CHAPTER IV

RESULTS AND DISCUSSION

4.1 Characterization of Non-Photo-Crosslinked Chitosan Scaffolds

4.1.1 Morphology

From SEM micrographs of cross-section of the scaffolds (Fig 4.1), it is clearly shown that different freezing temperatures gave different pore morphology. The lower temperature gave the smaller pore size (Fig 4.1 (X axis)) which suggested that the difference in freezing temperature led to the difference in heat transfer rate. Lower freezing temperature induces smaller ice crystal, therefore a smaller pore size was formed [64]. This similar result was also reported by Chung *et al.* on the scaffold from alginate/galactosylated chitosan scaffold with pore size of ~8-210 μm. In addition, in our study, it was found that only the scaffolds prepared at the freezing temperature of -10°C had three-dimensional interconnecting round pores, with diameters of 30-50 μm (Fig. 4.1A) while the pore structure of the scaffold obtained at -80°C and -196°C was columnar (Fig. 4.1B and 4.1C- cross-section in Y axis). A thin boundary shown in the middle of Fig. 4.1B and C were caused by two opposite freezing directions what met together at the center of the scaffolds. A similar channel structure was observed in porous gelatin scaffolds [65]. However, in their case, the columnar pores throughly connected (no boundary in the middle). Three-dimension diagrams represented the chitosan scaffold fabricated at various temperatures are shown in Fig. 4.2.

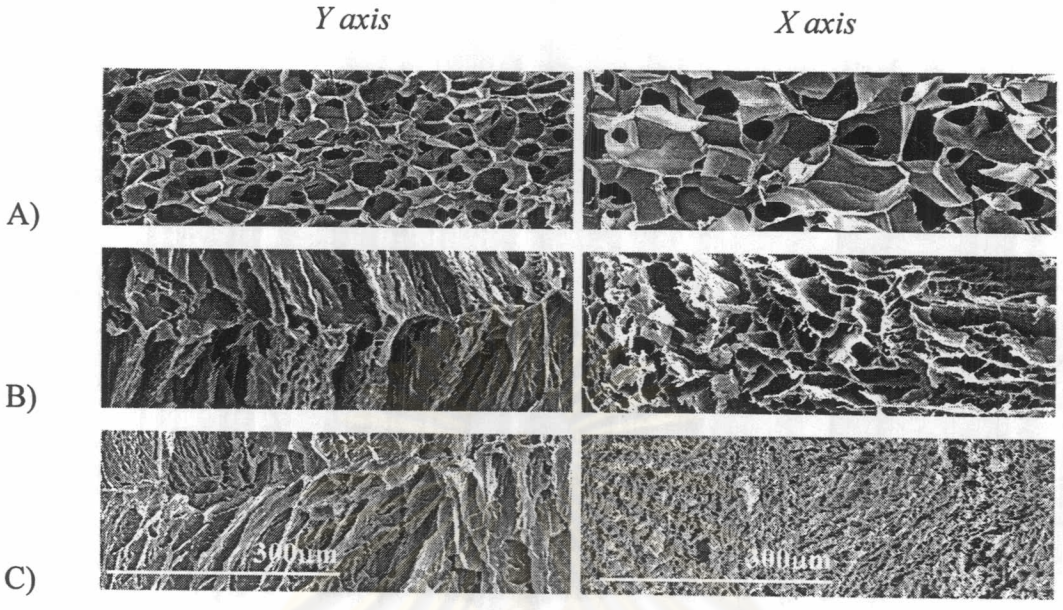


Figure 4.1 SEM micrographs (cross-section) of 3%wt chitosan scaffolds prepared from different freezing temperatures: A) -10°C, B) –80°C and C) -196°C.

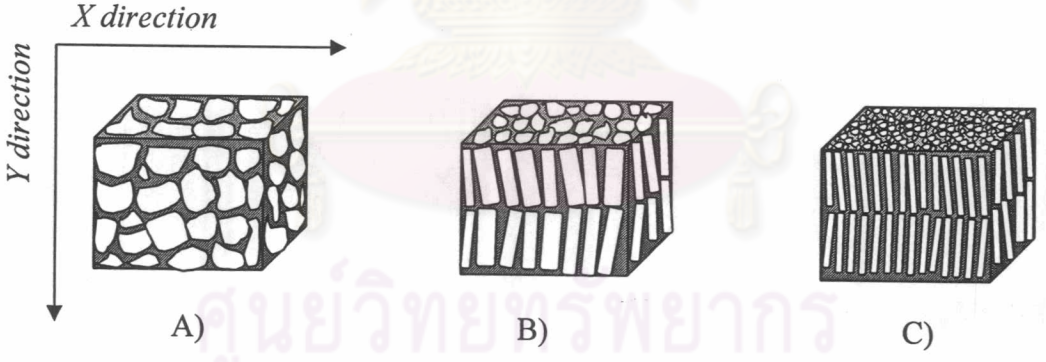


Figure 4.2 A diagram representing pore morphologies of 3%wt chitosan scaffolds prepared from different freezing temperatures: A) -10°C, B) –80°C and C) -196°C.

As it is well known that crystalline structures of shrimp and squid chitosan are different as α and β forms, respectively. However, there were no significant differences in pore size and morphology of the scaffold prepared from shrimp and squid chitosan (Fig. 4.3).

As well as the chitosan type, chitosan concentration did not significantly affect the pore size and morphology. In addition, the re-hydration step, to get rid of the remaining acetic acid, did not change the mean pore diameter of the scaffolds (Fig. 4.3). Overall, the results clearly show that the freezing temperature is the only factor that has a significant influence on the pore morphology of the scaffold.

