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Development of Fluorescent Particles for Bio-related Application

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A Report Submitted in Partial Fulfillment of the Requirements
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Project Title	DEVELOPMENT OF FLUORESCENT PARTICLES FOR BIO-RELATED APPLICATION
By	Miss Duangkamon Suttipat and Miss Wanchalach Sathienthammanee
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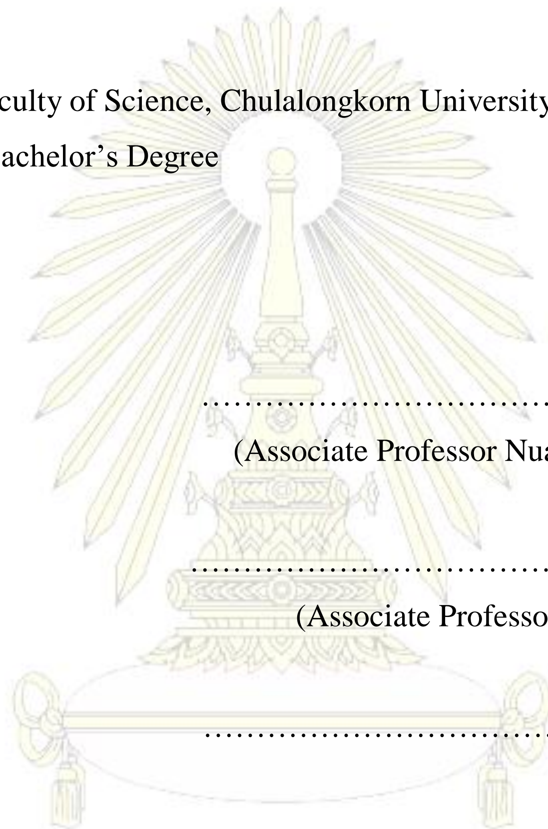

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บทคัดย่อ

ส่วนที่ 1

ควอนตัมดอทที่สามารถละลายน้ำได้และมีความเข้ากันได้ดีทางชีวภาพถูกเตรียมโดยการติดโคพอลิเมอร์ของเมทาคริลิกแอซิดและ 2-เมทาคริโลอิลออกซีเอทิลฟอสโฟริลโคลีน (พีเอ็มเอเอ็มพีซี) ที่มีการปรับปรุงโครงสร้างด้วยโฟลิคแอซิดลงบนควอนตัมดอท ผ่านวิธีการแลกเปลี่ยนลิแกนด์โดยการใช้เอทาโนลามีนเป็นสารเปลี่ยนเฟส เพื่อที่จะได้ควอนตัมดอทติดพีเอ็มเอเอ็มพีซีติดโฟลิคแอซิด ความสำเร็จของการตรึงโฟลิคแอซิดบนพอลิเมอร์และการมีอยู่ของพีเอ็มเอเอ็มพีซีติดโฟลิคแอซิดล้อมรอบควอนตัมดอทถูกยืนยันโดยเทคนิคเอฟที-ไออาร์ ตามที่วิเคราะห์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านพบว่า ควอนตัมดอทติดพีเอ็มเอเอ็มพีซีติดโฟลิคแอซิดมีความเสถียรสูง เป็นทรงกลม และมีขนาดที่มีการกระจายตัวแบบโมโนดิสเปอร์ส เท่ากับ 5±0.8 นาโนเมตร ซึ่งมีขนาดใหญ่ขึ้นเล็กน้อยเมื่อเทียบกับควอนตัมดอทที่สังเคราะห์เริ่มต้น (3.8±0.6 นาโนเมตร) ซึ่งผลการทดลองนี้มีความสอดคล้องกับการเกิดเรดชิฟต์เพียงเล็กน้อยของการเรืองแสงฟลูออเรสเซนต์จาก 597 นาโนเมตรไปที่ 606 นาโนเมตร (ควอนตัมดอทติดพีเอ็มเอเอ็มพีซีติดโฟลิคแอซิด) โครงสร้างแบบแกนกลาง-เปลือกหุ้มของควอนตัมดอทติดพีเอ็มเอเอ็มพีซีติดโฟลิคแอซิด ถูกวิเคราะห์ด้วยกล้องจุลทรรศน์แบบแรงอะตอม นอกจากนี้ควอนตัมยิวด์ ของควอนตัมดอทที่ทำให้เสถียรด้วยพีเอ็มเอเอ็มพีซีและพีเอ็มเอเอ็มพีซีติดโฟลิคแอซิด มีค่ามากกว่าควอนตัมดอทที่ทำให้เสถียรด้วยเมอแคพโตโพรไพโอนิคแอซิดซึ่งเป็นต้นแบบของควอนตัมดอทที่ละลายน้ำ แสดงให้เห็นถึงการปรับปรุงการเรืองแสงของควอนตัมดอทด้วยพอลิเมอร์ ผลการทดลองที่ได้คาดว่า ควอนตัมดอทที่มีการปรับปรุงสามารถถูกพัฒนาสำหรับการถ่ายภาพเซลล์อีพิเดอร์มอยด์คาร์ซิโนมาเซอร์วิเคิล ที่มีการแสดงออกของตัวรับโฟเลตมากกว่าปกติ

