CHAPTER IV

RESULTS AND DISCUSSION

There are basically three components to fulfill the process: a high voltage supplier, a capillary tube with a pipette or needle of small diameter, and a metal collecting screen. In the electrospinning process a high voltage is used to create an electrically charged jet of polymer solution or melt out of the capillary. Before reaching the collecting screen, the solution jet evaporates or solidifies, and is collected as an interconnected web of small fibers [Deitzel JM *et al.,* 2001, Fong H *et al.,* 2001]. One electrode is placed at the tip of the capillary and the other attached to the grounded collector. The electric field is subjected to the end of the capillary tube that contains the solution fluid held by its surface tension. This induces a charge on the surface of the liquid. Mutual charge repulsion and the contraction of the surface charges to the counter electrode causes a force directly opposite to the surface tension [Tang X *et al,.* 1997]. As the intensity of the electric field is increased, the hemispherical surface of the fluid at the tip of the capillary tube elongates to form a conical shape known as the Taylor cone [Taylor GI *et al,* 1969]. Further increasing the electric field, a critical value is attained with which the repulsive electrostatic force overcomes the surface tension and the charged jet of the fluid is ejected from the tip of the Taylor cone. The discharged polymer solution jet undergoes an instability and elongation process, which allows the jet to become very long and thin. Meanwhile, the solvent evaporates, leaving behind a charged polymer fiber.

Electrospinning is one of the widely used techniques to produce small fibers, yet the process is relatively complex because of many parameters such as solvent system, solution viscosity, solution conductivity, applied voltage, etc. [Reneker DH *et al.,* 1995] The products from electrospinning technique processes many special characteristics, such as high surface area, high porosity with interconnecting pores. The topology of electrospun products is similar to the extracellular matrix (ECM) and can enhance the

migration and proliferation of cells. Thus electrospun materials have been used in various biomedical applications which range from drug delivery devices to tissue engineering scaffolds. [Fong, H *et al.,* 1990] When considering the type of materials used in biomedical applications, natural polymers are often preferred because of better biocompatibility and biodegradability which makes them more suitable for the human body than synthetic polymers. [Li. M *et al.,* 2005] However, electrospinning natural polymers are often more complex than synthetic polymers because of their poor processability. [Wnek *et al.,* 2003]

Gelatin is a form of denatured collagen, it exhibits excellent biocompatibility and biodegradability and has been widely studied in medical applications, including wound dressings and tissue engineering of bone, skin and cartilage. [Balakrishnan *et al.,* 2005] Though few studies have been done on the method of electrospinning of gelatin, because organic solvents such as 2,2,2-trifluoroethanol (TFE) or formic acid must be used in order to produce fibers. [Zhang *et al.*, 2005][Ki C.S. *et al.,* 2005]

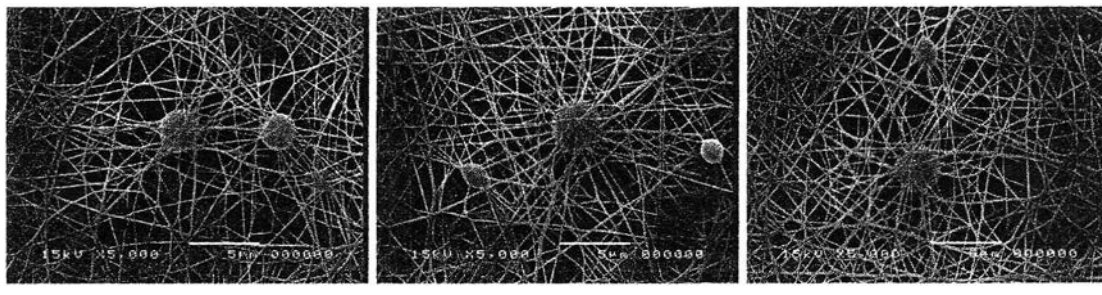
The main disadvantage of these organic solvents mentioned above is the toxicity of residue solvent which leaves water as the ideal solvent to dissolve gelatin. However, it was believed that electrospinning of aqueous gelatin was not possible, even above in room temperature. [Zhang Y *et al.*, 2005] Therefore an incorporation of secondary solvent such as acetic is considered as an alternative, since it can be diluted or neutralized by water or oven dry to remove residue solvents. [Choktaweesap N. et al., 2007]

With the target use in biomedical applications, blending with another biopolymer should increases the compatibility and enhances the properties of the nanofiber mats produced. The material in focus is hyaluronic acid which is one of the components of extracellular matrix (ECM). It consists of 2-acetamide-2-deoxy-α-D-glucose and β-D-glucuronic acid residues linked by alternate (1-3) and (1-4) glycoside bonding. It has been reported to possess high water absorption and water retention capacity, and can also influences cell adhesion, migration and proliferation. [Young S.C *et al.,* 1999] Doillon *et al.,* had investigated the effectiveness of hyaluronic acid in wound healing,

both with *in vitro* and *in vivo*. They reported that hyaluronic acid is effective in enhancing fibroblasts movement into a collagen sponge and in depositing collagen fibers during the early stage of wound healing. In addition, the action of hyaluronic acid in water homeostasis could favor the tissue hydration, which has positive effect on wound healing. [King S.R. *et al.*, 1991]

4.1 Effect of Different Solvent System on the Electrospun Fibers

The complication in electrospinning hyaluronic acid solution is that it has extremely high viscosity and it takes an amount of time to completely dissolve, even in distilled water. With only a small addition of hyaluronic acid to the spinning solution, the viscosity increases dramatically.