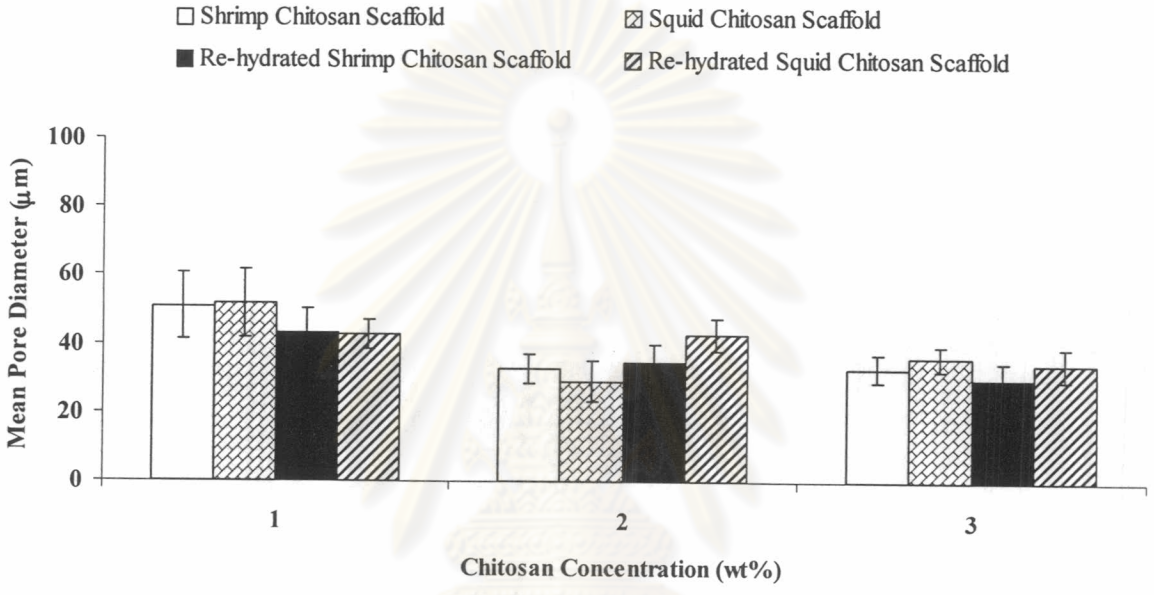


Figure 4.3 Effect of chitosan concentration and type of chitosan on average pore diameter of chitosan scaffolds fabricated at –10°C before and after re-hydration.

4.1.2 %Porosity

The scaffold porosity is defined by the amount of air within sponge. Fig 4.4 shows the effect of chitosan concentration and freezing temperature on the porosity of the sponge obtained from shrimp chitosan. The freezing temperatures did not affect %porosity, whereas lowering the chitosan concentration resulted in higher %porosity, due to less amount of substance. The chitosan scaffolds with 95 to 98% porosity could be prepared, making them suitable for tissue engineering application. The porosity of squid chitosan was similar to that of shrimp chitosan (data not shown).

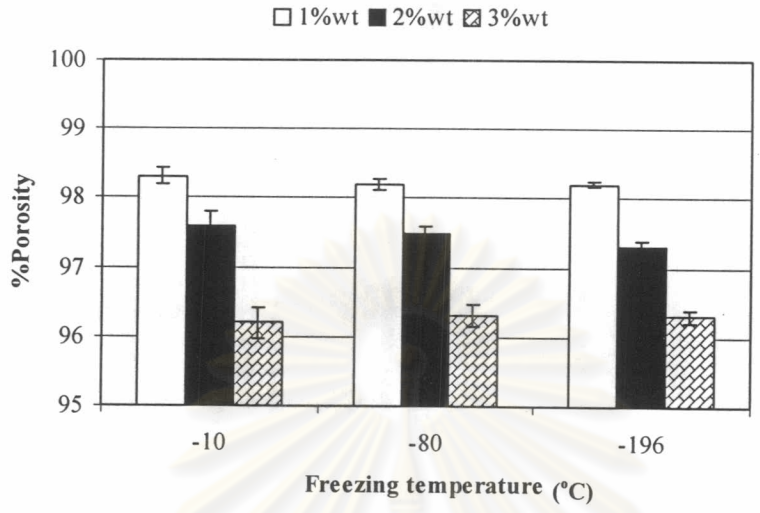


Figure 4.4 Effect of shrimp-chitosan concentrations (1, 2 and 3 %wt) and freezing temperature (-10, -80 and –196 °C) on %porosity.

4.1.3 Mechanical Property

The scaffolds prepared were subjected to compression test. The resulting scaffold from 1% solution was not investigated because it was apparently too fragile for the test. The compressive moduli of all other scaffolds prepared from both shrimp and squid chitosan are displayed in Fig. 4.5. There was no significant difference in the compression modulus between chitosan from two sources. As expected, the compressive modulus of the scaffold increased when the concentration of the solution used to prepare the scaffold increased.

Considering the effect of freezing temperature, the increasing compressive modulus was obtained when lowering the freezing temperature. This could be a result of the different morphology obtained from the different freezing temperature (-10, -80, and -196 °C). The columnar structure of the scaffolds fabricated at –80°C and –196°C seemed to yield a higher resistance to force than the round pore obtained at –10°C.