คำสำคัญ: ควอนตัมดอทที่มีความเข้ากันทางชีวภาพ, การถ่ายภาพเซลล์, พอลิเมอร์ที่มีทั้งประจุบวกและลบ, ตัวรับโฟเลต

ส่วนที่ 2

งานวิจัยนี้ทำการสังเคราะห์อนุภาคแอมฟิฟิลิกไคโทซานซึ่งเป็นอนุภาคไคโทซานที่ประกอบด้วยหมู่ที่ชอบน้ำและไม่ชอบน้ำ ซึ่งสามารถนำไปตรวจติดตามได้ด้วยกล้องจุลทรรศน์ฟลูออเรสเซนต์มาใช้เป็นพาหนะในการนำส่งยาหรือสารชีวภาพต่างๆ โดยทำการสังเคราะห์อนุภาคแอมฟิฟิลิกไคโทซาน 2 ชนิด อนุภาคชนิดแรก (Pyr-CS-HTAP) ประกอบด้วยหมู่ที่ไม่ชอบน้ำคือไพรีนและหมู่ที่ชอบน้ำคือ หมู่เอ็น-[(2-ไฮดรอกซิล -3-ไทรเมทิลแอมโมเนียม)]โพรพิล (เอชทีเอพี) โดยทำปฏิกิริยากับไกลซิดิลไทรเมทิลแอมโมเนียม คลอไรด์ เพื่อให้เกิดการรวมตัวเองเป็นอนุภาคได้ และชนิดที่สอง (Pyr-CS-mPEG) ประกอบด้วยหมู่ที่ไม่ชอบน้ำคือไพรีนและหมู่ที่ชอบน้ำคือพอลิเอทิลีนไกลคอลเมทิลอีเทอร์ (เอ็ม-พีอีจี) โดยทำปฏิกิริยากับพอลิเอทิลีนไกลคอลเมทิลอีเทอร์ที่มีหมู่ปลายเป็นหมู่คาร์บอกซิล ทำการพิสูจน์เอกลักษณ์ของอนุภาคทั้งสองได้ด้วยเทคนิคโปรตอนเอ็นเอ็มอาร์ และเทคนิคเอฟที-ไออาร์ และทำการตรวจวัดสัณฐานวิทยาของอนุภาคด้วยเทคนิคทีอีเอ็มและเอสอีเอ็ม พบว่าอนุภาค Pyr-CS-HTAP มีขนาดอนุภาคเท่ากับ 0.77± 0.186 µm นาโนเมตร มีลักษณะเป็นทรงกลมซึ่งเกิดจากการรวมกลุ่มกันของอนุภาคที่มีขนาดเล็ก จากนั้นนำอนุภาคชนิดแรกไปห่อหุ้มสารเคอคิวมินที่สามารถให้สัญญาณฟลูออเรสเซนต์ แล้วนำไปวิเคราะห์ด้วยเทคนิคกล้องจุลทรรศน์แบบคอนโฟคอลชนิดที่ใช้เลเซอร์ในการสแกน จากผลการทดลองพบว่าเคอคิวมินสามารถถูกห่อหุ้มอยู่ภายในอนุภาคได้โดยผ่านการเกิดแรงไฮโดรโฟบิกและ π-π interaction ระหว่างไพรีนกับเคอคิวมินได้