(A) (B) (C)

Figure 4.1 SEM images at magnification 3,500x of gelatin/hyluronic acid electrospun fibers of various acetic acid concentration: A) 10%, B) 20%, C) 30

The solvent system used was various ratios of distilled water and glacial acetic acid. Because the electrospinning process was carried out in room temperature, glacial acetic acid was added to prevent gelatin from forming gel since the gelation temperature of gelatin is below 32 degree Celsius. Previous work had used glacial acetic acid as the only solvent for electrospinning solution of gelatin [Choktaweesap N. et al., 2007] but unfortunately the glacial acetic acid prevents hyaluronic acid from dissolving. Therefore

the appropriate ratio between glacial acetic acid and distilled water must be achieved to be able to both prevent gelatin from forming gel at room temperature and at the same time efficiently dissolve hyaluronic acid. From figure 4.1 A), B) and C) the SEM images showed that there were phase separations in the electrospinning solution. Observable from the round beads deposited on fibers. At acetic acid concentration of 40% the blend fibers can form without beads, suggesting complete dissolvability. At higher acetic acid concentration the solution forms gels and could not be electrospun.

4.2 Effect of Solution Concentration on the Electrospun Fibers

The total of 5 concentrations of electrospinning solutions was evaluated. The factors which affected the morphology of electrospun fibers that were investigated are viscosity and conductivity of the electrospinning solutions. From table 4.2 the values of viscosity and conductivity of each electrospinning solution are shown and the trend of both values can be observed in figure 4.2 and 4.3. The viscosity of electrospinning solution increases linearly, but the conductivity has a different trend.

Table 4.1 Conductivity and viscosity of spinning solution

Sample	Acetic Acid (%)	Gelatin (wt%)	HA (wt%)	Solution Conc.(%)	Viscosity (cP)	Conductivity (us/cm)
1	40	5	0.5	5.5	195	1647
2	40	10	0.5	10.5	480	1954
3	40	15	0.5	15.5	651	7400
4	40	20	0.5	20.5	961	8200
5	40	25	0.5	25.5	1311	8900

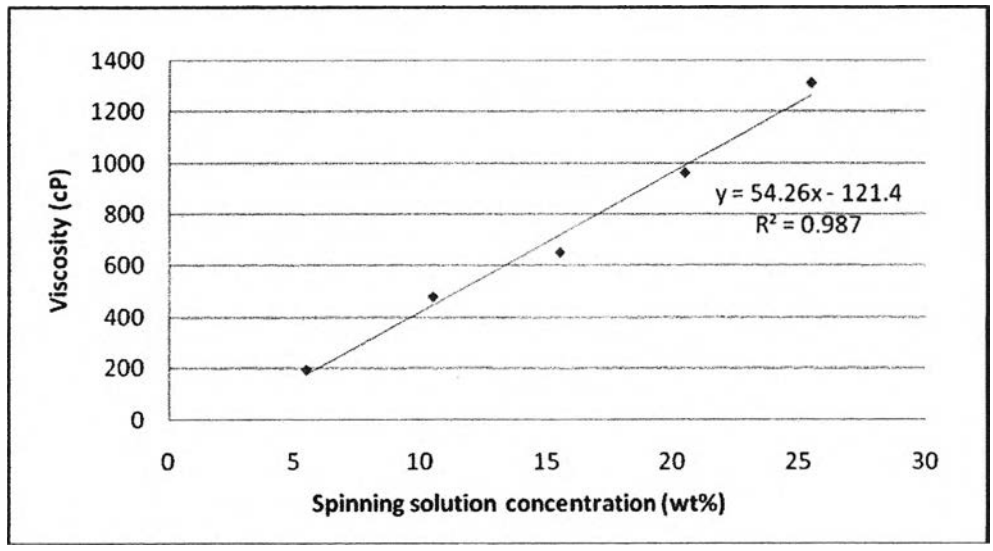


Figure 4.2 Graph showing viscosity of electrospinning solution.

