CHAPTER IV
SYNTHESIS AND CHARACTERIZATION OF *N*-SUCCINYL CHITOSAN HYDROGEL FOR MEDICAL APPLICATION

4.1 Abstract

N-Succinyl chitosan hydrogel (*N*-SCG) with various degrees of succinylation (DS) (~20% - ~70%) were simply synthesized by 2 steps. Firstly, 4 wt.% citric acid was directly mixed with 16 wt.% chitosan (in 4 wt.% acetic acid) and then consequently added various amount of succinic anhydride (1-15 mol per glucosamine unit, GlcN). It was observed that the presence of ionic interaction of chitosan and citric acid could promoted the direct inter succinyl linkage between succinic anhydride and chitosan detected by Fourier-transformed infrared spectroscopy (FT-IR).The 12 *N*-SCG shows the highest inter succinyl linkage that was indicated by the highest of FT-IR ratio at 1726 cm^{-1} and the lowest of swelling ratio as same as the weight loss in both pH 2.0 and 7.4. In accordance with FT-IR and swelling ratio, 12 *N*-SCG was much higher ability to absorb cationic crystal violet dye solution and susceptible to degrade in lysozyme than other conditions. However, the higher swelling ratio of 7 *N*-SCG showed much higher dye release rate than 12 *N*-SCG and this dye release characteristic was controlled at pH7.4. Apparently, an increase of DS in the range of 20 to 70% showed low toxicity to the L929 mouse fibroblast cells evaluated by indirect cytotoxicity.

(**Key-words**: Hydrogel, *N*-Succinyl chitosan, Physico-chemical properties, Dye absorption and release)

4.2 Introduction

Hydrogel are a hydrophilic polymers and a three dimensional network which is able to retain its structure and highly swell in aqueous environment without dissolution. Due to its several physical properties which are similar to a natural tissue, hydrogel has been focused for various medical applications such as wound dressing,

drug carrier and tissue engineering. A variety of biopolymers that have been modified into hydrogel and developed for these medical proposes were alginate (Zhang, *et al.,* 2008; Jeon, *et al.,* 2009), gelatin (Tabata and Ikada, 1999; Yamamoto et al., 2000; Hori, *et al.,* 2007) and chitosan (El-Sherbiny, *et al.,* 2005; Hong, *et al.,* 2007; Kandile, *et al.,* 2009),etc.

Chitosan or poly (*N*-acetyl-D-glucosamine-*co*-D-glucosamine) deriving from chitin or poly (*N*-acetyl-D-glucosamine) is one of the abundant natural biopolymers because it is major component of shells of crustaceans such as crabs, shrimps and crawfish. Because of their similar structure to glycosaminoglycans, including hyaluronic acid, chondrotin sulfate, dermatan sulfate, heparin sulfate, and keratin sulfate, which were originated, degraded and promoted the tissue healing in our body (Nair and Laurencin, 2007), chitosan have been extensively studied for many interesting bioactivities such as biocompatibility, biodegradability (Sashiwa, *et al.*,1990; Shigemasa, *et al.,* 1994) and would healing properties (Ueno, Mori and Fujinaga, *et al.,* 2001; Şenel and McClure; 2004). Moreover, the unique amino groups of chitosan cloud enhance their mucoadhesive (Borchard, *et al.,* 1996), antimicrobial (Zheng and Zhu, 2003), and antioxidant (Sun, *et al.,* 2007) properties. Chitosan hydrogel can be classified by two main strategies (Berger, *et al.,* 2004) base on crosslinker which are covalent crosslink and ionic crosslink. The covalently crosslinked hydrogel of chitosan has been fabricated into three methods. The first method is the chemical agent such as glutaraldehyde (Gupta and Jabrail, 2006), glyoxal (Khalid, *et al.,* 1999; Gupta and Jabrail, 2006) and genipin (Muzzarelli, 2009), etc. The second method is base on hybridization with other polymers such as chitosan hybrid with polyvinyl alcohol (Rodrigues, *et al.,* 2007) and the last one is base on interpenetration network method (Marsano, *et al.,* 2005). The ionically crosslinked hydrogel of chitosan has been produced by using the anionic agent such as tripolyphosphate; TPP (Sezer and Akbuğa, 1995; Bhumkar and Pokharkar, 2006), citrate and sulfate (Shu and Zhu, 2002) that can be interacted and crosslinked with the ammonium groups of chitosan. However, the solubility and some biological properties were decreased when chitosan is used in high pH range. To improve these properties, several chemical modifications of chitosan into water soluble chitosan

derivatives including alkylation, sulfonation, carboxyalkylation, carboxyacylation have been achieved.

Among these chitosan modifications, carboxyacylation is one of water-soluble chitosan derivatives synthesized by chitiosan and anhydrides. Various types of anhydrides have been used such as maleic anhydride, succinic anhydride, citarconic anhydride and phathalic anhydride, etc. Among these anhydrides, succinic anhydride has been widely used to produce *N*-succinyl chitosan (*N*-SC) due to its solubility in wide rage of pH (Sashiwa and Shigemasa, 1999) and has been proved for biocompatibility and *in vivo* low toxicity (Song, Onishi and Nagai, 1993). Due to its anionic characteristic, *N*-succinyl chitosan shows low interaction with tissue which developed for wound dressing (Kuroyanagi, *et al.*, 1994; Tajima, *et al.*, 2000), prolongs its life time in blood circulation (Kato, Onishi and Machida,2004) and chelates with various heavy metals by using lead (Sun and Wang, 2006) and cupper (Sun, Wang and Wang, 2007) as a template. Previously, *N*-SC has been fabricated by these covalent and ionic linkages. To form covalently crosslinked hydrogel, 1- ethyl-3- (3-dimethyl aminopropyl carbodiimide; EDC) (Yamaguchi, *et al.*, 1981) and gultaraldehyde (Suna and Wang, 2006) were used to form amide and imine linkages, respectively. To form ionically crosslinked hydrogel, tripolyphosphate (Rekha and Sharma, 2008) and calcium ion (Nobile, *et al.*, 2008) were used to form ionic linkage at residue ammonium and carboxylated group, respectively. Theoretically, covalent linkage (chemical bond) is stronger and more stable than ionic linkage (physical bond), however the limitation of the use of chemical crosslink agent is the toxicity.

Therefore, the aim of this present work is to produce the covalently crossliked hydrogel of *N*-SC by the use of succinic anhydride as a crosslinker promoted by the presence of citric acid. Citric acid is a weak organic acid naturally occurring in lime, lemon or orange (citrus fruit). Due to its low toxicity, citric acid is commonly used as a natural preservative and food additive in soft drink, meat, candies, etc. To serve *N*-SC hydrogel as biomaterials for medical propose, the physico-chemical characteristic, absorption-release characteristics, biodegradability and cytotoxicity of this synthesized *N*-SC hydrogel were further investigated.

4.3 Experimental

4.3.1 Materials

Crab chitosan (DD =83.61 %; H^1-NMR), Mw =50,000 Da) in flake form were supplied from Koyo Co.,Ltd.,(Japan). Succinic anhydride (Wako Pure Chemical Industries Co.,Ltd.,(Japan), crystal violet (Aldish, > 75% dye) and lysozyme from hen egg white from Fluka (85400 u/mg) were used. The other chemicals were reagent grade and used as received.

4.3.2 Preparation of *N*-succinyl chitosan hydrogel (*N*-SCG)

A viscous chitosan solution was firstly prepared by dissolving 4 g of chitosan flake in 25 ml of 4% v/v acetic acid solution and 1 g of citric acid was added consequently. To vary the degree of substitution (DS), various amount of succinic anhydride (1, 3, 5, 7, 9, 12 and 15 mol per glucosamine unit; GlcN) were used. Each amount of succinic anhydride was gradually mixed with chitosan/citric acid and minimum amount of distilled water was consequently added (10-160 ml) at each condition in order to dissolve all of succinic anhydride. This reaction was further reacted at 37°C for 24 h followed with solidified and washed with ethanol to get rid of unreacted succinic anhydride. The obtained solid *N*-SC products were re-dispersed in distilled water and adjusted with 0.5 M NaOH to pH 7-8 in order to separate the water soluble fraction. The water soluble of *N*-SC (*N*-SCW) fraction was precipitated in ethanol and washed several times with 70, 80 and 95 % ethanol to remove salt and dried in air, while *N*-SCG fraction was dialyzed against distilled water in order to remove salt for ~1 week by changing water every day and finally solidified in ethanol and keep in solid form.

4.3.3 Preparation of *N*-succinyl chitosan films (*N*-SCG)

The *N*-SCG films were prepared by casting 1 wt.% of *N*-SCG into polystyrene mold and then dried at 40 °C for 48 h. The dried *N*-SCG films were cut into circular discs (15 mm in diameter) and kept in desicator before experiment.

