Macromolecular Research, Vol. 16, No. 1, pp 57-61 (2008)

# Regioselective Succinylation and Gelation Behavior of Glycol Chitosan

**Keunsoo Jeong, Wonbum Lee, Jueun Cha, and Chong Rae Park***

*Carbon Nanomaterials Design Laboratory, Hyperstructured Organic Materials Research Center (HOMRC), Department of Materials Science and Engineering, Seoul National University, Seoul 151-744, Korea*

**Yong Woo Cho**

*Department of Chemical Engineering, Hanyang University, Ansan 426-791, Korea*

**Ick Chan Kwon**

*Biomedical Research Center, Korea Institute of Science and Technology, Seoul 126-791, Korea*

*Received August 28, 2007; Revised October 30, 2007*

**Abstract:** Chitosan is normally acylated and subsequently conjugated with drugs for biomedical applications. This study examined the relationship between the succinylation and gelation behaviors of glycol chitosan. Glycol chitosan was acylated with succinic anhydride under a wide variety of reaction conditions, such as different molar ratios of succinic anhydride to glucosamine, different methanol content in the reaction media, and different reaction temperatures. Among these reaction parameters, the methanol content in the solvent played an important role in determining the regioseletive succinylating site. N-succinylation and N-N cross-linking occurred regardless of the reaction conditions. However, O-succinylation was observed under specific conditions, i.e. a methanol content > 0.6 (v/v) and a reaction temperature > 25 °C. O-succinylation accelerated the N-O cross-linking of glycol chitosan, and led to gelation. The N-succinylated glycol chitosans were water-soluble, whereas the N- and O-succinylated glycol chitosans formed a gel. These physico-chemical structural differences in the succinylated glycol chitosans would definitely influence subsequent drug-conjugation reactions and consequently the drug loading and release kinetics.

*Keywords*: chitosan, biopolymers, regioselectivity, succinylation, gelation.

## Introduction

Chitin and chitosan have many valuable characteristics such as immuno-stimulatory ability, anticoagulant property, antibacterial action, wound-healing promotion ability, etc. Especially chitosan has recently been studied as a novel carrier material in drug or gene delivery systems.[1-10] But in such applications, chitosan has shown some inherent limitations of, for instance, the lack of water solubility[11] and the need of linker for higher drug loading. Various chemical modifications of chitosans have thus been adopted in order to widen their uses in various medical applications. Among such modificaitons, acylation is the easiest and most effective method to obtain the water solubility and the novel functionality that can be used for the conjugation of drugs. For example, carboxylic anhydride,[12,13] cyclic anhydride,[14,15] *n*-fatty acid chloride,[16] and *n*-fatty acid anhydride[17] have been used for the acylation of chitosans in previous works on the preparation of novel drug or gene delivery systems.

*Corresponding Author. E-mail: crpark@snu.ac.kr

However, the acylation behavior itself has not been systematically studied and hence the relationship between the acylation behavior and gel formation behavior has not been clearly understood. It is thus the aim of this work to investigate the acylation behavior, with viewpoints of not only the degree of acylation but also the regioselectiveness which can influence physico-chemical properties of the acylated chitosan. In this study, we used a glycol chitosan instead of chitosan to obtain water solubility, and succinic anhydride as an acylating agent. The glycol chitosan has two possible acylation sites as shown in Figure 1: N-acylation on C(2)-$NH_2$ group (A) and O-acylation on either C(3)-OH or C(8)-OH groups (B). We tried to identify acylation sites and to find their correlation with cross-linking and gelation behaviors.

## Experimental

**Materials.** Succinic anhydride (97 %, Aldrich, USA) and methanol (99.9 %, Mallinckrodt Baker Inc., France) were used as purchased without further purifications. Glycol chitosan (Sigma, USA) that has the degree of deacetylation of

ca. 85 % and the degree of polymerization of 2,500 with the nominal molecular weight of $2.5 \times 10^5$ was purified three times with methanol and dried *in vacuo*.

**Succinylation of Glycol Chitosan.** Glycol chitosan (0.2 g) was dissolved in 10 mL distilled water, and succinic anhydride was dissolved in methanol of a specified amount of 10, 15, and 20 mL, respectively. Two solutions were then mixed and stirred at a specified temperature of 10, 25, and 50 °C for 24 h, followed by dialysis against distilled water for 2 days, and lyophilization. The amount of succinic anhydride was varied in range of 1~15 equivalent mole to glucosamine of glycol chitosan. The sample was coded as T ▽▽ M △△ R ○○, in which T means the reaction temperature, M the amount of methanol, and R the molar ratio of succinic anhydride to glucosamine.