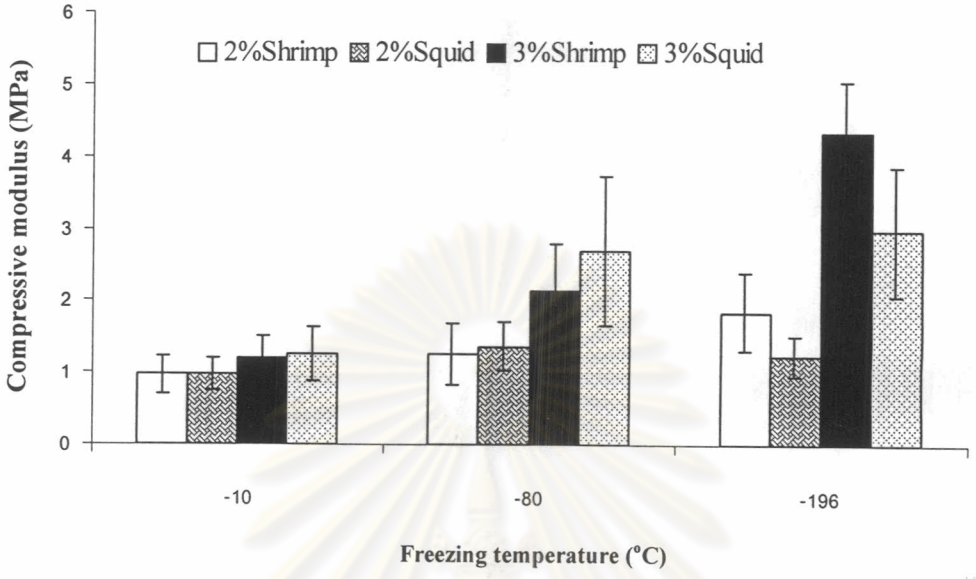


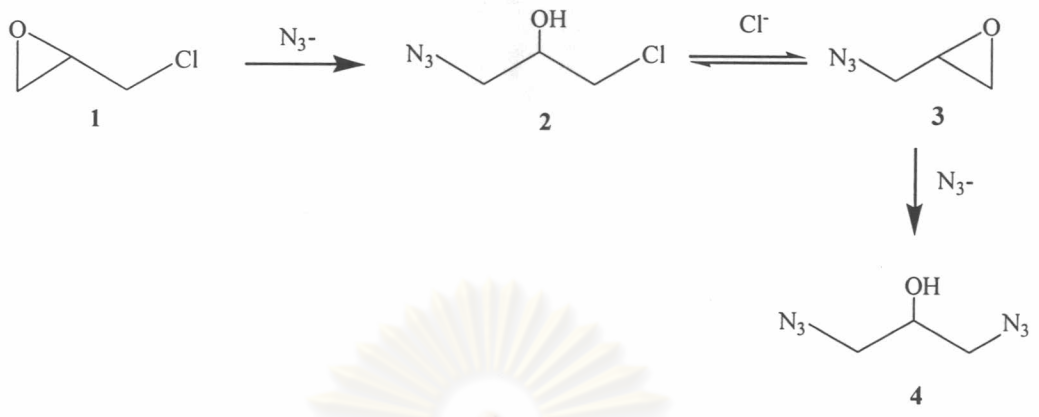
Figure 4.5 Effect of chitosan concentration and type of chitosan on compressive modulus of non-photo-crosslinked chitosan scaffolds.

4.2 Characterization of Photo-Crosslinked Chitosan Scaffolds

Crosslinking of the chitosan scaffold was carried out by means of adding 1,3-diazido-2-propanol (DAZ) into the chitosan solution (in 2% acetic acid). DAZ can be excited to produce nitrene radical by UV, thus can react with the chitosan chains. Synthesis of DAZ and characterization of the photo-crosslinked chitosan scaffolds are reported and discussed as follows.

4.2.1 Synthesis of 1,3-Diazido-2-Propanol

Scheme 4.1 shows the reactions between epichlorohydrin and sodium azide for the synthesis of DAZ. Epichlorohydrin (**1**) was ring-opened by azide ion yielding 1-azido-3-chloro-2-propanol (**2**). Ring closure of **2** resulted in the formation of glycidyl azide (**3**) and chloride ion (Cl^-). Ring-opening of the epoxide **3** by another mole of azide ion resulted in the formation of DAZ (**4**) [62].



Scheme 4.1 Ring-opening reactions of epichlorohydrin and azide ion.

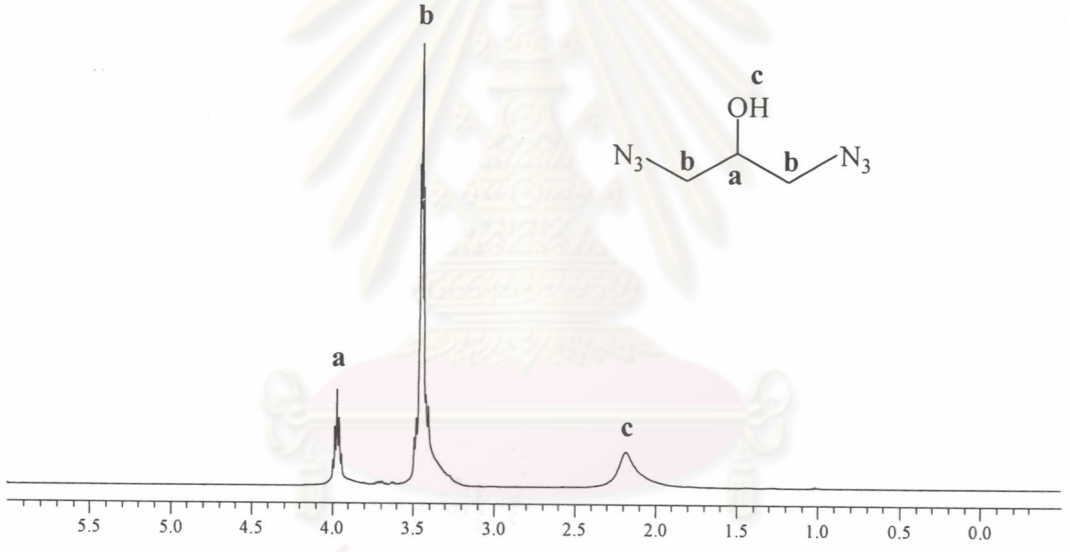


Figure 4.6 ^{1}H-NMR spectrum of DAZ.

Fig 4.6 shows ^{1}H-NMR signal of **4** in $CDCl_3$: 3.97 ppm (m, C<u>H</u>), 3.44 ppm (m, C<u>H</u>$_2$) and 2.18 ppm (s, O<u>H</u>). The physical appearance of DAZ was clear liquid and odorless.

Table 4.1 Elemental analysis result of DAZ.

Element (%)	Analytical calculation	Found
C	25.35	24.51
H	4.22	4.62
N	59.15	56.00

4.2.2 FT-IR Analysis of Photo-Crosslinked Chitosan Scaffolds

Characterization of the photo-crosslinked chitosan scaffold was carried out by FT-IR. Fig. 4.7 shows IR spectra of shrimp chitosan (SH) added with 50% mole of DAZ (or SH:DAZ = 1:0.5) with 0, 40 and 60 min of UV irradiation time. For pure chitosan (Fig 4.7 A), the strong and broad band at 3000-3500 cm^{-1} is assigned to the stretching vibration of –OH and –NH bands. Peaks at 2960-2850, 1642, 1565, 1380 and 1150-1070 cm^{-1} are assigned to C-H stretching, amide I, symmetric deformation of $-NH_3^+$, CH_3 vibration bands (in amide group), and C-O stretching bands, respectively. The NH_3^+ signal was observed instead of NH_2 (1590 cm^{-1}) because chitosan was dissolved in 2% acetic acid before analysis [66,67]. For the DAZ-added scaffolds (Fig 4.7 B, C and D), a strong band of stretching vibration azide appeared at 2108 cm^{-1}. To estimate the extent of crosslinking reaction, the peak height ratio of azide (2108 cm^{-1}) and amide I peaks (1642 cm^{-1}) were determined for the samples at various 0, 40 and 60 min of irradiation times. It was found that the peak ratio was reduced from 3.84, 2.68 to 1.40, when the irradiation time increased from 0, 40 and 60 min, suggesting that crosslinking by azide increased with irradiation time. Since the azide group can be decomposed by photolysis and thermolysis to release N_2, giving rise to a highly reactive nitrene group. The nitrene can react *via* several nonselective chemical reactions, including insertion into C-H bond, H atom abstraction, dimerization of NH to diimide (N_2H_2) [59]. All of these lead to crosslinking between the chitosan chains (Scheme 4.2).

