CHAPTER IV
CHITOSAN-OXANORBORNADIENE: A CONVENIENT CHITOSAN DERIVATIVE FOR CLICK CHEMISTRY WITHOUT METAL CATALYST PROBLEM

4.1 Abstract

Click chemistry is considered to be a good pathway to conjugate chitosan with functional molecules due to the ease of the reaction at room temperature. However, as chitosan forms a complex with metal ions, there is a problem with the existence of metal ions in the derivative. The present work demonstrates that chitosan-oxanorbornadiene can provide metal-free Click by showing the optimal condition to introduce oxanorbornadiene, with 80% substitution, and clarifies model reactions of chitosan with azido-modified substrates for the ligation of bioactive molecules.

Keywords: chitosan-oxanorbornadiene, metal free Click chemistry

4.2 Introduction

Chitosan, known for its specific properties –e.g., biocompatibility (Wang *et al.*, 2003), nontoxicity (Hu *et al.*, 2012), and biodegradability (Yamamoto and Amaike, 1997)– has had favorable reports in regard to its high value-added applications in the biological and biomedical fields.(Chang *et al.*, 2010; Lee *et al.*, 2011; Lee *et al.*, 2012) For chitosan derivative production, most of the approaches are based on the ligation of biological active, organic molecules (BSA (Masuko *et al.*, 2005), antibody (Lee *et al.*, 2012)) or inorganic molecules (magnetite (Shi *et al.*, 2008), silica (Deng *et al.*, 2011)). Thereby, the functionalizations of chitosan in aqueous and/or organic solvents by using conjugating/coupling reagents (Fangkangwanwong *et al.*, 2006; Hua *et al.*, 2011; Lee *et al.*, 2011) need to be considered. It is accepted that an ideal condition in modifying chitosan with bio-

active compounds should be done in aqueous at room temperature, and if possible, without the use of toxic coupling or conjugating agents.

Recently, Click chemistry has received much attention in macromolecular chemistry due to its high chemoselectivity in mild reaction conditions with a variety of functionalizations. The copper catalyzed Click chemistry based on the reaction between an azido- and an alkyno- group was first reported by Medal (Tornøe *et al.*, 2002) and Sharpless.(Kolb *et al.*, 2001) The bioorthogonality of the components avoids interaction with the environment of biological or biomedical systems.(Baskin and Bertozzi, 2007) For this reason, Click chemistry is a good reaction pathway to design bio-structured molecules, e.g. on the basis of chitosan.(Gao *et al.*, 2009; Deng *et al.*, 2011)

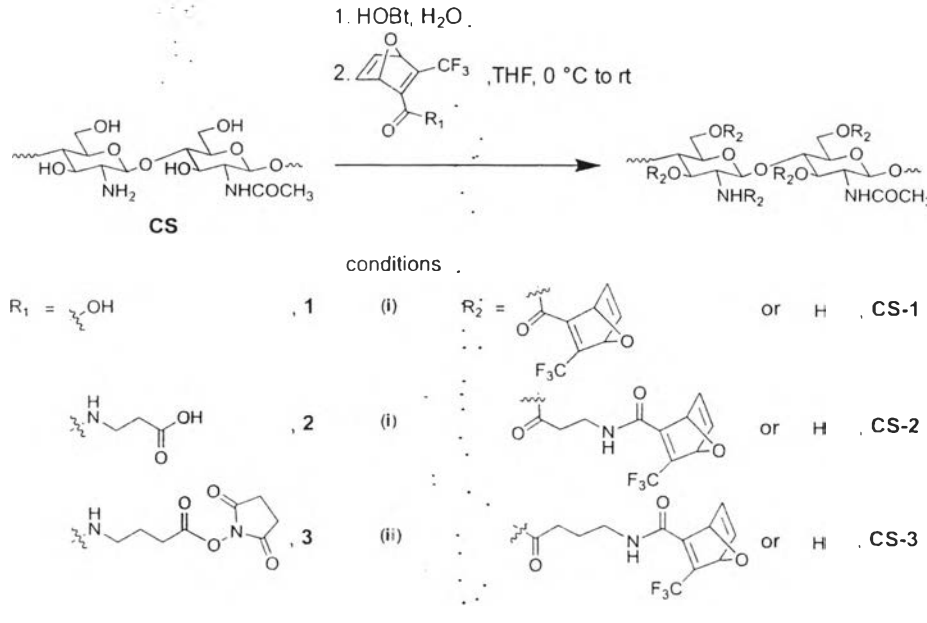
It is important to note that one of the specific properties of aminopolysaccharide chitosan is the complexation with metal ions (Schlick, 1986; Ogawa *et al.*, 1993; Mi, 2005), or Cu(I) catalyzed Click chemistry. Therefore, this might lead to Cu contamination after the reaction. In that case, the risks of metal toxicity may prevent its use in biomedical application.