**Characterization.** $^1$H-NMR spectra of succinylated glycol chitosan were recorded on a Bruker, Avance 500 MHz spectrometer. The sample was dissolved in $D_2O$ to make 1 wt% solution at room temperature. From $^1$H-NMR spectra, the degree of succinylation (DSu) was determined from the ratio of peak area of succinic H to that of C(2)-H in glycol chitosan. The succinylation sites were determined from NMR spectra. Elemental analysis was performed with EA1110 (CE Instrument, Italy), from which the degree of succinylation was also determined by calculating the C/N ratio.

## Results and Discussion

**Regioselective Succinylation Behavior.** Succinylation of glycol chitosan (GC) was carried out under 45 different kinds of reaction conditions. The regioselective succinylation behavior was accordingly identified with $^1$H-NMR spectra, and Figure 1 shows typically observed spectra.

Depending on the regioselective reaction site, there can be two kinds of succinylated glycol chitosans (SGCs), i.e. C(2)-N-only succinylated glycol chitosans (*N*-SGCs) and both C(2)-N- and either C(3)-O- or C(8)-O-succinylated glycol chitosans (N,O-SGCs). In both types of SGCs, multiple peaks due to the succinic protons can be found at 2.48 ppm (-NHCOC$\underline{H_2}$CH$_2$COO-) and 2.62 ppm (-NHCOCH$_2$C$\underline{H_2}$COOH) (see Figures 1(b) and (c)), which can not be found from the control sample of GC (see Figure 1(a)).[18] However, if there occur cross-linking reactions between unreacted C(2)-$\underline{NH_2}$ groups and C(2)-NHCOCH$_2$CH$_2$$\underline{COOH}$ of neighboring N-SGC molecules or N,O-SGC molecules (all denoted N-N cross-linking), then there should appear a singlet peak at up-

