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PREPARATION OF CHITOSAN INJECTABLE HYDROGEL FOR BONE TISSUE ENGINEERING

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A Thesis Submitted in Partial Fulfillment of the Requirements
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งานวิจัยนี้มีวัตถุประสงค์เพื่อเตรียมอนุพันธ์ของไคโตซานเพื่อใช้เป็นไฮโดรเจลฉีดได้สำหรับวิศวกรรมเนื้อเยื่อกระดูกด้วยวิธีอินคลูชันเจลเลชัน การที่นำไคโตซานเพื่อใช้เป็นไฮโดรเจลฉีดได้นั้น นอกจากไคโตซานสามารถละลายน้ำได้แล้ว ไคโตซานยังสามารถขึ้นรูปเป็นไฮโดรเจลภายใต้สภาวะร่างกายอีกด้วย แอลฟา-ไซโคลเด็กซ์ทรินได้ถูกเลือกเพื่อที่จะนำมาปรับปรุงสมบัติของไคโตซาน เนื่องจากแอลฟา-ไซโคลเด็กซ์ทรินมีปริมาณของหมู่ไฮดรอกซิลมากที่สามารถเหนี่ยวนำให้ไคโตซานสามารถละลายน้ำได้ และมีช่องว่างที่ไม่ชอบน้ำที่สามารถทำหน้าที่เป็นจุดเชื่อมขวางด้วยวิธีอินคลูชันกับพอลิเอทิลีนไกลคอล การรวมกันระหว่างแอลฟา-ไซโคลเด็กซ์ทรินและไคโตซานสามารถสังเคราะห์ผ่านคาร์บอกซีเมทิลไคโตซานที่มีหมู่คาร์บอกซิลในโครงสร้าง ไฮโดรเจลสามารถเตรียมได้ด้วยวิธีอินคลูชันเจลเลชันโดยผสมระหว่างแอลฟา-ไซโคลเด็กซ์ทรินที่ต่อลงบนไคโตซานและพอลิเอทิลีนไกลคอล จากสเปกตรัมเทคนิคฟูริเรียร์ทรานสฟอร์มอินฟราเรดสเปกโทรสโกปีของคาร์บอกซีเมทิลไคโตซานกราฟต์แอลฟา-ไซโคลเด็กซ์ทรินได้พบพีค β-pyranyl vibration ของไคโตซาน และพีค α-pyranyl vibration ของแอลฟา-ไซโคลเด็กซ์ทรินที่ 898 และ 952 cm^{-1} ตามลำดับ นอกจากนี้โครงสร้างสารถูกยืนยันด้วยเทคนิคนิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโทรสโกปี พบว่าเกิดพีค H-1 ของแอลฟา-ไซโคลเด็กซ์ทริน และพีค H-2 ของไคโตซาน จากผลการทดลองแสดงได้เห็นว่า แอลฟา-ไซโคลเด็กซ์ทรินสามารถกราฟต์ลงบนคาร์บอกซีเมทิลไคโตซานได้ สัดส่วนโมลที่เหมาะสมระหว่างแอลฟา-ไซโคลเด็กซ์ทรินและพอลิเอทิลีนไกลคอลคือ 2 ถึง 4 และจำนวนที่แอลฟา-ไซโคลเด็กซ์ทรินร้อยลงบนสายโซ่พอลิเอทิลีนไกลคอลคือ 2.5 ความเข้มข้นของคาร์บอกซีเมทิลไคโตซานกราฟต์แอลฟา-ไซโคลเด็กซ์ทรินที่ใช้เตรียมไฮโดรเจลคือ ร้อยละ 15 โดยน้ำหนักต่อปริมาตร สารละลายผสมสามารถถูกดูดเข้าไปในหลอดฉีดยา และฉีดลงในแม่พิมพ์ จากนั้นสารละลายผสมถูกขึ้นรูปเป็นไฮโดรเจลใช้เวลา 450±10 นาที ลักษณะสัญฐานวิทยาของไฮโดรเจลถูกสังเกตด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด พบว่าปรากฏรูพรุน และรูพรุนที่เกิดขึ้นเป็นรูพรุนที่รูเชื่อมขวางกัน ซึ่งสามารถส่งถ่ายอาหาร อากาศไปยังเซลล์ได้ ดังนั้นไฮโดรเจลฉีดได้นี้น่าจะเป็นวัสดุตัวรองรับสำหรับวิศวกรรมเนื้อเยื่อกระดูก

ภาควิชา วัสดุศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาศาสตร์พอลิเมอร์ประยุกต์และเทคโนโลยีสิ่งทอ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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ปีการศึกษา 2557

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KEYWORDS: CHITOSAN WATER SOLUBLE / INCLUSION GELATION / INJECTABLE HYDROGEL / TISSUE ENGINEERING

KANYARAT SAEKHOR: PREPARATION OF CHITOSAN INJECTABLE HYDROGEL FOR BONE TISSUE ENGINEERING. ADVISOR: ASST. PROF. WANPEN TACHABOONYAKIAT, Ph.D., CO-ADVISOR: PROF. SITTISAK HONSAWEK, M.D., Ph.D., pp.

This research was purposed to prepare chitosan derivative as injectable hydrogel for bone tissue engineering via inclusion gelation. In order to apply chitosan as injectable hydrogel, chitosan should not only be soluble in water, but it also could form hydrogel under physiological condition. α-cyclodextrin (α-CD) was selected to modify onto chitosan. Since, it had tremendous hydrophilic hydroxyl groups which provided chitosan for water solubility as well as hydrophobic cavities which function as crosslinking points by inclusion complex with poly(ethylene glycol), PEG. The conjugation between α-CD and chitosan was performed via water soluble carboxymethyl chitosan precursor (CM-chitosan) which provided carboxyl groups for conjugation. Hydrogel was prepared by inclusion gelation of mixing between α-CD conjugated chitosan and PEG. CM-chitosan-g-α-CD showed FTIR characteristic peak of β-pyranyl vibration of chitosan and the characteristic peak of α-pyranyl vibration of α-CD at 898 and 952 cm^{-1}, respectively. For NMR spectra, CM-chitosan-g-α-CD showed peak H-1 of α-CD and H-2 of chitosan. The results indicated that α-CD was successfully conjugated onto CM-chitosan. The optimum molar ratio of α-CD to PEG was 2 to 4 and threading number was 2.5. The concentration of CM-chitosan-g-α-CD was 15%(w/v) to prepare hydrogel. The mix solution formed hydrogel within 450±10 min. The solution could be drawn into syringe and injected into mold. The morphology of hydrogel was observed by Scanning Electron Microscope. It was found that the hydrogel appeared porosity. The porosity was interconnecting pore in order to transport nutrients to cells. Therefore, this injectable hydrogel would be a novel candidate approaching for bone tissue engineering injectable scaffolds.

Department:	Materials Science	Student's Signature .....
Field of Study:	Applied Polymer Science and Textile Technology	Advisor's Signature .....
		Co-Advisor's Signature .....
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CHAPTER I
INTRODUCTION

1.1 Background

Once bone was broken and lost some bone matrix or presented small gap, the patient need to be operated by fixing the broken bone with metal plate and screw. However, the surrounding tissues cannot be well regenerated. Another way to well regenerate bone tissue was so-called tissue engineering [1]. Scaffolds represent important components for tissue engineering. The most common scaffolds which were biodegradable polymers being used consist of polyesters such as poly(lactic acid), poly(glycolic acid), polycaprolactone and polysaccharides such as alginate, chitin chitosan [2].

Nevertheless, In order to put those scaffolds at the defect site, the doctors have to undergo the operation. So, injectable hydrogel attracted our interests in order to avoid that operation. Injectable hydrogels exhibited a sol-to-gel transition in response to stimuli change [3, 4]. Injectable hydrogels have found a number of uses in biomedical and pharmaceutical applications such as drug delivery, cell growth and tissue engineering [5]. Injectability provides several desirable advantages such as minimally invasive delivery, delivery for a site-specific action and fill irregularly sized defect sites [3, 5]. Hydrogels can be prepared several ways by polymeric crosslinkings such as ionic interaction, chemical crosslink and so on. However, those crosslinkings would be prepared out of the body to remove the unreacted reactants before used. In order to prepare for injectable hydrogels, it is our interest to molecularly design the polymer matrix having the function which the crosslink process can be done in the body without any removal of undesired by-products. The gelation by inclusion was attacked our interest. Therefore, this research was proposed the first time on the

molecularly design of polymeric matrix and inclusion gelation for preparing injectable hydrogels with an approach for bone tissue engineering.

Chitosan was selected as polymer matrix because it was one of considering natural polymers. It was biodegradable, biocompatible, and non-toxic [6]. Chitosan had amino groups in its chemical structure, which induced cell adhesion and antibacterial [7]. However, chitosan was water insoluble [8] which was not suitable for applications in physiological conditions. Therefore, chitosan was chemically modified with some functional groups which provide both water solubility and possibility for injectable gelation. With an application for tissue engineering, the amino groups would be kept for inducing cell adhesion, thus, the chemical modification of chitosan would be performed at the hydroxyl groups of chitosan instead.

Cyclodextrins or CDs were selected to modify with chitosan. Because CDs have many hydroxyl groups, the introduction of CDs onto chitosan should improve its water solubility. One more interesting property, CDs are cyclic oligosaccharides with hydrophobic cavities that can form inclusion complex with linear polymer. The inclusion complex between linear polymer and CDs was well-known as pseudo-polyrotaxane [9], [4]. Therefore, the introduction of CDs onto chitosan main chain would be functioned as the crosslinking points for inclusion gelation with linear polymers such as poly(ethylene glycol). Based on mechanism of inclusion between α-CD and PEG was a kinetic interaction, the inclusion complex would be formed or dissociated during shearing or stirring, but completely formed complex while keep clam or static condition. This phenomenon was the motivation for our research idea proposed the first time for injectable hydrogel.

This research was aimed to prepare chitosan-g-α-CD injectable hydrogel for bone tissue engineering by inclusion gelation between α-CD and poly (ethylene glycol). However, both α-CD and chitosan were composed of similar saccharide units which were not suitable for conjugation. Therefore, the conjugation between α-CD and chitosan was performed via water soluble carboxymethyl chitosan precursor (CM-chitosan) which provided carboxyl groups for conjugation. Then the α-CD was conjugated onto CM-chitosan precursor through esterification reaction. The structures of CM-chitosan and CM-chitosan-g-α-CD were characterized by FTIR and NMR. Then, the chitosan-g-α-CD could form inclusion complex with polyethylene glycol (PEG) in order to form injectable hydrogel. Injectability and setting time to become hydrogel were investigated. Then, the dried gels were observed for their morphology and tested for biological assay.

CHAPTER II
LITERATURE SURVEY

2.1 Tissue engineering

Tissue Engineering is one of the most interesting in biomedical field because this method is a great potential for regeneration or repair of tissues which lost or degenerated tissues. The surrounding tissues can be well regenerated. Cells are placed in three-dimensional scaffold in environmental of extra cellular matrix (ECM). Cellular adhesion, spreading differentiation and growth depended on structural of the scaffolds. Therefore, scaffolds have a variety of required properties, including biocompatibility, biodegradability, non-toxic, interconnecting porosity for transport nutrients and gases to cell, appropriate surface of scaffold for cells growth. [2, 10] Materials have been used for scaffold, including ceramics and polymers. Polymers are the most choices to prepare scaffold because of their selective properties that can be controlled by variation of monomers. Both synthetic and natural polymers can be formed scaffold but the biocompatible polymers such as proteins (collagen, Gelatin), polysaccharides (chitin, chitosan, alginate), poly (lactic acid), polyethylene, poly (vinyl chloride), poly (glycolic acid) and polyester urethane etc. [1, 2] were considered for selective materials for preparing scaffolds

2.2 Injectable hydrogels

Injectable hydrogels are growingly interested in promising substrates for develop tissue engineering and drug delivery because of minimally invasive procedures, delivery for a site-specific action, fill irregularly sized defect sites, easily incorporated proteins, cells or drugs into polymer solution before administration and improved patient compliance and comfort. [11-13] The hydrogel exhibited a sol-to-gel transition in response to stimuli change such as pH [14], temperature [15] etc.

Injectable hydrogel have been proposed with several polymers such as PPO-PPO-PEO, poly (vinyl alcohol), alginate, Polyanhydrides, chitin and chitosan etc. [5]

2.2.1 Preparation of hydrogels

Many methods have been employed for preparing hydrogels.

2.2.1.1 Chemical crosslink

Hong et al. [3] prepared covalently crosslinked chitosan hydrogel at neutral pH and body temperature. Methacrylic acid (MA) and lactic acid (LA) were grafted onto chitosan chain by the combination between carboxyl groups and amino groups using carbodiimide as catalyst. Then CS-MA-LA aqueous solution was formed gel via crosslinking reaction between double bonds. The crosslinked reaction was done by radical polymerization using redox initiation which gel could be formed at 37°C within few minutes.

2.2.1.2 Thermosensitive gelation

Bhattarai et al. [15] prepared thermosensitive injectable hydrogel from PEG-grafted chitosan using genipin as crosslinker. The mechanism of thermoreversible sol-gel transition of PEG grafted onto chitosan can be described as follow. At low temperature, the hydrophilic groups of PEG onto chitosan-g-PEG had intermolecular hydrogen bond with water, leading to dissolvability of chitosan-g-PEG into water. Whilst, the hydrophobic interaction between polymer chains increased when increasing the temperature,, leading to reduction of chitosan segment mobility and form gel . Therefore, when the chitosan-g-PEG solutions were heated to 37°C, the solutions were formed gels. Again, when the temperature decreased below 10°C, the gels were transformed to the solution. In addition genipin was added into chitosan-g-PEG solutions for several hours to formed gel. The color of gel was changed from light yellow to dark blue since formation of network by the reaction of

chitosan fragments in PEG-g-chitosan with genipin. This work evaluated on gelation time, it was found that at higher concentrations of polymer solution the gelation time is faster than lower concentrations of polymer solution.

2.2.1.3 Inclusion gelation

Inclusion complex are especially the ones leading to supramolecular self-assemblies. The inclusion complex between linear polymer and CDs was called polyrotaxane. Polyrotaxane has bulky groups to avoid dethreading of CDs. The cutting of bulky groups will cause degradation. While, the inclusion complex with no bulky groups was called pseudopolyrotaxane which can be degraded easily by unthreading of CDs. [4, 16] Over the past decade, polyrotaxane have interesting in field of supramolecular science due to their unique properties and structures.

In previous work, the cyclodextrins formed inclusion complex with suitable linear polymers. An α-CD cannot form inclusion complex with low molecular weight of ethylene glycol, triethylene glycol etc. [17] An α-CD formed inclusion complex with poly(ethylene glycol) (PEG) and a variety of molecular weight of oligoethylene to give inclusion complexation in high yield, but β-CD and γ-CD did not formed complex with PEG of any molecular weight, since β-CD and γ-CD cavity are too large to fit with chain of PEG. On the other hand, β-CD and γ-CD formed complex with poly(propylene glycol) (PPG) to give inclusion complexation in high yield, even though α-CD did not formed complex with PPG of any molecular weight. Since the PPG has the hindrance of methyl groups to penetrate into cavity of α-CD. Therefore the size correlation between cavities of CDs and cross section area of linear polymer chain are very important to form inclusion complexation. [16, 18]

Harada et al. [17] studied complexes formation between Poly (ethylene glycol) (PEG) and α-cyclodextrin. PEG aqueous solution using the average molecular weight between 400 and 10000 was mixed with α-CD saturated aqueous solution at room temperature to obtain turbid and precipitated complexes. In the experimental, this report studied turbidity development of saturated α-CD solution mixed PEG solution in various molecular weights. It was found that PEG of molecular weight 1000 showed rapid precipitation. When increasing the molecular weights more than 1000, the rate of turbidity development decreased. This may because high molecular weights have low the number of chain end. Quantitative of α-CD formed complex with PEG was also studied. When high concentration of PEG was added into saturated α-CD solution, the number of α-CD threading on PEG increased. The number of α-CD more than 90% was included onto PEG chain. Two ethylene glycol units were threaded in each α-CD cavity. The length of two ethylene glycol units corresponds to the depth of the α-CD cavity (7 °A). In addition the complex was observed by X-ray diffraction. It was found that the complex formed between α-CD and PEG is crystalline and iso-morphous.

