CHAPTER IV
RESULTS AND DISCUSSION

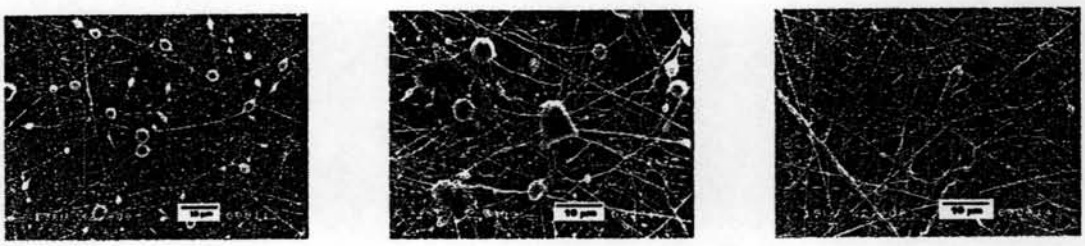
4.1 Preparation of the Electrospun Chitosan/THC Fiber Mats

In recent years, the electrospinning process has attracted a great deal of attention due to its ability to produce ultrafine fibres with diameters in the range of nanometers to sub-micrometers and high surface area to volume or mass ratio (Lu et al, 2001). Most of the previous research involving the electrospinning of chitosan has focused on using acetic acid solution or creating matrix polymer fibers consisting of chitosan and another polymer, such as poly (ethylene oxide) (PEO) Min et al, 2004). The electrospinning solutions were prepared at various conditions to find the optimization of the electrospinning condition. Chitosan is able to dissolve in acid solvent but difficult to electrospun due to its poor solubility and high viscosity of its aqueous solution. At low polymer concentrations, the solutions do not contain sufficient material to produce stable solid fibers. With increasing polymer concentration, the number of direct interchain associations of chitosan molecules in the solution increases rapidly causing a highly viscous gel, and rendering the solution unspinnable. Ohkawa K et al. (2004) found that trifluoroacetic acid or trifluoroacetic acid/dichloromethane co-solvent has ability to electrospun chitosan into fibers. There are two possible reasons why the electrospinning of chitosan is successful when using TFA: (I) TFA forms salts with the amino groups of chitosan [Hasegawaa et al, 2004] and this salt formation destroys the rigid interaction between the chitosan molecules, making them ready to be electrospun; (II) the high volatility of TFA is advantageous for the rapid solidification of the jet of the chitosan-TFA solution. But the electrospun fibers achieved from the use of pure TFA as a solvent, were interconnected fiber networks which had small beads. One possible approach to this optimization was to mix a volatile organic solvent with TFA. Dichloromethane (DCM) was chosen as a mixed solvent. When the mixing ratio was TFA: DCM = 70:30, the network morphology became more homogenous due to it might balanced the ratio between TFA and DCM to form enough the chain entanglement for resisting the force acting on the polymer jet. The small beads and interconnected fibers mostly

cannot be seen, indicating that a homogenous fiber network could be prepared under these conditions of solvent (Ohkawa et al, 2004). In this work, the solution parameters (i.e. polymer and THC concentration) that affect on the fiber morphology were studied by using TFA and DCM as solvents. (The mixing ratio of solvent between TFA and DCM was 70:30)

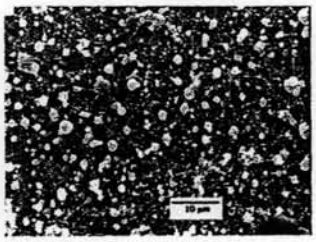
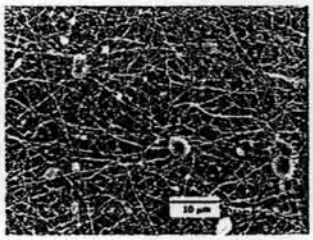
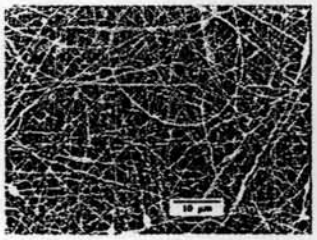
4.1.1 Effect of Polymer and THC on Morphological Appearance of the As-spun Fibers

The effect of polymer and THC concentration on morphological appearance of the as-spun chitosan fibers was investigated at a given apply voltage (25 kV) and collecting distance (20cm). The homogeneous chitosan fibers (average diameter, 130±10 nm) could be electrospun at 7wt.% chitosan concentration in TFA:DCM (70:30)(Sangsanoh et al,2006) which is a major effect to morphological appearance of the as-spun fibers. THC affects to the system of solution and influences to the chitosan concentration. In this work, the conditions of electrospinning solution were prepared by using the chitosan concentration in the range of 6.5 to 7.5wt.% and THC concentration was varied from 10 to 20wt.% .(compared with the weight of chitosan) in 70:30 (v/v) trifluoroacetic acetic (TFA): dichloromethane (DCM). Figure 4.1 shows the SEM images of each condition for fabricating the electrospun chitosan/THC fibers.



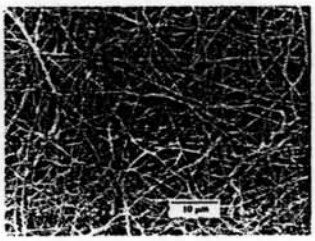
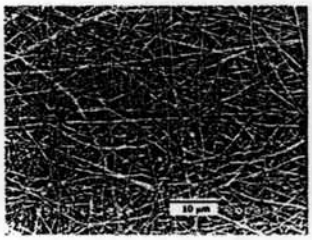
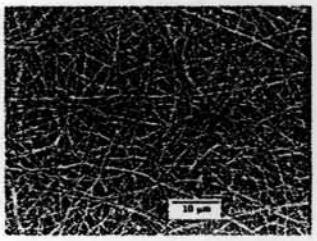
1a) 1b) 1c)

(SEM images of chitosan fibers at 6.5 wt.%)