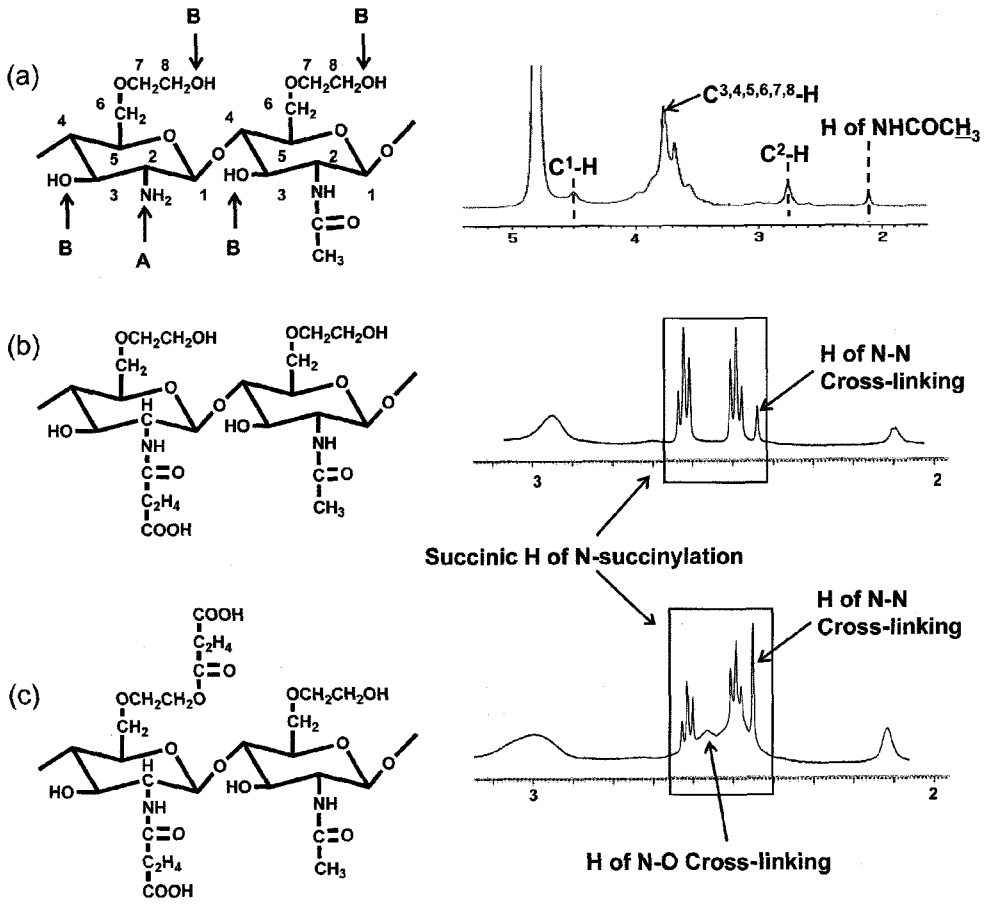



**Figure 1.** Chemical structure and the corresponding $^1$H-NMR spectra of (a) glycol chitosan, (b) N-succinylated glycol chitosan, and (c) N,O-succinylated glycol chitosan.

field. This could be confirmed by the predicted $^1$H-NMR spectra by the ChemDraw for the model compounds. So, the singlet peak showing up at 2.43 ppm in Figures 1(b) and (c) can be attributed to the protons of N-N cross-linking (-NHCOC$\underline{H_2}$C$\underline{H_2}$CONH-). In Figure 1(c), a broad peak is additionally recognized at around 2.56 ppm. From the observation that the integrated area of the peak at 2.62 ppm is smaller than that of the peak at 2.48 ppm, and the sum of the integrated areas of both peaks at 2.62 ppm and at 2.56 ppm is nearly the same with that of the peak at 2.48 ppm, it can be reasoned that the peak at 2.56 ppm results from O-succinylation causing N-O cross-linking between C(2)-NHCOCH$_2$-C$\underline{H_2}$COOH and either C(3)-$\underline{OH}$ or C(8)-$\underline{OH}$ groups (all denoted N-O cross-linking).

Figure 2 shows $^1$H-NMR spectra of all SGCs synthesized in this study. As shown in Figure 2(a), all SGCs prepared at 10 °C, i.e. T10 series samples, are selectively N-succinylated, irrespective of the variations in the amount of methanol and the molar ratios of succinic anhydride. It is worthwhile to mention that all these samples are water-soluble irrespective of N-N cross-linking. This indicates that N-N cross-linking does not lead to the gelation of SGCs. In contrast to

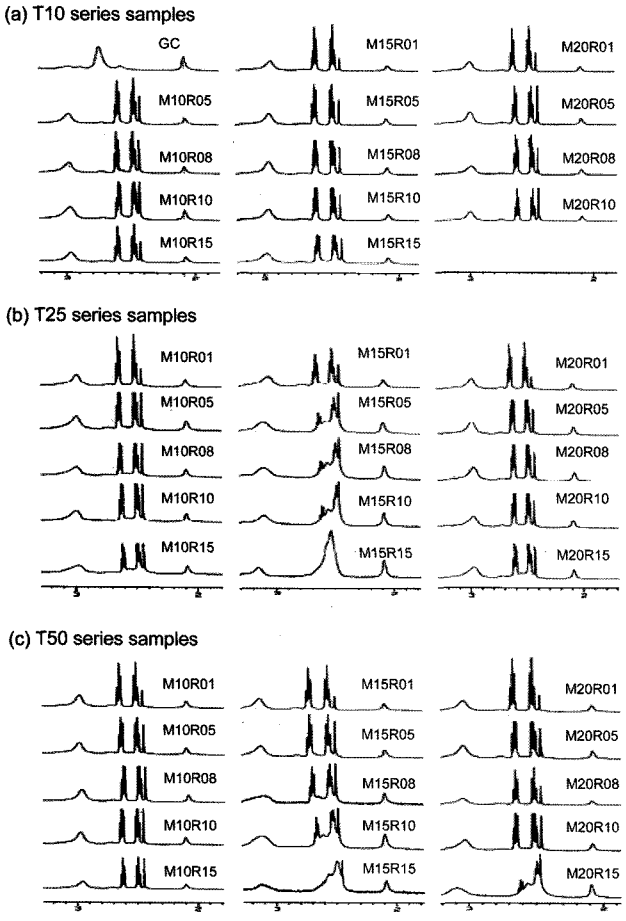



**Figure 2.** $^1$H-NMR spectra of the glycol chitosans succinylated under various reaction conditions.

the case of T10 series samples, T25 series and T50 series samples exhibit both N-N and N-O cross-linkings depending on the amount of the added methanol. It is particularly interesting to note in these two series samples that the presence of methanol of 15 and 20 mL in the reaction system results in both N-N and N-O cross-linkings (see Figures 2(b) and (c)). Here again, we can recognize that molar ratios of succinic anhydride and reaction temperatures are not so influential. In the case of temperature effect, it seems that the lower temperature the more favorable N-succinylation. Further, that such samples are all gelled during dialysis, which implies that N-O cross-linkings tend to give rise to the gelation of N,O-SGCs.