คำสำคัญ : ไคโทซาน, ควอเทอไนเซชั่น, แอมฟิฟิลิก ไคโทซาน, การห่อหุ้ม, เคอคิวมิน

Abstract

Part 1

Water-soluble and biocompatible quantum dots (QDs) were prepared by grafting folic acid-functionalized poly(methacrylic acid)-*ran*-(2-methacryloyloxyethyl phosphorylcholine) on QDs via ligand exchange method by using ethanolamine as a novel phase transfer agent to obtain QDs-PMAMPC-FA. The success of FA immobilization and the presence of PMAMPC-FA around the QDs were confirmed by FTIR. As revealed by transmission electron microscopy, the QDs-PMAMPC-FA were highly stable, spherical and had monodispersed size of 5.2±0.8 nm, slightly larger than the as-synthesized QDs (3.8±0.6 nm). This result was consistent with a small red-shift of fluorescence emission from 597 nm (as-sythesized QDs) to 606 nm (QDs-PMAMPC-FA). The core-shell structure of QDs-PMAMPC-FA was also observed from AFM analysis. Moreover, quantum yields of QDs stabilized by PMAMPC and PMAMPC-FA were higher than QDs stabilized by mercaptopropanoic acid (QDs-MPA), as water-soluble model QDs, demonstrating the improvement of photoluminescence of QDs by polymer modification. It is anticipated that these modified QDs can be developed for cellular imaging with specific targeted epidermoid cervical carcinoma (CaSki) cell having overexpressed folate receptor.

Keywords : biocompatible quantum dots, cell imaging, zwitterionic polymer, folic acid receptor

Part 2

This research focused in synthesis of amphiphilic chitosan particle that can be detected with fluorescent microscope. The particles were to be used as carrier of drug or bioactive compounds. Two types of amphiphilic chitosan particle were prepared. The first type (Pyr-CS-HTAP) had pyrene as hydrophobic entity and *N*-[(2-hydroxyl-3-trimethyl ammonium)]propyl (HTAP) from reaction with glycidyltrimethylammonium chloride (GTMAC) as hydrophilic entity. The second type (Pyr-CS-mPEG) had pyrene as hydrophobic entity and mPEG from reaction with mPEG-COOH as hydrophilic entity. Both types of particle were characterized by NMR and FT-IR and their morphology were observed with TEM and SEM. It was found that Pyr-CS-HTAP formed round particles with size of 0.77± 0.186 nm through aggregation of smaller particles. Pyr-CS-HTAP particle was then used to encapsulate curcumin, which could act as fluorescent dye. Investigation through CLSM showed that curcumin was successfully encapsulated via hydrophobic interaction and π-π interaction between aromatic rings of pyrene and curcumin.

Keywords : chitosan, quaternization, amphiphilic chitosan, encapsulation, curcumin

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CONTENT

	Page
ABSTRACT (THAI)	iv
ABSTRACT (ENGLISH)	v
ACKNOWLEDGEMENTS	vi
CONTENTS	vii
LIST OF FIGURES	x
LIST OF SCHEMES	xii
LIST OF ABBREVIATIONS	xiii
PART I	1
CHAPTER I INTRODUCTION	2
1.1 Objectives	5
CHAPTER II EXPERIMENTAL SECTION	7
2.1 Materials	7
2.2 Synthesis of Quantum Dots (QDs)	7
2.3 Preparation of QDs Stabilized by PMAMPC-SH (QDs-PMAMPC) via Ligand Exchange Method	8
2.3.1 Synthesis of Thiol-terminated PMAMPC (PMAMPC-SH)	9
2.3.2 Preparation of QDs Stabilized by PMAMPC (QDs-PMAMPC) Method	10
2.4 Preparation of QDs stabilized by Folic Acid-modified PMAMPC-SH (QDs-PMAMPC-FA) via Ligand Exchange Method	10
2.4.1 Immobilization of FA on PMAMPC-SH (FA-PMAMPC)	10
2.4.2 Preparation of QDs-PMAMPC-FA by Ligand Exchange Method	11
2.5 Preparation of QDs Stabilized by Mercaptopropanoic Acid (QDs-MPA) via Ligand Exchange Method	11
2.6 Cell Study	11
2.7 Characterizations	11

ภาควิชาเคมี
คณะวิทยาศาสตร์
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER III RESULTS AND DISCUSSION 13
3.1 Synthesis and Characterization of PMAMPC-SH 13
3.2 Synthesis and Characterization of QDs-PMAMPC-FA 18

CHAPTER IV CONCLUSION 25
References 26

PART II 29
CHAPTER V INTRODUCTION 30
5.1 Objectives 33

CHAPTER VI EXPERIMENTAL SECTION 34
6.1 Materials 34
6.2 Equipments 34
6.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy 34
6.2.2 Fourier Transform -Infrared Spectroscopy (FT-IR) 34
6.2.3 Scanning Electron Microscopy (SEM) 34
6.2.4 Transmission Electron Microscopy (TEM) 35
6.2.5 Confocal Laser Scanning Microscopy (CLSM) 35
6.3 Methods 35
6.3.1 Synthesis of Phthaloylchitosan (PhCS) 35
6.3.2 Synthesis of Poly(ethylene glycol)methyl ether Terminated with Carboxyl Groups (mPEG-COOH) 35
6.3.3 Preparation of Amphiphilic Chitosan Particles 35
6.3.3.1 Particles Having Pyrene as Hydrophobic Entity and Amino Group as Hydrophilic Entity. (Pyr-CS-NH_2) 35
6.3.3.2 Particles Having Pyrene as Hydrophobic Entity and HTAP Group as Hydrophilic Entity. (Pyr-CS-HTAP) 36
6.3.3.3 Particles Having Pyrene as Hydrophobic Entity and mPEG-COOH as Hydrophilic Entity. (Pyr-CS-mPEG) 36