4.3.4 Characterization

4.3.4.1 Structural Characterization

Fourier-transformed infrared spectroscopy (FT-IR) was used to characterize the structure of all the *N*-SCW and *N*-SCG products. The products were purified in absolute ethanol before measuring. The sample pellets were prepared by mixing the exact weight of precipitated sample and KBr powder (5:80w/w) recorded on a Thermo Nicolet Nexus 670 FT-IR spectrometer at a resolution of 4 cm^{-1} and 32 scans over the wavenumber range of 400-4,000 cm^{-1}.

The chemical structure of chitosan and all of the *N*-SCW and *N*-SCG products on the values of succinyl groups substituted at the *N*-positions along the chitosan chains (i.e., the degree of substitution, DS) was analyzed by INOVA Varian 500 mHz nuclear magnetic resonance (NMR) equipped with the VnmrJ software. CD_3COOD in D_2O (2 wt.%) was used as a deuterated solvent for chitosan,D_2O for all of the *N*-SC products and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as the internal standard (0.0 ppm).The DS values of all of the *N*-SC products were calculated according to the following equation (Sashiwa and Shigemasa,1999):

$$DS\ (\%) = \frac{\text{Intergrated area at (2.44 - 2.52) ppm}}{4 \times \text{Intergrated area at (2.70 - 3.00) ppm}} \times 100, \qquad (1)$$

4.3.4.2 Swelling and Weight Loss Characteristics

The swelling ratio of various DS of *N*-SCG films was determined by incubation *N*-SCG films in 20 ml of buffer solutions at 37 °C, 50 rpm for 24 h, after that *N*-SCG films were removed, rinsed with distilled water, carefully blotted the excess of buffer solution and weighted immediately. The swelling characteristic of *N*-SCG films was performed in both citric/HCl buffer solution pH 2.0 (0.03 M citric/0.0082 M HCl), and phosphate buffer solution (PBS) pH 7.4 (1.3 mM$NaHPO_4$/8.8mM Na_2HPO_4).The ionic strength of these solutions was adjusted to 0.15 M by adding appropriate amounts of NaCl. The weight loss of *N*-SCG films was consequently determined after drying the swollen *N*-SCG films in an oven at 40 °C for overnight. The swelling ratio and weight loss of *N*-SCG films were calculated according to the following equations:

$$\text{Swelling ratio} = \frac{W_s - W_o}{W_o}, \quad (2)$$

and

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

Where W_s is the wet weight of the swollen *N*-SCG films (mg), W_0 is the dry weight of original *N*-SCG films (mg) and W_d is the dry weight of *N*-SCG films after incubation in buffer solution.

4.3.5 Dye absorption and release characteristics

The absorption and release characteristics of various DS of *N*-SCG films were studied with cationic crystal violet dye (Figure 1.). The dye stock solution at concentration 0.0221M was firstly prepared by dissolving crystal violet dye in ethanol and further diluting in distilled water to 0.221 mM for using as working solution. The absorption characteristic of *N*-SCG films was performed by immersing various DS of *N*-SCG films in 40 ml of 0.221mM of dye solution at 37°C and 50 rpm. At different time interval from 0 min to 2880 min, 1 ml of the dye solution was taken out.

The release characteristic of dye loaded *N*-SCG films were subsequently determined by the total immersion method in both of citric/HCl buffer solution at pH 2.0 and PBS at pH 7.4. At the same condition, the dye loaded *N*-SCG films were immersed in 40 ml of buffer solutions, 1.0 ml of buffer solution was also taken out and 1 ml of fresh buffer solution was added subsequently to maintain the same volume of solution at a predetermined time interval between 0 and 1440 min. Additionally, the actual amount of dye in the dye loaded *N*-SCG films was determined by immersing the remaining released dye of *N*-SCG films in citric/HCl buffer solution at pH 2.0.

The concentrations of taken dye solution were detected by UV-vis at maximum wavelength (λ_{max}) 594 nm calculated against calibration curve. The experiments were done in triplicate and the mean values were calculated and taken as the percentage of dye absorption and accumulate release based on weight of *N*-SCG films (i.e., mg of released dye /mg of *N*-SCG film ×100).

4.3.6 Biodegradability

The various DS of *N*-SCG films were test on biodegradability by using hen egg white lysozyme as an enzyme. The various DS of *N*-SCG films were incubated in PBS or 10 mg/l of lysolyme/PBS at 37°C from time interval from 0-2 days. At specific time, the *N*-SCG films were taken out, rinsed with distilled water to remove residual salt and dried in oven at 40°C for overnight. The weight loss was measured as equation (3) and the same sample was re-incubated in the fresh buffer solution and/or lysolyme/PBS before measuring the weight loss again. Finally, the lysozyme degradability of *N*-SCG films was calculated by the difference between the weight loss in PBS and lysozyme/PBS solution at the same time interval.

4.3.7 Indirect cytotoxicity evaluation

The various DS of *N*-SCG films were test on indirect cytotoxicity by adaptation test from the ISO 10993-5 standard test method in a 24-well tissue-culture polystyrene plate (TCPS; Nunclon^{TM}, Denmark) using mouse fibroblasts (L929) as reference cells. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., USA), supplemented with 10 wt.% fetal bovine serum (FBS; Invitrogen Corp., USA), 1 wt.% L-glutamine (Invitrogen Corp., USA) and 1 wt.% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)].To produce extraction media, various DS of *N*-SCG films were sterilized by 75% EtOH for 0.5 h before immersing in serum-free medium (SFM; containing DMEM, 1 wt.% L-glutamine, 1 wt.% lactalbumin, and 1 wt.% antibiotic and antimycotic formulation) and then incubated for 1 and 7 days. To allow cell attachment, L929 cells were separately cultured in 24-well TCPS at 40000 cells/well in serum-containing DMEM for 16 h. The cells were then starved with SFM for 24 h before replacing SFM with an extraction medium and re-incubated for 1day. Finally, the viability of the cells cultured by each of the extraction media of various DS of *N*-SCG films for 1 and 7 days was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the viability of the cells cultured by fresh SFM was used as control. The experiment was done in triplicate. The statistical analysis of different

data sets was performed using student t-test and statistical significance was accepted at a 0.05 confidence level ($p < 0.05$).

4.4 Results

4.4.1 Characterization of interaction between chitosan and citric acid

FT-IR spectra of chitosan and chitosan/citric acid are shown in Figure 4.2., original chitosan showed characteristic peaks at 3445,1640,1570,1375 and 1070 cm^{-1} assigned to O-H and N-H stretching, amide I, amide II, amide III and skeletal vibration involving the C-O stretching, respectively (Zhang, *et al.*, 2003). It was observed that characteristic peak of chitosan/citric acid at 1570 cm^{-1} increased significantly due to the formation of ionic interaction between ammonium (NH_3^+) groups of chitosan and carboxylated (COO^-) groups of citric acid (Bhumkar and Pokharkar,2006). Because of the cationic character, chitosan can be formed the ionic complxation with several polyanionics such as chitosan/alginate (Lawrie *et al.*, 2007), chitosan/polyaspartic acid (Zheng *et al.*, 2007), and chitosan/gum kondagogu (Naidu, *et al.*, 2009) and/or with various types of anions such as chitosan/triphosphate (Bhumkar and Pokharkar, 2006), chitosan/sulfate and chitosan/citrate (Shu and Zhu, 2002), etc. To confirm this electrostatic interaction between chitosan and citric acid, this chitosan/citric acid mixture was also confirmed by adjusting pH of chitosan/citric acid solution with 0.1 M HCl solution. The result suggested that the turbid solution of chitosan (pKa ~ 6.3) and citric acid (pKa ~ 3.15-6.4) at initial state (pH 4.0) was gradually turned to clear solution when the pH of solution was gradually decreased until pH ~2.5 as shown in Figure 4.3. It could be verified that the ionic interaction between chitosan and citric acid presented at initial chitosan/citric acid condition (16 wt. % chitosan/4 wt. % acetic acid and 4 wt. % citric acid) was destroyed by an excess amount of acid (H^+) due to the conversion of COO^- groups of citric acid to COOH groups.