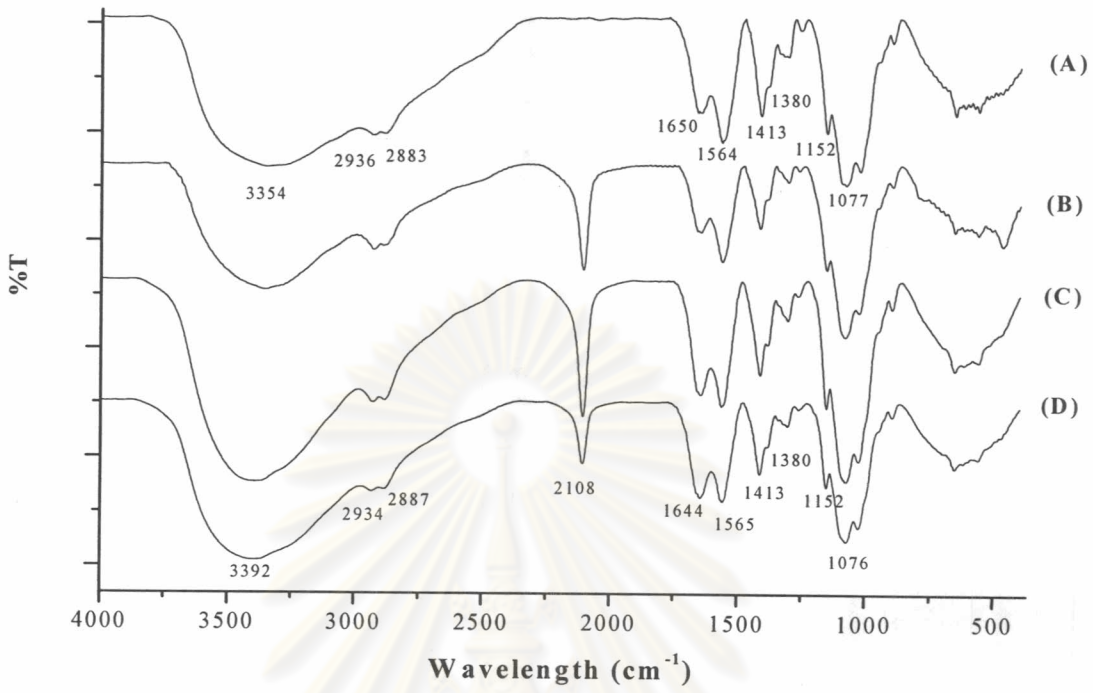
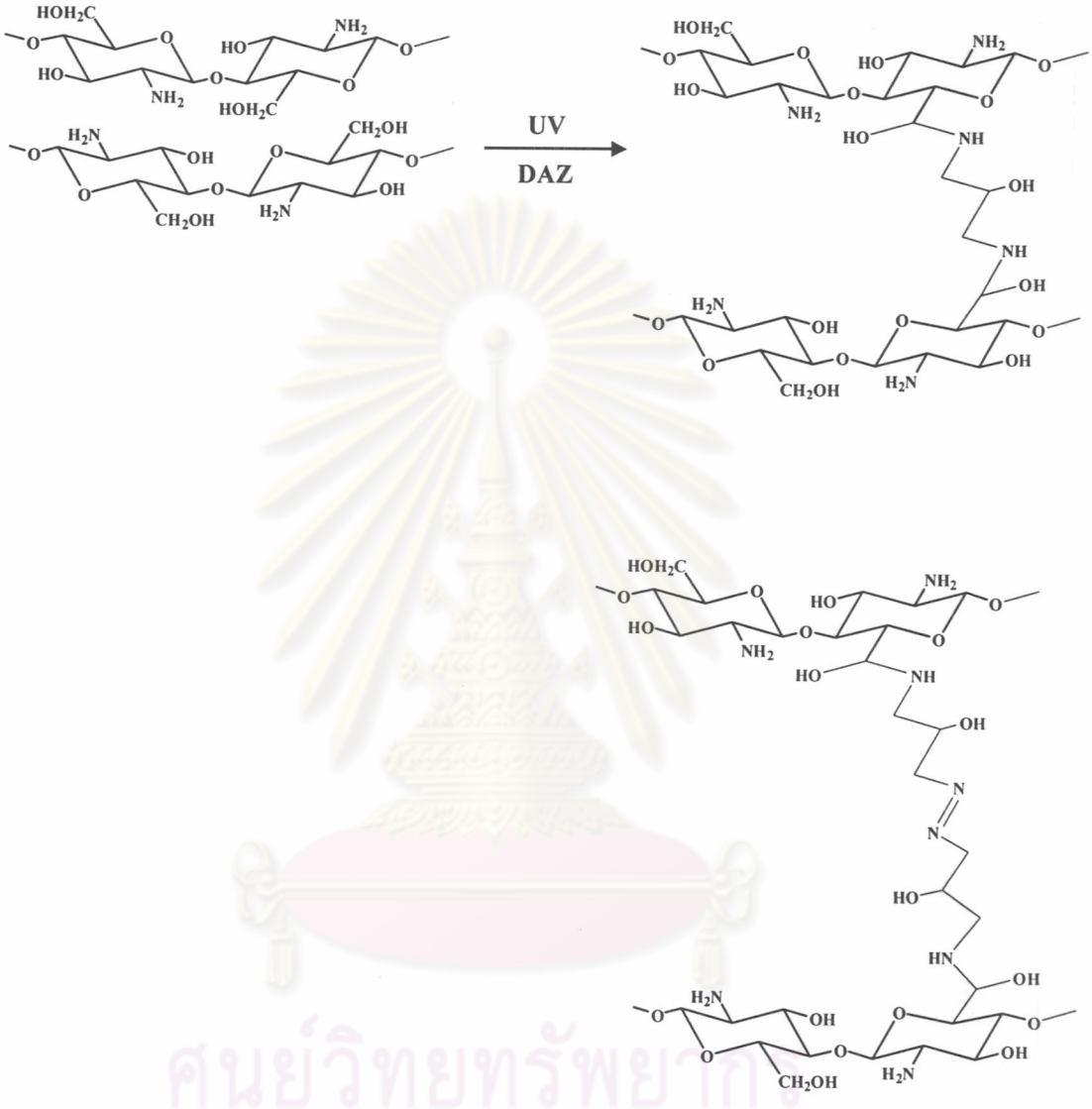


Figure 4.7 FT-IR spectra of A)shrimp chitosan (SH), B)-D)SH:DAZ (1:0.5 mole ratio) at 0, 40 and 60 min of UV irradiation time, respectively.