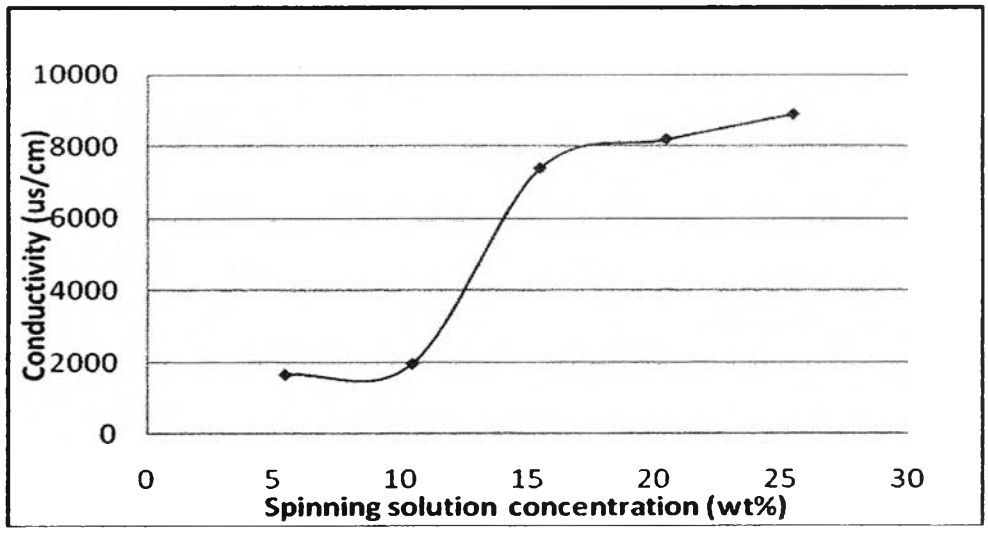
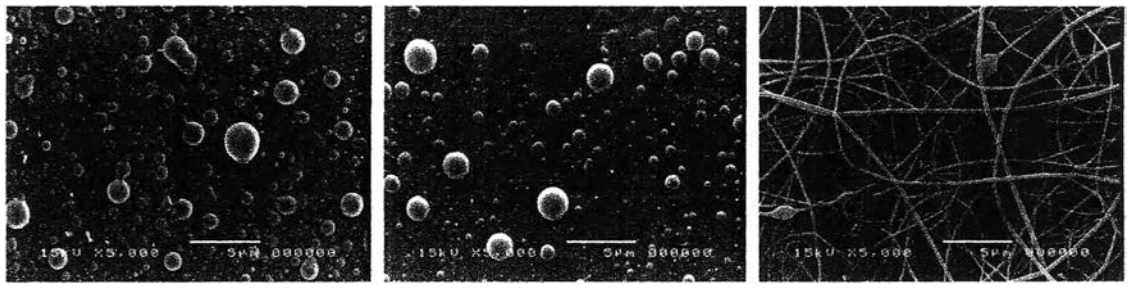


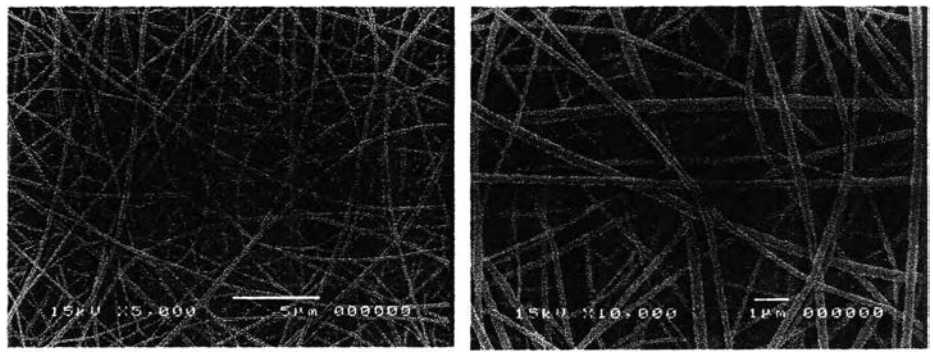
Figure 4.3 Graph showing conductivity of electrospinning solution.



(A) (B) (C)

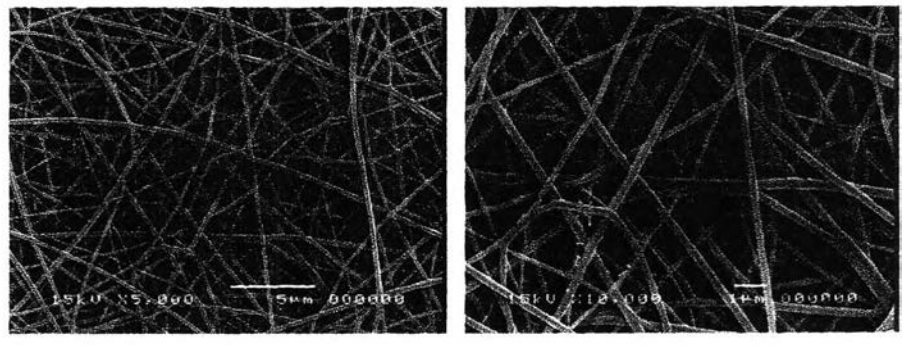
Figure 4.4 SEM images of electrospun gelatin/hyaluronic acid fibers various gelatin:hyaluronic ratio A) 10/1, B) 20/1, C) 30/1

With gelatin:hyaluronic acid ratio of 10/1 and 20/1 the polymer concentration is too low to provide enough chain entanglements which can be observed by the low viscosity and the SEM images (figure 4.4), droplets of polymers are present instead of fibers. The reason being could be because of the low conductivity of the electrospinning solution (figure 4.3). At gelatin:hyaluronic acid ratio equals to 30/1 (figure 4.4 C) the conductivity is high enough to produce fibers but the fiber diameters have high variation with the inclusion of beaded fibers. The beaded fibers could be the results from the low viscosity, because continuous jet could not form during the electrospinning process. With increasing gelatin content, the elecrospun fibers increase in diameters (figure 4.6).



(A) (B)

Figure 4.5 SEM images of electrospun gelatin/hyaluronic acid at gelatin:hyaluronic acid equals to 40/1 at various magnification: A) 5,000x and B) 10,000x.

