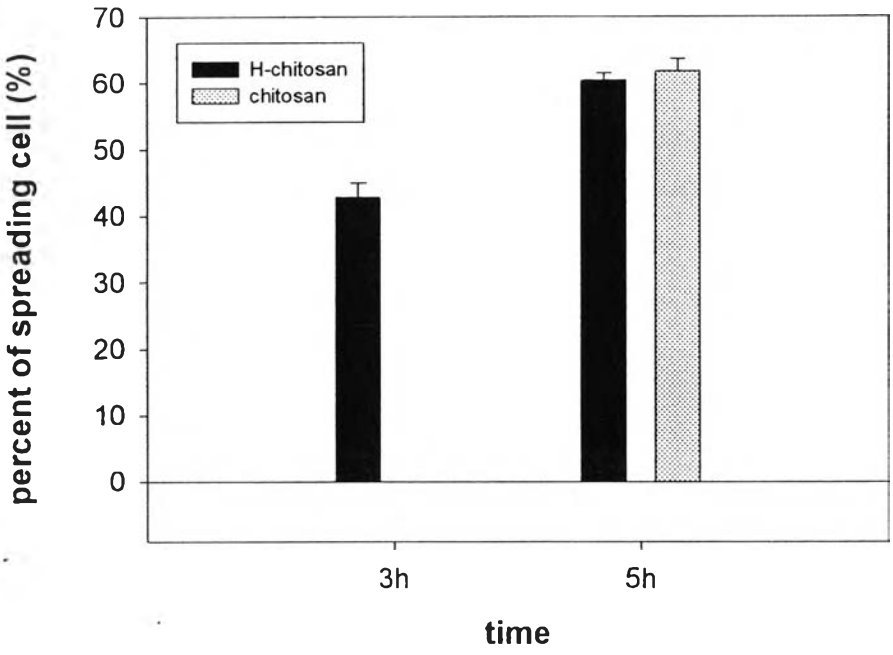


Figure 3.8 Cell spreading of L929 cells on H-chitosan and chitosan films.