4.4.2 Characterization of *N*-SCW and *N*-SCG

4.4.2.1 Structural Characterization

According to this synthesis pathway, the yield percentage of both *N*-SCW and *N*-SCG were obtained as summary in Table 4.1. The result showed

that the yield percentage of *N*-SCG obtained in the range of ~35% - ~44% was lower than that of the *N*-SCW obtained in range of ~55% - ~61%. The structure of both fractions was characterized by using FT-IR and ^{1}H-NMR. Compared to chitosan and chitosan/citric acid (Figure 4.4.), the FT-IR spectra of *N*-SCW and *N*-SCG showed the new characteristic peak at 1726 cm^{-1} assigned to the carbonyl group of amide bond (succinyl linkage) between chitosan and opened succinic anhydride and appeared in all conditions except 1 *N*-SC condition, while the characteristic peak at 1660 cm^{-1} assigned to the amino group of amide I and/or amide linkage appeared in all *N*-SCW and *N*-SCG conditions. As Figure 4.5., FT-IR spectra of *N*-SCW and *N*-SCG showed the peak ratio at 1660 cm^{-1} which was greater than the peak ratio at 1726 cm^{-1}(using peak at 1070 cm^{-1} as reference peak ;Shigemasa, *et al.*,1996) and the peak ratio at 1660 $cm^{-1}$of *N*-SCG tended to increase with an increase in an amount of succinic anhydride, while the peak ratio at 1660 $cm^{-1}$of *N*-SCW was fluctuated with an increase in an amount of succinic anhydride because this peak position was represented to all of amino groups at amide I and amide (inter, intra linkage and salt form) positions. Apparently, the peak ratio at 1726 cm^{-1} of *N*-SCG tended to increase with an increase in an amount of succinic anhydride and the highest ratio of this peak was found at 12 *N*-SCG and this peak ratio of *N*-SCG was also greater than that of *N*-SCW showed the same value with an increase in an amount of succinic anhydride. As previously studied, the FT-IR spectra of *N*-SCW showed the characteristic peak at 1648 cm^{-1} assigned to the succinyl substitution group in its salt form and no peak at 1726 cm^{-1} (Yan, *et al.*,2006;Vanichvattanadecha *et al.*,2010). As a result of FT-IR, most of inter succinyl linkages are shown at *N*-SCG fraction, while most of their salt form of succinyl group and some of intra succinyl linkages due to the presence of peak at 1726 cm^{1} are shown at *N*-SCW fraction.

^{1}H-NMR spectra of chitosan shows the characteristic signal at chemical shift 2.06-2.08 ppm (H of acetyl groups), 3.15 ppm (H2 of glucosamine, GlcN), 3.3-4.3 ppm (H2 of *N*-acetyl glucosamine, GlcNAc; H3, H4, H5 and H6 of GlcNAc and GlcN) and 4.55 ppm (N**H**$COCH_3$) (Zhang, *et al.*, 2003). In comparison with chitosan, the characteristic signal of *N*-SCW and *N*-SCG of succinyl substitution group are chemical shift at 2.44-2.52 ppm assigned to the H of $NH_2C\mathbf{H}_2C\mathbf{H}_2COOH$, 2.70-3.00 ppm assigned to the H2 of GlcN (Kato, Onishi and Machida, 2000) and 4.46-4.52

ppm assigned to the H of N**H**$COCH_2CH_2COOH$ (Aiping, *et al.*, 2006; Yan, *et al.*, 2006) as shown in Figure 4.6. The DS values of the *N*-SC products can be calculated from the ^{1}H-NMR signals according to Equation (1). As shown in Figure 4.7., the DS of *N*-SCG was obtained from ~20% (1 *N*-SCG) to ~70% (15 *N*-SCG) while the DS of *N*-SCW was obtained from ~30% (1 *N*-SCG) to~ 90% (15 *N*-SCG) with an increase in amount of succinic anhydride from 1 to 15 molar ratio. According to FT-IR and NMR results, the mechanism of both *N*-SCW and *N*-SCG formation could be proposed as shown in Figure 4.8.

4.4.2.2 Swelling and Weight Loss Characteristic of N-SCG Films

The swelling and weight loss characteristic of *N*-SCG films were performed in both acidic and basic buffer solutions at 37 °C as shown in Figure 4.9. At pH 2.0, the swelling ratio of *N*-SCG films was impossible to determined at 1*N*-SCG due to its all dissolution at this pH, however the swelling ratio tended to decrease from ~31.36 at 3 *N*-SCG to ~21.57 at 15 *N*-SCG, while the swelling ratio at pH 7.4 tended to increase with an increase in DS from ~8.31at 1 *N*-SCG to ~29.73 at 5 *N*-SCG and tended to decrease to ~25.68 at 15 *N*-SCG. Comparing between pH 2.0 and pH 7.4, low DS of *N*-SCG films showed greater swelling ratio than that at pH 7.4, while the high DS of *N*-SCG showed greater swelling ratio at pH 7.4 than that at pH 2.0. It was suggested that the swelling characteristic of *N*-SCG of low DS of *N*-SCG containing many of NH_2 groups in their structure can be greater protonated and expelled each other at low pH than that of high DS of *N*-SCG. Contrary to low DS, high DS of *N*-SCG containing many of succinyl linkages in their structure can be greater converted into COO^- groups and expelled each other at pH 7.4. However, the decrease of swelling ratio in both pHs when an increase in DS was found at 12 *N*-SCG (~19.19 at pH2.0 and ~22.30 at pH 7.4). This swelling ratio results were consistent with the highest FT-IR ratio at 1726 cm^{-1} referred to the highest ratio of inter succinyl linkages at this 12 *N*-SCG condition. As Figure 4.10, the weight loss of *N*-SCG films was found from ~12.80% to ~56.06% at pH 2.0 and ~5.29% to ~20.14% at pH 7.4. It was also found that the lower weight loss of *N*-SCG films was observed at the lower swelling ratio at each pHs condition due to the lower dissolution from the solution penetration into their structure.

4.4.3 Dye Absorption and Release Characteristic of *N*-SCG Films

The cationic crystal violet dye was used as an ionic drug model for absorption and release characteristic of *N*-SCG films. Firstly, this dye was absorbed on the various DS of *N*-SCG films in dye solution and then the dye loaded *N*-SCG films were further released in buffer solutions pH 2.0 and pH 7.4. The higher DS of *N*-SCG was much higher absorbed crystal violet dye than the lower DS of *N*-SCG as shown in Figure 4.11. The lowest DS of *N*-SCG (1 *N*-SCG) can be absorbed crystal violet dye ~10.48%, while the highest DS of *N*-SCG (12 *N*-SCG) can be absorbed crystal violet dye ~41.17%. It can be concluded that the absorption of crystal violet dye at higher DS of *N*-SCG was greater than that of lower DS of *N*-SCG because higher DS of *N*-SCG containing more succinyl linkages can be greater converted to COO^- groups in an aqueous solution and ultimately interacted with cationic groups of crystal violet dye by electrostatic interaction.

The release characteristic of dye loaded *N*-SCG films was shown in Figure 4.12. and Figure 4.13. It was found that the crystal violet dye was gradually released at buffer solution pH 7.4, while it was rapid release at buffer solution pH 2.0. The release profiles of this dye in both pHs also suggested that the higher amount of crystal violet dye absorption the higher release amount of crystal violet dye was observed. However, the dye loaded of 7 *N*-SCG films was much higher release rate and higher release an amount of dye in both pH 2.0(~60.01%) and pH 7.4(~35.67%) than that at dye loaded of 12 *N*-SCG films (pH2.0 ~29.79%, pH7.4 ~27.77%)because the swelling characteristic of 7*N*-SCG films was higher than that at 12 *N*-SCG films in both pHs which can be highly expanded their structure and then highly released an amount of crystal violet dye in these solutions. At pH 2.0, the crystal violet dye showed low ionic interaction with the all of *N*-SCG films due to the repulsion of cationic charges between protonated amino groups of and *N*-SCG and crystal violet dye. Comparison with that in pH 2.0, the crystal violet dye was gradually released in pH 7.4 because the anionic charges of COO^- groups were interacted with the cationic charges of crystal violet dye then the *N*-SCG films can sustain the release rate of crystal violet dye. It can be suggested that the release characteristic of dye loaded *N*-SCG films was controlled by DS of *N*-SCG, swelling degree of *N*-SCG films and the pH of buffer solution.

4.4.4 Biodegradability

The biodegradability of *N*-SCG films was studies by measurement of the change of their weight in lysozyme solution at concentration 10 mg/l lysozyme/PBS which is the range of concentration of lysozyme in human serum founded in range of 4 -13 mg/l (Muzzarelli, 1993). As shown in Figure 4.14. and Figure 4.15., the weight loss of *N*-SCG in PBS was lowest at 1 *N*-SCG (~14.44%) and was highest at 7 *N*-SCG (~22.34%) for incubation time within 2 days corresponded to the swelling behavior of *N*-SCG films at this pH condition. Contrary to the weight loss of *N*-SCG films in PBS, the weight loss of *N*-SCG films in lysozyme/PBS was significantly higher than that in PBS and also the weight loss of *N*-SCG films increased with an increase in the DS of *N*-SCG by the weight loss was lowest at 1 *N*-SCG (~32.54%) and highest at 12 *N*-SCG (~ 82.07%) for incubation time within 2 days. This result suggested that the inter succinyl linkage was susceptible to degrade in lysozyme/PBS corresponding to the susceptibility of amide bond of methacrylic acid grafted chitosan (Hong, *et al.*, 2007) amide and/or ester bond of methacrylate alginate (Jeon, *et al.*, 2009) to lysozyme degradation. It can be concluded that the degradability of *N*-SCG films in PBS was depended on the swelling degree caused by hydrolytic reaction. Unlike the degradability of *N*-SCG films in PBS, the degradability of *N*-SCG film in lysozyme/PBS was depend on the DS caused by both of hydrolytic reaction occurred at the glycocidic linkage and enzymatic reaction occurred at the glycocidic linkage and amide bond (Hong, *et al.*, 2007).