The present work focuses on a new pathway to obtain metal-free Click chemistry for chitosan. In the past, Agard et al. reported a good example of the metal-free Click reaction called "the Strain-Promoted Azide-Alkyne Cycloadditon (SPPC)" which can be further conjugated with peptides.(Agard *et al.*, 2004) In this work, Oxanorbornadiene moieties, which were first reported by Rutjes(van Berkel *et al.*, 2007; van Berkel *et al.*, 2008), are considered to be effective functional molecules to bring in a reaction with azido-modified substrates, resulting in a triazole linkage between chitosan and the substrates. An attractive point of chitosan-oxanorbornadiene is the reaction progress at room temperature without any additives or catalysts. Recently, Krause et al. have successfully linked the modified polysaccharide alginate with cyclic RGD-pentapeptides via this oxanorbornadiene based metal-free Click technique. The regio- and chemoselectivity of the cycloaddition, resulting in different products, were investigated by using ^{19}F-NMR.(Mazeau *et al.*, 1994; Lertworasirikul *et al.*, 2004) To the best of our knowledge, an oxanorbornadiene-based Click reaction with aminopolysaccharide chitosan has not yet been reported.

4.3 Results and Discussion

Scheme 1 shows the introduction of trifluoromethylated oxanorbornadienes (1-3) to chitosan (CS). All reaction conditions were performed by dissolving CS in water containing 1.2 equivalent HOBt.(Fangkangwanwong *et al.*, 2006) To study the reaction efficiency of the oxanorbornadiene derivatives, three types of oxanorbornadienes (1-3)

Scheme 4.1 Synthesis of chitosan-oxanorbornadiene with; (i) EDC, DMAP, 1 d, and (ii) DiPEA, 1 d. Purification by intense dialysis against an aqueous NaCl solution and DI water.



having different spacer lengths were considered. Typically, the trifluoromethyl group shows a ^{19}F-NMR chemical shift at ~ -61 ppm, as shown in the case of 1 (Figure 4.1a). For 1 and 2, the conditions were similar to each other based on the use of a conjugating agent (EDC) and DMAP to obtain CS-1 and CS-2, respectively. Figure 4.1b and 4.1c confirm the success of the reaction.

The FTIR spectra of CS-1 and CS-2 show a new peak at 1740 cm^{-1} and an increase of the peak at 1550 cm^{-1}. The changes in peak intensity were clarified by the curve fitting technique to confirm that the substitution of oxanorbonadiene resulted

in ester and amide bonds (Figure H1b and H1c, Table H1). The percentage of substitution, which could be easily quantified by ^{1}H-NMR (Figure H3A-B), were found to be only 15% for both cases.