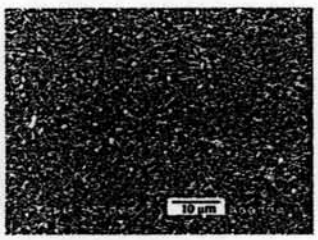
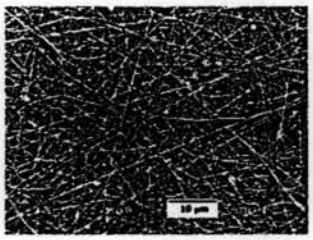
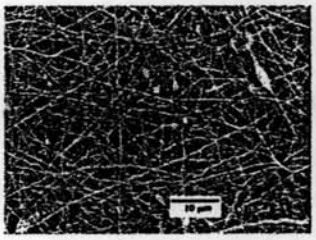
2a) 2b) 2c)

(SEM images of chitosan fibers at 6.7 wt.%)

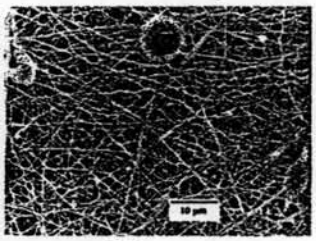
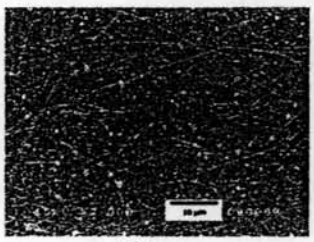
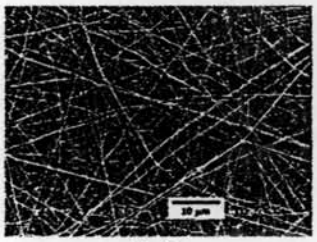
3a) 3b) 3c)

(SEM images of chitosan fibers at 6.9 wt.%)

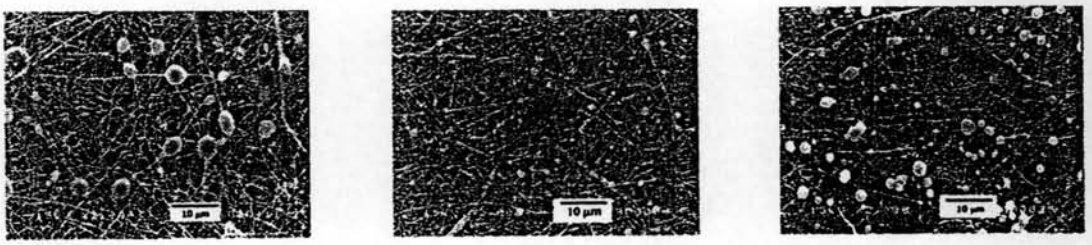
4a) 4b) 4c)

(SEM images of chitosan fibers at 7.1 wt.%)

5a) 5b) 5c)

(SEM images of chitosan fibers at 7.3 wt.%)



6a) 6b) 6c)

(SEM images of chitosan fibers at 7.5 wt.%)

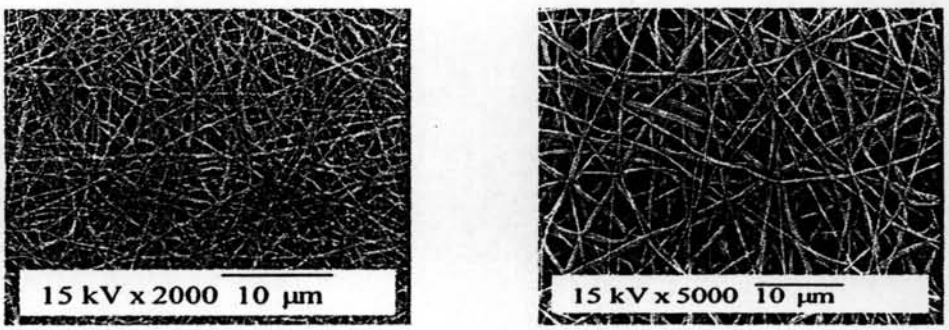
Figure 4.1 Morphological images of the electrospun chitosan/THC fiber mats at various condition of electrospinning solution: 10 wt.% (magnification, 2000x, a), 15 wt.% (magnification, 2000x, b) and 20 wt.% (magnification, 2000x, c).

When the chitosan concentration was lower than 6.9wt.%, distinct bead like structures were observed on the morphological appearance of fibers. Solution with low concentrations did not have chain entanglement to withstand the force acting on polymer jet. Once the charged jet was broken up, surface tension resulted in the formation of discrete droplets. For SEM images of 6.9wt.% chitosan concentration, the beaded-fibers were predominantly decrease because the chain entanglement high enough to prevent the breaking of charge jet. When the chitosan concentration further increased from 6.9wt.%, the bead fraction could be seen due to the solution being too viscous that it resists any deformation during the whipping process, the lack in continuous flow through the needle and breaks, instead of undergoing drawing. When compared the SEM micrographs of 6.9wt.% chitosan concentration at each THC concentration, the electrospun chitosan/THC fibers at 6.9wt.% chitosan and 20wt.% THC concentrations could be observed the beaded free-fibers. Base on the good quality of the obtained as-spun fibers, 6.9wt.% chitosan and 20wt.% THC concentration in TFA:DCM 70:30(v/v) was used as the optimum solution for fabricating the electrospun chitosan/THC fibers under an electrical potential of 25 kV applied over a collection distance of 20cm. The morphological appearance of the electrospun chitosan/THC fibers had an average fiber diameter 300 ± 10 nm, while the pure chitosan fibers had the average fiber diameter 130 ± 10 nm (Both fibers were fabricated at same experimental parameters), indicating that THC

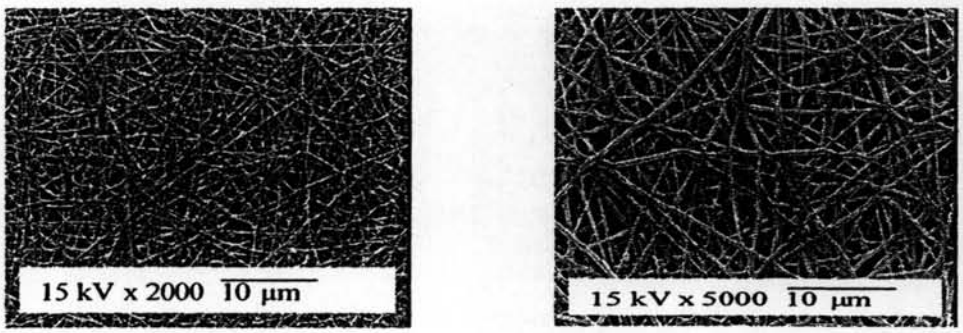
might be incorporated in the resulting fibers from electrospinning solution of 6.9wt.% chitosan and 20wt.% THC concentration in TFA: DCM 70:30(v/v). After consecutive spinning for 36 h, the thickness of the obtained mats was 54 ± 5 μm.