A similar effect of methanol on a selective N-acylation has been reported, although the mechanism has not been clarified. However, our results are somewhat different with those of earlier works. That is, earlier works reported that when methanol of 30-50 mL was added to a reaction system at room temperature, N-acylation took place[2,11,13,15] exclusively. However, our result is that N,O-succinylation occurs exceptionally when methanol of 15 mL is added. The role of this particular amount of methanol in N,O-succinylation is not clear at this stage.

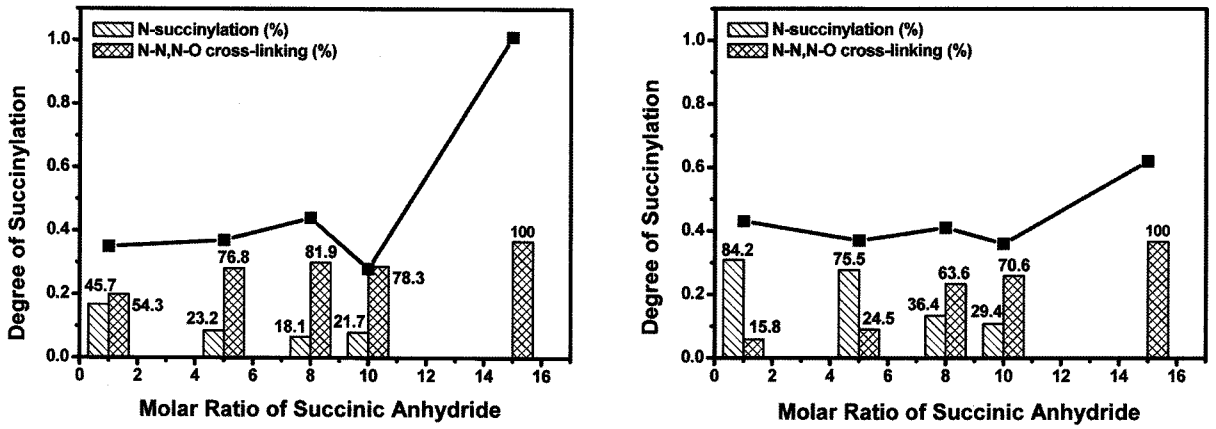
The degree of succinylation (DSu), measured by both $^1$H-NMR spectroscopy and elemental analysis, is listed in Table I. There is slight difference in the measured values between the two different techniques. The DSu value is in range of 0.3-1.0, but common values are about 0.4, nearly independent of reaction conditions. When a DSu value is differentiated into the proportions of the pure N-succinylation and the cross-linkings of both N-N and N-O types, we can recognize in Figure 3 that, as the molar ratio of succinic anhydride increases, the proportion of pure N-succinylation decreases whereas that of the cross-linkings increases in both T25 and T50 series samples.

**Regioselective Succinylation and Gelation Behavior.** To find a relationship between the gelation behavior of SGCs and the regioselective succinylation, we divided the total degree of cross-linkings including both N-N and N-O types into its components, i.e. N-N and N-O succinylations, respectively. It is clearly seen from Figure 4 that both the degree of total cross-linking and the degree of N-O cross-linking exhibit a similar changing tendency with increasing molar ratio of succinic anhydride. It is worthwhile to note that the degree of N-N cross-linking remains at a nearly fixed value irrespective of the amount of succinic anhydride. We found that the gel is spontaneously formed, irrespective of reaction temperature, when the molar ratio of succinic anhydride exceeds the value of 5 for T25 series sample and 8 for T50 series sample (boxed area in Figure 4). Recalling that N-N cross-linked SGCs are all water-soluble, we can easily learn that the degree of N-O cross-linking is the most influential factor in the gelation of SGCs. Earlier Hirano *et al.* reported that the gel of SGCs is formed when