Harada et al. [19] prepared polyrotaxane consisting of monodisperse α,ω-diaminopoly(ethylene glycol) (PEG-BA) (MW=1248) and α-cyclodextrins (α-CDs). Then the complex was blocked with 2,4-dinitrofluorobenzene at chain end of each polyrotaxane in order to prevent dethreading. In the experimental, the inclusion complex of α-CD and PEG-BA was prepared. PEG-BA aqueous solution was added into α-CD saturated aqueous solution at room temperature. The inclusion complex was characterized by using ^{1}H-NMR, ^{13}C-NMR and 2D NOSEY NMR spectroscopy. From ^{1}H-NMR and ^{13}C-NMR in DMSO were found broad peak of CD, PEG and dinitrophenyl group of polyrotaxane. 2D NOSEY NMR spectrum showed peak of H-3 and H-5 of CD which are located inner cavity of CD, correlate with proton of CH_2 of PEG, whereas peak of H-1, H-2 and H-4 of CD which are outer

cavity of CD, do not correlate with proton of PEG. From this result indicated that α-CDs was threaded on PEG chain. In addition, the number of CD threaded on PEG was calculated to determine the molar ratio of α-CD to PEG by comparing with the integration of proton NMR of PEG and the integration of H-1 of α-CD. But the peaks were broad because of bulky blocking groups. Therefore, the calculation to find the molar ratio of α-CD to PEG was modified to be easier by elimination of bulky end groups This method was also checked by ^{1}H-NMR spectroscopy in NaOD.

Li et al. [20] studied sol-gel transition condition and time of gelation for inclusion complex between α-cyclodextrin and high molecular weight of poly (ethylene glycol) (PEG). In this experiment, α-CD aqueous solution was mixed with aqueous solution of various molecular weight of PEG. The mixed solution was sonicated and left out at room temperature for at least 90 hrs. α-CD formed inclusion complex with PEG at suitable concentrations on each conditions. Gelation can be formed only at high concentration of α-CD and PEG. Because α-CD threaded only at the chain ends of PEG chains to form gel. Time for gelation of α-CD and PEG (MW=20,000) aqueous solution was a function of PEG α-CD concentrations. It was found that the time of gelation decreased as the concentrations of PEG or α-CD increased. The gelation time of α-CD-PEG (MW=20,000) aqueous solution was also a function of PEG molecular weight. It was found that gelation time increased as the PEG molecular weight increased. Since, PEG with high molecular weight performed chain entanglement. Therefore, PEG chains have a difficulty to include into cavity of α-CD.

Li et al. [21] studied on supramolecular self-assembled hydrogel. Cyclodextrins have inner hydrophobic cavity, which can form inclusion complex with suitable hydrophobic linear polymers. The structure is like necklace. In this report, hydrogels were prepared via self-assembled between PEO-PHB-PEO triblock copolymers and α-CD. It was found that, suitable concentration to form gels was 13 %(w/v)of PEO-PHB-PEO copolymers and 9.7 %(w/v). of α-CD at room temperature, the α-CD was threaded on chain ends of PEO segment. PHB has hydrophobicity and high crystallinity, thus, hydrophobic interaction between PHB blocks were formed self-assembly which could be induced supramolecular hydrogels.

2.2.2 Application of injectable hydrogels via inclusion gelation method

Injectable hydrogels have found a number of uses in biomedical and pharmaceutical applications, such as drug delivery, cell growth and tissue engineering. These hydrogels can be used in simple pharmaceutical formulations which can be prepared by mixing the hydrogel with drugs, proteins, or cells.

Li et al. [22] studied supramolecular polymer based cyclodextrins for drug and gene delivery using injection method. α-CDs threaded over Poly (ethylene oxide) chain to form inclusion complexation. The gels were found a unique property which was thixotropic and reversible. This property gets the gels forming injectable by a fine needle. The viscosity of gels reduced and reverted to original within hours when there was no disturbed. Drugs or genes were added into the gels in a syringe, then injected in tissue and released the drugs or genes.

Tan et al. [5] studied injectable hydrogel for tissue engineering application. An approach of injectable hydrogels for tissue regeneration, first cells are mixed with hydrogel precursors which are subsequently transplanted into the body by injection using needle. The Hydrogels provide the initial structural support and

retain cells in the defect site for cell growth, metabolism and new extracellular matrix synthesis, while hydrogel gradually degraded.

2.3 Biopolymer

2.3.1 Chitosan

Chitosan is a biopolymer which is the second most polysaccharide next to cellulose. Normally chitosan is a derivative from of chitin by deacetylation with alkaline or enzyme which is found in shells of shrimp and crab and the exoskeletons of cephalopods.[10, 23, 24] Chitosan, a linear amino polysaccharide copolymer, is composed of β-1,4-D-glucosamine and β-1,4-*N*-acetyl-glucosamine units [25] as follow in Figure 2.1. Generally, chitosan need to have %deacetylation more than 40% [10]. Chitosan has found a number of uses in biomedical such as tissue engineering [5], wound dressing [26], drug and gene delivery [22]. Because chitosan is biodegradable, biocompatible, non-toxic [23, 27] and antibacterial [28]. Chitosan is only soluble in dilute acidic aqueous solution which limits some of its applications such as solubility in water. [29] Chitosan has two reactive group which were reactive amino groups and reactive hydroxyl groups. Therefore, chitosan can be modified with various other function groups.

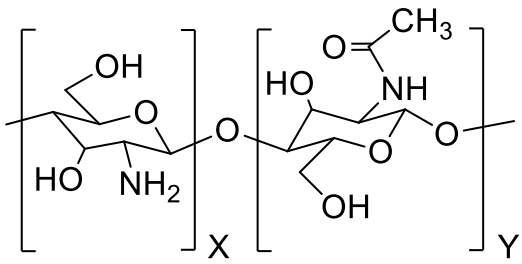


Figure 2.1 Chemical structures of chitosan.

- Carboxymethyl chitosan

Tokura et al. [30] studied on water soluble chitin derivative. Carboxymethyl-chitin prepared by suspending chitin with NaOH solution at -20°C overnight. After that the frozen alkali-chitin was suspended in isopropanol at room temperature, and monochloroacetic acid was dropwised. The mixture solution was mix with mechanical stirrer. The product was filtered and washed with ethanol several times. In the result, the degree of substitution of carboxymethyl chitin was evaluated as 0.6-0.8 under their reaction condition from elemental analysis and potentiametric titrate. For ^{13}C-NMR spectrum of carboxymethyl chitin in D_2O showed peak C-6 of *N*-acetylglucosamine at 63 ppm which decreased and showed new peak at 60 ppm of substituted carbon atom.

Zheng et al. [31] synthesized and characterized O-carboxymethyl chitosan. Preparation of CM-chitosan, chitosan was add into sodium hydroxide and isopropanol as solvent to swell and alkalize at -20°C for 24 h. Monochloroacetic acid was dissolved in isopropanol, then dropwised into the reaction mixture and reacted at 50°C for 7 h. The reaction was stopped by adding 70% ethanol. The product was filtered and rinsed with 70% ethanol several times. The primary product was the sodium salt of CM-chitosan. The CM-chitosan was prepared by immersing in 70% ethanol and adding 32% HCl. Finally the CM-chitosan product was freeze dried. From FTIR spectrum of sodium salt of CM-chitosan showed new absorption peaks at 1407 cm^{-1} and 1598 cm^{-1}, assigned to the symmetry and asymmetry stretch vibration of COO^-, respectively. In addition, the structural of sodium salt of CM-chitosan was confirmed from peak at 1068 cm^{-1} corresponding to C-O-C stretching. The C-O stretching peak at 1030 cm^{-1} responding to the primary hydroxyl group disappears, it indicated that verifying carboxymethyl was substituted onto hydroxyl groups of chitosan. Furthermore, ^{1}H-NMR spectrum of CM-chitosan showed peak of H-2 of

glucosamine ring at 3.61 ppm, H-2 of glucosamine ring with amino group substitution at 3.61 ppm, H-3, 4, 5, 6 of glucosamine ring at 3.7-3.9 ppm and H-1 of glucosamine ring at 4.75 ppm. The proton signal of CM-chitosan was showed around 4.0-4.1 ppm. For ^{13}C-NMR spectrum of CM-chitosan, peak at 55.2 and 178.9 ppm were carbonyl carbon O-CH2 and COOH carboxyl group, respectively. And the *N*-substitution is not presented because the peak at 47 and 168 ppm, assigned to *N*-CH_2 and COOH were not observed in this spectrum, respectively. This result indicated that the carboxymethyl was linked to chitosan backbone at hydroxyl groups of chitosan.

2.3.2 Cyclodextrins

Cyclodextrins (CDs) are widely used in many fields such as pharmaceutical, agrochemical, analytical, food, cosmetic and biochemical etc.[32] CDs are a number of cyclic oligosaccharides of α-1,4-D-glucopyranoside. Typical of CDs are divided in three majors which were consist of 6, 7 and 8 membered ring of glucose molecules as shown in Figure 2.2. [33, 34]

Thus:

- α-CD : six membered glucose ring molecule
- β-CD : seven membered glucose ring molecule
- γ-CD : eight membered glucose ring molecule

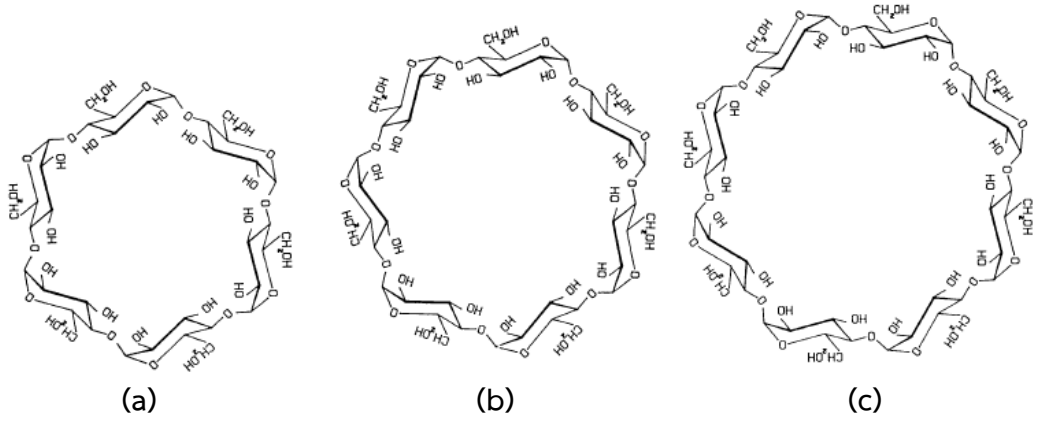


Figure 2.2 Chemical structures of a) α-CD, b) β-CD and c) γ-CD.

Cyclodextrins has hydrophobic cavity which is a unique properties. CDs can form inclusion complex with suitable linear polymer by hydrophobic interaction between hydrogen of polymer chain and hydrogen of CDs. This phenomenon is characterized by 2D-NMR. Previous research, Harada et al. [16] characterized correlation of inclusion complex between α-CD and poly (ethylene glycol). From 2D NOSEY NMR result, it was found that peak of H-3 and H-5 of α-CD correlated with the resonance of proton (CH2) of PEG. For H-1, H-2 and H-4 are located outer of α-CD cavity. They do not correlate with proton (CH2) of PEG. This result indicated that PEG chain could include into cavity of α-CD.

CHAPTER III

EXPERIMENTAL

3.1 Materials and chemicals

3.1.1 Chitosan

A chitosan flake, degree of deacetylation (DD) 85%, was purchased from Seafresh Industry Public Company Limited, Bangkok Thailand, and used as a polymer matrix for preparing injectable hydrogel.

3.1.2 Carboxymethyl chloride

An analytical reagent grade of carboxymethyl chloride was purchased from Merck, Darmstadt, Germany, and used as a reactant for chemical modification of chitosan to synthesize water soluble chitosan precursor of carboxylmethyl chitosan (CM-chitosan).

3.1.3 Alpha – cyclodextrin

An analytical reagent grade of α-cyclodextrin (α-CD) was purchased from Junsei Chemical Co. Ltd, Japan and used as a reactant for chemical modification to carboxylmethyl chitosan precursor to synthesize water soluble CM-chitosan-g-α-CD.

3.1.4 Poly(ethylene glycol)

An analytical reagent grade of Poly(ethylene glycol), molecular weight 1000 and 4000 Da, were purchased from Merck KgaA, Darmstadt, Germany, and used as a crosslinker which can form inclusion complex with α-cyclodextrin to generate hydrogel.

3.1.5 Sodium hydroxide

An analytical reagent grade of sodium hydroxide was purchased from Ajax Finechem, New Zealand and used to prepare sodium salt of carboxylmethyl chitosan.

3.1.6 1-Hydroxybenzotriazole hydrate (HOBT)

A synthesis grade of 1-Hydroxybenzotriazole hydrate was purchased from Sigma-Aldrich Co., Missouri, USA, and used as a proton donor for synthesizing α-cyclodextrin conjuated onto chitosan main chain.

3.1.7 *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC · HCl)

A synthesis grade of *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride was purchased from Sigma-Aldrich Co., Missouri, USA., and used as a coupling agent for synthesizing chitosan-g- α-cyclodextrin.

3.1.8 Ethanol

A commercial grade of ethanol was purchased from RCI Labscan Co. Ltd., Bangkok, Thailand, and used to purify the product or eliminate sodium hydroxide form chitosan derivative.

3.1.9 Distilled water

Distilled water was used in the whole experiment. Sterile water was used in biological experiment.

3.2 Equipments and Instruments

The whole of equipment and instruments which being used in this research were showed in Table 3.1 and Table 3.2

Table 3.1 Equipment and instruments used to prepare CM-chitosan, CM-chitosan-g-α-CD and injectable hydrogel via inclusion gelation.

Equipment and Instruments	Model	Company/City/Country
Magnetic stirrer	-	-
Mechanical stirrer	IKA C-MAG HS7	-
Water bath	-	Memert/Germany
Lyophilizer	Beta 1-8 LD plus	Christ/Germany
Aspirator	Eyela aspirator A-25	Tokyo Rikakikai Co., Ltd/Tokyo/Japan
Three-necked round bottle flask	500 ml	Duran/Wertheim/Germany

Table 3.2 Equipment and instruments used for characterization and testing.

Equipments and Instruments	Model	Company/City/Country
Fourier transform infrared spectroscopy (FTIR)	Nicolet 6700	Thermo Scientific/Wisconsin/USA
Nuclear magnetic resonance spectroscopy (NMR)	Ultrashield 500 plus	Bruker/ USA
Scanning electron microscope (SEM)	JSM-6400	JEOL Ltd/Tokyo/Japan
Digital micrometer	S-Dial S229	Sylvac, Crissier, Switzerland

3.3 Experimental procedure

3.3.1 Synthesis of water soluble chitosan derivatives

3.3.1.1 Synthesis of carboxymethyl chitosan (CM-chitosan) precursor

This procedure was prepared by adapting the method of Tokura et al. [30]. Chitosan (10 g., 0.06 mol) was suspended in 60 ml of freshly prepared 60% sodium hydroxide solution including 0.2% sodium dodecylsulphate (SDS) into three-necked round bottom flask. The reaction flask was put into water bath at 0°C for 3 h. After that, a 180 ml of isopropanol was added into the alkali chitosan solution at room temperature. Carboxymethyl chloride (12 g., 0.13 mol) was dissolved in isopropanol 60 ml and added dropwise into the reaction mixture for 2 h. The reaction temperature was raised to 60°C under mechanical stirring at approximately 400 rpm. The product was filtered and washed with ethanol. The residue was extracted with 1.5 liters of distilled water at room temperature. The water soluble CM-chitosan was obtained after dialysis against distilled water and freeze-dried.

3.3.1.2 Synthesis of CM-chitosan-g-α-cyclodextrin (CM-chitosan-g-α-CD)

CM-chitosan (0.5 g., 2 mmol) was dissolved in distilled water (50 ml) into 100 ml beaker. The α-CD aqueous solution was prepared by dissolving α-CD (3.45 g., 4 mmol), HOBT (0.95 g., 8 mmol) and EDC · HCl (1.35 g., 7 mmol) in distilled water (30 ml) into another beaker. The α-CD aqueous solution was dropwised into CM-chitosan aqueous solution and stir at room temperature for 24 hours. The obtained product was dialyzed against distilled water and freeze-dried.