4.1.2 Effect of Crosslinking and Neutralization on Morphological Appearance of the As-spun Fibers

After fabricating the electrospun chitosan/THC fiber mats, they were crosslinked with GTA vapor (for 1 h) in order to prevent dissolution of mats in aqueous buffer solution. The SEM images (Figure 4.2b) shows the crosslinked electrospun chitosan/THC fiber mats. And then the crosslinked electrospun chitosan/THC fiber mats were further neutralized with saturated Na_2CO_3 to prevent fusion of the fibers. When compared the SEM images between the electrospun chitosan/THC fiber mats and the post-neutralized and crosslinked electrospun chitosan/THC fiber mats, the SEM images (Figure 4.2c) shows that the almost fibers unchanged significantly from the original fibers (Figure 4.2a).



a)



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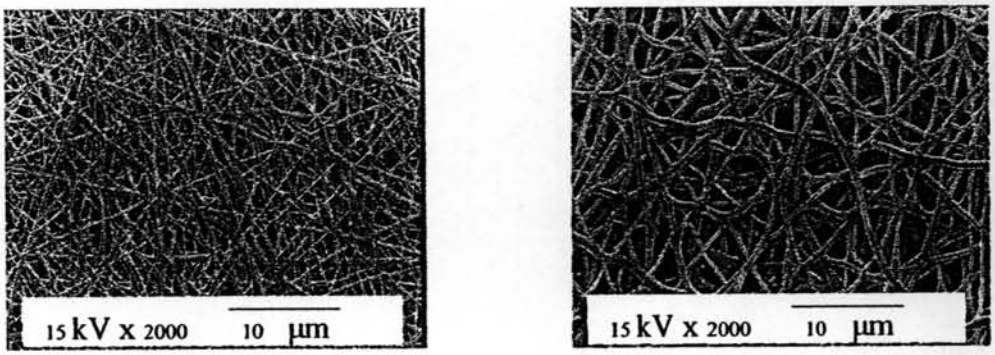


c)

Figure 4.2 The SEM images a) the electrospun chitosan/THC fiber mats b) the crosslinked electrospun chitosan/THC fiber mats c) the post-neutralized and crosslinked electrospun chitosan/THC fiber mats with magnification, 2000x and 5000x, respectively).

Figure 4.3 shows selected SEM images of the crosslinked electrospun chitosan/THC fiber mats that were neutralized by immersing the mats in 5M Na CO_3 (a) and in 5M NaOH (b) aqueous solution for 3 h at ambient condition. When the crosslinked electrospun Chitosan/THC was treated with the NaOH aqueous solution, its initial fibrous structure (see Figure 4.3b) was lost. From the literature (Sangsanoh et al, 2006), the chitosan nanofibers would became either partially or completely dissolved after neutralization with NaOH solution because the salt residues dissolved($-NH_3^+CF_3COO^-$), leaving NH_3^+ groups on the chitosan chains. Some of these groups would be deprotonized with –OH ions to leave $-NH_2$ groups, while others would became hydrated. Figure 4.3c shows a selected SEM image of the electrospun chitosan/THC fiber mats that was made by submerging the mats in 5M $NaCO_3$ aqueous solution. Evidently, the fibrous structure of the mats was intact after treatment since the leaving NH_3^+ groups on the chitosan chains would be rapidly deprotonized with CO_3^{2-} ions to become HCO_3^- . Moreover, the detached proton could further react with HCO_3^- ions to finally obtained carbonic acid, H_2CO_3. Due to the excess amount of $NaCO_3$ (s) in aqueous solution, the salt residues could be neutralized until no residues are available.