4.4.5 Indirect Cytotoxicity Evaluation

The cytotoxicity of *N*-SCG films was determined by indirect cytotoxicity evaluation. The extraction medium of various DS of *N*-SCG films that had been incubated for 1 and 7 days was used as a culture medium for the L929 reference cells and compared with that of fresh medium. It was obviously shown that the cell viability after culturing the cells with the extraction medium with various DS of *N*-SCG films exhibited the cell viability in the range of ~88.17% - ~103.37% for 1 day of incubated medium and slightly increased of a number of cells viability in the range of ~97.20% - ~110.59% for 7 days of incubated medium (Figure 4.16.). Apparently, the releasing by-product of *N*-SCG films (i.e., some dissolution part of

N-SCG) can promote this cell growth. Therefore, this synthesized *N*-SCG which was the DS from ~44% to~ 70% can be served as a non-toxic material.

4.5 Conclusion

The *N*-SCG was successfully synthesized by the succinylaion of chitosan and succinic anhydride directly covalent crosslinked to the chitosan chains after the ionic interaction of chitosan and citric acid. It can be verified by ^{1}H-NMR that the DS of *N*-SCG was increased with an increase in an amount of succinic anhydride. The increase of inter succinyl linkage of *N*-SCG was confirmed by an increase of FT-IR ratio at carbonyl group of amide bond (1726 cm^{-1}) and the decrease of swelling ratio which an increase the DS. Because of their anionic properties, the higher DS of *N*-SCG films was much higher absorbed cationic crystal violet dye due to their higher conversion of succinyl linkages to ended carboxylate groups and much higher release an amount of this dye in the medium. However, the release also depends mainly on both of pH of media and degree of swelling. Moreover, this succinyl linkage of *N*-SCG films was greatly susceptible to the lysozyme degradation. As a result of their swelling, absorption, release, biodegradability and cytotoxicity, this *N*-SCG may be continuously developed as novel controllable biodegradable materials.

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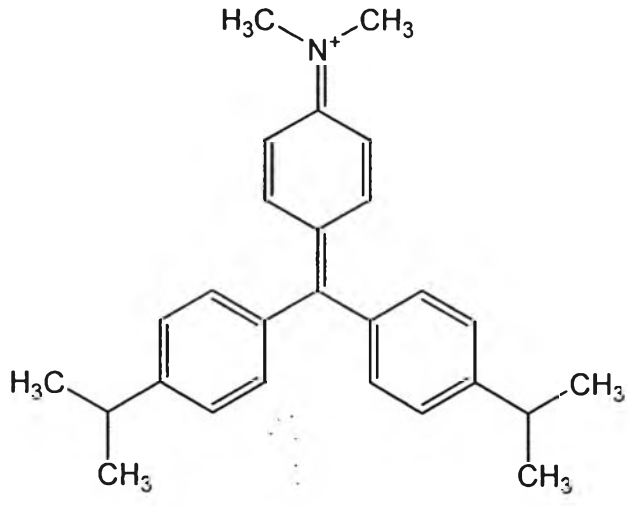

Figure 4.1 Chemical structure of crystal violet dye.

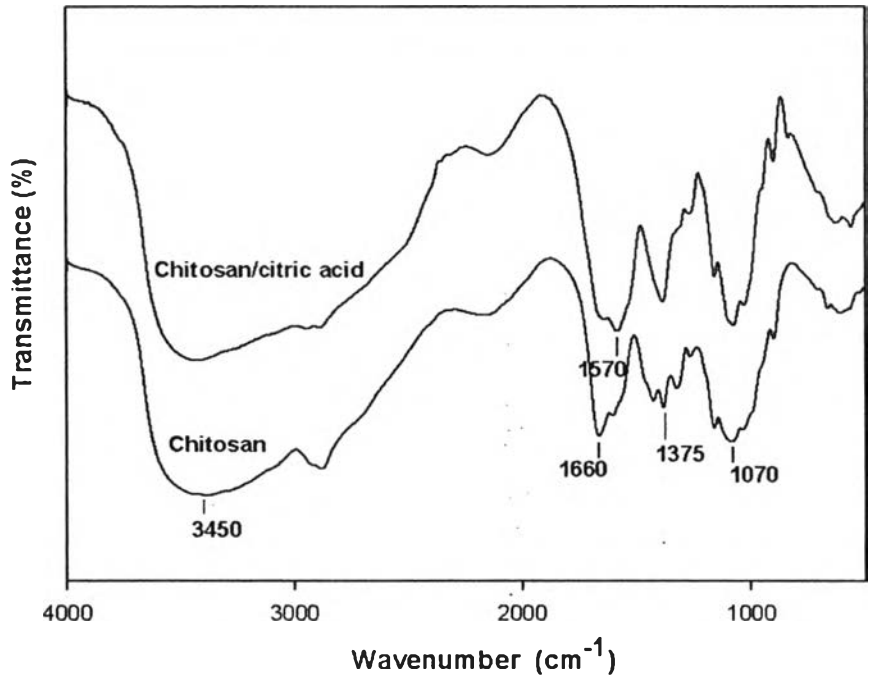

Figure 4.2 FT-IR spectra of chitosan (a) and chitosan-citric acid (b).

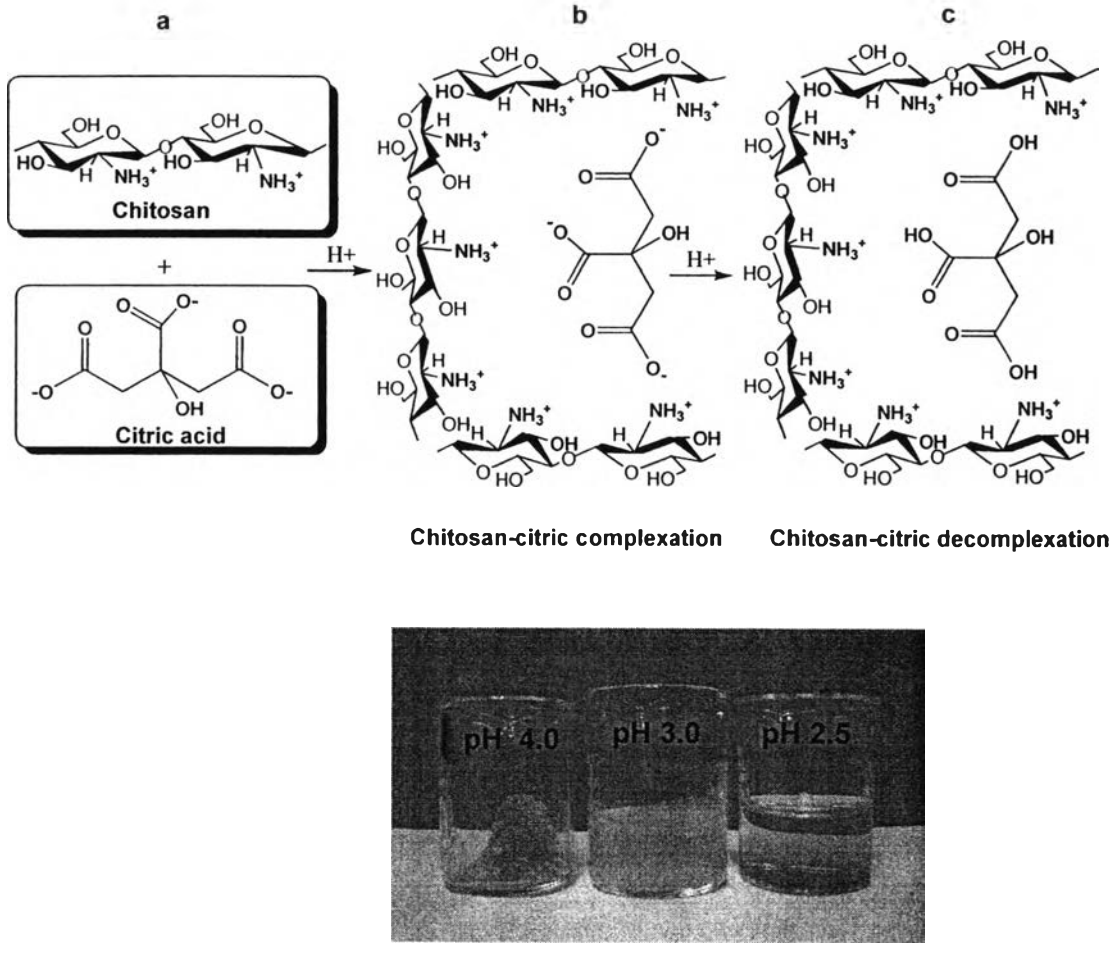

Figure 4.3 Chitosan-citric acid complexation and decomplexation.