Scheme 4.2 Possible products from photo-crosslinking between chitosan and DAZ by UV irradiation.

4.2.3 Degradation of Chitosan by UV Irradiation

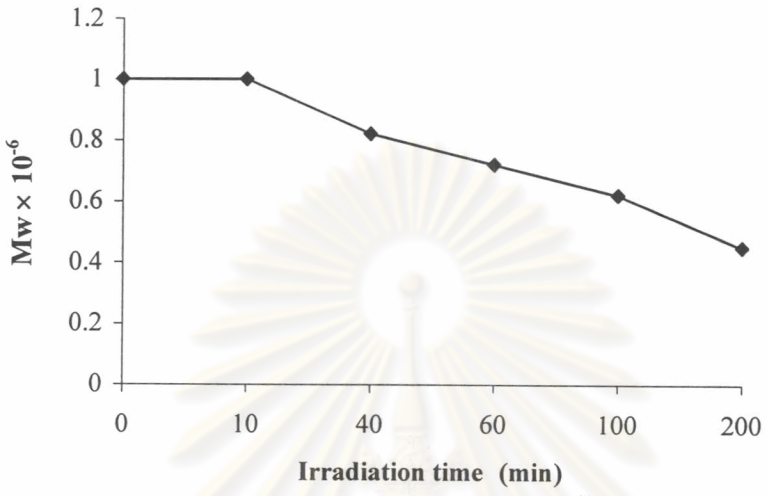


Figure 4.8 Effect of irradiation time on the degradation of shrimp chitosan.

It is generally known that chitosan can be degraded by several ways such as enzymes, acid hydrolysis, hydrogen peroxide treatment and photo-radiation [68]. In this work, the chitosan scaffolds were treated with UV, therefore, the degradation of shrimp chitosan was investigated by GPC. Fig 4.8 shows the reduction of molecular weight that clearly started after 10 min of UV irradiation. The molecular weight continuously dropped and reached one half of the original molecular weight after approximately 200 min. Sionkowska *et al.* explained the chain scission of chitosan by UV irradiation caused by cleavage at glycosidic bond that linked two glucosamine units.

4.2.4 Degree of Crosslinking

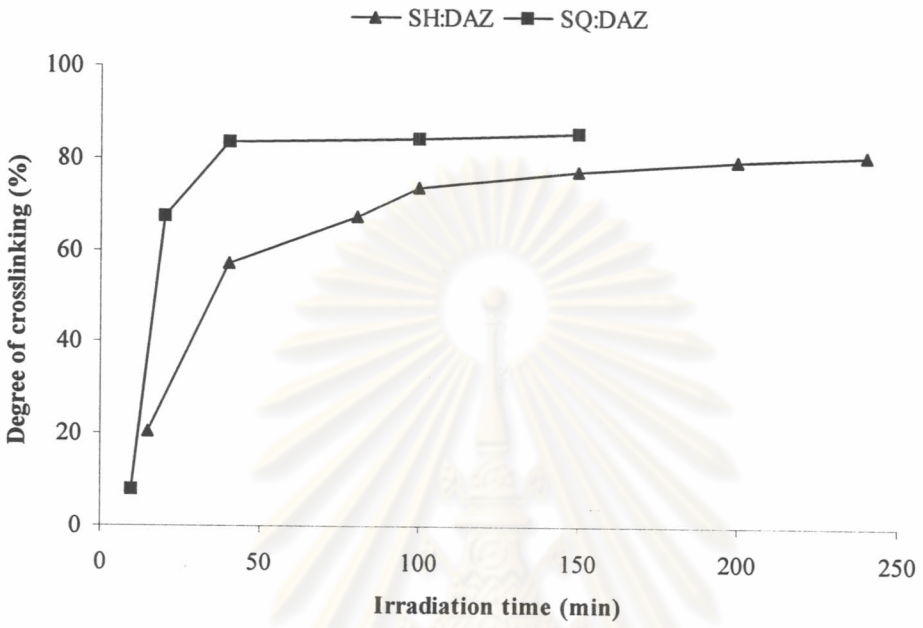


Figure 4.9 Degree of crosslinking of DAZ-added chitosan from shrimp (SH) and squid (SQ) (the mole ratio of chitosan:DAZ = 1:1) at different irradiation times.

The insoluble residue was related degree of crosslinking, due to the fact that crosslinked polymer had a lower solubility than the non-crosslinked one. As shown in Fig 4.9, the crosslinking in DAZ-added squid chitosan reached a steady amount within 40 min of irradiation, whereas that of the DAZ-added shrimp chitosan required a longer irradiation time (> 100 min). The explanation for this observation is not yet known. But it could be related to the molecular weight of squid chitosan which is 10% higher than that of shrimp chitosan.

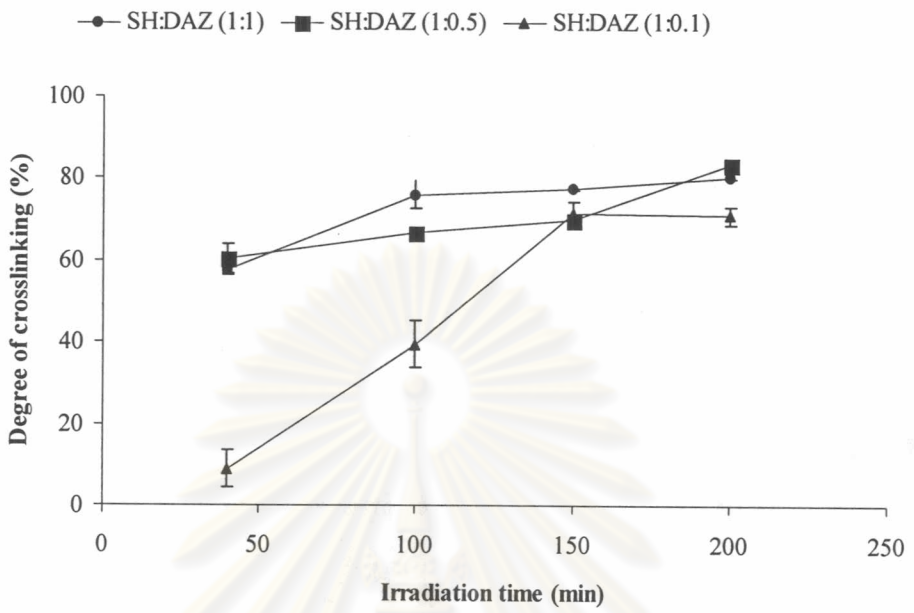


Figure 4.10 Degree of crosslinking of various mole ratios of shrimp chitosan (SH) to DAZ (1:1, 1:0.5 and 1:0.1) at various irradiation times.