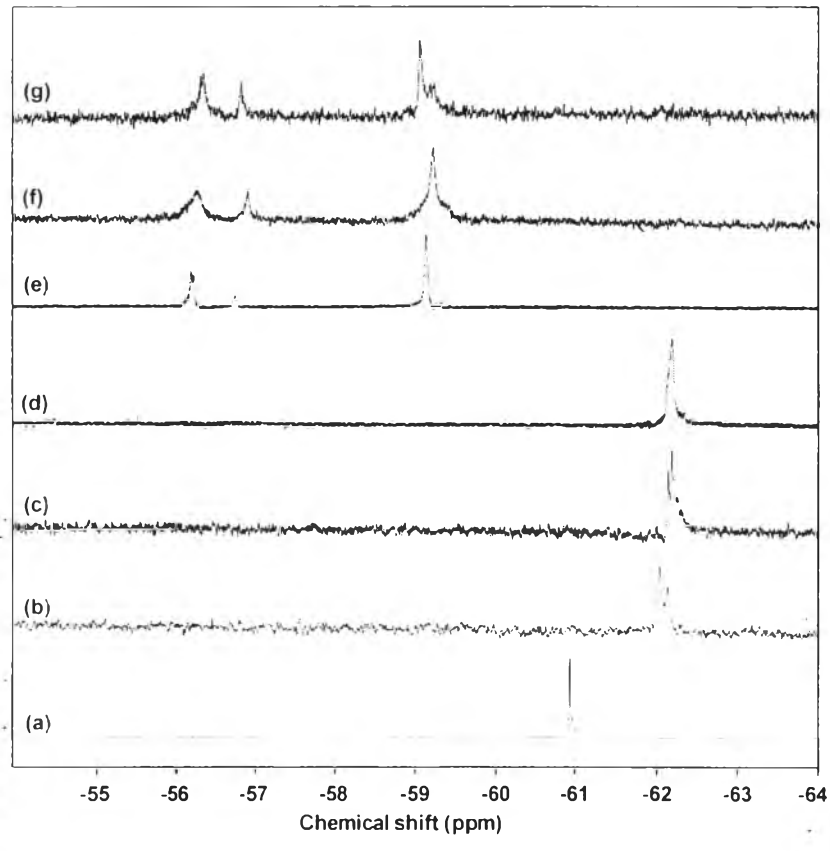
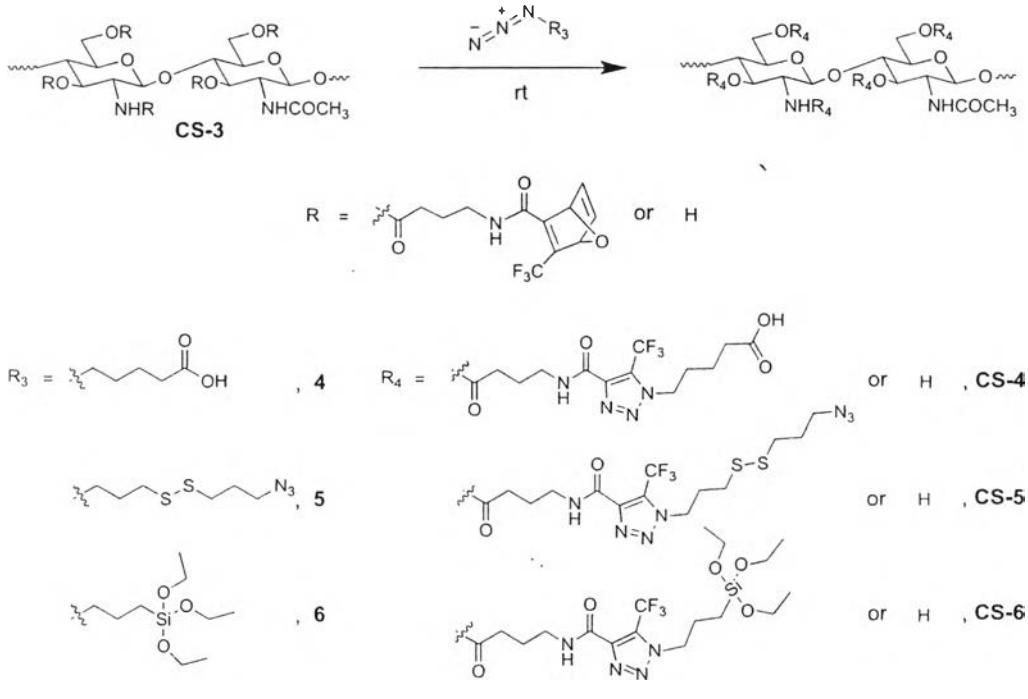


Figure 4.1 ^{19}F-NMR spectra of (a) 1, (b) CS-1, (c) CS-2, (d) CS-3, (e) CS-4, (f) CS-5, and (g) CS-6 in 2% CD_3COOD/D_2O.

In order to increase the percentage of substitution of CS, a NHS-active ester oxanorbornadiene derivative, 3, was prepared prior to obtaining CS-3 in alkaline condition (DiPEA) without EDC (Figure 4.1d, Figure H1d, and Table H1). ^{1}H-NMR confirms the percentage of substitution for CS-3 to be the highest at ~80 (Figure H3C). This suggests how reactive NHS-ester species play an important role in the introduction of oxanorbonadiene to CS. In other words, in the cases of CS-1 and CS-2, the low yield might come from the side reaction of *O*-acylurea resulting in *N*-acylurea (Figure H4).

Scheme 4.2 Cu-free cycloaddition of azido-modified substrates; 5-azidopentanoic acid, 4; 1,2-*bis*(3-azidopropyl)disulfane, 5; and (3-azidopropyl)triethoxysilane, 6; to CS-oxanorbornadiene, CS-3, yielding chitosan conjugation products CS-4, CS-5, and CS-6 in 2% CD_3COOH/H_2O.