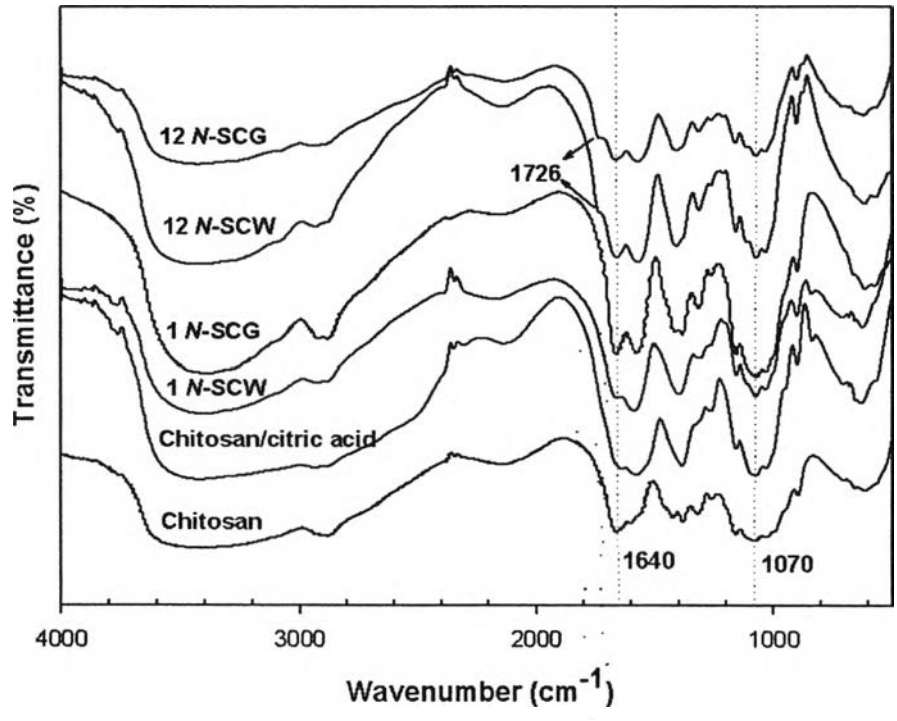

Figure 4.4 Comparison FT-IR spectra of chitosan, chotosan citric complex, *N*-SCG and *N*-SCW.

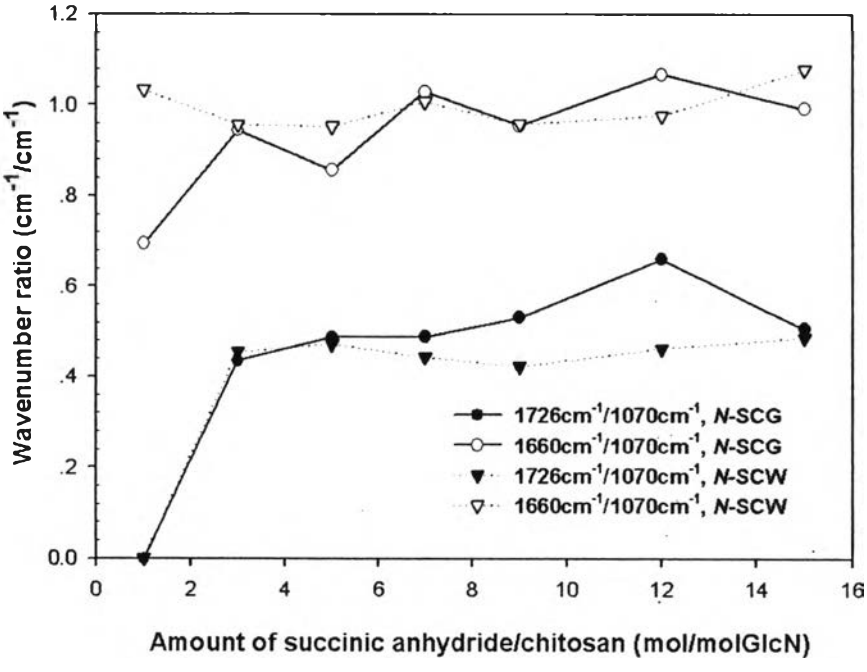

Figure 4.5 FT-IR ratio of *N*-SCW and *N*-SCG.

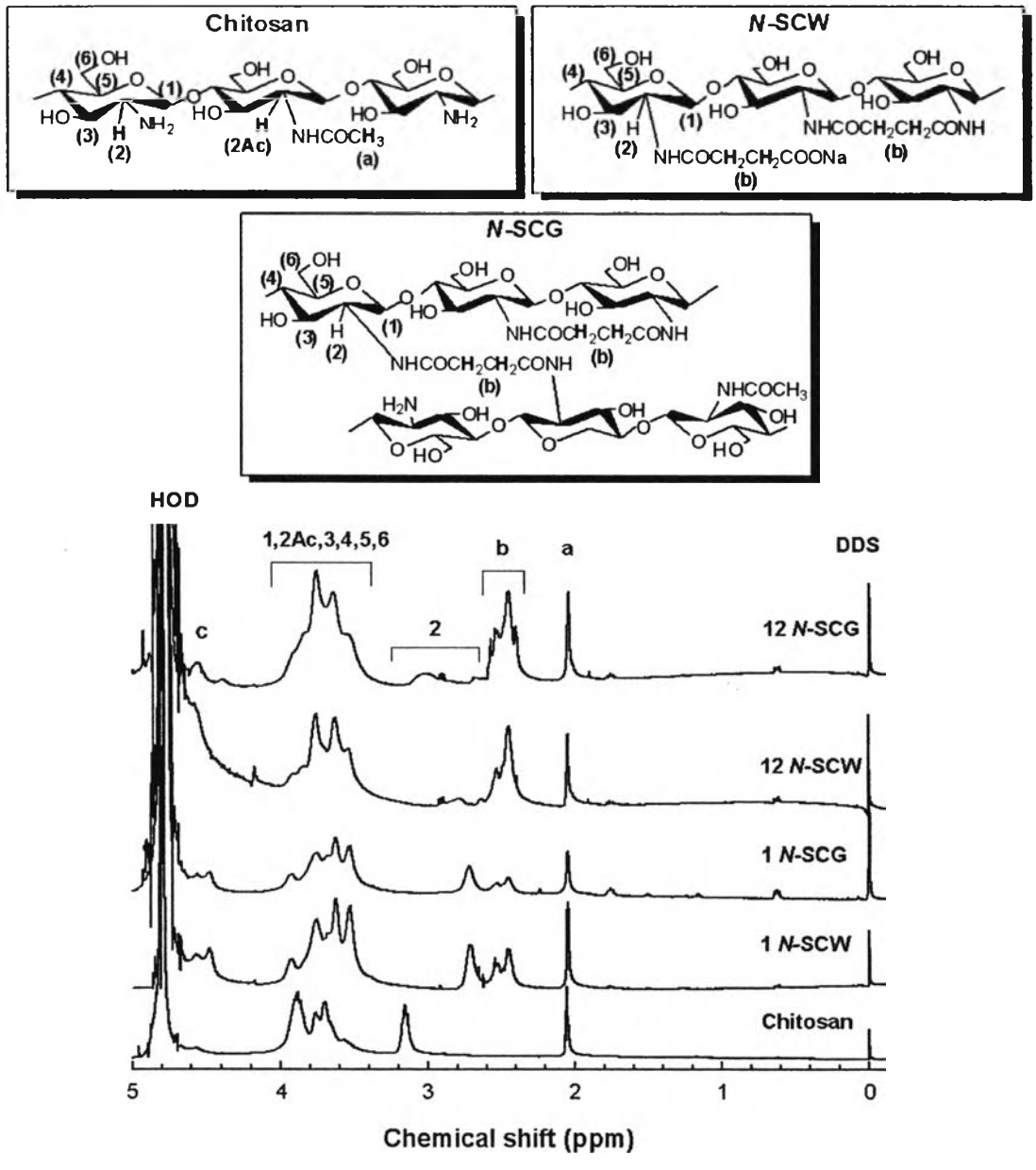

Figure 4.6 ^{1}H-NMR spectra of chitosan and *N*-SC products.