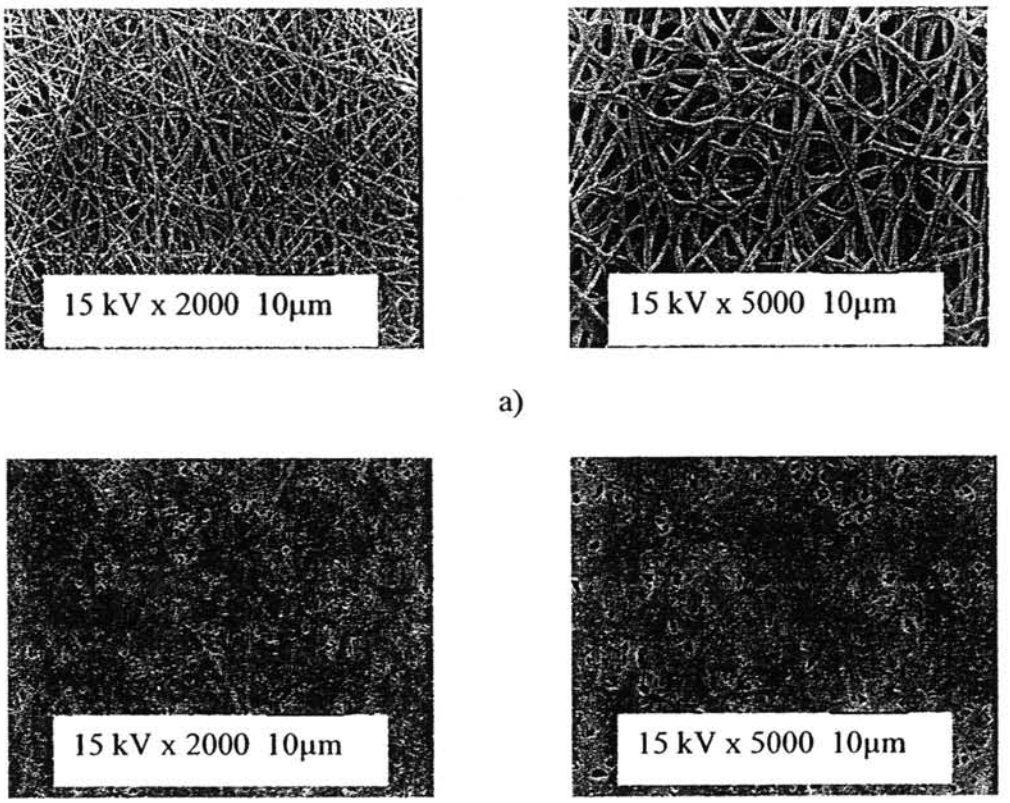
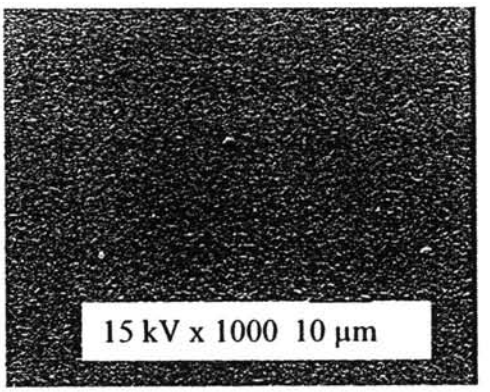
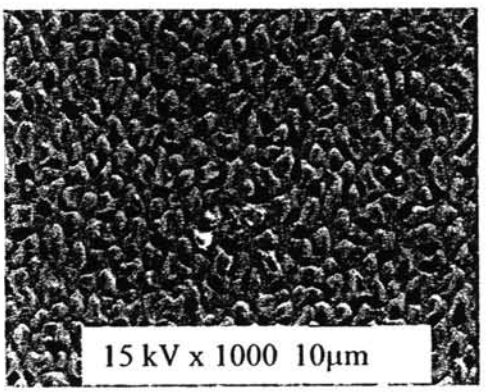


Figure 4.3 Selected SEM images of the crosslinked electrospun chitosan/THC fiber mats after neutralization with 5M $NaCO_3$ (a) and 5M NaOH (b) aqueous solution for 3 h at ambient condition.

Both the neat and THC-containing chitosan solutions were also fabricated into films by solution-casting technique. The surface morphology of the obtained films is shown in Figure 4.4. Evidently, the upper surface of the chitosan films was rough while the surfaces of the chitosan/THC films were significantly different, there was a bumpy structure on the surface of the films (see Figure 4.4b) obtained from the solution containing 20wt.% THC. It was believed to be a result of phase separation during the drying of films.



a)



b)

Figure 4.4 Selected SEM of as-cast chitosan films from (a) neat 4%. w/v chitosan solution in 70:30 v/v TFA: DCM and (b) the chitosan solution that contained 20wt.% THC (base on the weight of chitosan).

4.2 Physical Characterization of the Electrospun Chitosan/THC Fiber Mats

4.2.1 The Degree of Swelling of the Post-neutralized and Crosslinked Electrospun Chitosan/THC Fiber Mats and Chitosan/THC Films

The electrospun chitosan/THC fibers dissolved completely in acetate buffer when without the crosslinking with GTA vapor (50% aqueous glutaraldehyde) at 37 °C, So this research work, the electrospun chitosan/THC fiber mats were crosslinked and further neutralized to prevent dissolution and fusion of the electrospun chitosan/THC in acetate buffer, respectively. The effect of crosslinking

and neutralization on the swelling behavior of electrospun chitosan/THC fibers was also investigated (see Figure 4.5). For electrospun chitosan/THC fiber mats, the degree of swelling increased gradually with initial submersion period (20 min) and increased rapidly during 40 to 60 min. After submersion time 60 min, the swelling started to level off with the highest degree of swelling being about 206.94%. On the other hand, the swelling of films (thickness = 42 ± 3) increased rapidly during the first 60 min and then became unchanged along submersion time with the highest degree of swelling being about 168.80%.

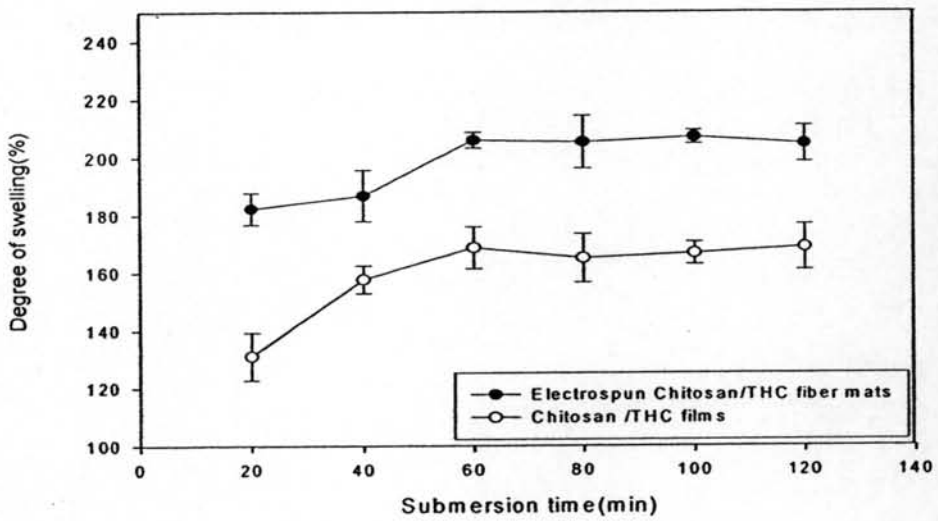


Figure 4.5 Degree of swelling in acetate buffer as a function of submersion time for the electrospun chitosan/THC fiber mats (after crosslinking and neutralization with saturated Na_2CO_3 aqueous solution for 30 min) and solution-cast chitosan films (after crosslinking and neutralization with 5M NaOH aqueous solution for 3 h).