The spacer chain length in R_1 is of interest whether it shows any effect to the reaction or not. Here, the short chain length of the oxanorbornadiene-NHS ester, 3a (supporting information), was also applied by using condition (ii). It was found that derivatization was not successful. The results indicated that oxanorbonadiene with a certain chain length is necessary as it might help minimize the steric hindrance in the reaction. In the condition (ii), the substitution of oxanorbornadiene on chitosan could be controlled at ~20%, ~70%, and ~80%, by varying the content of oxanorbornadiene 3 for 0.5, 1.5, and 3 equivalent to chitosan, respectively. It should be noted that a higher degree of oxanorbonadiene, lead to a decrease in solubility of the derivative in water.

In order to apply Click chemistry for chitosan functionalizations, a series of azido-modified substrates, i.e. 4, 5, and 6, were used as model compounds. The

success of the metal-free cycloaddition between CS-3 and 4, 5, and 6 will then be a guideline for coupling chitosan with amino acids, peptides, and antibodies, as well as being used for surface modification with inorganic particles and nanoparticles. The ligation of CS-3 with 4, 5, and 6 was carried out by using metal-free cycloaddition (Scheme 4.2) to obtain CS-4, CS-5, and CS-6, respectively. After mixing both compounds in 2% (v/v) acetic acid aqueous solution at room temperature, the cycloaddition was monitored by using ^{1}H-NMR. The success of the reaction could be traced by the disappearance of the chemical shift at $\delta \sim -62$ ppm belonging to oxanorbornadiene in ^{19}F-NMR (Figure 4.1e-g). After the reaction, a new set of ^{19}F signals could be identified. This is related to the regioisomeric form (1,4 cycloaddition (*trans*) and 1,5 cycloaddition (*cis*)) of triazole, which shows a signal at $\delta \sim -59$ for *trans* and a signal at $\delta \sim -56$ for *cis*. This result is relevant to the report by Krause et al. which showed *cis* and *trans* triazoles of monomeric model substrates (azido valeric acid and Boc-protonated ε-azido-lysine) and alginate. Another possible way to trace the reaction is the appearance of methine protons of the furan by-product as well as the disappearance of methine protons of oxanorbonadiene in ^{1}H-NMR. For example, in the case of CS-4, the appearance of chemical signals at 6.4 and 7.4 ppm, as well as disappearance of signals at 5.6, 5.7, and 7.3 ppm confirms the successful click addition of 4 to CS-3 (Figure 4.2B).

Figure 4.2C shows the plots between the ratios of furan and oxanorbonadiene and the reaction time. It is clear that azido-carboxylic acid, 4, performs the best to accomplish the reaction within 3 days whereas azido-disulfide substrate, 5, and azido-silane substrate, 6, show the completion of the reaction after more than 10 days. The reason why 4 shows higher reactivity might be related to good solubility in water. In addition, the bulkiness of 5 and 6 might hinder the ability to Click the oxanorbornadiene. Especially in the case of 5, a crosslink via two units of triazoles might be possible as can be traced from disappearance of N_3 group (2090 cm^{-1}) in FT-IR spectrum (see supporting information Figure 4.2d). The disappearance of the azido peak at 2090 cm^{-1} during the reaction could confirm the conversion of this metal-free Click reaction, while the original finger print of the chitosan-oxanorbornadiene derivative remained unchanged (Figure H2).