3.3.2 Structural characterization

3.3.2.1 Structural characterization of CM-chitosan and chitosan-g-α-cyclodextrin

3.3.2.1.1 Fourier Transform Infrared Spectroscopy (FTIR)

The functional group of CM-chitosan and chitosan-g-α-cyclodextrin were characterized by Attenuated Total Reflectance Fourier Transform Infrared Spectrometer, ATR-FTIR, (Nicolet 6700, Thermo Scientific, Waltham, USA) as shown in Figure 3.1. The samples were divided to small pieces and placed on the ATR probe. This method is able to characterize the samples directly. The FTIR spectra were used to specify the functional groups. FTIR spectrum of each sample was collected from 3900-550 cm^{-1} at resolution of 8 cm^{-1} with number of scan 64 scans.

Figure 3.1 Fourier Transform Infrared Spectroscopy (Nicolet 6700, Thermo Scientific, Waltham, USA)

3.3.2.1.2 Nuclear magnetic resonance spectroscopy (NMR)

The chemical structure of CM-chitosan and chitosan-g-**α**-cyclodextrin were characterized by Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and 13-Carbon nuclear magnetic resonance spectroscopy (^{13}C-NMR) (Bruker, USA) as shown in Figure 3.2 using at 500 MHz. All samples were dissolved in D_2O as solvent except only chitosan was dissolved in CD_3COOD/D_2O.

Figure 3.2 Nuclear Magnetic Resonance Spectroscopy (Bruker, USA).

3.3.2.1.3 Degree of substitution

Degree of substitution of CM-chitosan was investigated by using ^{1}H-NMR and calculated following this equation;

$$\text{DS of CM-chitosan} = \frac{I_{methylene\ proton}}{I_{H-2\ of\ chitosan}} \qquad (1)$$

Where;

$I_{\text{methylene proton}}$ = integral area from ^{1}H-NMR peak of methylene proton around 3.96-3.93-ppm

$I_{H\text{-}2\ of\ chitosan}$ = integral area from ^{1}H-NMR peak of H-2 of chitosan at 4.47 ppm

Degree of substitution of CM-chitosan-g-α-CD was investigated by using ^{1}H-NMR and calculating follow as;

$$\text{DS of CM-chitosan-g-}\alpha\text{-CD} = \frac{\frac{I_{H-1\ of\ \alpha-CD}}{6}}{I_{H-2\ of\ chitosan}} \qquad (2)$$

Where;

$I_{H\text{-}1\ of\ \alpha\text{-CD}}$ = integral area from ^{1}H-NMR peak of H-1of α-CD at 5.03 ppm

$I_{H\text{-}2\ of\ chitosan}$ = integral area from ^{1}H-NMR peak of H-2 of chitosan at 2.91 ppm

3.3.3 Investigation of the optimum ratio between CM-chitosan-g-α-CD and poly (ethylene glycol) (PEG) to prepare hydrogel

The optimum ratio between CM-chitosan-g-α-CD and PEG was investigated by calculating from ^{1}H-NMR. The molar ratio of CM-chitosan-g-α-CD to PEG were varied to 0.5, 1, 2, 4 and 8 by fixing mole of CM-chitosan-g-α-CD (20 μmole). The molar ratio of CM-chitosan-g-α-CD to PEG were calculated follow as;

$$\text{Molar ratio} = \frac{g_{CM-chitosan-g-\alpha-CD}}{MW_{CM-chitosan-g-\alpha-CD}} \times \text{DS} \qquad (3)$$

Where;

$g_{\text{CM-chitosan-g-}\alpha\text{-CD}}$ = gram of CM-chitosan-g-α-CD

$MW_{\text{CM-chitosan-g-}\alpha\text{-CD}}$ = Molecular weight of CM-chitosan-g-α-CD

DS = Degree of substitution of CM-chitosan-g-α-CD

The threading number of each molar ratio was calculated from ^{1}H-NMR. The relation between molar ratio and threading number was plotted. The inclusion was formed in the excess CM-chitosan-g-CD or PEG, since the excess reagents did not be removed from the mixture. Therefore, the appropriate molar ratio to form inclusion complex should be the plateau region which represented the equilibrium. The threading number calculated follow as;

$$\text{Threading number} = \frac{\frac{I_{\alpha-CD}}{6}}{\frac{I_{PEG}}{4\,x\,repeating\ unit}} \qquad (4)$$

Where;

$I_{\alpha\text{-CD}}$ = integral area from ^{1}H-NMR peak of H-1 of **α**-CD at 5.03 ppm

I_{PEG} = integral area from ^{1}H-NMR peak of H of PEG (-CH_2-CH_2-) around 3.68-3.62 ppm

3.3.4 Preparation of injectable hydrogel via inclusion gelation

Hydrogel was prepared by mixing PEG in a saturated aqueous solution of CM-chitosan-g-**α**-CD until the solution was homogenous. The molar ratio between CM-chitosan-g-**α**-CD and PEG to form inclusion gelation was selected from the appropriate ratio obtaining form 3.3.3. In this part, the CM-chitosan-g-**α**-CD was functioned as polymeric matrix, whilst PEG was acted as inclusion crosslinker. The optimum concentration of the CM-chitosan-g-**α**-CD for preparing injectable hydrogel was defined from the maximum concentration that the mixture can flow through syringe and needle number 18 which was around 15%(w/v). The mixture was injected into molds and investigated for its setting time.

3.3.4.1 Investigation of hydrogel setting time

The obtained mixture was drawn into syringe, injected to test tube and keep clam at 37°C. The setting time was defined as the duration time of changing from viscous fluid to hydrogel. This experiment was done in triplicate.

3.3.5 Characterization of hydrogel

3.3.5.1 Morphology of hydrogel

The morphology of hydrogel preparing by inclusion gelation between CM-chitosan-g-α-CD and PEG was observed by Scanning Electron Microscope (SEM) (JSM-6400 and Link ISIS Series 300, JEOL Ltd., Tokyo, Japan) as shown in Figure 3.3. The hydrogel was freeze-dried and coated with gold via sputtering technique to resist charging on the specimen surface. Each specimen was observed under magnification 35x, 100X and 500X, depending on the size of porosity.

Figure 3.3 Scanning Electron Microscope (JSM-6400, JEOL, Tokyo, Japan).

3.3.6 Biological property

3.3.6.1 Initial cell attachment and proliferation assay

In this research, sarcoma osteogenic cell line ($SaOs_2$) was used. $SaOs_2$ cells ($1.0x10^5$ cells/hydrogel) were seeded onto the hydrogels and cultured in proliferation medium (α-MEM supplemented with 15% fetal bovine serum (FBS)) at 37 °C, 5% CO_2. The numbers of cells were measured after cultivation for one day, three, five, and seven days using a DNA determination assay. The brief processes and the methods of DNA determination assay are described as follows. The cell samples were lysed in sodium citrate-buffered saline solution (pH 7.4) containing sodium dodecylsulfate at 37 °C overnight. Subsequently, 100 μL of cell lysate was mixed with a fluorescent dye solution (Hoechst 33258 dye) in a 96-well black plate. The fluorescence intensities of the mixed solutions were spontaneously measured at the excitation and emission wavelengths of 355 and 460 nm, respectively. The standard curve between the DNA and cell numbers was prepared using cells of known numbers. This procedure was prepared by a reported method of Thitiset et al. [35]

CHAPTER IV
RESULTS AND DISCUSSION

The goal of this research is to prepare injectable hydrogel via inclusion gelation for bone tissue engineering by using chitosan as polymer matrix. Injectable hydrogel for bone tissue engineering has many advantages such as minimally invasive procedures, delivery for a site-specific action, fill irregularly sized defect sites etc [11, 12]. Chitosan is one of the most natural polymer which is selected for using in many fields of biomedical applications [10]. However, chitosan cannot be dissolved in water which is the main drawback for using in some biomedical applications, especially for injectable hydrogel. In order to apply chitosan as injectable hydrogel, chitosan should not only be soluble in water or physiological body fluid, but it also could form hydrogel under physiological condition. Therefore, chitosan was chemically modified with some functional groups which provide both water solubility and possibility for injectable gelation. α-cyclodextrin (α-CD) was selected to modify onto chitosan. Since, it had tremendous hydrophilic hydroxyl groups which provided chitosan for water solubility. Besides, α-CD is a cyclic oligosaccharide composed of six α-D-glucopyranoside units linking with α-gylcosidic linkage, leading to generate the hydrophobic cavities within the cyclic molecules. Those hydrophobic cavities would function as crosslinking points by inclusion complex between α-CD and linear polymers such as poly(ethylene glycol), PEG. Therefore, PEG in this research would function as inclusion crosslinker. Therefore, the conjugation of α-CD onto chitosan main chain was attacked our interest to provide water soluble chitosan derivative which composed of function of forming gel by inclusion complex. However, both α-CD and chitosan were composed of similar saccharide units which were not suitable for conjugation. Therefore, the conjugation between α-CD and chitosan was

performed via water soluble carboxymethyl chitosan precursor (CM-chitosan) which provided carboxyl groups for conjugation.

Hydrogel was prepared by inclusion gelation of mixing between α-CD conjugated chitosan and poly (ethylene glycol) (PEG). Based on mechanism of inclusion between α-CD and PEG was a kinetic interaction, the inclusion complex would be formed or dissociated during shearing or stirring, but completely formed complex while keep clam or static condition. This phenomenon was the motivation for our research idea to apply for injectable hydrogel. Therefore, the injectable hydrogel was used as scaffolds for bone tissue engineering. The inclusion complex between α-CD conjugated chitosan and PEG can be formed and dissociated during accepting shearing force by injection through the needle (viscouse fluid), and then the inclusion gelation would be completely formed at the bone defect site (hydrogel).

4.1 Synthesis of water soluble chitosan derivatives

4.1.1 Synthesis of water soluble carboxymethyl chitosan (CM-chitosan) precursor

Carboxymethyl chloride was reacted with hydroxyl groups of chitosan via S_N2 reaction to obtain carboxymethyl chitosan (CM-chitosan) precursor, because functional groups of α-CD has only hydroxyl groups which cannot be conjugated onto hydroxyl or amino groups of chitosan. First step chitosan was soaked in NaOH (aq) to swell and alkalize for 3 hours in ice bath which the nucleophiles were activated at hydroxyl groups of chitosan, whilst amino groups were not. Then carboxymethyl chloride in isopropanol was gradually dropwised into the alkali chitosan solution and the reaction was carried out at 60 ºC for 3 hours to obtain CM-chitosan. The substitution would occur at hydroxyl groups, due to the nucleophiles

of amino group positions was less reactive than hydroxyl group positions. The reaction pathway was shown in Figure 4.1.

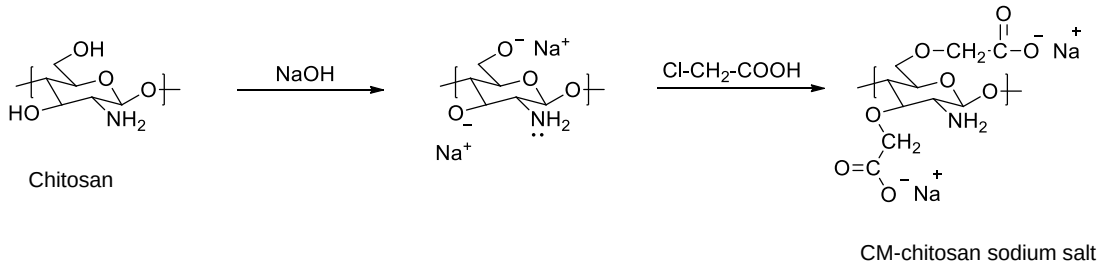


Figure 4.1 Synthesis pathway of CM-chitosan.

4.1.2 Synthesis of CM-chitosan-g-α-cyclodextrin

CM-chitosan-g-α-CD was synthesized by esterification between carboxyl groups of CM-chitosan and hydroxyl groups of α-CD using EDC·HCl as coupling agent and HOBT as proton donor. The esterification could occur at room temperature. The reaction started by HOBT protonated the amino groups of chitosan to support CM-chitosan to dissolve in water as well as activated EDC·HCl and CM-chitosan via donating proton. An EDC·HCl reacted at carboxyl groups of CM-chitosan to obtain O-acylisourea active ester intermediate which was unstable and then reacted with hydroxyl groups of α-CD to obtain water soluble CM-chitosan-g-α-CD and water soluble isourea as by-product. The by-product and the remained reactants would be removed by dialysis. The reaction was shown in Figure 4.2.

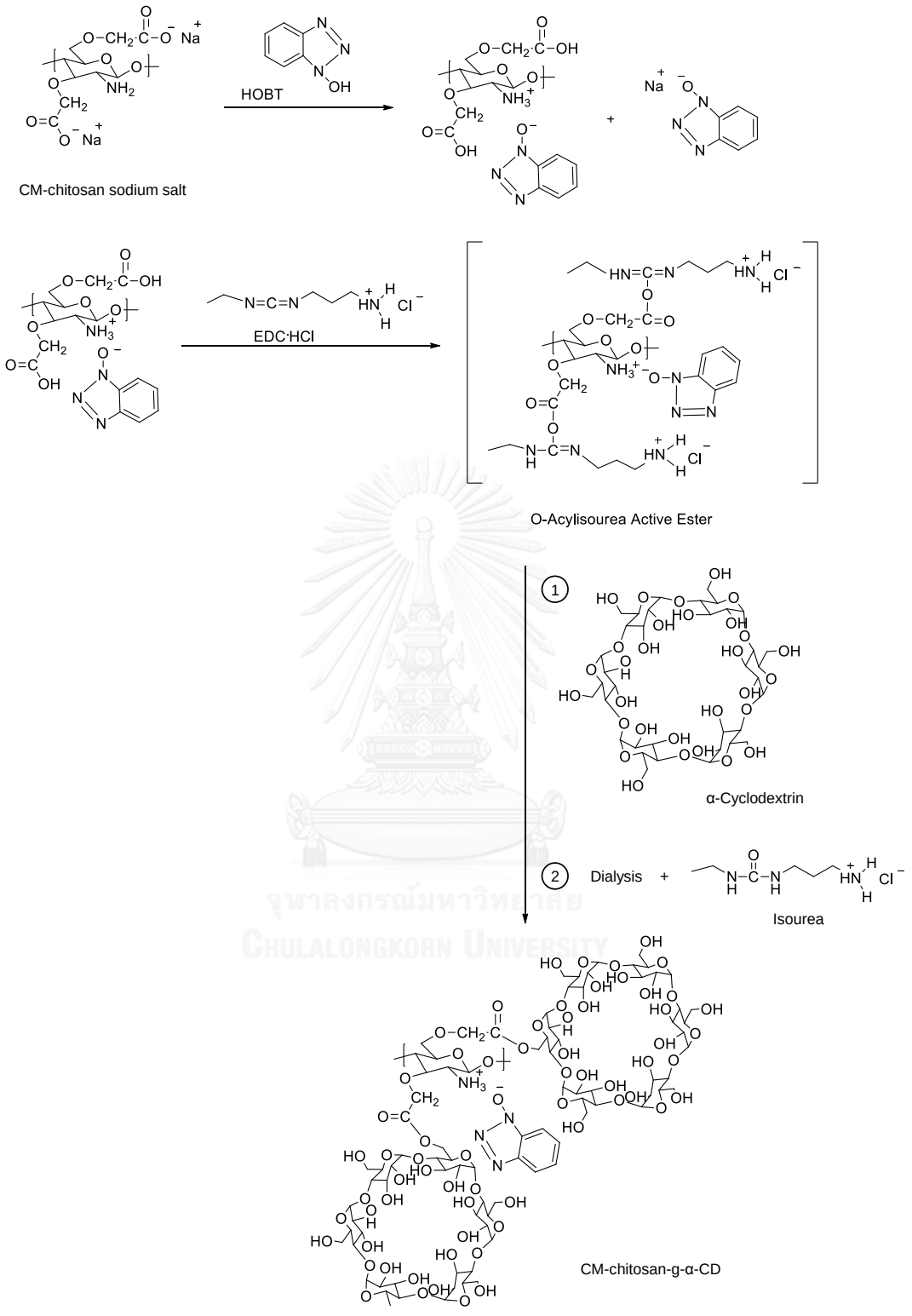


Figure 4.2 Synthesis pathway of CM-chitosan-g-**α**-CD.