4.2.2 The Weight Loss of the Post-neutralized and Crosslinked Electrospun Chitosan/THC Fibers and Chitosan/THC Films

The effect of crosslinking and neutralization on the weight loss of electrospun fibers and films was investigated.(see Figures 4.6) After the treatment, the loss in the weight of the fiber mat samples which submerged in acetate buffer increased rapidly during the first 2 weeks and increased gradually until it level off after 7 weeks. When compared with the weight loss of the solution-cast film samples,

it increased during the first 5 weeks in submersion, after 5 weeks no significant change was observed. For investigation, the electrospun fibers exhibited a greater weight loss than that of films due to the highly porous nature of the electrospun fiber. Figure 4.7 shows a selected SEM image of the electrospun fibers after submersion in acetate buffer for 9 weeks.

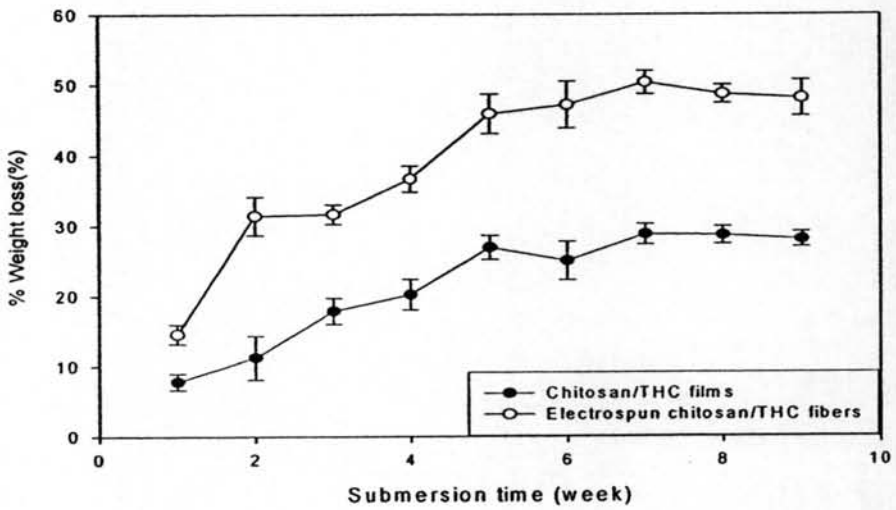


Figure 4.6 Weight loss in acetate buffer as a function of submersion time for the electrospun chitosan/THC fiber mats (after crosslinking and neutralization with saturated Na_2CO_3 aqueous solution for 30 min) and solution-cast chitosan films (after crosslinking and neutralization with 5M NaOH aqueous solution for 3 h).

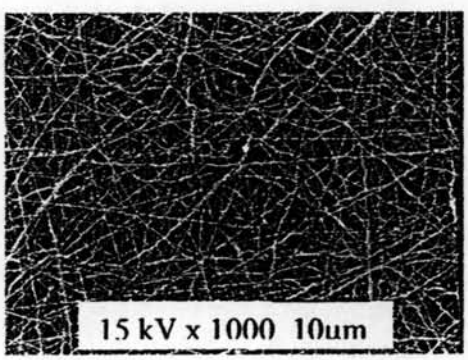


Figure 4.7 A selected SEM image of the electrospun chitosan/THC fiber mats after submersion in acetate aqueous solution for 9 weeks.

4.3 Release of Model Drug from the Post-neutralized and Crosslinked Electrospun Chitosan/THC Fiber Mats and Chitosan/THC films

The actual amount of model drug within the samples needs to be measured prior to investigating the release characteristics of the model drug from the post-neutralized and crosslinked electrospun chitosan/THC fiber mats. Table 1 show the actual amount of drug (THC) present in the electrospun fibers and films for both total immersion and transdermal diffusion through pig skin techniques [reported as % drug assay or the percentage of the initial content of drug loaded in the spinning and casting solution (20 wt.% based on the weight of chitosan powder)]. The drug loaded in the post-neutralized and crosslinked as-spun fibers presented about 95.05 % and 92.19% for total immersion and transdermal diffusion through pig skin techniques, respectively. While the drug loaded in the post-neutralized and crosslinked as-cast films presented about 98.76% and 97.96% for total immersion and transdermal diffusion through pig skin techniques, respectively. The release characteristic of the model drug from the electrospun chitosan/THC fiber mats and chitosan/THC films was carried out by total immersion and transdermal diffusion through pig skin. Both experiments were conducted by using acetate buffer as the transfer medium at a controlled temperature of 37°C (stimulated the environment of human skin).The accumulative amount of drug released (report as the percentage of the actual amount of drug present in the drug-loaded samples) from the chitosan/THC fiber mats and chitosan/THC films based on the total immersion method is shown in figure 4.8 and based on transdermal diffusion through a pig skin method is shown in Figure 4.9 Based on the result was shown in figure 4.8, the total amount of THC released from the drug loaded as-spun chitosan mats (at 10 h) was about 81.39%, while that from the drug loaded as as-cast chitosan films was 65.71%.The accumulative release of THC from the drug loaded as-spun chitosan mats increased continuously with immersion time and leveled off at long immersion time. On the other hand, the accumulative release of THC from the drug loaded as-cast chitosan films increased rapidly at initial immersion time and increased gradually after 45 min immersion time. For drug delivery system, one of the factors controlling the release of a drug is the swelling behavior of the hydrogel carrier. The porosity of carrier contributed to

the swelling in an aqueous medium. When the chitosan matrix began to swell, molecules of THC were solvated and diffused out from the matrix to the aqueous medium, indicating that the mechanisms for the release characteristic of THC were the swelling of the fiber mats and diffusion of THC molecules. The amount of THC released from the as-spun chitosan mats was greater than that from the as-cast chitosan film, because the as-spun fibers had higher porous structure and degree of swelling than those of films.

In the transdermal diffusion through a pig skin method (Figure 4.9), the amount of THC from the drug-loaded samples increased monotonically with diffusion time, but the rate of the drug released was much less when compared with that in the total immersion method since the transport of the drugs through the pig skin is the rate-determining step for both cases. The total amount of THC released from the drug-loaded as-cast chitosan films (at 10 h) was about 2.35%,while that released from the drug-loaded as-spun chitosan fibers was about 18.59%. In analogy to the total immersion method, the electrospun chitosan/THC fiber mats exhibited much greater release of the model drug when compared with the chitosan/THC films.