**Table I. Degree of Succinylation as Determined from $^1$H-NMR Spectroscopy and Elemental Analysis**

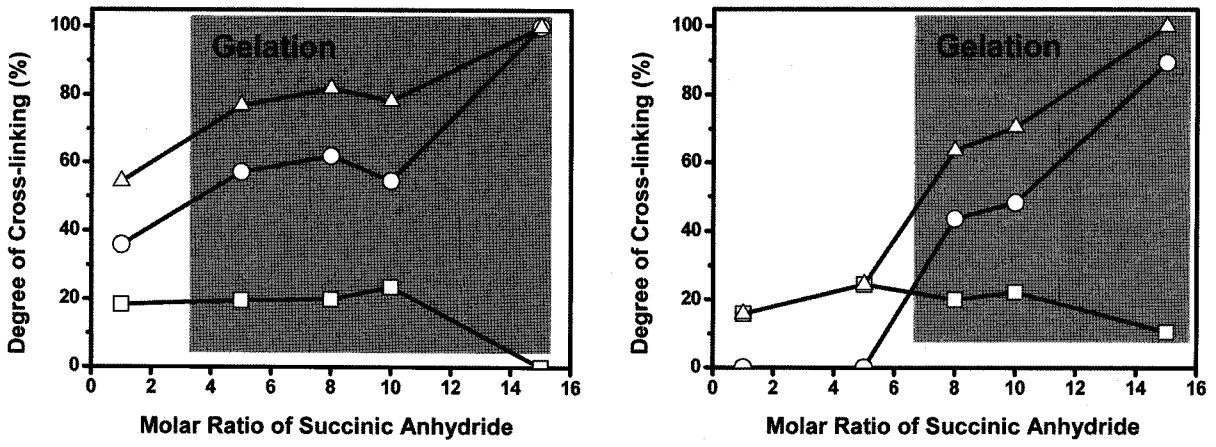
| Sample Code | R01 | R05 | R08 | R10 | R15 |
|---|---|---|---|---|---|
| T10M20 | 0.39(0.40) | 0.37(0.39) | 0.44(0.46) | 0.43(0.42) | |
| T10M15 | 0.43(0.39) | 0.38(0.40) | 0.44(0.42) | 0.46(0.43) | 0.41(0.45) |
| T10M10 | | 0.44(0.42) | 0.46(0.50) | 0.45(0.47) | 0.43(0.44) |
| T25M20 | 0.38(0.49) | 0.40(0.49) | 0.38(0.45) | 0.40(0.49) | 0.39(0.45) |
| T25M15 | 0.35(0.25) | 0.37(0.28) | 0.44(0.27) | 0.28(0.36) | 1.01(0.45) |
| T25M10 | 0.35(0.50) | 0.33(0.47) | 0.34(0.47) | 0.35(0.52) | 0.45(0.50) |
| T50M20 | 0.37(0.52) | 0.37(0.57) | 0.36(0.53) | 0.36(0.52) | 0.52(0.51) |
| T50M15 | 0.43(0.29) | 0.37(0.25) | 0.41(0.31) | 0.36(0.31) | 0.62(0.22) |
| T50M10 | 0.32(0.46) | 0.35(0.50) | 0.36(0.46) | 0.37(0.47) | 0.36(0.47) |

*Parenthesized values are determined from elemental analysis.




(a) (b)

**Figure 3.** Degree of succinylation of (a) T25M15 series and (b) T50M15 series samples as a function of the molar ratio of succinic anhydride to glucosamine of glycol chitosan.




(a) (b)

**Figure 4.** Degree of N-N (□), N-O (○), and total (△) cross-linking of (a) T25M15 series and (b) T50M15 series samples as a function of the molar ratio of succinic anhydride to glucosamine of glycol chitosan.

water-soluble N-SGCs go through a condensation reaction in the presence of carbodiimide.[15] But our result shows that the gel of SGCs can be easily formed, without using any kind of catalyst like carbodiimide, when the amount of

methanol is 15 mL in the reaction system. This finding may be easily utilized to increase the drug loading because a drug can be loaded by both chemical conjugation with succinic carboxyl groups and physical uptake within the gel.

## Conclusions

Succinylated glycol chitosan was prepared by reacting glycol chitosan with succinic anhydride under various reaction conditions. It was found that, when methanol content in the reaction system is below 10 mL, N-selective succinylation was favored irrespective of different reaction conditions. But, when methanol of above 15 mL was added into a solvent at above 25 °C, both N- and O-succinylation occurred. As for gelation behavior, the degree of N-O cross-linking turned out to be the most influential factor. When we utilize the extended knowledge of the regioselective succinylation and the gelation behaviors of SGCs, it may be possible to up-load more drug on to a drug-carrier based on SGCs.

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