4.2 Structural characterization of water soluble chitosan derivatives

4.2.1 FT-IR analysis

FTIR spectroscopy has been used to determine the structure of chitosan, CM-chitosan and CM-chitosan-g-α-CD. FTIR spectra of chitosan, CM-chitosan and CM-chitosan-g-α-CD were showed in Figure 4.3. Chitosan showed absorption peaks of hydroxyl group at 3369 cm^{-1}, C–H stretching at 2873 cm^{-1}, N–H bending at 1597 cm^{-1} and C-O stretching at 1066 cm^{-1} [31]. When chitosan was modified with carboxymethyl chloride, the carboxyl groups and ether linkage were formed. CM-chitosan spectrum showed absorption peaks of hydroxyl group at 3355 cm^{-1}, C–H stretching at 2876 cm^{-1} and sodium salt of carboxyl groups at 1585 cm^{-1}. According to Chen et al., the sodium salt of carboxyl groups showed the absorption peak around 1598 cm^{-1} whilst H-form of carboxyl groups showed the absorption peak around 1741 cm^{-1}. [36] In addition, this spectrum appeared new absorption peaks of sodium salt of carboxyl group at 1585 cm^{-1} and 1406 cm^{-1}, assigned to the asymmetry and symmetry stretch vibration of COO^{-}, respectively [31]. CM-chitosan showed high fraction of carboxyl and amino groups indicated that substitution of carboxymethyl groups onto chitosan backbone mostly on –OH position. Because of α-CD and chitosan were carbohydrates, they had some similar FTIR absorption peaks. When α-CD grafted on CM-chitosan, most IR spectra absorption peaks of α-CD would be overlapped with chitosan. Therefore the IR spectrum of CM-chitosan-g-α-CD was similar to chitosan. However, the characteristic peak of β-pyranyl vibration of chitosan would be observed at 898 cm^{-1} whilst the characteristic peak of α-pyranyl vibration of α-CD would be observed at 952 cm^{-1} [37, 38]. Both β-pyranyl vibration of chitosan (865 cm^{-1}) and α-pyranyl vibration of α-CD (949 cm^{-1}) appeared in the spectrum of CM-chitosan-g-α-CD, indicating that α-CD was successfully conjugated onto chitosan.

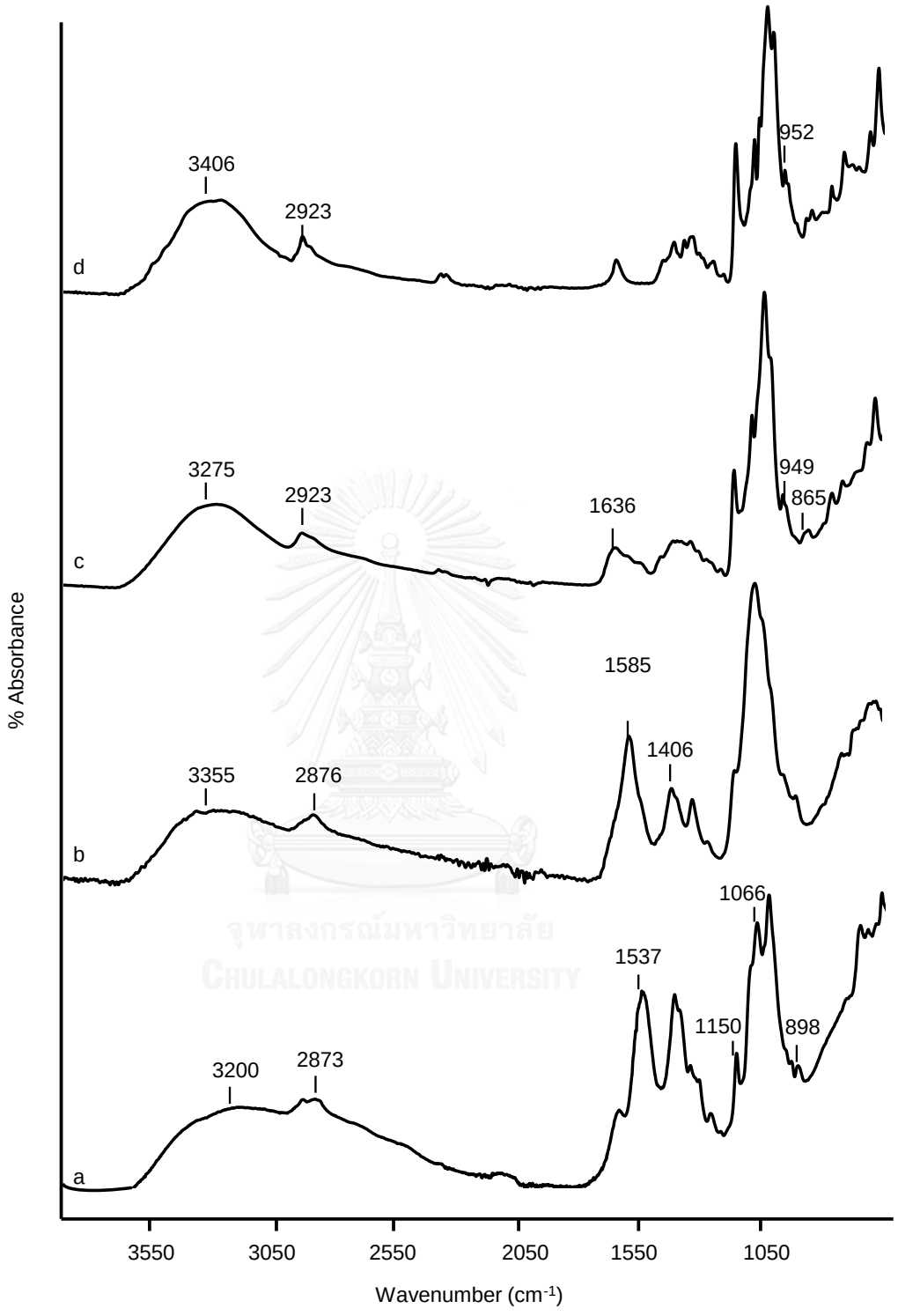


Figure 4.3 FTIR spectra of a) chitosan, b) CM-chitosan, c) CM-chitosan-g-**α**-CD and d) **α**-CD.

4.2.2 NMR analysis

Furthermore, the structure of CM-chitosan and CM-chitosan-g-α-CD were characterized by ^{1}H-NMR spectroscopy as shown in Figure 4.4. ^{1}H-NMR spectrum of chitosan in CD_3COOD/D_2O (Figure 4.4a) was shown as follows (ppm): 3.19 (H-2 of chitosan), 3.95-3.76 (H-3, H-4, H-5 and H-6 of chitosan) [39]. Because CD_3COOD/D_2O was used as solvent of chitosan, it was found that H-1 peak of chitosan was shifted to overlap with solvent peak. The spectrum of CM-chitosan in D_2O (Figure 4.4b) was shown as follows (ppm): 4.47 (H-1 of chitosan), 2.72 (H-2 of chitosan) and 3.77-3.53 (H-3, H-4, H-5 and H-6 of chitosan). This spectrum appeared new peak which was methylene proton at 3.96-3.93 ppm [31, 40]. The ^{1}H-NMR spectrum of CM-chitosan-g-α-CD in D_2O (Figure 4.4c) was showed peaks of H-2, H-3, H-4, H-5, H-6 of α-CD overlapped with H-3, H-4, H-5, H-6 of chitosan at 3.96-3.59 ppm. H-1 of α-CD and H-2 of chitosan was represented at 5.03 ppm and 2.91 ppm, respectively. Besides, peaks of H of aromatic of OBT were also observed as counter ions at7.79, 7.66, 7.4. From this result, it indicated that α-CD was substituted onto chitosan backbone.

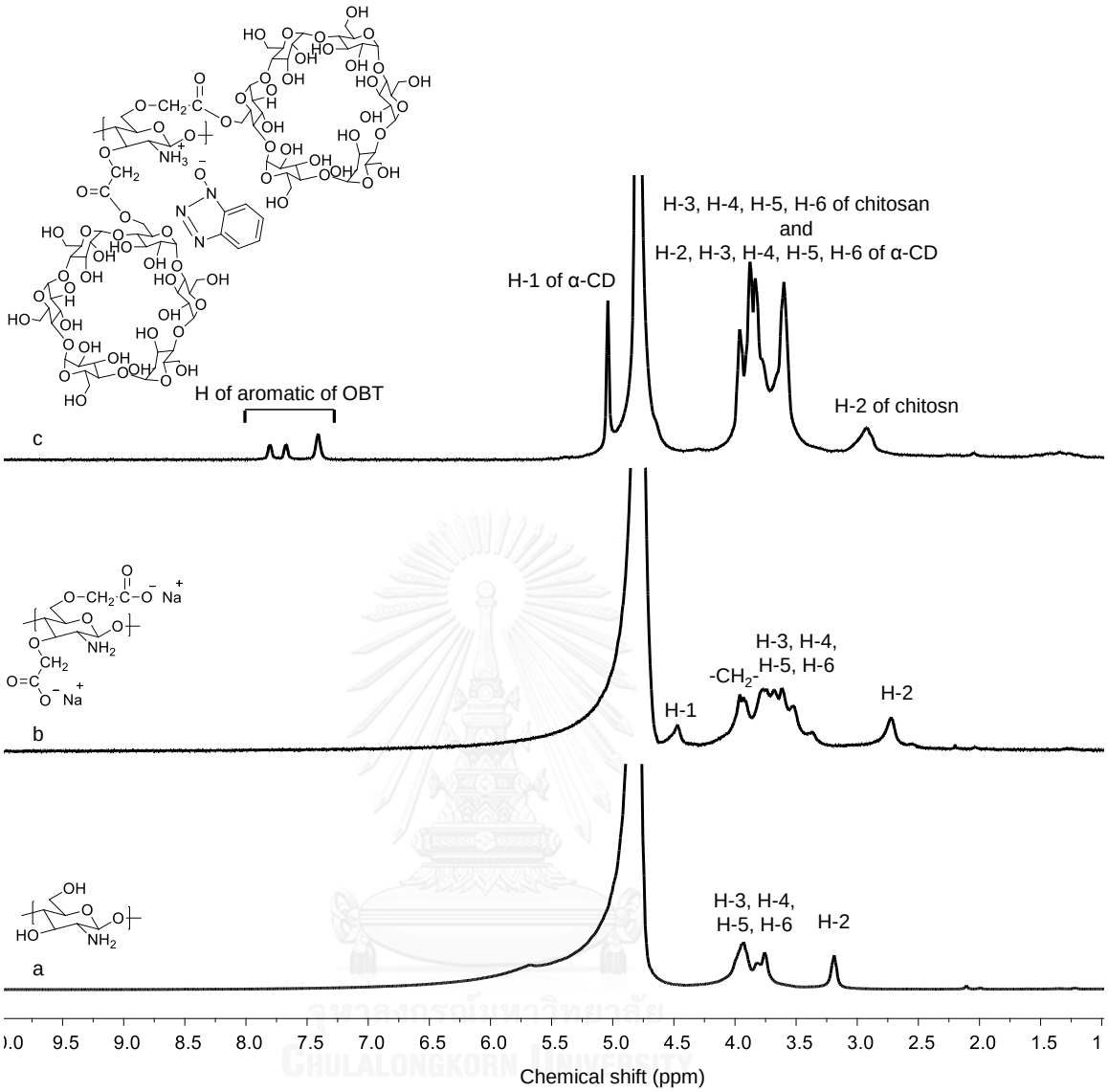


Figure 4.4 ^{1}H-NMR spectra of a) Chitosan in CD_3COOD/D_2O, b) CM-chitosan in D_2O and c) CM-chitosan-g-**α**-CD.

In addition, the structure of CM-chitosan and CM-chitosan-g-α-CD were confirmed by ^{13}C-NMR as shown in Figure 4.5. The ^{13}C-NMR spectrum of chitosan in CD_3COOD/D_2O (Figure 4.5a) was shown as follows (ppm): 180 (C=O of CD_3COOD as solvent and C=O of *N*-acetylglucosamine ($NHCOCH_3$)), 98 (C-1), 76 (C-4), 74 (C-5), 70 (C-3), 60 (C-6), 55 (C-2) and 21 (-CH_3 of CD_3COOD as solvent) [31]. The ^{13}C-NMR spectrum of CM-chitosan in D_2O was described in Figure 4.5b (ppm); 102 assigned to C-1 of chitosan and 78 assigned to C-4 of chitosan. The decrement of C-4 peak may be due to the substitution influence at C-3 of chitosan. Besides, CM-chitosan showed ^{13}C-NMR spectrum (ppm) at 74 (C-5 of chitosan), 70, 69 (C-3 of chitosan) which was split into 2 peaks, indicating partial substitution occurred at C-3 positions, 60 (C-6 of chitosan) which was decreased, indicating the substitution at C-6 position, 56 (C-2 of chitosan), 52 (O-CH_2 of carboxymethyl group) and 177 (-COO- of carboxyl group) [31, 41]. From these spectra indicated that carboxymethyl groups were substituted onto chitosan at hydroxyl groups at C-3 and C-6 positions. On the other hand, the *N*-carboxymethyl sustituent peaks of *N*-CH_2 and COOH would be observed at 47 and 168 ppm, respectively [31, 40, 41]. However, those *N*-CH_2 and COOH were not observed in this experiment, confirming that the substitution occurred only at the *O*-substitution at C-3 and C-6. The ^{13}C-NMR spectrum of CM-chitosan-g-α-CD in D_2O described in Figure 4.5c (ppm): 143-101 (C of aromatic OBT counter ions), 101 (C-1 of α-CD), 100 (C-1 of chitosan), 56-81 (pyranose C of α-CD overlapped with those of chitosan), 43 (O-CH_2) which was shifted from CM-chitosan spectrum, indicated that α-CD was successfully conjugated onto chitosan backbone.

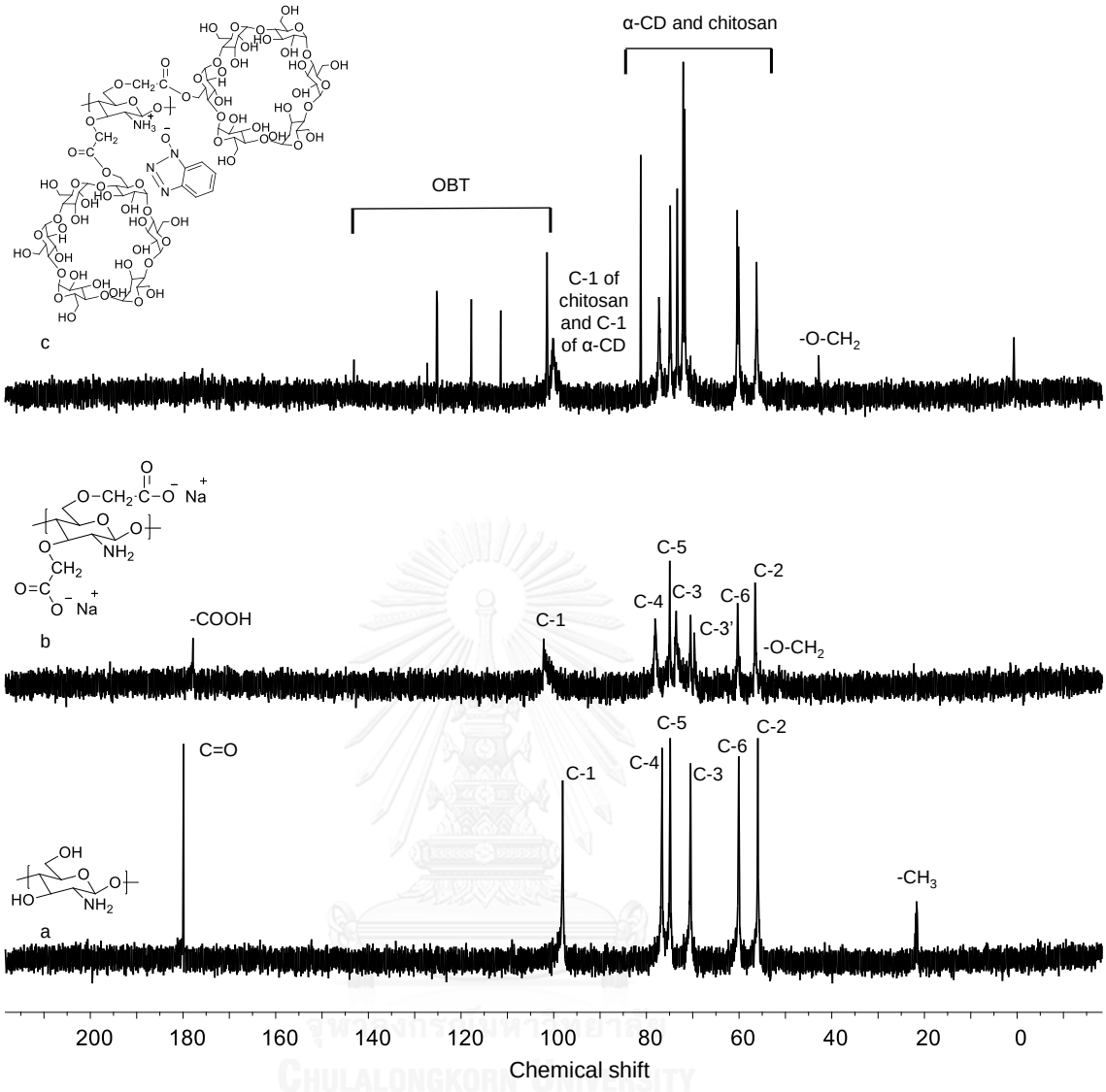


Figure 4.5 ^{13}C-NMR spectra of a) chitosan in CD_3COOD/D_2O, b) CM-chitosan and c) CM-chitosan-g-**α**-CD in D_2O.