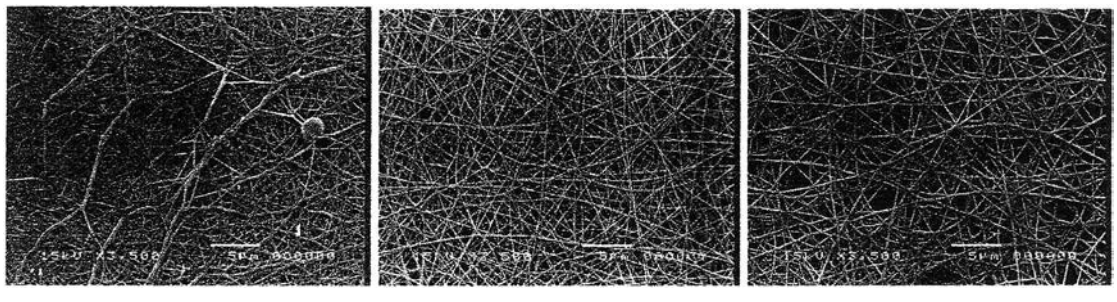


(A) (B)

Figure 4.6 SEM images of electrospun gelatin/hyaluronic acid at gelatin:hyaluronic acid equals to 50/1 at various magnification: A) 5,000x and B) 10,000x.

4.3 Effect of Collecting Distance on the Electrospun Fibers

Collecting distance also plays an important part in determining the characteristics of electrospun fibers. From the SEM images of figure 4.7, short collecting distances shown in figure 4.7 A) beads and irregular fiber diameters were observed also, the fibers collected were larger in diameters and fused together at the fibers junctions. The reason for this is because with short collecting distance the solution jets exciting from the tip of the Taylor cone were deposited onto the collector before the solvents could vaporize off. The residue solvents will increase the diameters of fibers collected and also fuse the fibers together on the collector. At the collecting distance of 20 cm, as shown in figure 4.7 B), consistent fiber diameters are observed including round fibers without fiber fusing at the touching points. This suggests that at 20 cm collecting distance, the solvents were sufficiently vaporized off the polymer jets before they were deposited on the collector. At longer collecting distance, figure 4.7 C), the SEM images showed smaller amount of fibers and larger fiber diameters deposited. The reason for this is unclear, but from the observation of the electrospinning process, a large amount of fibers were deposited at nearby objects rather than the collector, which is further away. It is possible that small fiber diameters were deposited on nearby objects and leave the fibers with larger diameter which have more electrical charge to deposit on the collector.

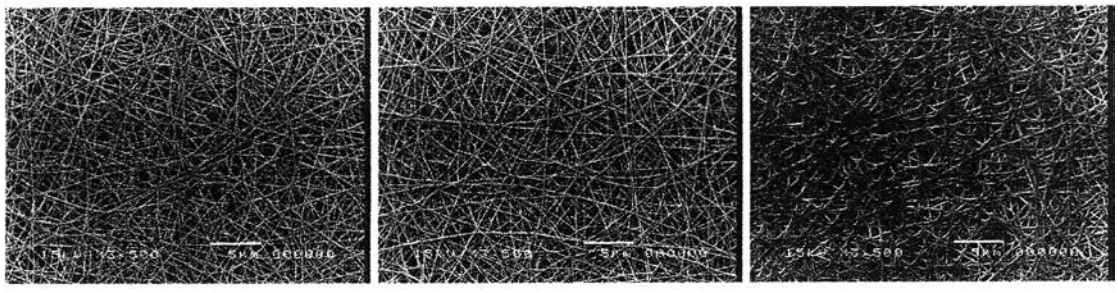


(A) (B) (C)

Figure 4.7 SEM images of gelatin/hyaluronic acid electrospun fiber mats at different collecting distance: A) 15 cm, B) 20 cm, C) 25 cm

4.4 Effect of Applied Voltage on the Electrospun Fibers

The differences in applied voltage on the electrospinning process were investigated. From figure 4.8 A) the applied voltage of 18 kV were used, the resulting fibers showed regular fiber diameters but during the electrospinning process the needle was often blocked as the result of hardening of the solution accumulated on the tip of the needle. From figure 4.8 B) the applied voltage of 20 kV were used, similar fibers were obtained as with fewer frequency of blocked needle. When the applied voltage was increased to 22 kV as shown in figure 4.8 C) larger fiber diameters were observed along with some fused fibers indicating the presence of residue solvents. The reason for this in still unclear but form the observation of the electrospinning process, with high applied voltage, the fibers will travel at a high velocity which gives less time for the solvent to vaporized. Therefore, the residue solvent resulted in large and fused fibers.



(A) (B) (C)

Figure 4.8 SEM images of gelatin/hyaluronic acid electrospun fiber mats at different collecting distance: A) 15 cm, B) 20 cm, C) 25 cm

4.5 Cross-linking Process

There are many known chemicals which can be used to cross-link gelatin, for example formaldehyde, glutaraldehyde, water soluble carbodiimide and dextran dialdehyde. However, studies have shown that glutaraldehyde is by far the most widely used, due to its high efficiency in stabilizing collagenous derivative materials. [Khor E., 1997] The cross-linking mechanism of gelatin by glutaraldehyde involves the reaction of free amino groups of lysine or hydroxylysine amono acid residues of the polypeptide chains with the aldehyde functional groups of glutaraldehyde [Olde DL *et al.*, 1995] creating cross-linking network which gives the material its water resistivity. Without this cross-linking process, the electrospun fiber mats dissolved almost instantly when come in contact with water.