Table 4.1 The actual amount of THC present in the fiber samples (reported as the percentage of the initial content of drug loaded in the spinning and casting solution)

The actual amount of THC (mg)	Initial content of THC (mg)	The actual amount of THC Based on the original amount of THC loaded (%) from electrospun fibers for total immersion method
1.398	1.453	96.26
1.431	1.528	93.62
1.478	1.551	95.33
Average1.436	1.511	95.05
The actual amount of THC (mg)	Initial content of THC (mg)	The actual amount of THC Based on the original amount of THC loaded (%) from films for total immersion. method
7.48	7.544	99.10
7.76	7.886	98.45
8.06	8.16	98.76
Average 7.766	7.863	98.76
The actual amount of THC (mg)	Initial content of THC (mg)	The actual amount of THC Based on the original amount of THC loaded (%) from electrospun fibers for transdermal diffusion through pig skin technique
1.425	1.551	91.90
1.441	1.566	92.02
1.486	1.604	92.65
Average 1.451	1.558	92.19
The actual amount of THC (mg)	Initial content of THC (mg)	The actual amount of THC Based on the original amount of THC loaded (%) from films for transdermal diffusion through pig skin technique
7.47	7.65	97.62
7.61	7.76	98.09
7.88	8.028	98.16
Average 7.65	7.81	97.96

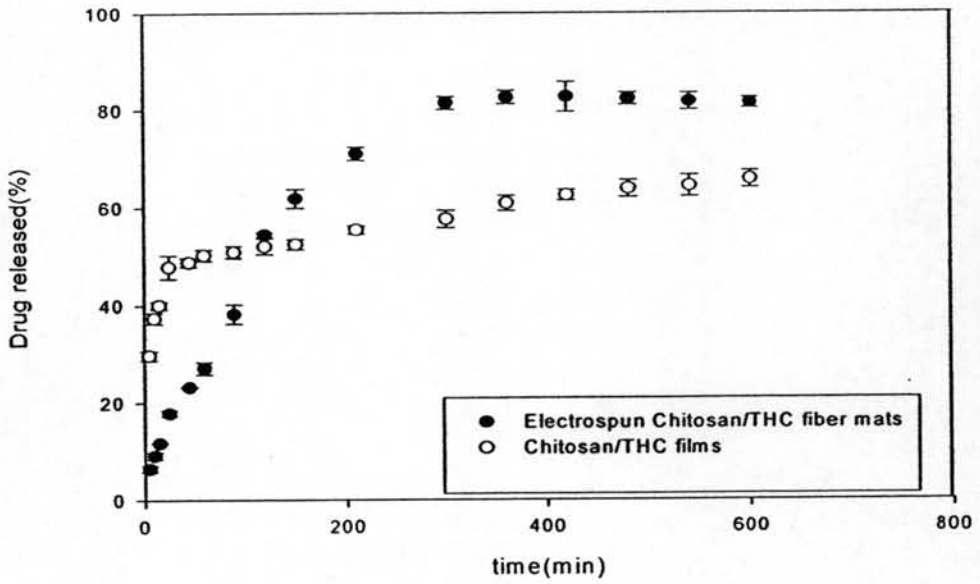


Figure 4.8 The accumulative amount of THC released from the drug samples base on the total immersion method.

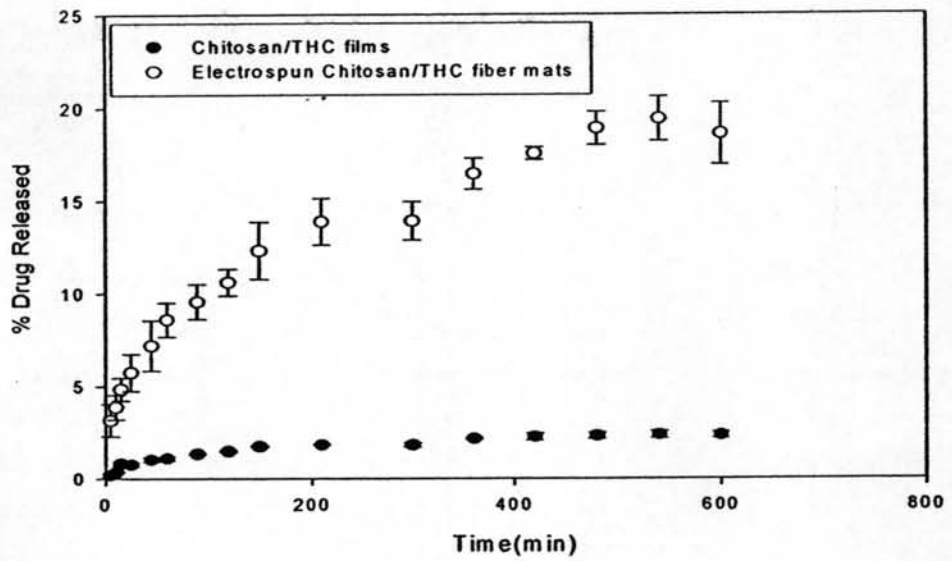


Figure 4.9 The accumulative amount of THC released from the drug samples base on the transdermal through pig skin method.

4.4 Release Kinetic of Model Drug from the Post-neutralized and Crosslinked Electrospun Chitosan/THC Fiber Mats and Chitosan/THC films

The release kinetic of drugs from drug delivery carrier is often characterized using an equation of the following form:

$$\frac{M_t}{M_\infty} = kt^n \qquad (3)$$

where Mt is the accumulative amount of drugs released at an arbitrary time t, $M\infty$ is the accumulation amount of drugs released at an infinite time, n is an exponent characterizing the mechanism with which the release kinetics can be described, and k is the rate of release of the drugs that incorporates physical characteristics of the matrix/drug system as well as some physical contributions from the measurement methods (namely in the case of the transdermal diffusion through a pig skin method which involves the diffusion of the drugs through a pig skin).