4.2.3 Degree of substitution

The degree of carboxymethyl groups substituted onto chitosan backbone was calculated by the ratio of the integral area of methylene proton (3.96-3.93 ppm) and the integral area of H-1 of chitosan (4.47 ppm). Normally, H-1 of chitosan peak is next to D_2O peak on the right. Thus, degree of substitution of CM-chitosan was 1.36 and molecular weight of CM-chitosan was calculated as 246.18 per unit.

The degree of substitution of **α**-CD conjugated on CM-chitosan was calculated by the ratio of the integral area of H-1 of **α**-CD (5.03 ppm) and the integral area of H-2 of chitosan (2.91 ppm). In this case integral area of H-2 of chitosan was used to calculate instead of H-1 owing to the overlap H-1 peak of chitosan with D_2O peak. Therefore degree of substitution of **α**-CD conjugated on CM-chitosan was 0.27 and molecular weight of CM-chitosan-**α**-CD was calculated as 503.76 per unit.

4.3 Investigation the optimum ratio between CM-chitosan-g-α-CD and PEG to prepare hydrogel

Preparation of hydrogel via inclusion gelation needs to find the optimum ratio of **α**-CD and PEG in order to form inclusion complex. The molar ratio of **α**-CD and PEG were investigated by fixing mole of CD (20 μmole) and decrease mole of PEG. Therefore the molar ratio of **α**-CD to PEG were increased from 0.5, 1, 2, 4, to 8. PEG with molecular weight of 1000 (PEG1000) and 4000 (PEG4000) were used. After that the mixed solution was characterized by ^{1}H-NMR and calculated the threading number as follow in equation (3). From the result (Figure 4.6), it was found that at the start, there was low content of **α**-CD. The molar ratio of **α**-CD and PEG was not appropriated to form inclusion complex. When mole of PEG decreased leading to

molar ratio of α-CD to PEG increased. PEG chains had more mobility to form inclusion complex with α-CD as graph sloping upward indicating the mole of PEG higher than α-CD . At the equilibrium point when mole of PEG decreased to the point could form inclusion complex was represented as plateau of graph, indicating the equilibrium mole ratios to form inclusion complex. After this points, the graph sloping upward again indicating the mole of α-CD higher than PEG . For PEG1000, the plateau appeared in molar ratio between 2 and 4, the threading number was 2.5 indicated that 2.5 α-CD rings possibly threaded onto one PEG chain. The α-CD had functioned as inclusion crosslinker with degree of crosslinking around 2.5. Whilst PEG molecular weight 4000, the plateau appeared in molar ratio between1 and 2, the threading number was calculated to be 1 indicated that 1 α-CD ring possibly threaded onto one PEG chain, thus, it could not be crosslinked since the good crosslinker should have more than bifuntional groups. When molecular weight of PEG increased from 1000 to 4000, threading number of α-CD onto PEG4000 was less than that of PEG1000. Because the amount of chain end decreased as the molecular weight increased. High molecular weight of PEG had a difficulty and less possibility to penetrate into α-CD cavities [20]. Therefore, PEG1000 was used as inclusion crosslinker to prepare injectable hydrogel.

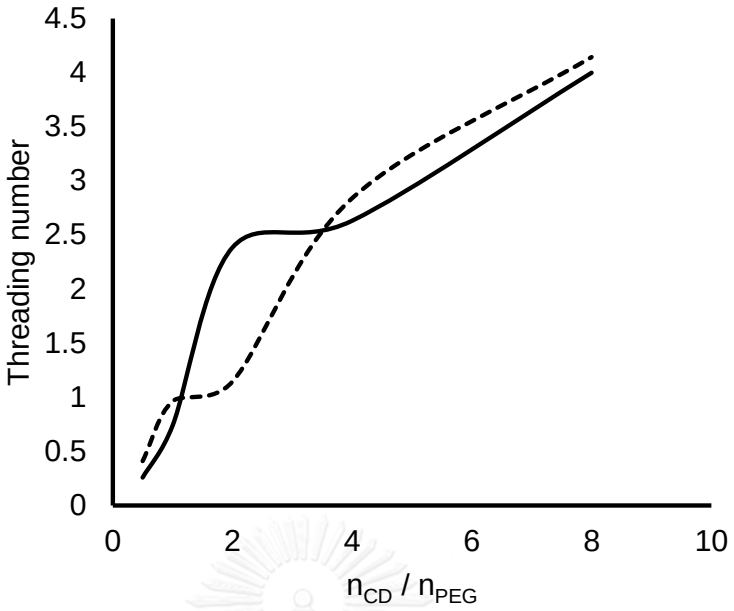


Figure 4.6 Threading number of various molar ratios of α-CD to PEG (—PEG1000 and ---- PEG4000).

4.4 Preparation of injectable hydrogel via inclusion gelation

Hydrogel was prepared by mixing PEG in a saturated aqueous solution of CM-chitosan-g-α-CD. The solution was tested to suck into needle number 18. It was found that CM-chitosan-g-α-CD was dissolve in water until concentration was around 15%(w/v), which was the highest concentration that could flow through needle number 18 as shown in Figure 4.7a.

4.4.1 Investigation of setting time of hydrogel

Injectable hydrogel need to be thixotropic and reversible properties. When the hydrogel was disturbed, the viscosity of hydrogel decreased. But the viscosity of gels reverted to original within hours when there was no disturbed [22]. The injectable mixture solution was prepared by mixing PEG1000 in a saturated aqueous solution of CM-chitosan-g-α-CD 15%(w/v). Then the solution was suck into syringe, then injected in test tube at 37°C (body temperature) (Figure 4.7b). After

injection, the solution could flow (Figure 4.7d). After that the solution was left clam for a while, so the hydrogel was formed inclusion complex which cannot flow (Figure 4.7e). The hydrogel was observed as yellow and soft. The setting time was measured to be 450±10 min (n=3).

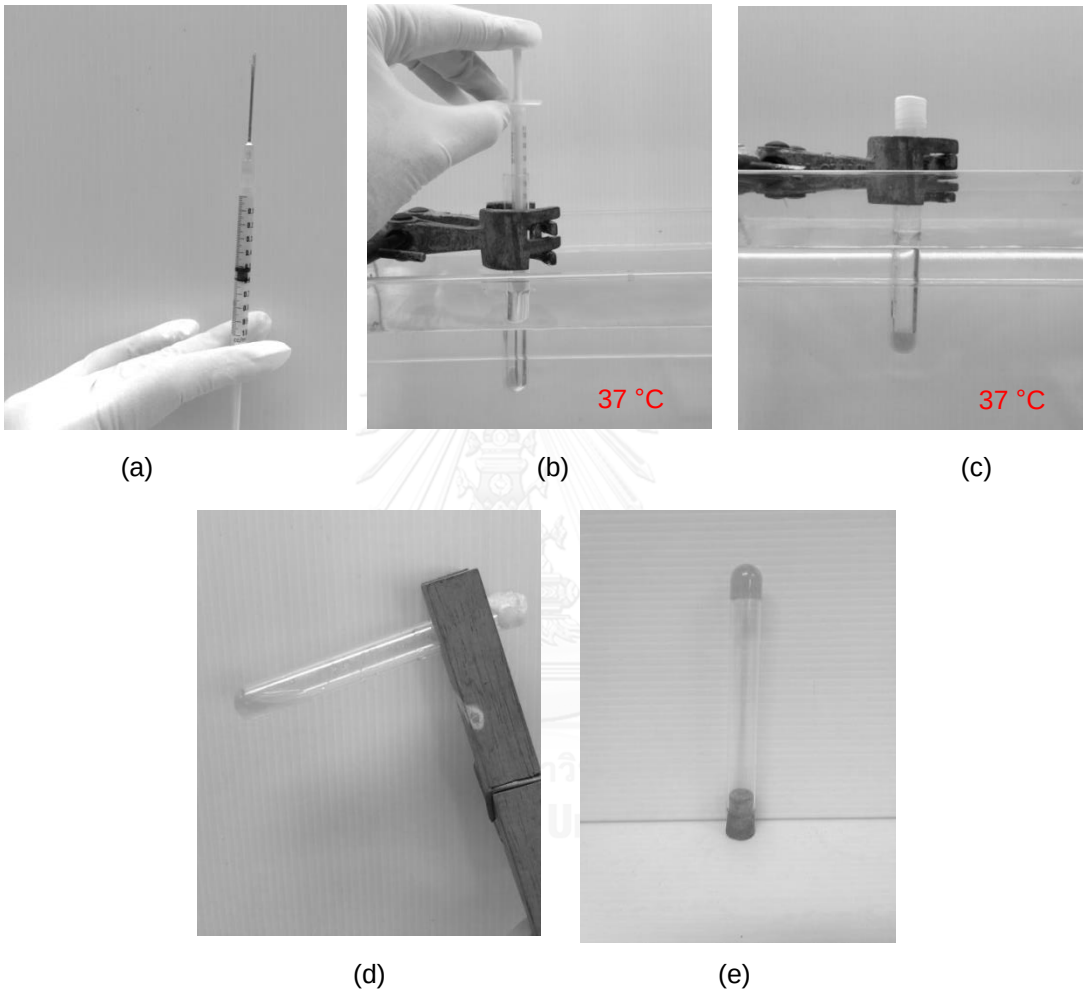
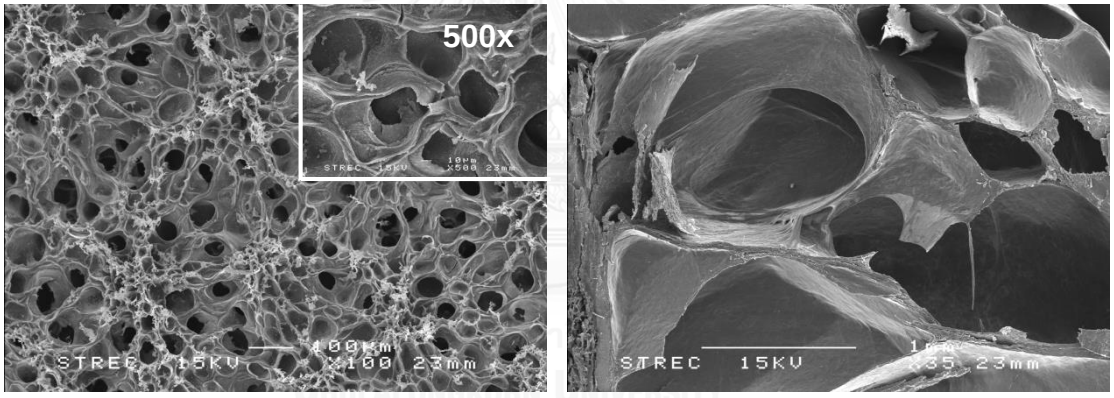


Figure 4.7 Representative appearance of a) mixed solution in syringe, b) injected mixed solution at 37°C, c) hydrogel at 37°C, d) mixed solution after injected and e) hydrogel left for setting at 37°C.

4.5 Characterization of hydrogel

4.5.1 Morphology of hydrogel

The morphology of freeze-dried gel prepared by inclusion complex between CM-chitosan-g-α-CD and PEG1000 was observed by scanning electron microscopy (SEM) as shown in Figure 4.8. The surface of freeze-dried gel was shown in Figure 4.8a. It was found that the hydrogel had porosity and open pore. At magnification 500x, it was found that the porosity was interconnected pores in order to transport oxygen, nutrients and waste products into and out of the cells. For cross section of hydrogel was shown in Figure 4.8b. It was found that the hydrogel had porosity and big open pores which were also interconnecting.



(a) Surface 100x (b) Cross section 35x

Figure 4.8 Morphology of hydrogel a) surface and b) cross section.

4.6 Initial cell attachment and proliferation assay

Sarcoma osteogenic cell line ($SaOs_2$) cells were seeded and cultured on hydrogel for 1, 3, 5, and 7 days. Cell attachment and proliferation from selected wells of the plate were measured by DNA assay as shown in Figure 4.9. Initially, $SaOs_2$ cells were seeded on hydrogel 100,000 cells. For the first day, the $SaOs_2$ cells were adhered onto hydrogel around 55,000 cells. After that the $SaOs_2$ cells continuously proliferated up to 56,000, 70,000 and 160,000 cells for 3, 5 and 7 days, respectively. It was observed that $SaOs_2$ cells adhered and proliferated well on the hydrogel. It indicated that the hydrogel was compatible and non-toxic to the $SaOs_2$ cells. The positive control was also evaluated on cell culture well plate with the same procedure. This phenomenon also showed the higher efficiency of cell culture onto 3D porous structure of hydrogel than 2D cell culture plate.

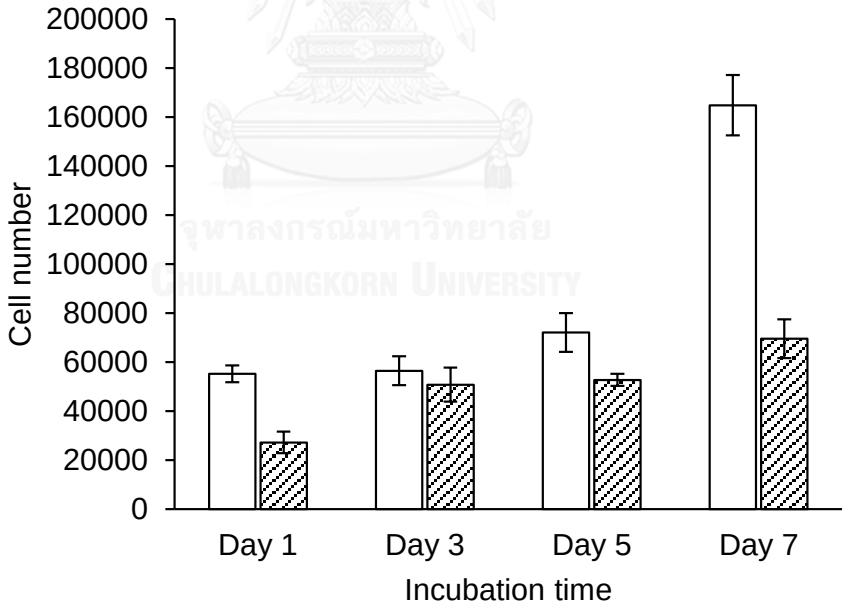


Figure 4.9 Cell number of SaOs2 cells attachment and proliferation on hydrogel at (☐) hydrogel and (▨) control.

CHAPTER V
CONCLUSIONS

5.1 Conclusions

5.1.1 Carboxymethyl chitosan (CM-chitosan) precursor was successfully synthesized via etherification between chitosan and carboxymethyl chloride under alkaline condition with degree of carboxymethyl substitution of 1.36. FTIR analysis showed the absorption band of chitin together with the sodium salt of carboxyl groups. CM-chitosan showed high fraction of carboxyl and amino groups indicated that substitution of carboxymethyl groups onto chitosan backbone mostly on –OH position. In order to confirm the chemical structure, ^{1}H-NMR and ^{13}C-NMR were used to investigate. ^{1}H-NMR showed the additional peak of methylene (-CH_2) protons of carboxymethyl groups and ^{13}C-NMR spectra confirmed that carboxymethyl groups were substituted onto chitosan at hydroxyl groups at C-3 and C-6 positions.

5.1.2 α-CD was successfully conjugated onto CM-chitosan main chain via esterification between carboxyl groups of CM-chitosan and hydroxyl groups of α-CD using EDC · HCl as coupling agent and HOBT as proton donor. FTIR analysis showed both absorption band of β-pyranyl vibration of chitosan and α-pyranyl vibration of α-CD in the spectrum of CM-chitosan-g-α-CD, indicating that α-CD was successfully conjuagated onto chitosan. The structure of CM-chitosan-g-α-CD was confirmed by ^{1}H-NMR and ^{13}C-NMR, the specific peaks of α-CD together with chitosan were observed in both ^{1}H-NMR and ^{13}C-NMR with the shift of the (O-CH_2) ^{13}C-NMR peak, indicating that α-CD was successfully conjugated with carboxyl groups of CM-chitosan that influenced to the shift of the neighbor carbon peak. Degree of α-CD substitution to CM-chitosan was 0.27.