After the cross-linking process, the gelatin/hyaluronic acid fiber mats developed yellowish color which is result of aldimine linkages (CH=N) between free amino groups of protein and glutaraldehyde. [Akin H *et al.*, 1995] There are also reports on the slightly shrunken dimensions of gelatin membranes after cross-linking [Harland RS *et al.*, 1989], therefore in this experiment the electrospun gelatin/hyaluronic fiber mats were bound tightly to stainless frame using metal clips to prevent the shrinkage due to fiber contraction from the cross-linking reaction.

The cross-linking process was investigated in four different time intervals of: 30 min, 1, 2 and 4 hour. However, with different cross-linking time the resulting fibers showed an increase in fiber diameter and increase the variation in fiber diameters. In the cross-linking process, when the required time was reached the fiber mats were immediately removed and immersed in sodium bimetasulfite to stop the cross-linking reaction. Otherwise, if leave unattended, the residue glutaraldehyde vapor which remained within the pores of the fiber mats could continue cross-linking and alter the fiber mats' properties.

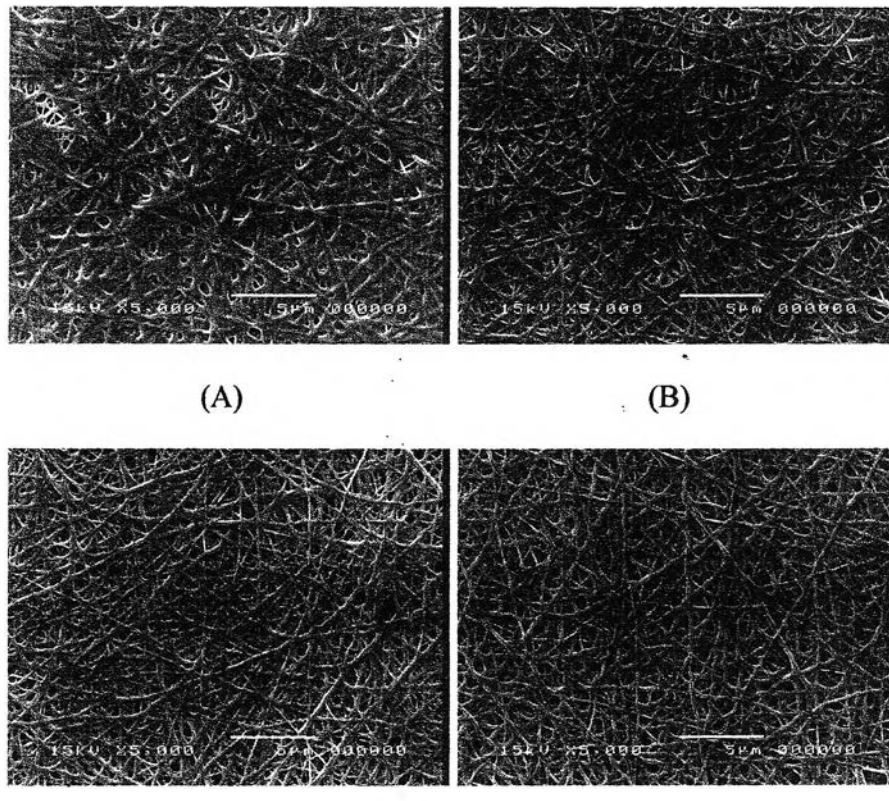


Figure 4.9 SEM images of electrospun gelatin/hyaluronic acid fibers at different cross-linked time intervals: A) 30 min, B) 1 hr, C) 2 hr, D) 4hr

4.5.1 Infrared (IR) Analysis

To investigate the cross-linking process, infrared spectrum was measured and distinctive change in wave number and intensity of peaks were analyzed. From figure 4.10 spectrum b), the representative bands can be assigned as follows [Haxaire K *et al.*, 2003]: the intense groups of bands extending from 1500 to 1800 cm^{-1} are super-positions of amides I and II bands and of various carbonyl and carboxyl νCaO bands. The bands extending between 950 and 1100 cm^{-1} are mainly resulted from different

vibrations of the pyranose ring, corresponding to νC–OH. The shoulder at 1155 cm^{-1} can be assigned to νC–O–C.

The FTIR spectra obtained for the Gel–HA electrospun mat showed strong increases in intensities of amide (1630, 1543, 1450 cm^{-1}) which indicated that there was an increase in amide functional group, possibly from the cross-linking process since glutaraldehyde can react with the functional group amine to form amide linkages.