Various mole ratios of shrimp chitosan and DAZ on degree of crosslinking at various irradiation time was studied. Fig. 4.10 shows that the degree of crosslinking was higher when the irradiation time increased at every mole ratio studied. In addition, when a higher amount of DAZ was used, the higher degree of crosslinking and shorter crosslinking irradiation time were obtained. However, after 150 min, a crosslinking limit was reached, where the degree of crosslinking of 70%-80% was obtained, for all samples studied.

4.2.5 Shape Retention

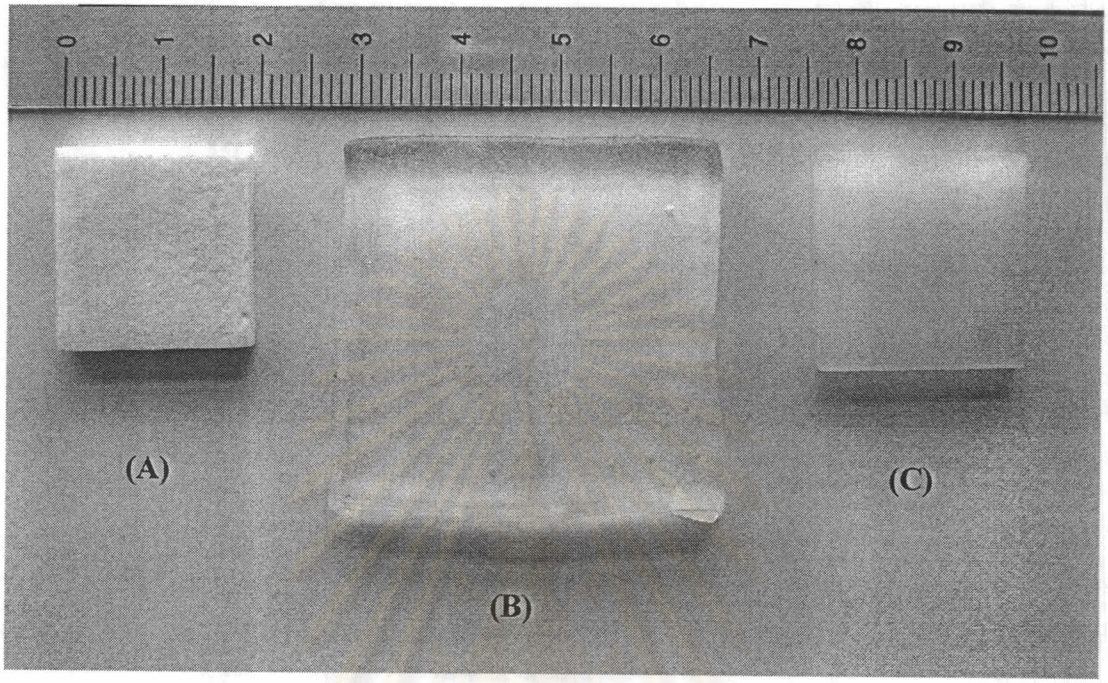


Figure 4.11 Images of shrimp chitosan scaffolds; (A) before, (B) after swelling in pH 5, and (C) photo-crosslinked chitosan (SH:DAZ = 1:0.5) at 40 min irradiation after swelling at pH 5.

As generally known, chitosan can swell in fluid, especially at lower pH. Certainly, the size of swollen pore can change from an original size which may be an obstacle to cell attachment and growth. The factors such as pH, mole ratio of SH:DAZ and UV irradiation time that can affect shape retention of pore structure in chitosan scaffolds were studied. Fig. 4.11 shows the image of the scaffolds from shrimp chitosan before (A), after swelling at pH 5 (B), and from the photo-crosslinked scaffold (C) after swelling at pH 5. The photo-crosslinked chitosan scaffold retained their shape to almost its original size. Contrary to the non-crosslinked chitosan, it expanded rapidly and lost its shape after 1 day of soaking.

Considering factor of mole ratio SH:DAZ at pH 5, it was found that %dimension change decreased when the SH:DAZ and the UV irradiation time were increased (Fig. 4.12). With the mole ratios of 1:1 and 1:0.5, the dimension increased 30-80 %, while at the mole ratio of 1:0.1, % dimension increased to >100%. These

evidences therefore confirmed that the morphology of photo-crosslinked chitosan can be kept almost constant in acidic condition usage.

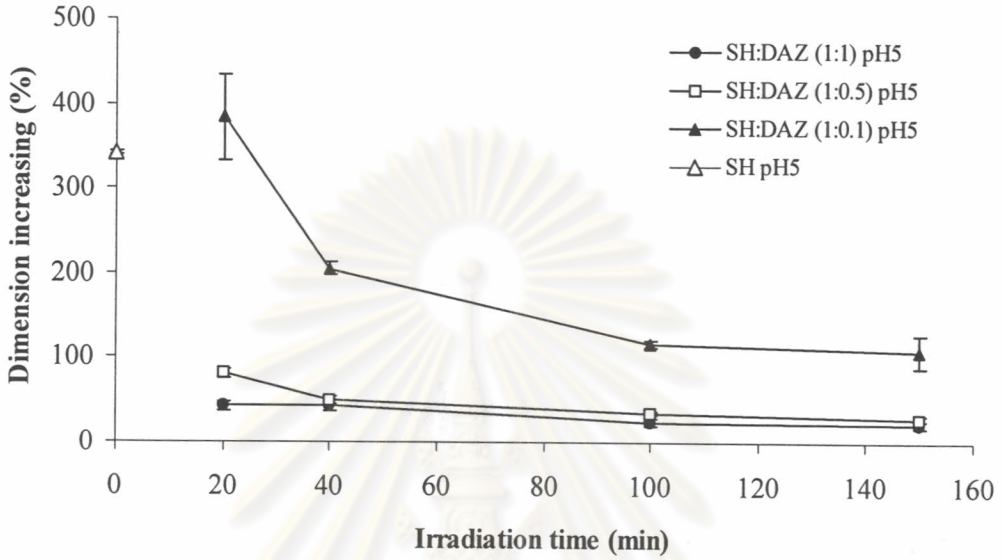


Figure 4.12 Effect of mole ratios of SH:DAZ (1:1, 1:0.5 and 1:0.1) and irradiation time on shape retention of scaffolds swelling at pH 5 (comparing the results to original chitosan).