5.1.3 The optimum ratio between CM-chitosan-g-α-CD and PEG to prepare hydrogel was investigated by finding the equilibrium mole ratio using ^{1}H-NMR. It was found that the equilibrium mole ratio of CM-chitosan-g-α-CD and PEG1000 was between 2 and 4 having threading number of 2.5, whilst the equilibrium mole ratio of CM-chitosan-g-α-CD and PEG4000 was between 1 and 2 having threading number of 1. When molecular weight of PEG increased from 1000 to 4000, threading number of α-CD onto PEG4000 was less than that of PEG1000. Because the amount of chain end decreased as the molecular weight increased, leading to less possibility to penetrate into α-CD cavities. The threading number was considered as a crosslinking points, therefore, the gelation possibly formed with the crosslinking points over than two. Thus, PEG1000 was selected to prepare injectable hydrogel instead of PEG4000.

5.1.4 Injectable hydrogel was prepared by mixing PEG in a saturated aqueous solution of CM-chitosan-g-α-CD with the maximum concentration of 15%(w/v) that could flow through needle number 18 . The injectable hydrogel need to have thixotropic and reversible properties. The inclusion complex between α-CD conjugated chitosan and PEG can be formed and dissociated during accepting shearing force by injection through the needle (viscous fluid), and then the inclusion gelation would be completely formed at the defect site (hydrogel). Therefore, the injectable hydrogel was used as scaffolds for bone tissue engineering. The hydrogel was observed as yellow and soft. The setting time was measured to be 450±10 min.

5.1.5 The morphology of freeze-dried gel was observed by SEM. The interconnecting porous structures were found in both surface and cross-section which was suitable for transport nutrient and air to cells.

5.1.6 Initial cell attachment and proliferation assay was tested by using sarcoma osteogenic cell line ($SaOs_2$). Cell number was evaluated using a DNA

determination assay. $SaOs_2$ cells adhered and proliferated well on the hydrogel. It indicated that the hydrogel was compatible and non-toxic to the $SaOs_2$ cells.

Suggestions:

1. Since the temperature seems to be the main factor to the mobility of PEG to form inclusion complex with α-CD, thus, the optimum or equilibrium mole ratios of α-CD to PEG should be investigated by ^{1}H-NMR under various temperature.

2. The setting time was also influenced by the mobility of the PEG molecules, thus, the setting time at various temperature should be further investigated.

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APPENDIX

Appendix A

FTIR spectra

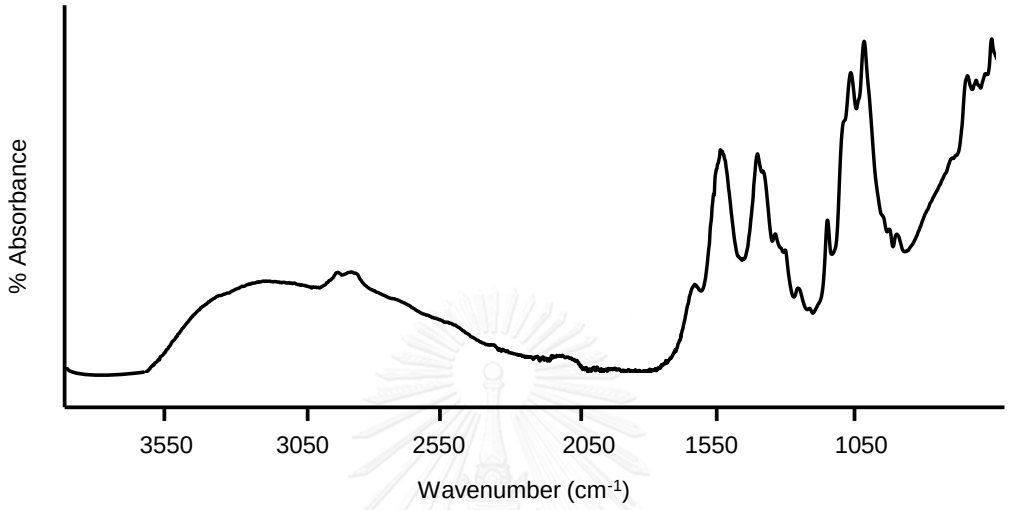

Figure A1 FTIR spectrum of chitosan.

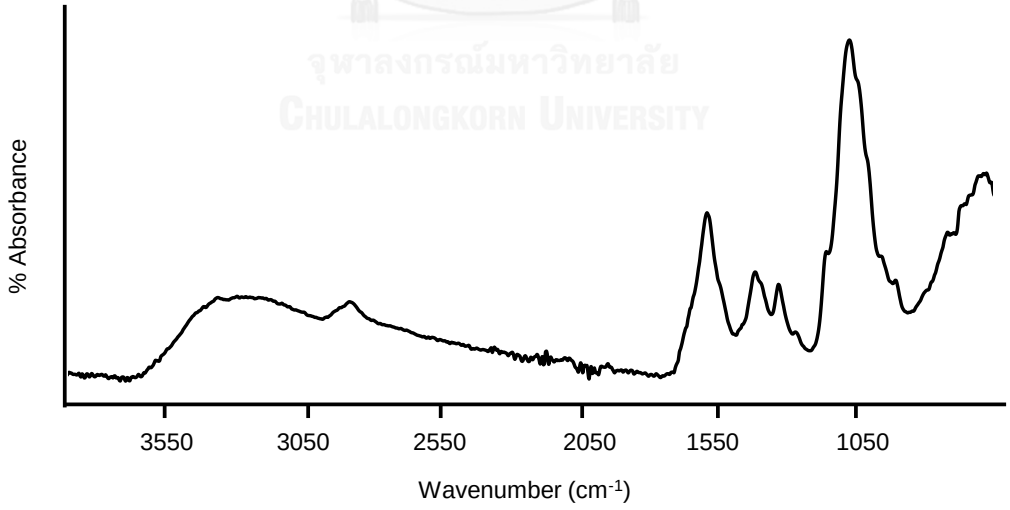

Figure A2 FTIR spectrum of CM-chitosan.

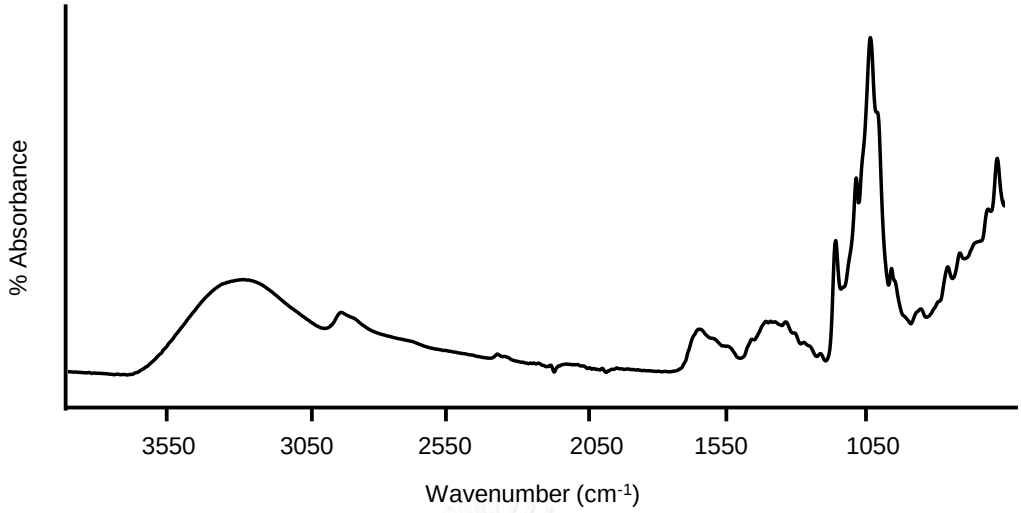

Figure A3 FTIR spectrum of CM-chitosan-g-**α**-CD.

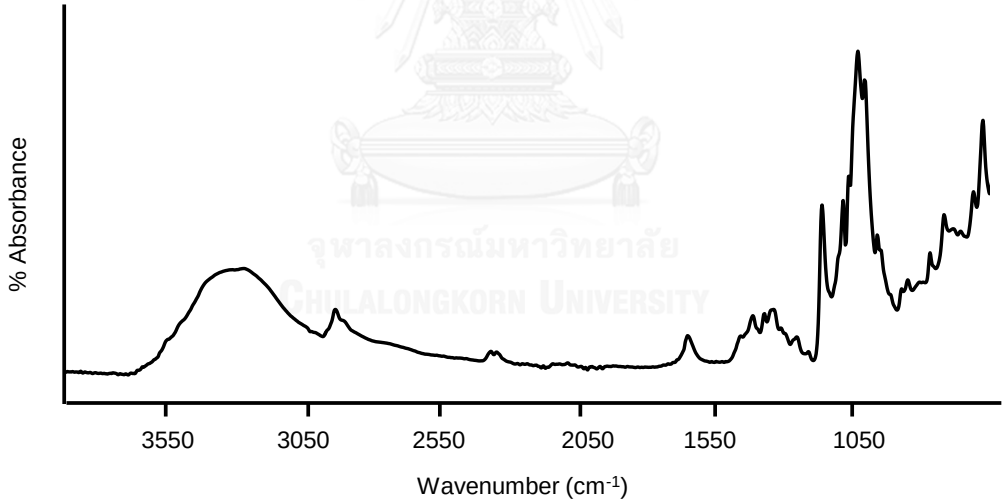

Figure A4 FTIR spectrum of **α**-CD.

Appendix B

NMR spectra

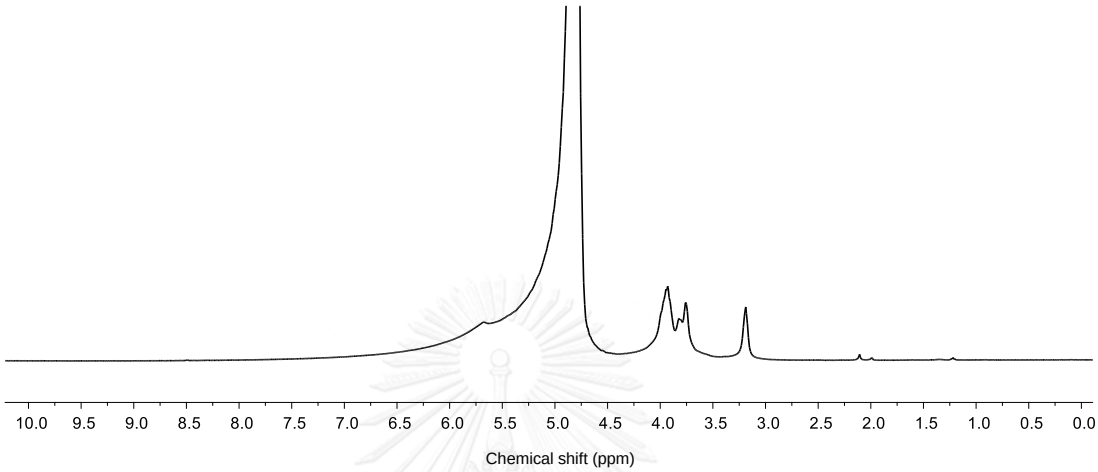

Figure B1 ^{1}H-NMR spectrum of chitosan.

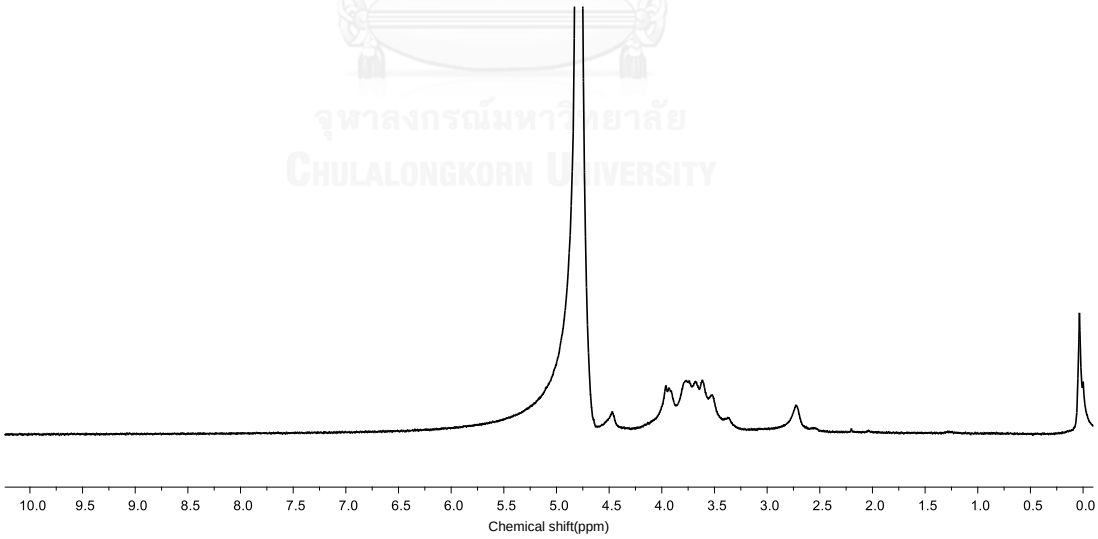

Figure B2 ^{1}H-NMR spectrum of CM-chitosan.

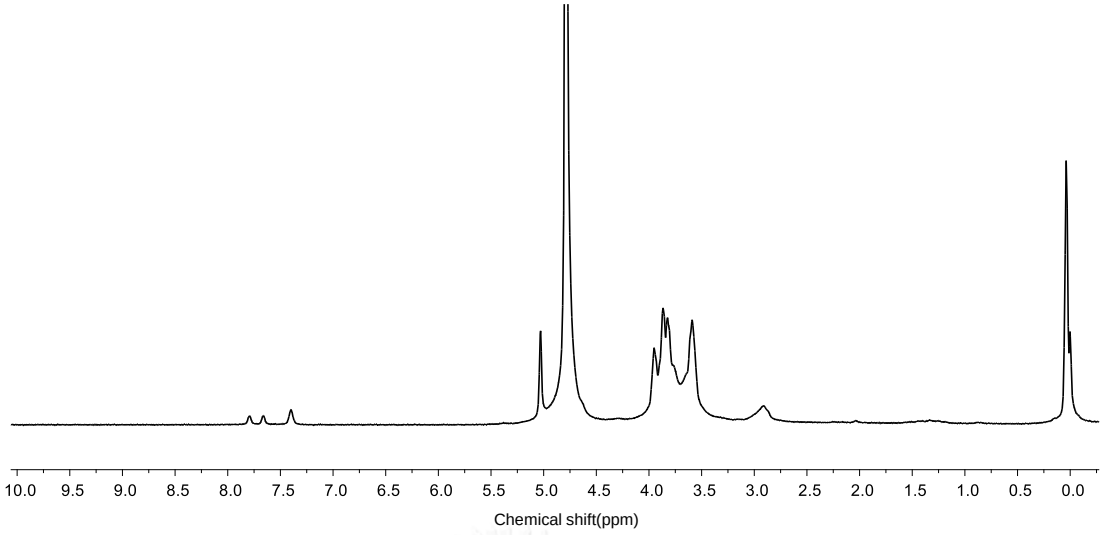

Figure B3 ^{1}H-NMR spectrum of CM-chitosan-g-**α**-CD.

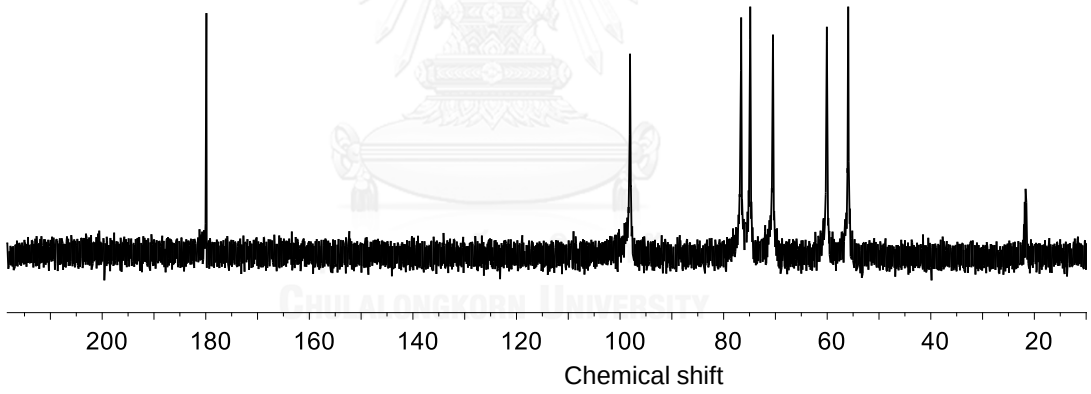

Figure B4 ^{13}C-NMR spectrum of chitosan.