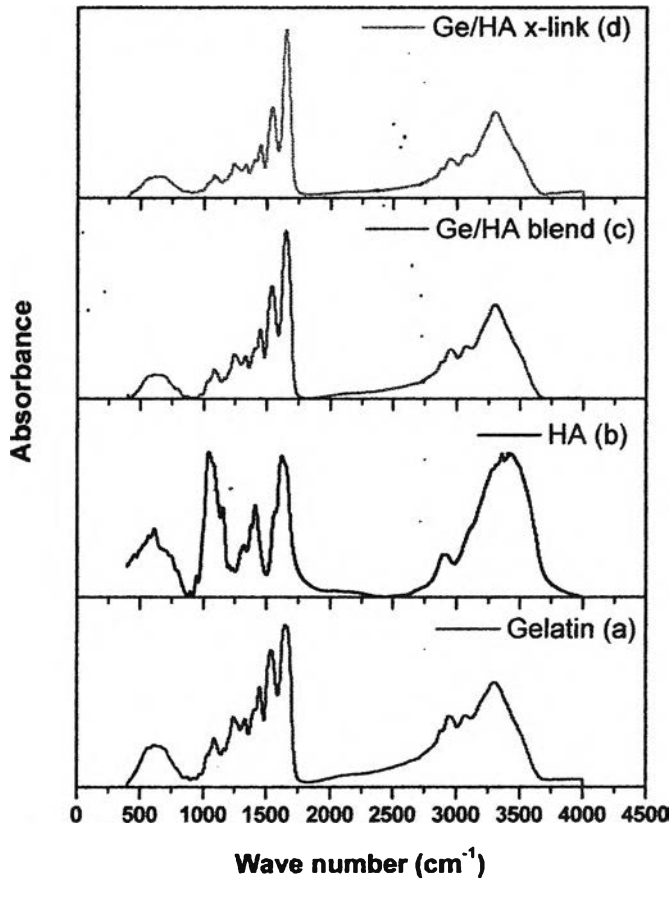


Figure 4.10 Infrared spectrum of: a) gelatin powder, b) hyaluronic acid powder,C) gelatin/hyaluronic acid electrospun fibers, d) cross-linked gelatin/hyaluronic acid electrospun fibers

4.6 Thermal Degradation Temperature

There are not much differences in the TG results (figure 4.14) between the pure gelatin powder, and gelatin/hyaluronic blend electrospun fibers because there are too small amount of hyaluronic acid content to make any significant change. Also, similar results are obtained with cross-linked gelatin/hyaluronic electrospun fibers, the crosslinking process only prevent the fiber mats from dissolving in water but it did not increase the degradation temperature of the cross-linked electrospun mats (figure 4.14). The degradation temperature of gelatin is approximately 280 degree Celsius, and hyaluronic acid is approximately 220 degree Celsius. The degradation temperature of gelatin/hyaluronic blend electrospun fibers is approximately 280 degree Celsius, not much different form that of pure gelatin. All sample shows the same desorption of physisorbed water at approximately 80 – 90 degree Celsius.

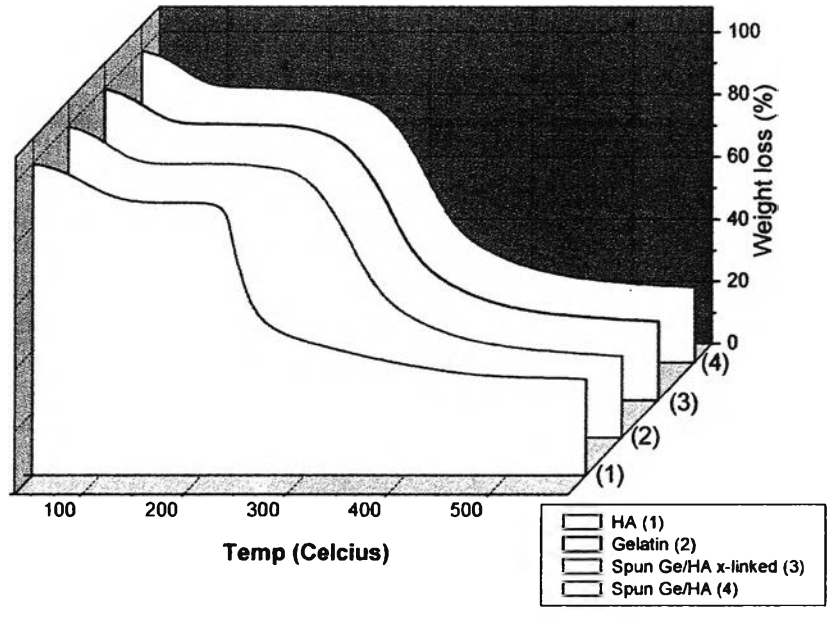


Figure 4.11 Thermal degradation profiles

4.7 Water uptake

The water uptake and retention ability is one of the most important functions of HA in skin tissues. Its water high water uptake ability suggests that hyaluronic acid may be involved in the maintenance of the extracellular space, facilitate the transport of iron solutes and nutrients, and preserve tissue hydration. [H. Lui *et al.*, 2006]

At different cross-linking time intervals the fiber mats showed similar trends. There was a high increase in percentage swelling from 30 min to 1 hr. For 30 min and 1 hr cross-linked fiber mats, the percentage swelling decreased from the immersion time of 2 hr and leveled off at the immersion time of 4 hr. While the 2 hr and 4 hr cross-link fiber mat showed a stable percentage swelling from the immersion time of 1 hr onward. The factors attributed to this behavior is because at 30 min and 1 hr cross-linked intervals the fibers were not fully cross-linked and some parts were allow to dissolve in water. Thus the percentage swelling was decreased with increasing immersion period.