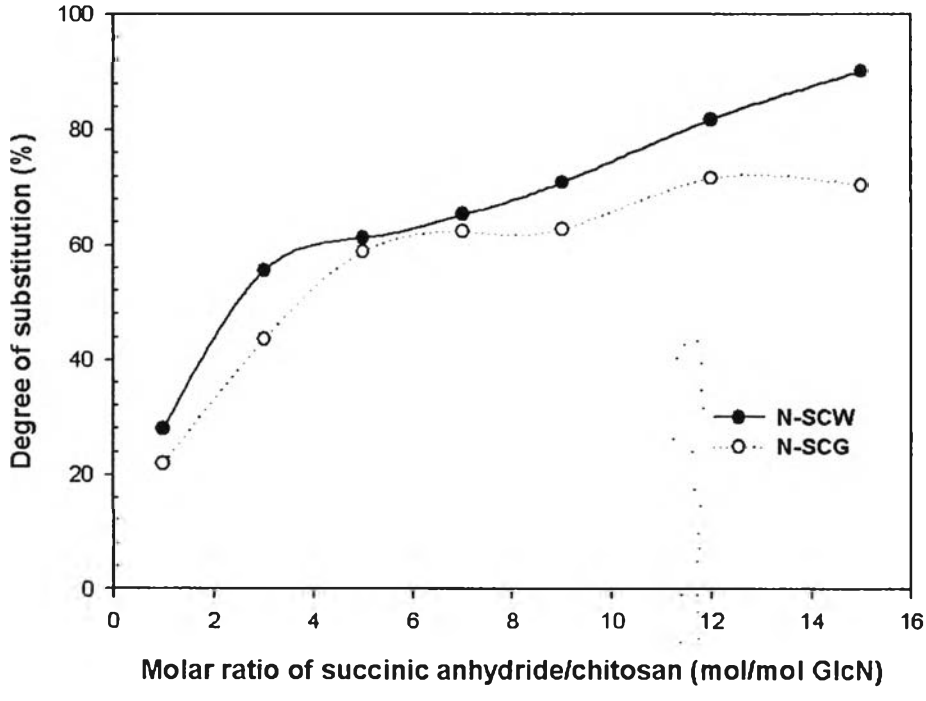

Figure 4.7 Degree of substitution of *N*-SCW and *N*-SCG.

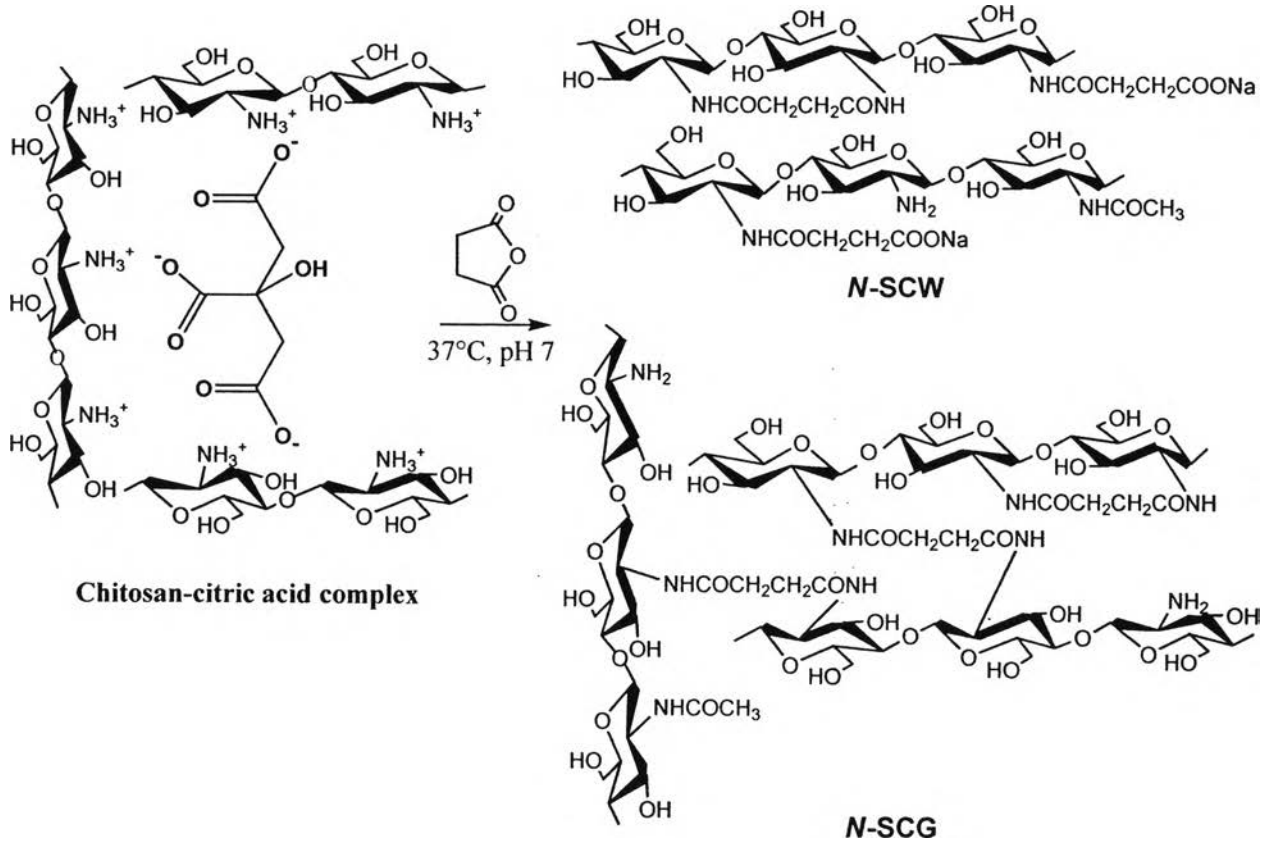

Figure 4.8 The propose mechanism of *N*-SCW and *N*-SCG formations.

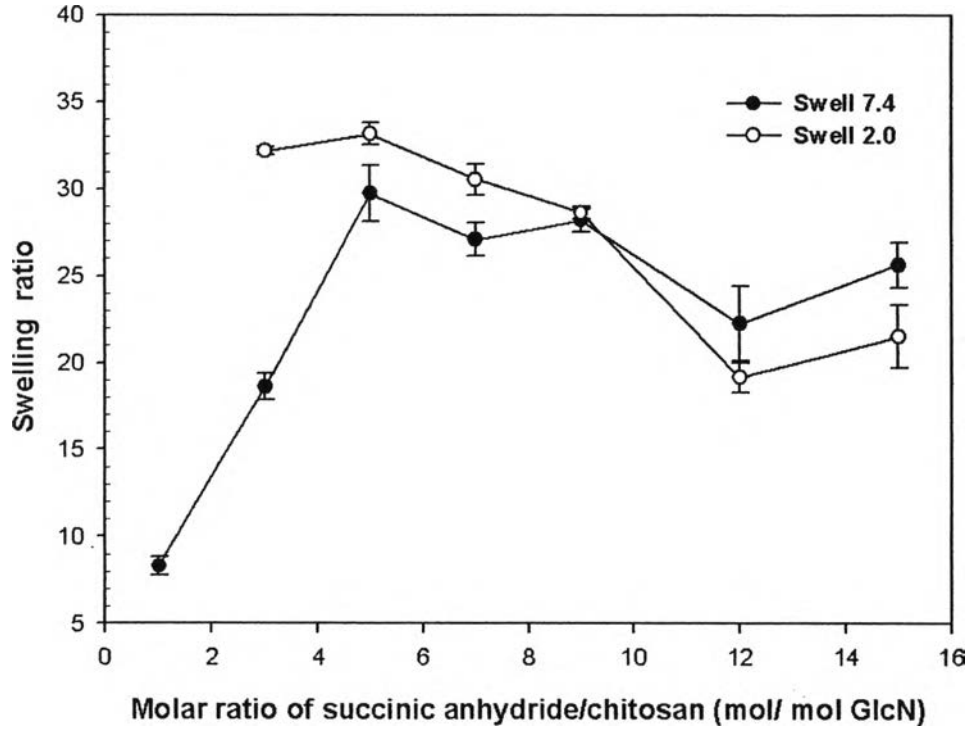

Figure 4.9 The swelling ratio of *N*-SCG films in pH 2.0 and pH 7.4 buffer solution.

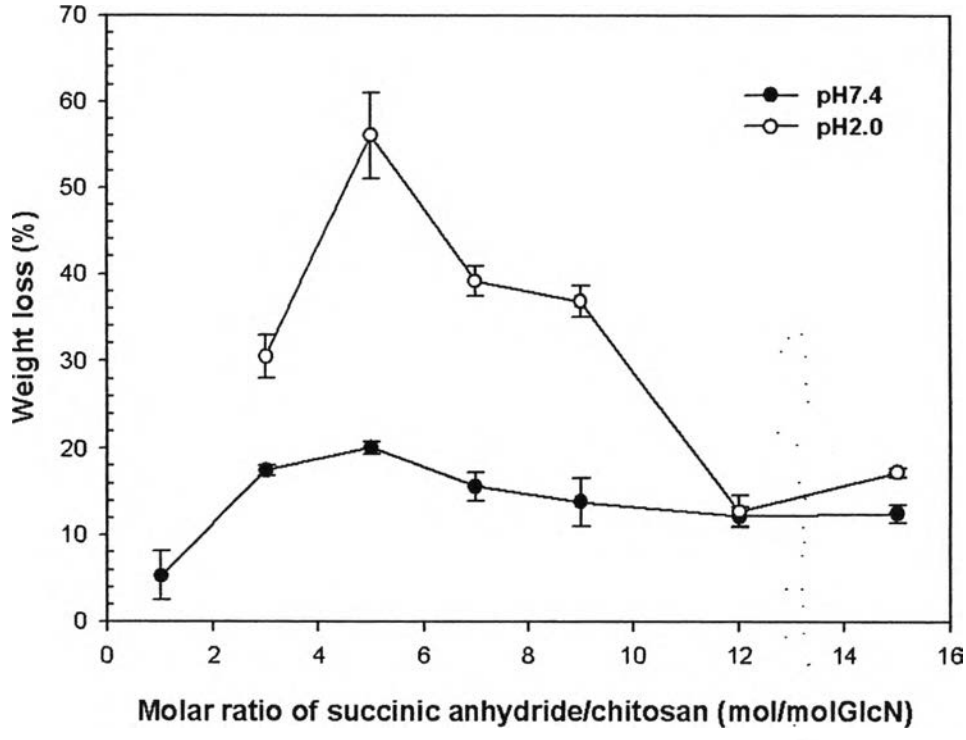

Figure 4.10 The weight loss of *N*-SCG films in pH 2.0 and pH 7.4 buffer solution.