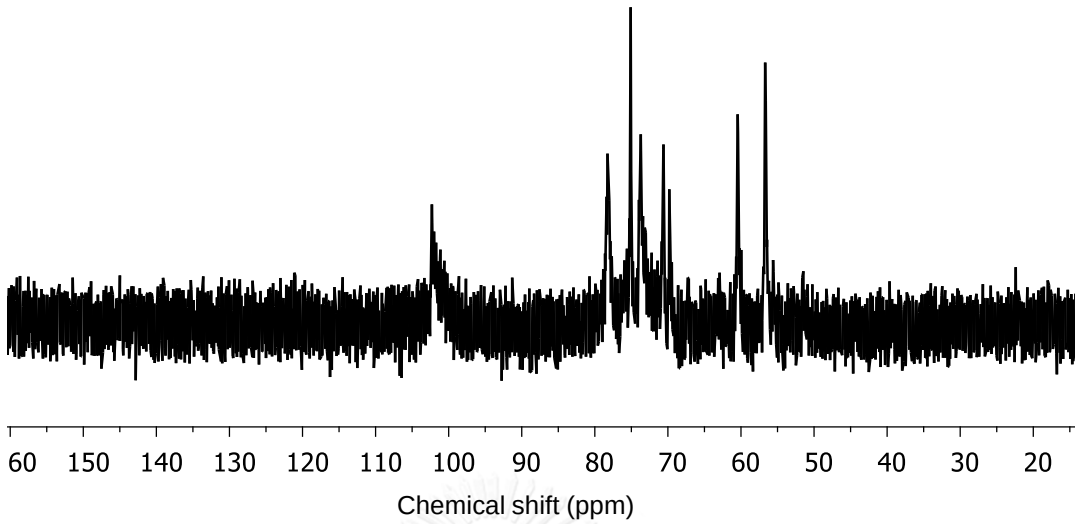

Figure B5 ^{13}C-NMR spectrum of CM-chitosan.

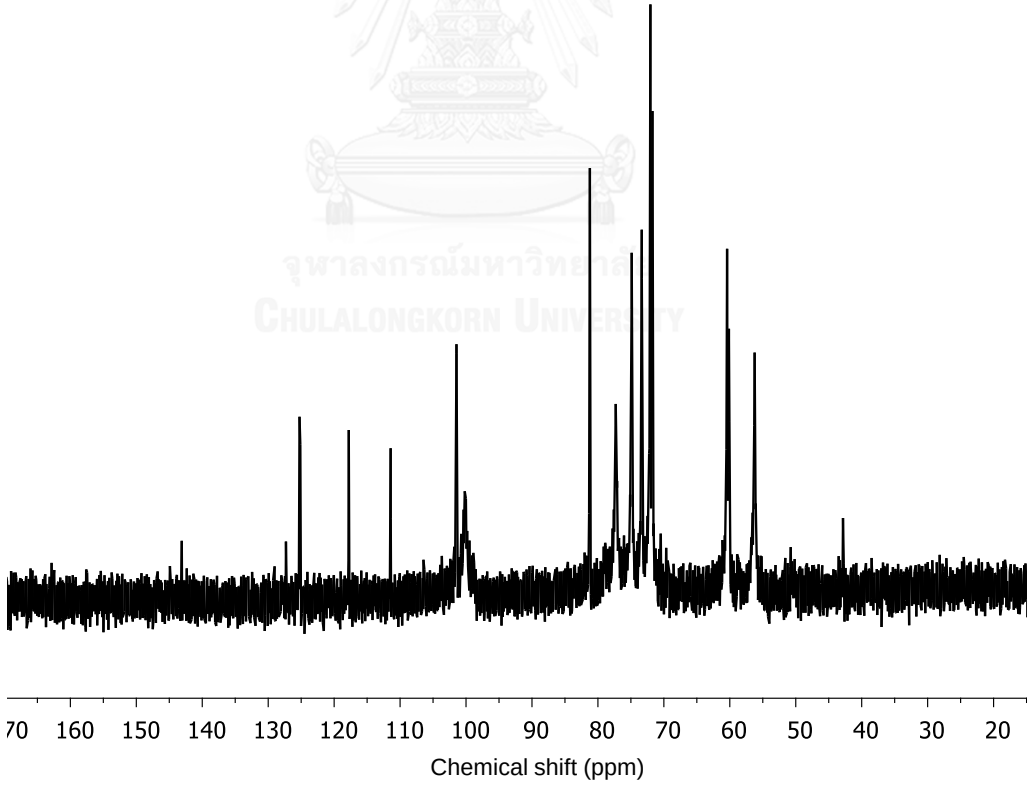

Figure B6 ^{13}C-NMR spectrum of CM-chitosan-g-**α**-CD.

Appendix C

Calculating of threading number of α-CD threaded on PEG chain

Threading number of α-CD threaded on PEG chain was calculated as follow;

$$\text{Threading number} = \frac{\frac{I_{CD}}{6}}{\frac{I_{PEG}}{4\,x\,repeating\ unit\ of\ PEG}}$$

Where;

$I_{\alpha\text{-CD}}$ = integrated from H-1 of α-CD by ^{1}H-NMR at 5.03 ppm

I_{PEG} = integrated from H of PEG by ^{1}H-NMR around 3.68-3.62 ppm

Table C1 Threading number with various molar ratios by using PEG MW 1000

Molar ratio (n_{CD}/n_{PEG})	$I_{\alpha\text{-CD}}$	I_{PEG}	Threading number
0.5	1	58.98	0.26
1	1	20.59	0.74
2	1	6.36	2.38
4	1	5.76	2.63
8	1	3.79	4.00

Table C2 Threading number with various molar ratios by using PEG MW 4000

Molar ratio (n_{CD}/n_{PEG})	$I_{\alpha\text{-CD}}$	I_{PEG}	Threading number
0.5	1	148.14	0.39
1	1	63.04	0.98
2	1	53.01	1.12
4	1	21.35	2.81
8	1	14.63	4.73

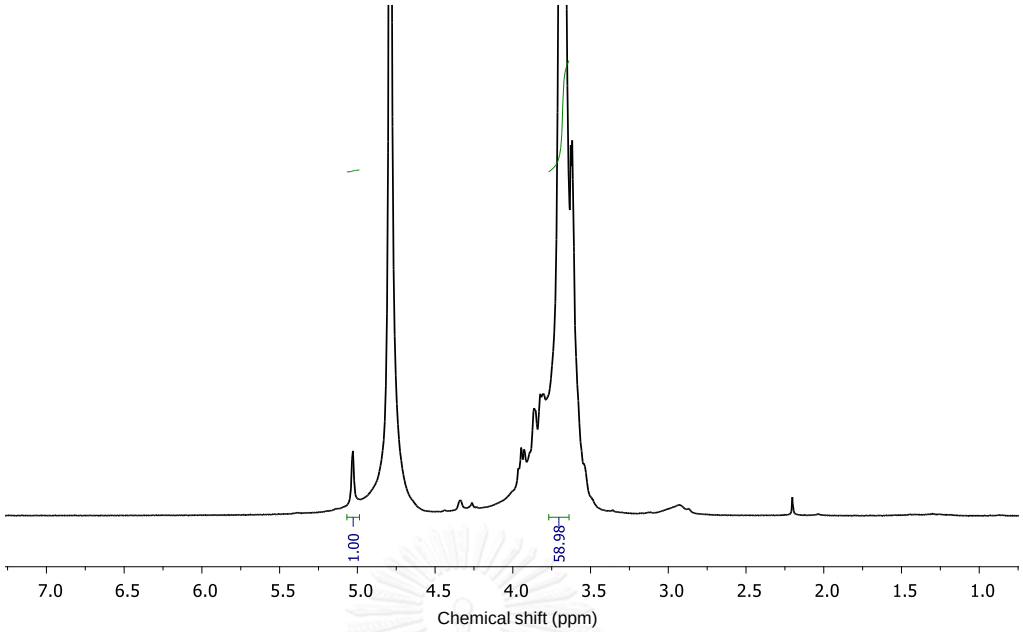

Figure C1 ^{1}H-NMR spectrum of 0.5 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 1000).

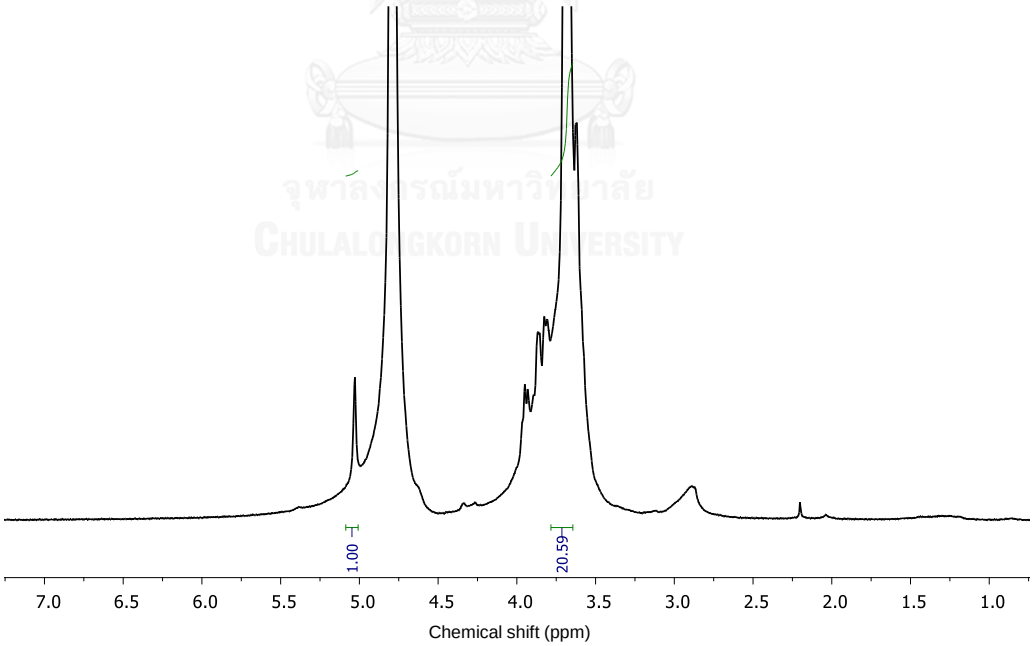

Figure C2 ^{1}H-NMR spectrum of 1 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 1000).

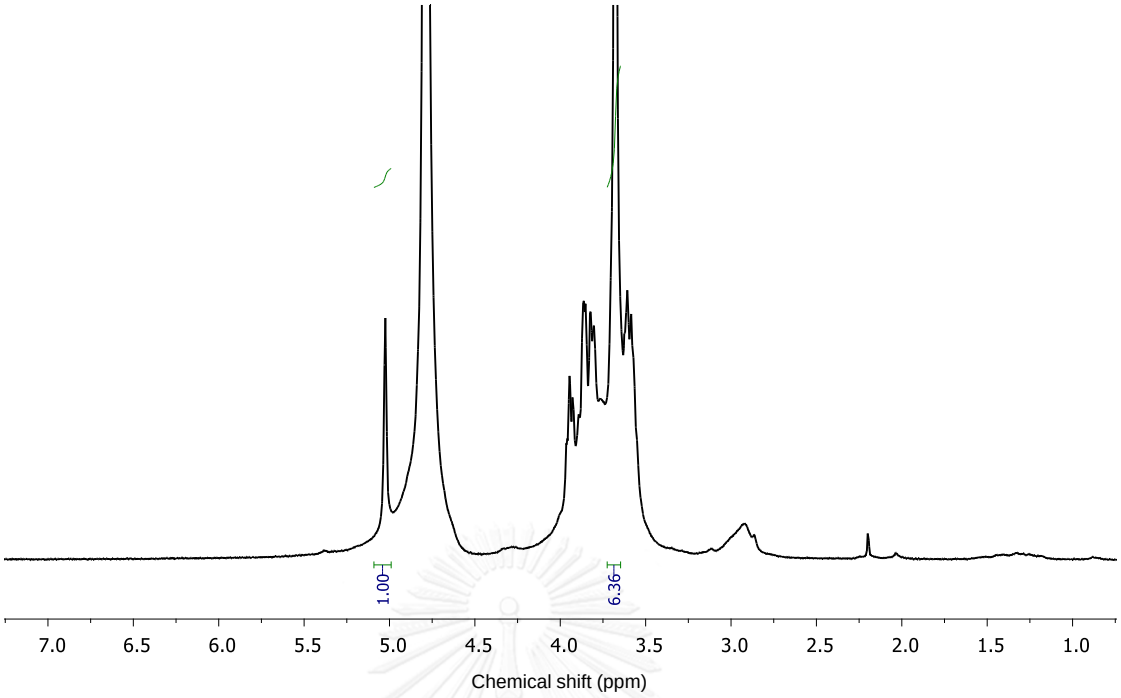

Figure C3 ^{1}H-NMR spectrum of 2 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 1000).

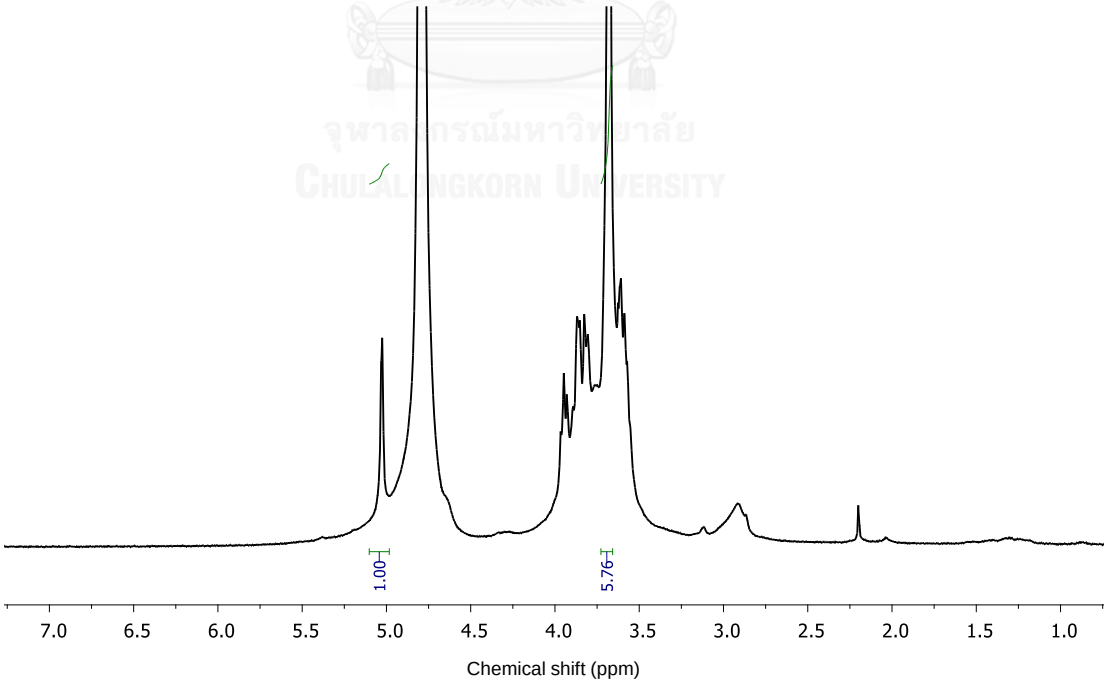

Figure C4 ^{1}H-NMR spectrum of 4 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 1000).