At neutral pH (Fig 4.13), there were no significant difference in %dimension change even the mole ratio of SH:DAZ and UV irradiation time were increased. The untreated chitosan scaffold was stable only in neutral pH, whereas the one made from photo-crosslinked chitosan was stable in either lower or neutral pH. Moreover, they can retain their overall size throughout 4 weeks which is a good result for a wide range of clinical usage application.

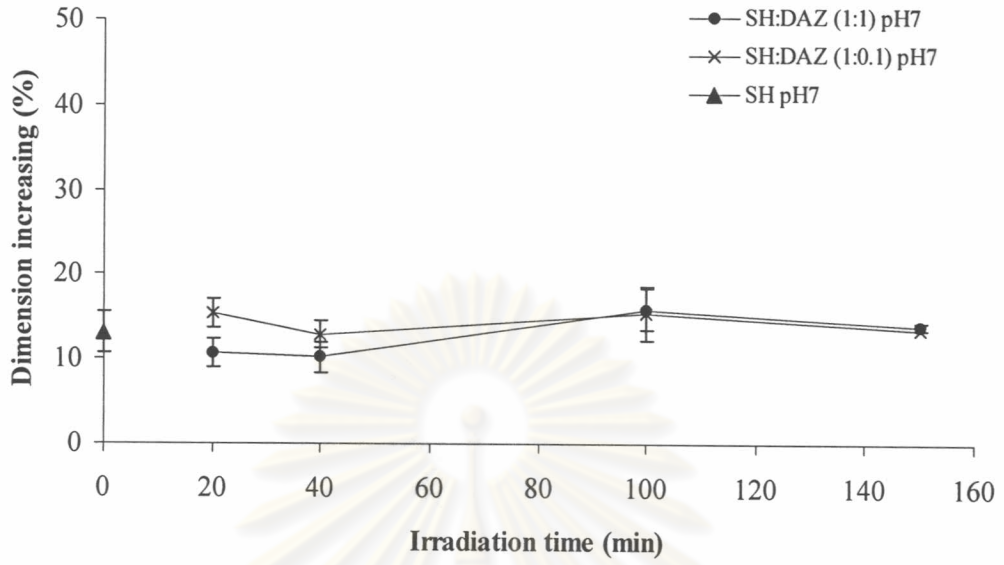


Figure 4.13 Effect of mole ratios of SH:DAZ (1:1 and 1:0.1) and irradiation time on shape retention of scaffolds swelling at pH 7 (comparing the results to original chitosan).

4.2.6 Morphology of Photo-Crosslinked Chitosan Scaffolds

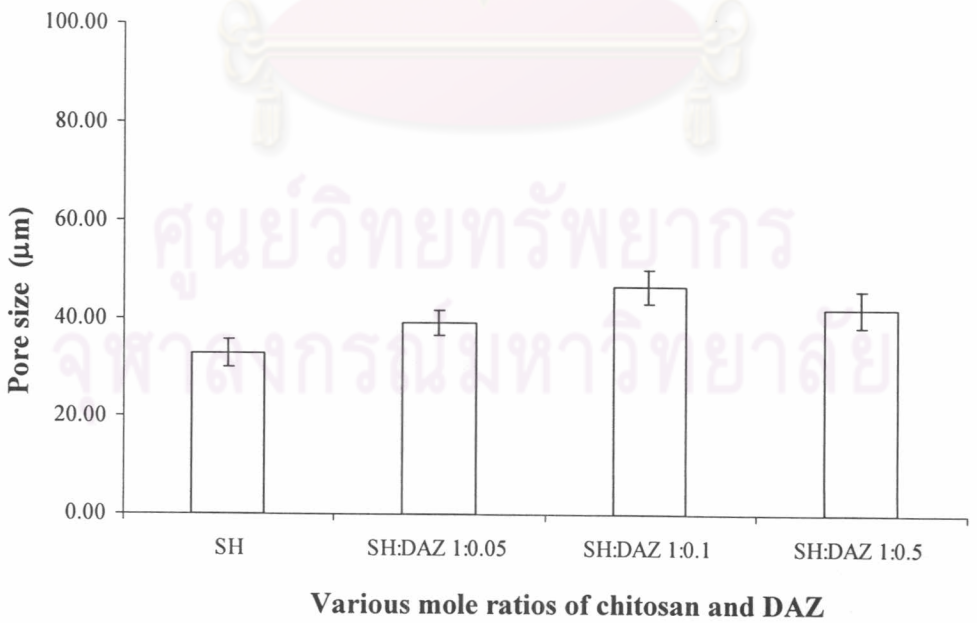


Figure 4.14 Pore size of chitosan and photo-crosslinked chitosan with various mole ratios (1:0.05, 1:0.1 and 1:1) at UV irradiation time 40 min.

In addition, from Fig 4.14, the pore sizes of photo-crosslinked chitosan were about 40 μm for all samples having various DAZ amounts, and they were not significantly different from the non-photo-crosslinked chitosan (35 μm). Therefore, it can be concluded that DAZ did not affect on ice crystal formation during freezing process of the scaffold. Also, DAZ did not affect the morphology of photo-crosslinked chitosan. The pore structure of photo-crosslinked chitosan was the same interconnected round pores as obtained from the non- photo-crosslinked chitosan (data not shown).