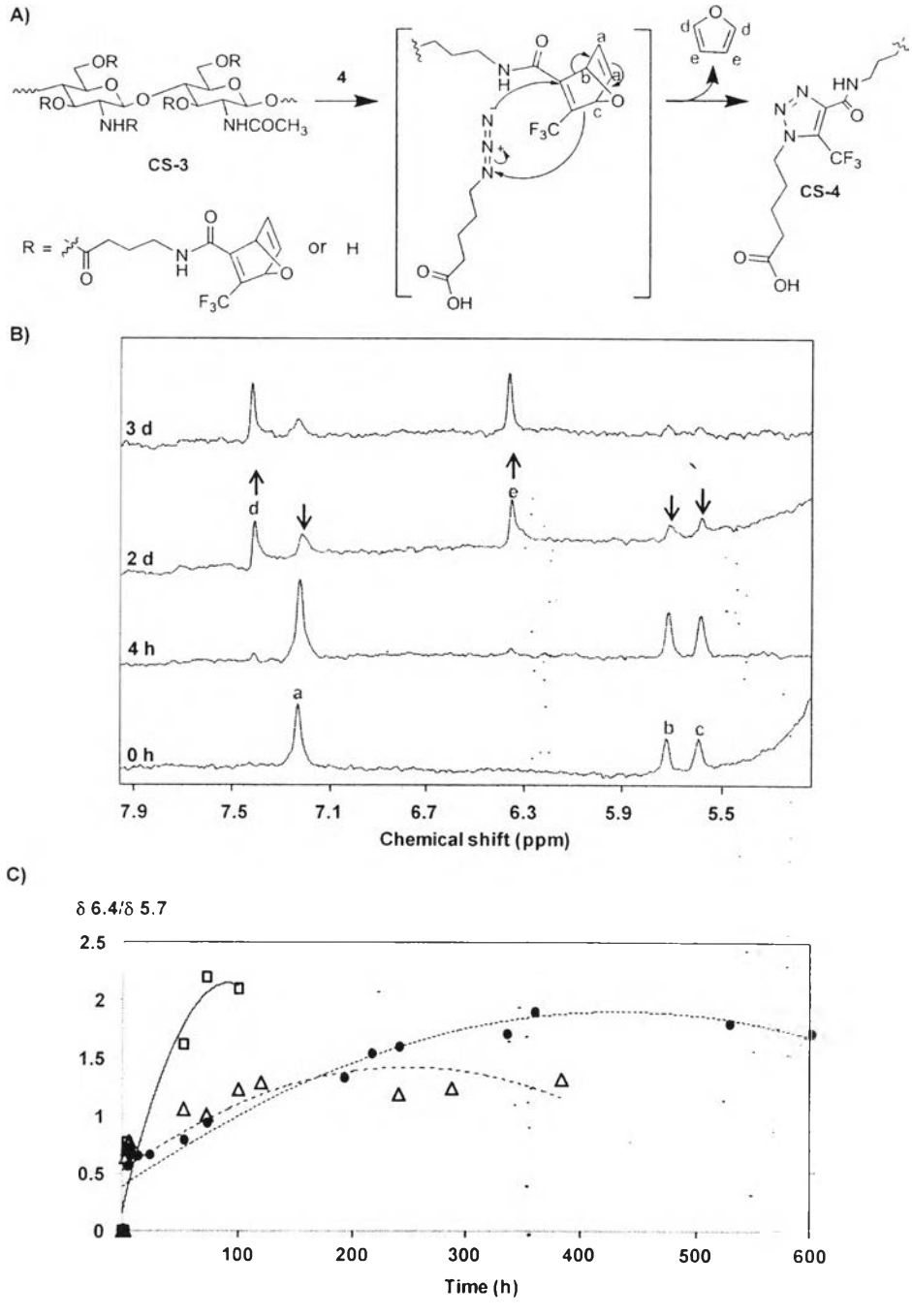


Figure 4.2 (A) Mechanism of cycloaddition between CS-3 and 4, (B) ^{1}H-NMR spectra of ligation progress in 2% CD_3COOD/D_2O over time between CS-3 and 4, and (C) Ratio of furan and oxanorbornadiene (integral ratio of δ 6.4/δ 5.7) over the reaction time detected by ^{1}H-NMR based on the integration; (□) from CS-3 and 4, (Δ) from CS-3 and 5, and (●) from CS-3 and 6.

4.4 Conclusions

In summary, novel chitosan derivatives, chitosan-oxanorbornadienes, were successfully synthesized enebling metal free Click chemisty. The hydroxyl and the amine groups at the polymeric chain acted as nucleophiles to perform the reaction with the NHS-oxanorbornadiene. The high degree of oxanorbornadiene substitution on chitosan (~80%) could be accomplished when oxanorbonadiene was used with a certain spacer chain length in the form of a NHS-active ester, 3. A series of water-based model reactions at room temperature between chitosan-oxanorbonadiene (CS-3) and azido- carboxylic acid derivative, disulfide derivative, and silane derivative proved a successful triazole linkage. Consequently, the chitosan oxanorbornadiene derivative, combined with metal-free Click chemistry, is a convenient derivative to provide simple ligation to other functional molecules like polymers or inorganic particles (e. g. magnetite, gold, silica particles) and makes chitosan useful in advanced applications, especially in the biomedical field.

4.5 Acknowledgments

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