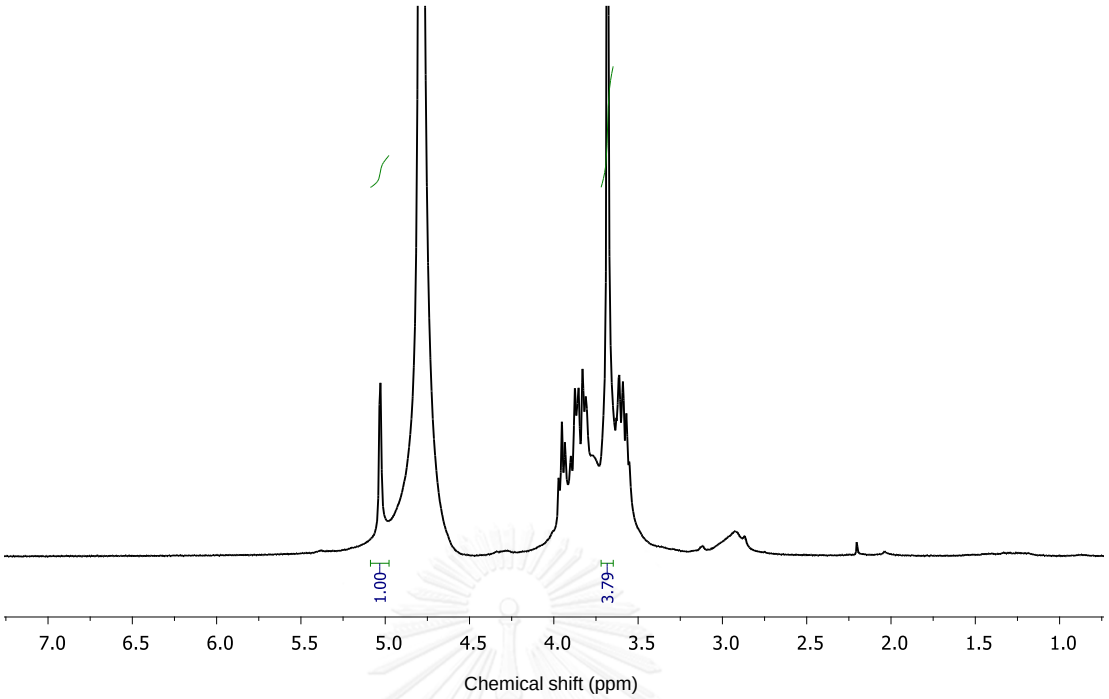

Figure C5 ^{1}H-NMR spectrum of 8 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 1000).

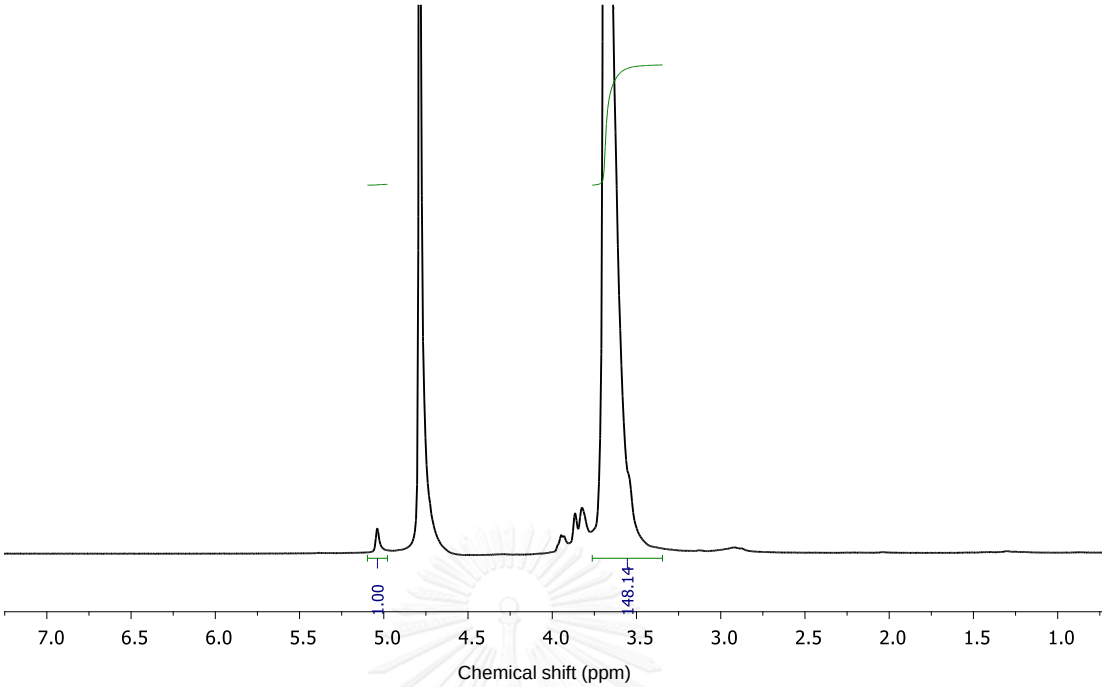

Figure C6 ^{1}H-NMR spectrum of 0.5 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 4000).

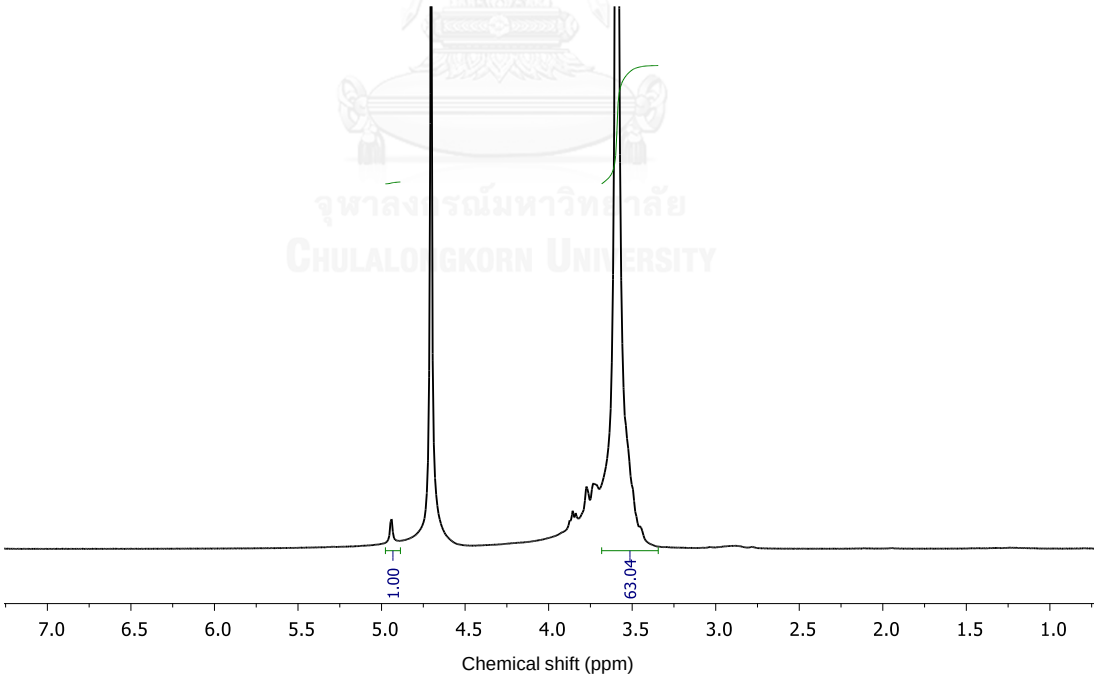

Figure C7 ^{1}H-NMR spectrum of 1 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 4000).

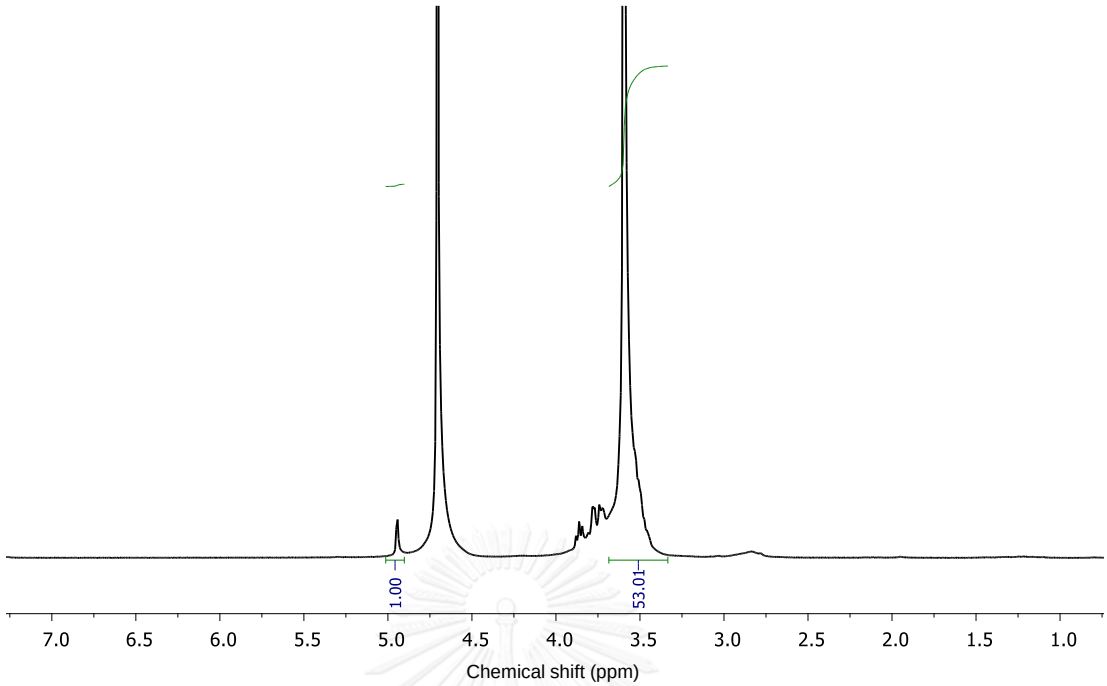

Figure C8 ^{1}H-NMR spectrum of 2 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 4000).

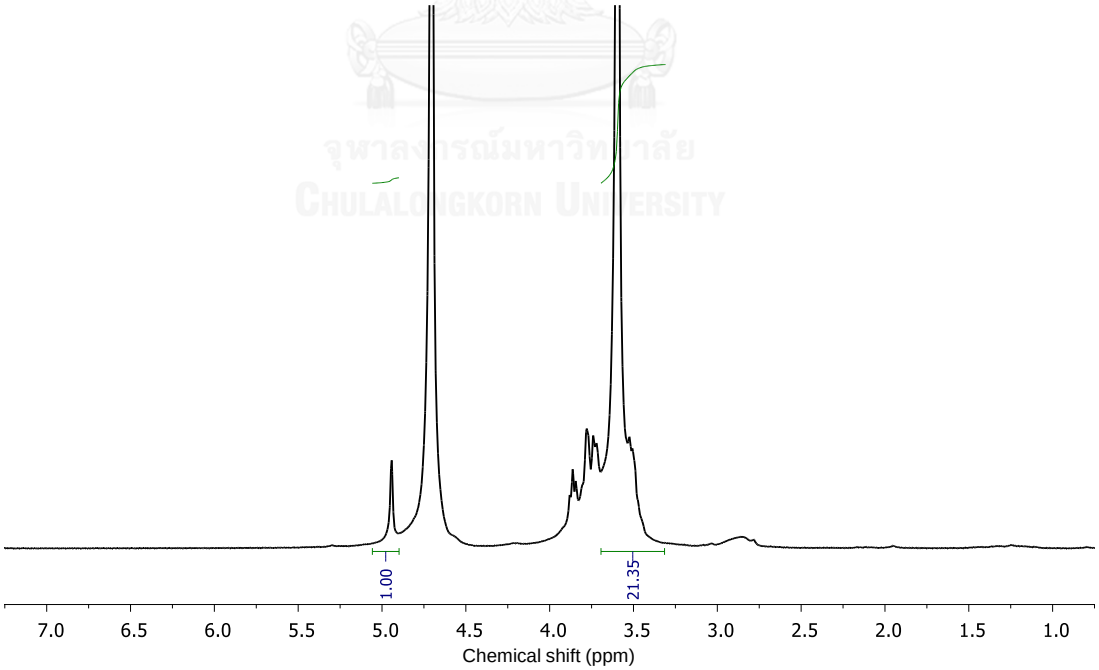

Figure C9 ^{1}H-NMR spectrum of 4 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 4000).

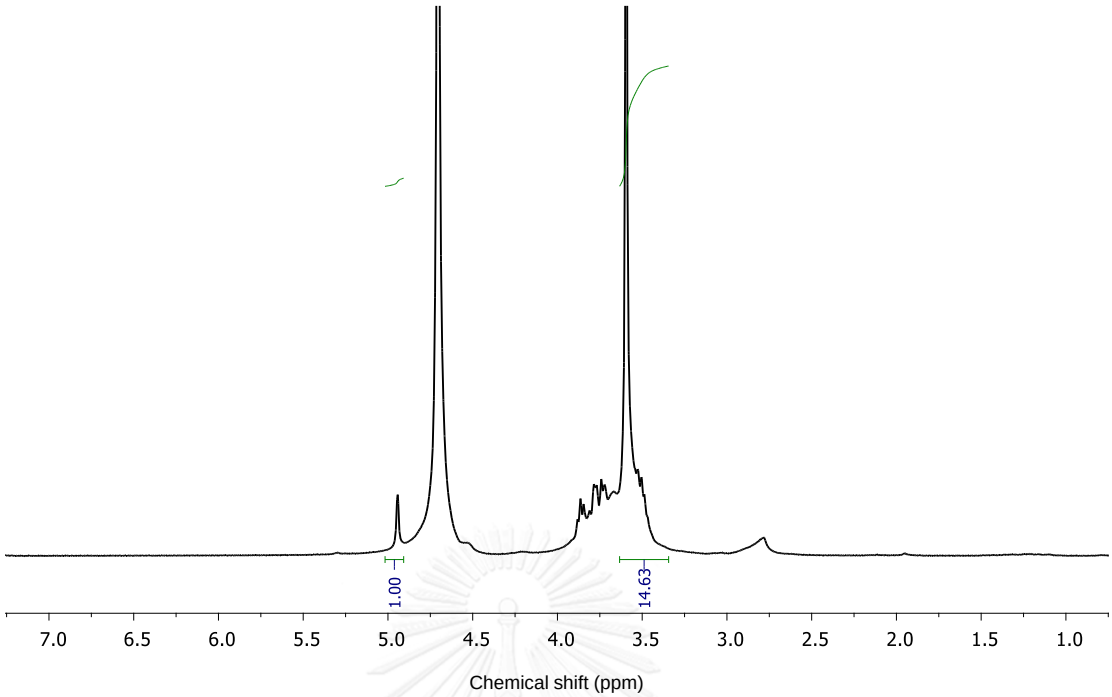

Figure C10 ^{1}H-NMR spectrum of 8 molar ratio for calculating threading number of **α**-CD threaded on PEG chain (PEG = 4000).

VITA

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Publication:

International Proceedings

K. Saekhor, W. Tachaboonyakiat, Synthesis of Water Soluble Chitosan-g-α-Cyclodextrin, Proceedings of The 5th research symposium on petrochemical and materials technology and The 20th PPC symposium on petroleum, petrochemicals and polymers (Petromat and PPC SYM 2014), Queen Sirikit National Convention Centre, 22 April 2014, pp. 623-627 (2014).

International Conferences

1. K. Saekhor, W. Tachaboonyakiat "Synthesis of water soluble chitosan-g-α-cyclodextrin with an approach for oil trapment" 10th International Conference of the Asian Pacific Chitin Chitosan Symposium (APCCS 2013), October 4-8, 2013, Yonago Convention Center "BIGSHIP", Yonago, Tottori Prefecture , Japan

2. K. Saekhor, W. Tachaboonyakiat, "Synthesis of Water Soluble Chitosan-g-α-Cyclodextrin" The 5th research symposium on petrochemical and materials technology and The 20th PPC symposium on petroleum, petrochemicals and polymers (Petromat and PPC SYM 2014), Queen Sirikit National Convention Centre, 22 April 2014.

3. K. Saekhor, W. Tachaboonyakiat, "Synthesis of chitosan conjugated cyclodextrins with an approach for oil absorbent" The 2014 IUPAC World Polymer Congress (MACRO 2014), Chiangmai International Convention and Exhibition Centre, 6-11 July 2014.

4. K. Saekhor, W. Tachaboonyakiat, "Synthesis of chitosan-cyclodextrins with an approach for oil absorbent" NUT-CU Materials Science and Technology Colloquium 2014, Nagaoka University of Technology (NUT), Nagaoka, Niigata, Japan, 17-18 November 2014.

International Internship

1. Japan-Asia Youth Exchange Program in Science" (SAKURA Exchange Program in Science) supported by JST (Japan Science and Technology Agency), Nagaoka University of Technology (NUT), Nagaoka, Niigata, Japan, 4-24 November 2014.