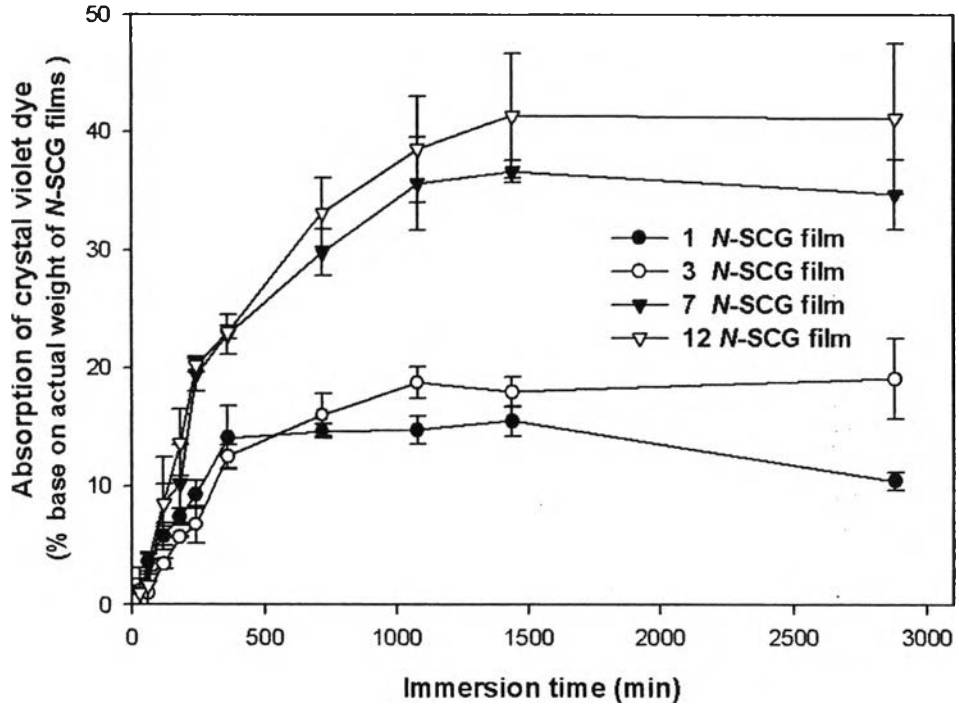

Figure 4.11 The crystal violet dye absorption characteristic of *N*-SCG films.

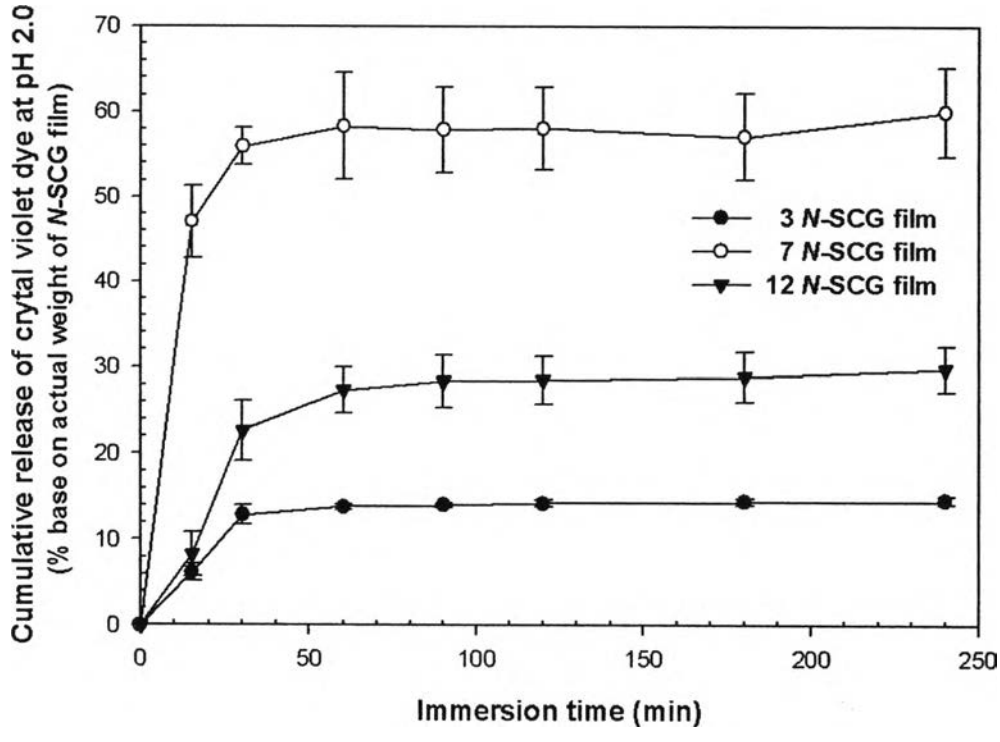

Figure 4.12 The release characteristic of crystal violet dye loaded *N*-SCG films at pH 2.0 buffer solution.

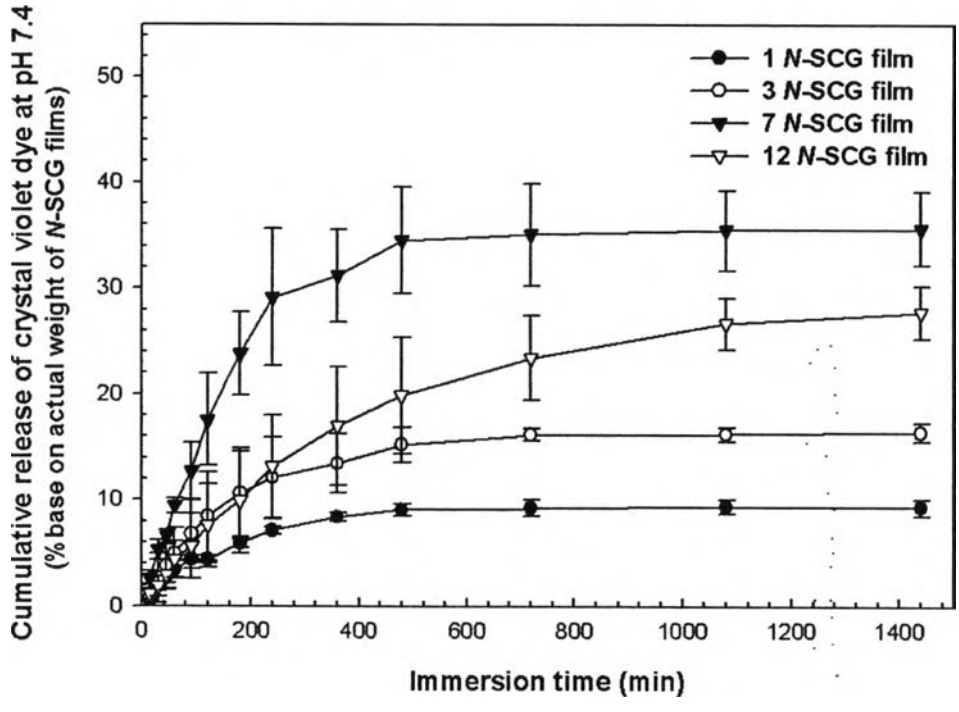

Figure 4.13 The release characteristic of crystal violet dye loaded *N*-SCG films at pH 7.4 buffer solution.