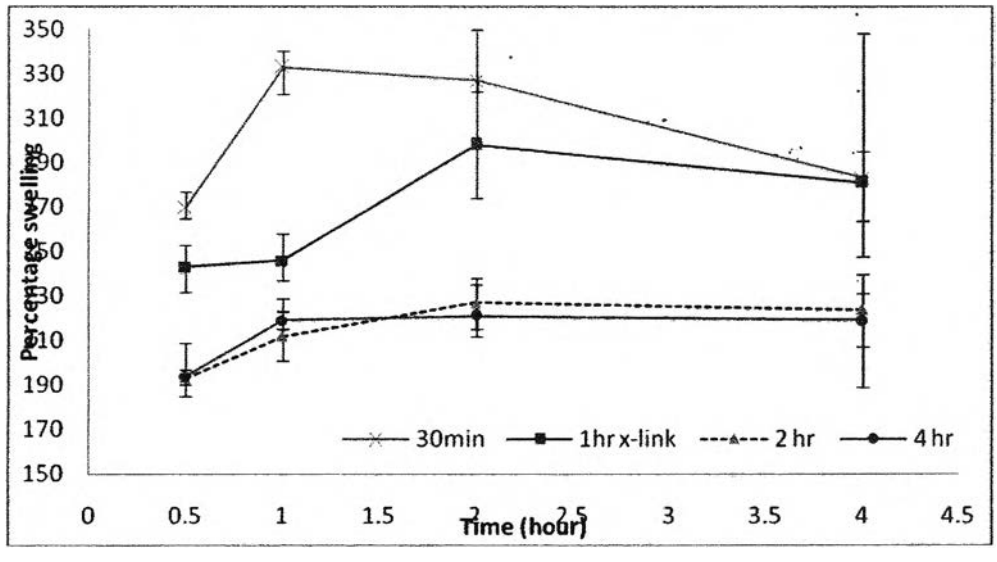


Figure 4.12 Degree of swelling of 30min, 1hr, 2 hr, 4 hr cross-link gelatin/hyaluronic acid electrospun fiber mats

4.8 Tensile Test

To test the strength of the cross-linked material to evaluate for its possibilities in using as tissue scaffolds and wound dressing, the cross-linked fiber mats were subjected to tensile tests. The fiber mats were cut into 1x 10 cm pieces and immersed in distilled water at room temperature for 1 hr to allow it to swell to its full capacity. As shown in figure 4.14 the percentage strain at break decreases as the cross-linking time increase. Low percentage strain at break means that the fiber mats broke at low extension distance, with increasing cross-linking density the fiber mats losses its elasticity. Because of more cross-linking density, the fiber mats becomes tougher and stiffer, as shown by the Yong's modulus values in figure 4.15. Although there are no dramatic changes in the Young's modulus values, but the slight increase can be observed.

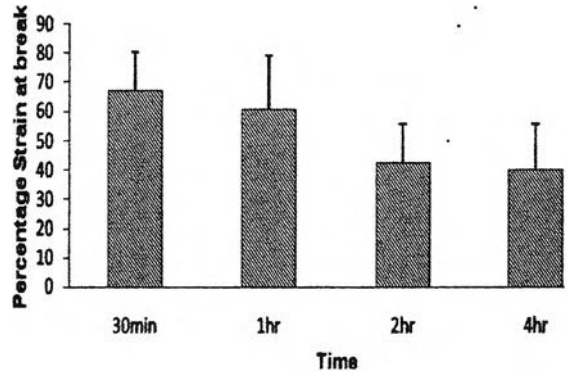


Figure 4.13 Percentage strain at break of 30 min, 1 hr, 2 hr, 4 hr cross-linked gelatin/hyaluronic acid electrospun fiber mats

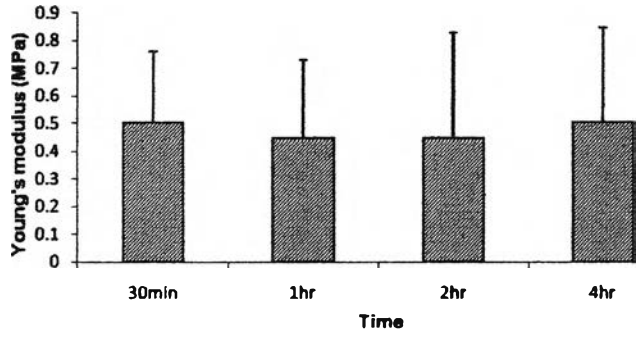


Figure 4.14 Young's modulus of 30 min, 1 hr, 2 hr,4 hr cross-linked gelatin/hyaluronic acid electrospun fiber mats

4.9 Biocompatibility Test

The 4 hr cross-linked fiber mats were chosen as the optimum condition and were subjected to cytotoxicity test by using mouse fibroblast (L929) cells. Prior to cytotoxicity test the fiber mats were immersed in solution of sodim bimetasulfite to remove the residue cross-linking agent, and wash 2-3 times in distilled water to clean the fibers. The amount of viable cells compared to control (Polystyrene Culture Plate) was within the acceptable range and could be concluded that the materials were not toxic.