For n = 0.5, the release mechanism can be described as Fickian diffusion (Verreck et al, 2003). For Fickian diffusion, a straight line is expected when the fractional accumulative amount of drug released (i.e. $Mt/M\infty$) is plotted as a function of $t^{0.5}$. The results from the transdermal diffusion through a pig skin method for both the drug-loaded as-spun chitosan fiber mats and the as-cast chitosan films could be described with such an equation, indicating the Fickian diffusion type of the release mechanism of these drugs. The results from such analyses (i.e. parameter k and r^2, which signifies the goodness of the fit) are summarized in table 2. Apparently, the rate parameter k for drug-loaded as-spun chitosan fiber mats was 0.0088 $min^{-0.5}$, while that of the drug-loaded as-cast chitosan films was 0.0017 $min^{-0.5}$ (the rate parameters were calculated from Eq.3 at saturation period of drug release). The rate parameter of drug-loaded as-spun chitosan fiber mats is higher value than that of the drug-loaded as-cast chitosan films. It implied that THC diffuse out from drug-loaded as-spun chitosan fiber mats faster than that from the drug-loaded as-cast chitosan films which correspond to the release profile.

Table 4.2 Analyses of the release kinetics of model drug from drug-loaded as-spun chitosan mats and as-cast chitosan films based on the Fickian diffusion type of release mechanism. The experimental results were based on the transdermal diffusion through a pig skin method

Type of sample	Rate parameter $k(min^{-0.5})$	r^2
Drug-loaded as-spun chitosan fiber mats With 20 wt.% Tetrahydrocurcumin (THC)	0.0088	0.99
Drug-loaded as-cast chitosan films With 20 wt.% Tetrahydrocurcumin (THC)	0.0017	0.98

4.5 Indirect Cytotoxicity Evaluation

The potential for use of the electrospun chitosan/THC fiber mats was assessed by indirect cytotoxicty assay. This experiment was conducted by using mouse fibroblasts (L929) as the reference cell lines, the extraction medium from each fiber mat specimen was used to culture the cells. The viability of the cells was compared with the viability of the cells that were cultured with fresh SFM (i.e., control). Figure 4.10 shows the relative absorbance illustrating the viability of the cells that were cultured with the extraction medium from various types of specimens at 1 day and 7 days as well as that of the control for 24 h. For both 1 day and 7 days of extraction, the viability of L929 cell lines that were cultured with the extraction medium from all of fiber mats at given time exhibited either closet value as that of the control. These results indicated that all of the electrospun chitosan/THC fiber mats did not release cytotoxic substances in the culture medium towards mouse fibroblasts (L929). Suwantong et al. (2006) reported the viability of the cells that were cultured with the extract medium 24 h exhibited values that were either equal to or greater than that of the control, implied that samples were not toxic, at least the 24 h culturing period. Although the toxicity of glutaraldehyde is widely well known (Scobbie et al, 1995), its toxicity was minimized by the cross-linking technique with GTA vapor.

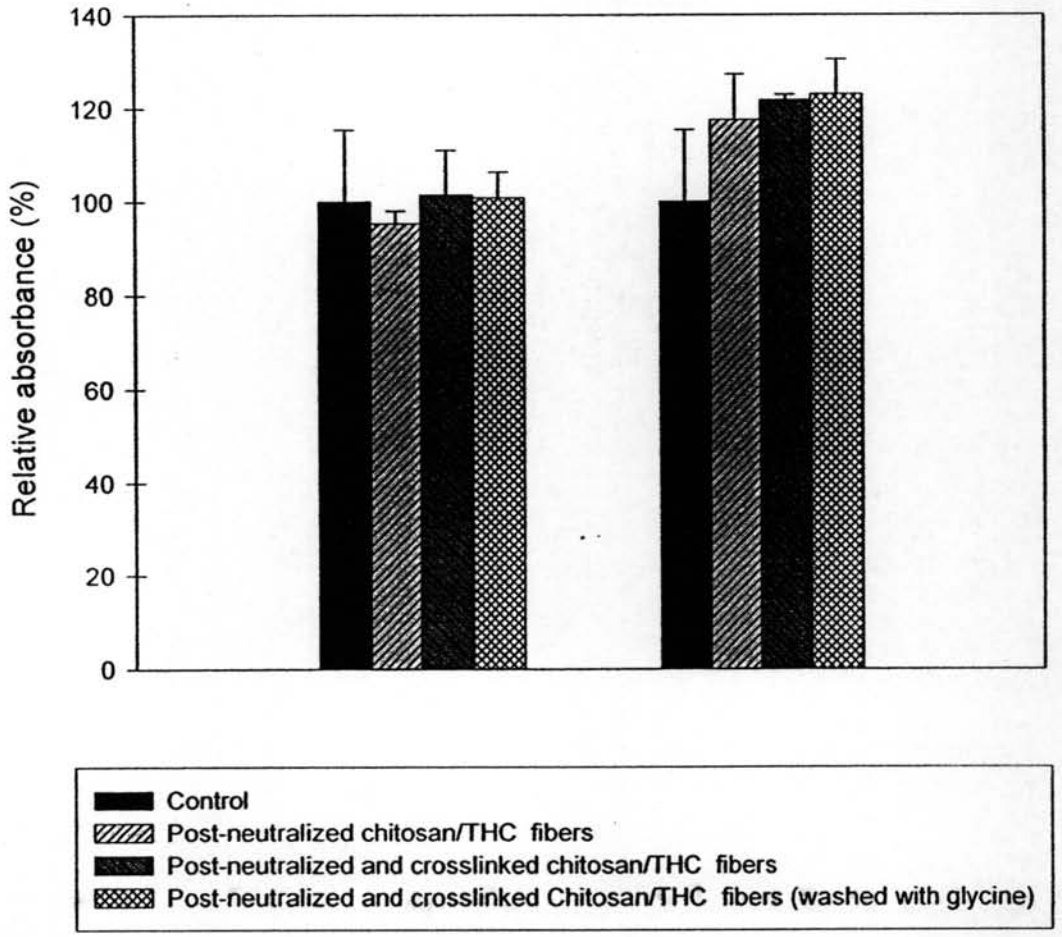


Figure 4.10 The relative absorbance illustrating the viability of the cells that were cultured with the extraction medium at 1 day and 7 days from various types of specimens as well as that of the control.