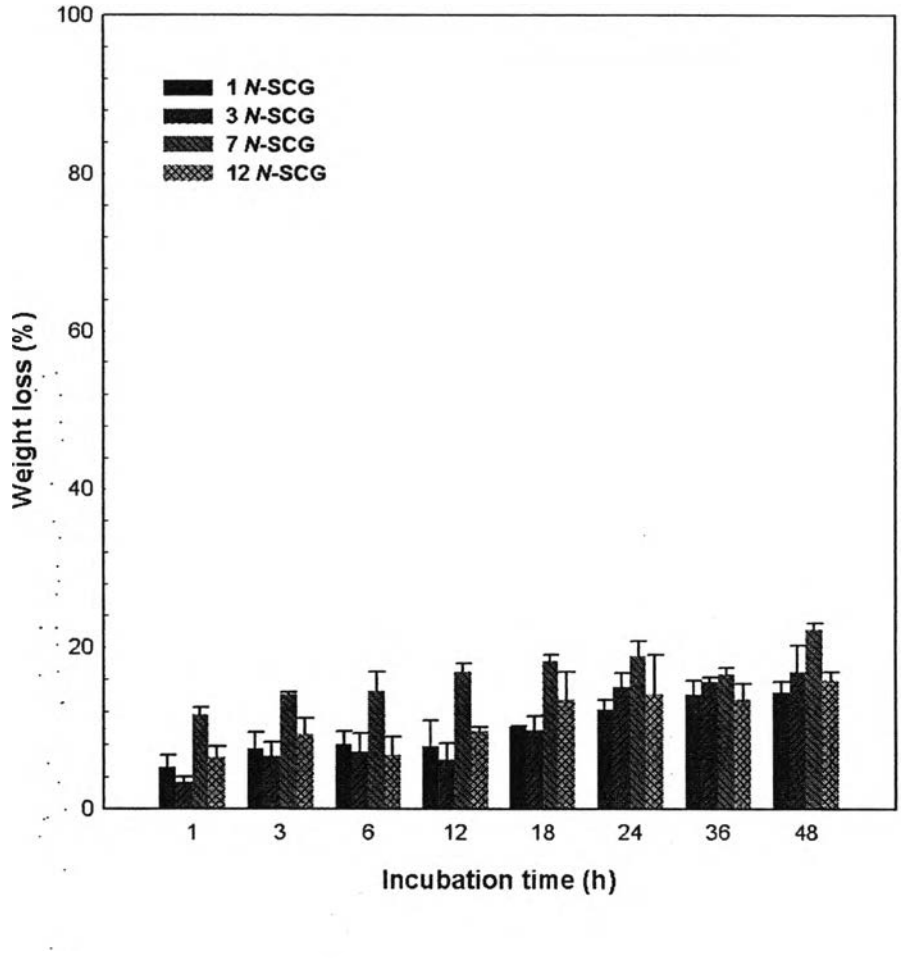

Figure 4.14 The degradability of *N*-SCG films in PBS.

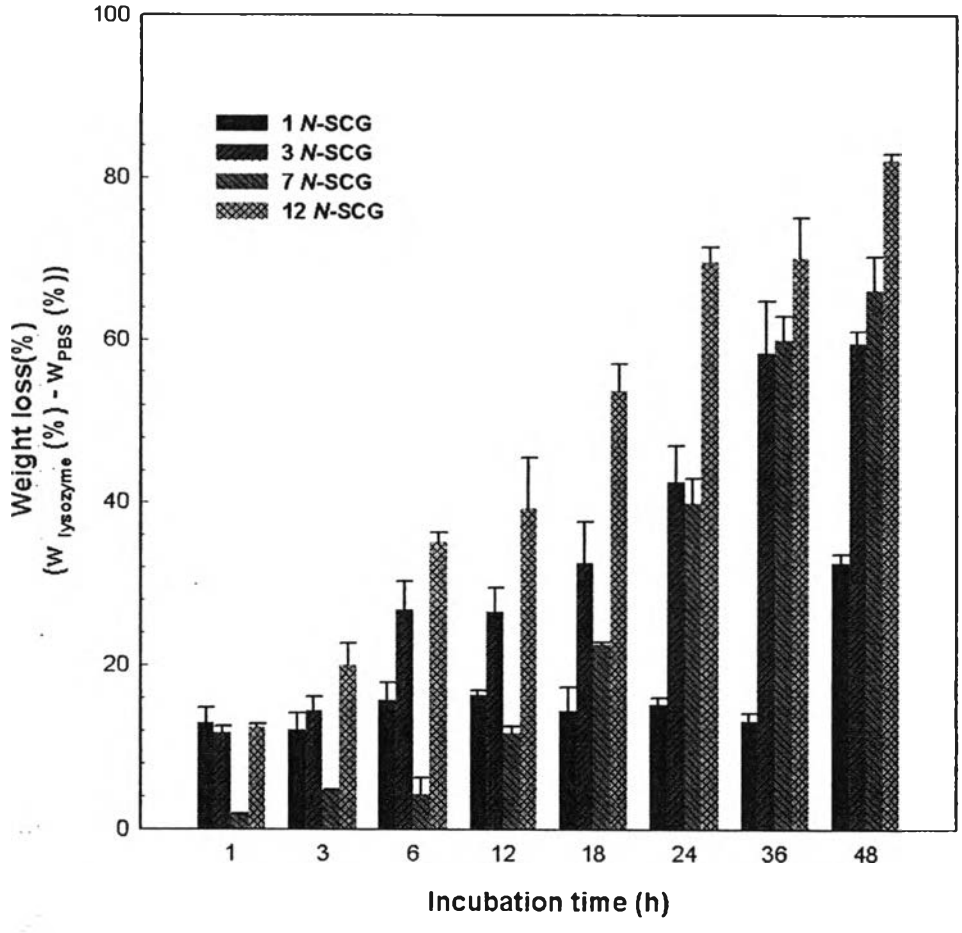

Figure 4.15 The degradability of *N*-SCG films in lysozyme/PBS.

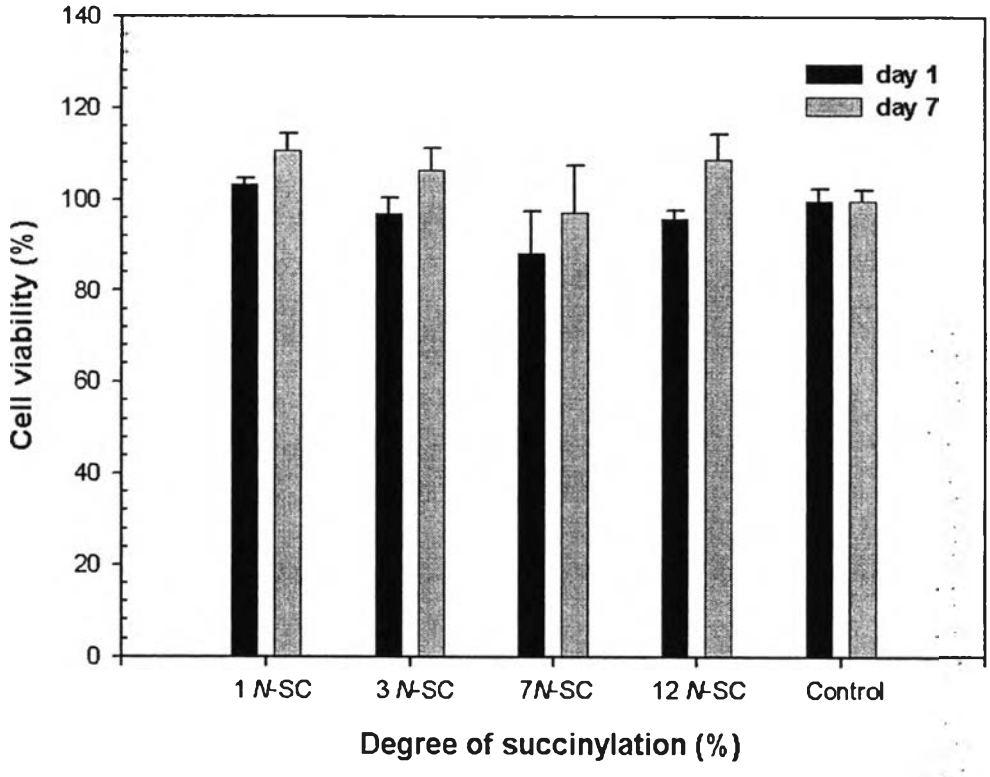

Figure 4.16 Indirect cytotoxicity evaluation of *N*-SCG films against mouse fibroblasts cells.

Table 4.1 Synthesis conditions and characteristics of *N*-succinyl chitosan (*N*-SC) products.

Samples	Succinic anhydride : NH_2 group (mol/mol)	Amount of water[a] (ml)	Yield[b] (%)		DS[c] (%)	
			N-SCW	*N*-SCG	*N*-SCW	*N*-SCG
Chitosan	-	-	-	-	-	-
1 *N*-SC	1:1	10	60.55 ± 4.82	39.45 ± 4.82	27.98	21.96
3 *N*-SC	3:1	30	58.89 ± 0.59	41.11 ± 0.59	55.50	43.58
5 *N*-SC	5:1	60	64.91 ± 2.24	35.09 ± 2.24	61.27	58.90
7 *N*-SC	7:1	90	56.75 ± 4.36	43.25 ± 4.36	65.40	62.45
9 *N*-SC	9:1	120	56.42 ± 1.79	43.57 ± 1.79	70.97	62.81
12 *N*-SC	12:1	140	56.81 ± 1.36	43.19 ± 1.36	81.83	71.73
15 *N*-SC	15:1	160	56.66 ± 1.87	43..34 ± 1.87	90.18	70.54

[a] Minmimum amount of water that can dissolve succinic anhydride at each condition; [b]An average values (n=3), calculated by (g of *N*-SCW or *N*-SCG /g of total *N*-SC ×100); [c] Degree of substitution, calculated from ^{1}H-NMR.