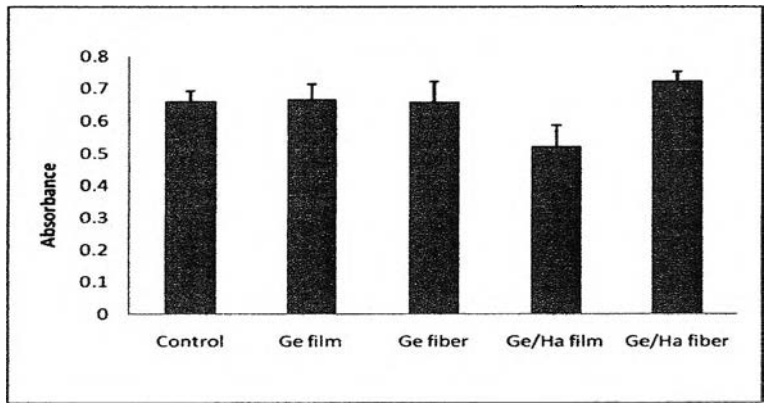


Figure 4.15 Cytotoxicity test

4.10 Cell Attachment and Proliferation

To evaluate the cell effectiveness and the possibilities of the cross-linked electrospun Ge/HA blend nanofiber mats mouse fibroblast cells (L929) were cultured on the selected materials which were gelatin film, electrospun cross-linked gelatin fiber mats, Ge/HA film and electrospun cross-linked Ge/HA blend fiber mats, glass cover slips were used as control. Because gelatin react with MTT test method and give absorbance even without the cultured cells, propidium iodide fluorescence dye were used in order to enable cell counting under optical microscope. Each sample were seeded with 10,000 cells of mouse fibroblast (L929) cells.

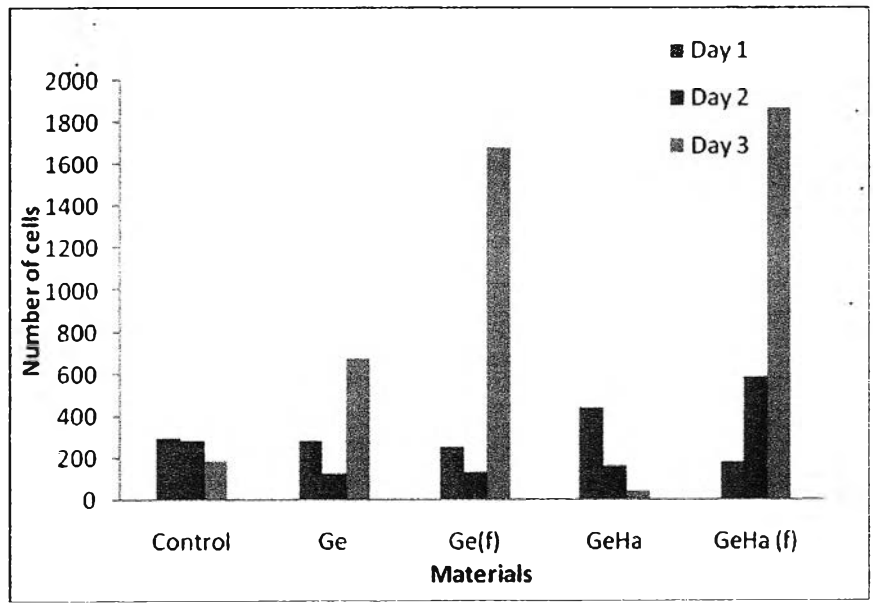


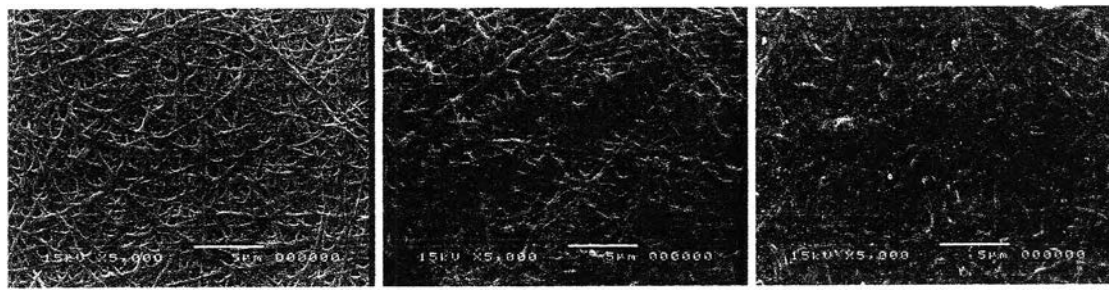
Figure 4.16 Number of viable cells on cultured materials

From figure 4.16 the number of viable cells counted on day 1 of all materials were similar with the control except for a visible increase found when Ge/HA film were used. it can be concluded that the electrospun Ge/HA fibers showed a significant increase in the number of viable cells on day 3 of culture. While on day 2 gelatin film

film showed a decrease in the number of viable cells which could be the result of toxicity of the films from residue solvents and cross-linking agent. On day 3 the number of viable cells on the materials which were fiber rose dramatically with electrospun Ge/HA fibers having the higher number of viable cells than electrospun gelatin fibers. It could be concluded that the fiber-like morphology of the electrospun fibers can promote cell growth and the addition of hyaluronic acid to gelatin matrix can enhance cell growth also. For the attachment properties of the materials, the test results were inconclusive.

4.11 Degradation

Because both gelatin and hyaluronic acid are biodegradable polymers, the degradation behavior of them must be studied. The electrospun Ge/HA fiber mats were immersed in DMEM culture media and incubated at 37°C for a period of 1, 2 and 3 days, then they were taken out of the DMEM culture media and washed with distilled water. The samples were left to dry and the morphology of the fibers were observed using scanning electron microscope.



(A) (B) (C)

Figure 4.17 SEM images of degrading electrospun Ge/HA fiber mats: (A) Day 1, (B) Day 2, (C) Day 3

From day 1 of immersion the fiber mats showed small amount of degradation, the fiber structure is still intact. The degradation was visible in day 2 where the fiber structure starts to disappear and was distorted on day 3.