4.6 FTIR Analysis

Figure 4.11 shows the absorbance spectra each type of as-spun chitosan before and after neutralization with the Na_2CO_3 aqueous solution in comparison with that of as-receive THC powder. The characteristic absorption peaks of each pre-neutralized chitosan fiber mats were observed at 1672 and 1530 cm^{-1} for the stretching of the protonated amino($-NH_3+$) groups. The significant absorption peak at 1675 cm^{-1} and the three absorption peaks around 840-720 cm^{-1} are indicative of the presence of trifluoroacetic acid in electrospun chitosan fibers as amine salts

(Hasegawa et al, 1992). On the other hand, each post-neutralized chitosan fiber mats exhibited sharp absorption peaks at 3400, 3300 cm^{-1} corresponding to the stretching of $-NH_2$ groups (Min et al, 2004), but the peaks appeared aboard here due to the contributed peaks of O-H stretching and hydrogen bonds (Banerjee et al, 2002), and 1580 cm^{-1} for the amino group (Qu et al, 2000). The FTIR of the THC-loaded electrospun chitosan fibers could not be clearly observed the spectra of THC structure. It was contributed to a low amount of THC incorporated in the electrospun fibers, and the spectra of C=O absorption and –OH vibrations of intermolecularly-bonded OH group are repetitive the peak of all post-neutralized chitosan fibers. Evidently, FT-IR results confirmed the regeneration of the amino groups after the neutralization treatment which the post-neutralized and crosslinked chitosan/THC displayed a lower absorbance because some amino groups were involved in the crosslinking process. Meanwhile, based on the spectrum of post- neutralized and crosslinked chitosan fiber mats, it shows a reduction of the peak at 1580 cm^{-1}, in comparision with each post-neutralized chitosan fiber mats, which is due to the loss of the free amine during crosslinking and there is a significant new peak near 1670 cm^{-1}, which can be attributed to an imine bond (C=N) (Ngah et al, 2006). The loss of the free amine indicated that the electrospun chitosan fibers exhibited a Schiff base imine functionality (Jessica et al, 2007) and also reported by Tual et al. The mechanism of crosslinking with glutaraldehde was shown in Figure 4.12. Obliviously, a similar peak can be observed for pre-neutralized and crosslinked chitosan fiber mats but then it is a stronger band than that of post- neutralized and crosslinked chitosan fiber mats due to an imine bond(C=N) and the protonated amino ($-NH_3+$) groups.

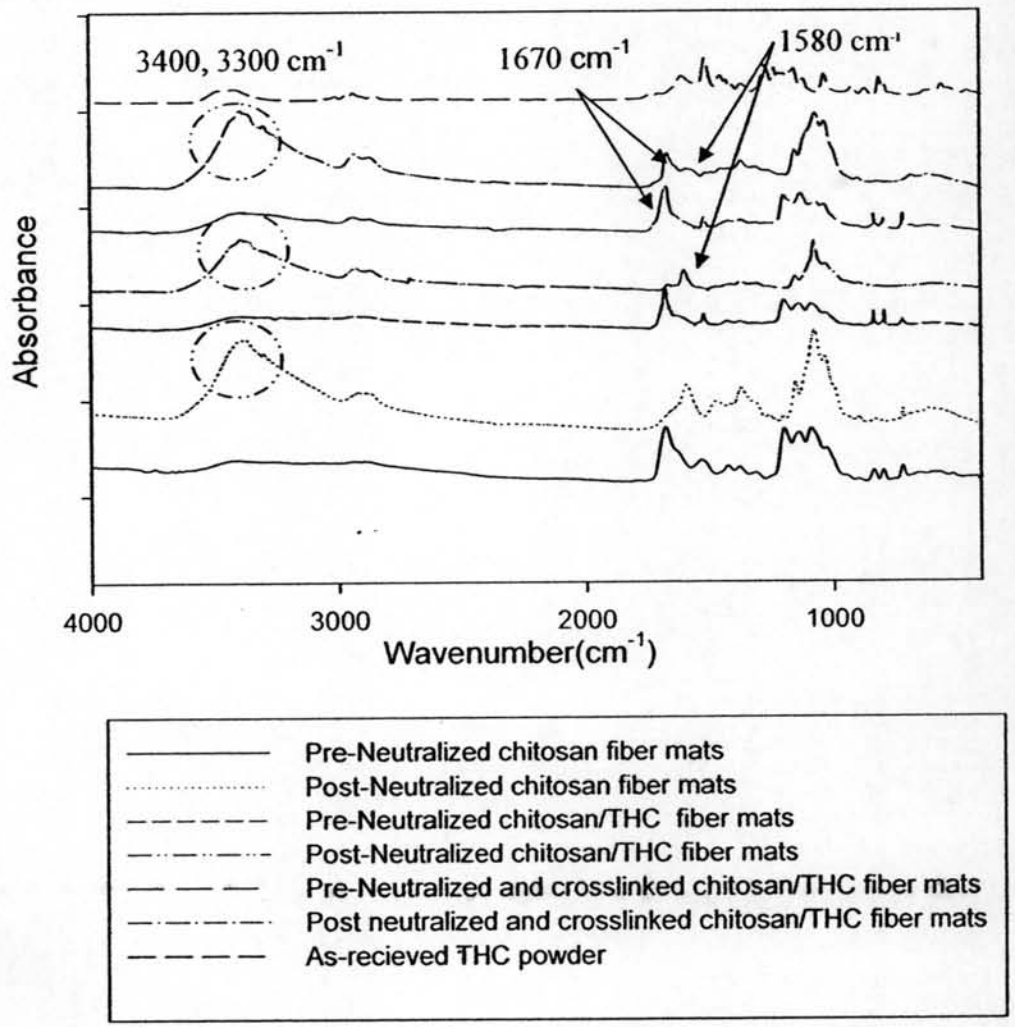


Figure 4.11 FTIR spectra of the pre- neutralized chitosan, the post-neutralized chitosan, the pre-neutralized chitosan/THC fiber mats, the post-neutralized chitosan/THC, the pre-neutralized and crosslinked chitosan/THC, and the post-neutralized and crosslinked chitosan/THC fiber mats.