4.2.7 Compressive Modulus of Photo-Crosslinked Chitosan Scaffolds

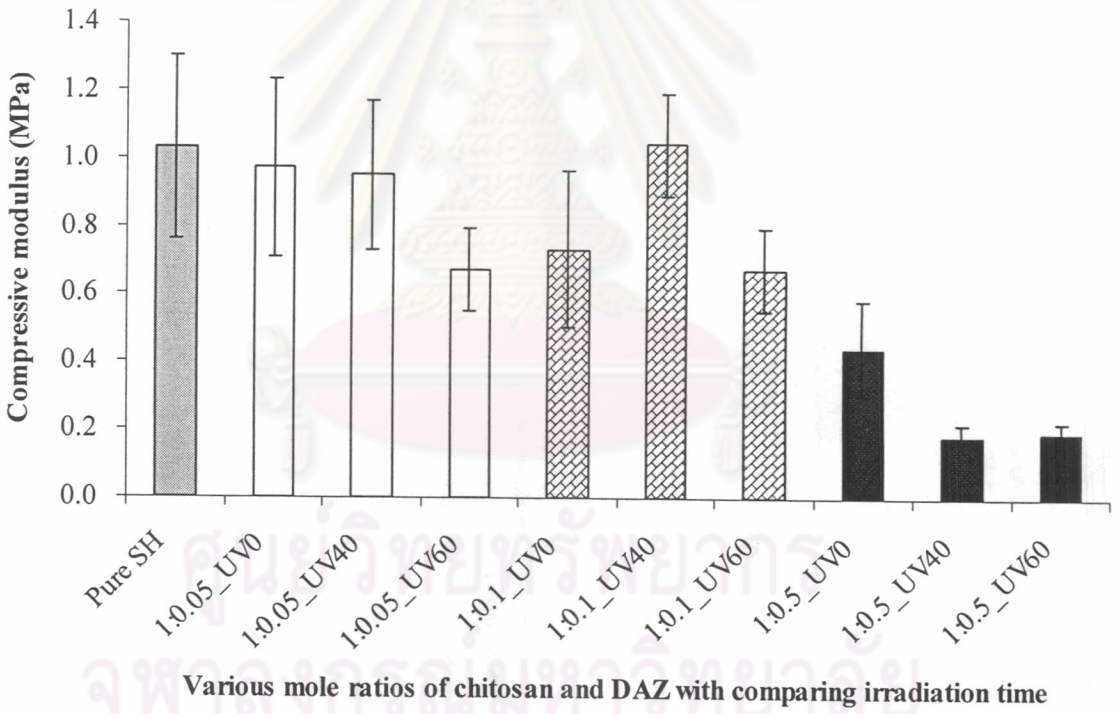


Figure 4.15 Effect of mole ratios of SH and DAZ, and irradiation time on compressive modulus.

Table 4.2 %Degree of crosslinking of SH:DAZ (mole ratios 1:0.05, 1:0.1 and 1:0.5) at 40 and 60 min irradiation time.

UV irradiation time (min)	Degree of crosslinking (%)		
	SH:DAZ (1:0.05)	SH:DAZ (1:0.1)	SH:DAZ (1:0.5)
40	2.0	8.9	60.2
60	2.2	16.2	66.9

The compressive moduli of photo-crosslinked chitosan at various mole ratios of DAZ and irradiation time were compared with scaffold from pure shrimp chitosan (SH) (Fig 4.15). At one point DAZ was suspected to cause lowering of mechanical strength of the scaffolds. The addition of plasticizer to a crystalline polymer has an effect on the reduction of mechanical properties of polymer such as stiffness, hardness, and brittleness [69]. Therefore a set of scaffolds mixed with DAZ but without exposure to light (UV0) were also analyzed for the compression test. It was found that the higher amount of DAZ added, the scaffold had a lower compressive modulus. This indicates that, DAZ acts as a plasticizer.

However, at mole ratios of 1:0.05 and 1:0.5, compressive modulus decreased when irradiation time increased. It can be suggested that photo-degradation of chitosan occurred during longer irradiation time which data already shown in section 4.2.2. Opposite to at mole ratio of 1:0.1, compressive modulus increased at 40 min irradiation time and slightly dropped with a longer irradiation time (60 min). This also showed the competition between photo-degradation and crosslinking of chitosan but influence of crosslinking was superior to degradation.

4.2.8 Cytotoxicity

The cytotoxicity of samples was evaluated using direct contact test with L929 cells from mouse. The results are shown in Fig 4.16 A to D. The high density polyethylene (HDPE) is known to be non-cytotoxic, so it was used as a negative control, while natural rubber is known to be cytotoxic and was used as a positive control.

The cells spread in A) HDPE, C) SQ:DAZ (1:1) UV 15 min and D) SQ:DAZ (1:1) UV 40 min showed normal morphology after 48 h of incubation. They were well attached and stained red. These results indicate that both photo-crosslinked chitosan scaffolds were non-cytotoxic against the L929 cells.

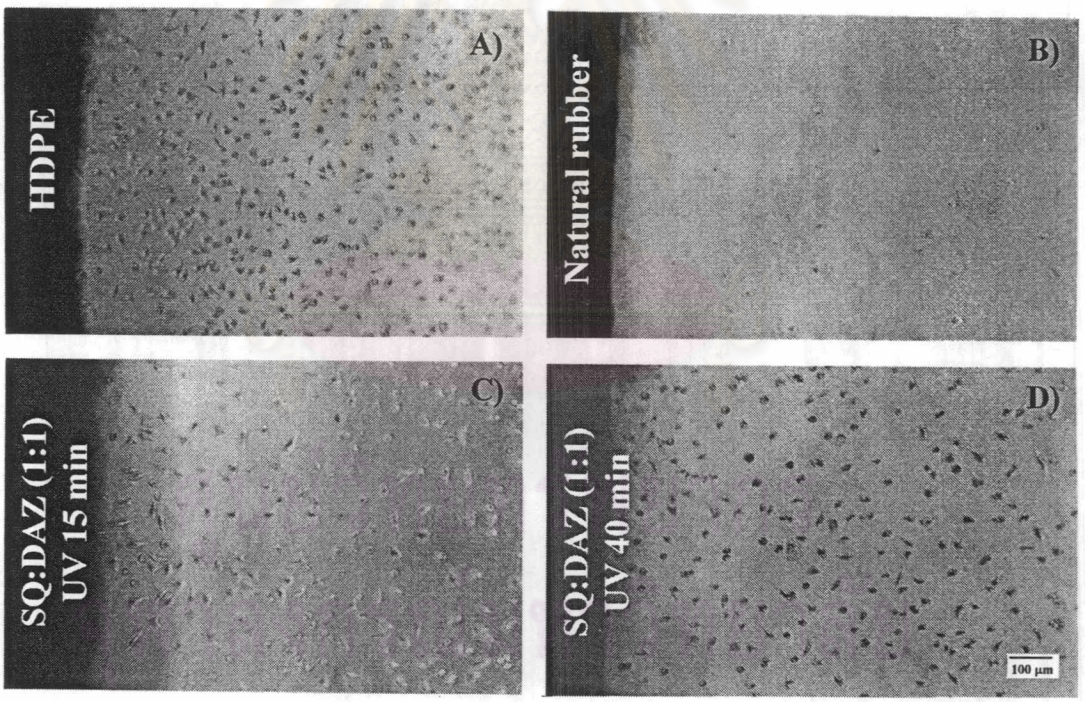


Figure 4.16 Optical micrographs (at original magnification x 100) of neutral red stained L929 cells after 48 h incubation in direct with: A)HDPE (negative control), B)natural rubber (positive control), C)SQ:DAZ (1:1) UV 15 min, and D)SQ:DAZ (1:1) UV 40 min. Material surfaces are seen as the area on the left.