6.3.4 Encapsulation of Curcumin by Amphiphilic Chitosan Particles 37

CHAPTER VII RESULTS AND DISCUSSION 38
7.1 Synthesis of Phthaloylchitosan (PhCS) 38
7.2 Synthesis of Poly(ethylene glycol)methyl ether Terminated with Carboxyl Groups (mPEG-COOH) 41
7.3 Preparation of Amphiphilic Chitosan Particles 42
7.3.1 Particles Having Pyrene as Hydrophobic Entity and Amino Group as Hydrophilic Entity. (Pyr-CS-NH_2) 42
7.3.2 Particles Having Pyrene as Hydrophobic Entity and HTAP Group as Hydrophilic Entity. (Pyr-CS-HTAP) 46
7.3.3 Particles Having Pyrene as Hydrophobic Entity and mPEG-COOH as Hydrophilic Entity. (Pyr-CS-mPEG) 50
7.4 Encapsulation of Curcumin by Amphiphilic Chitosan Particles 51

CHAPTER VIII CONCLUSION 52
References 53
Vitae 54

ภาควิชาเคมี
คณะวิทยาศาสตร์
จุฬาลงกรณ์มหาวิทยาลัย

List of Figures

Figure		Page
3.1	^{1}H NMR of PMAMPC copolymer……………………………………………	14
3.2	UV-vis spectra of PMAMPC and PMAMPC-SH……………………………….	15
3.3	IR spectra of PMAMPC………………………………………………………	15
3.4	(a) Fluorescence spectra of QDs and QDs-PMAMPC, and QDs-PMAMPC after storage for 2 months in the dark, (b) UV-Vis spectrum of QDs…………..	16
3.5	Physical appearance of the as-synthesized QDs under ambient light (left)and black light (right)………………………………………………………………..	16
3.6	Physical appearance of QDs-PMAMPC after ligand exchange process and mixing with PMAMPC aqueous solution using (a) aminopropanol and (b) ethanolamine as phase transfer agents (b) (left: under ambient light and right: under black light).	17
3.7	IR spectra of (a) QDs, and (b) QDs-PMAMPC…………………………………	18
3.8	IR spectra of (a) PMAMPC, and (b) PMAMPC-FA……………………………	19
3.9	(a) Fluorescence emission spectra of QDs, FA, and QDs-PMAMPC-FA and (b) physical appearance of QDs-PMAMPC-FA (left: under ambient light and right: under black light).	20
3.10	IR spectra of (a) QDs, (b) QDs-PMAMPC, and (c) QDs-PMAMPC-FA………	21
3.11	TEM images of (a) QDs, (b) QDs-PMAMPC, and (c) QDs-PMAMPC-FA……	21
3.12	AFM images of (a) QDs-PMAMPC (Inset: zoom of QDs-PMAMPC) and (b) QDs-PMAMPC-FA…………………………………………………………….	22

Figure | Page

3.13 Viability with Ca SKi cervical cancer cells in the presence of QDs-MPA, QDs-PMAMPC, and QDs-PMAMPC-FA………………………………………………. 24

5.1 Signal change of loading drug and releasing drug. [4]………………………….. 31

5.2 (a) Structure of f-TMC and (b) Result from fluorescent microscope in bronchial epithelial labeling f-TMC. [5]…………………………………………………… 32

5.3 (a) Structure of TPE-CS and (b) Result of fluorescent microscope of HeLa cell with TPE-CS. [6]………………………………………………………………… 32

7.1 ^{1}H NMR spectra of (a) chitosan and (b) PhCS…………………………………. 40

7.2 FT-IR spectra of (a) chitosan and (b) PhCS…………………………………….. 41

7.3 ^{1}H NMR spectra of (a) mPEG and (b) mPEG-COOH……………………… ….. 42

7.4 ^{1}H NMR spectra of the product after (a) bromination, (b) azidation, (c) click reaction between pyrene and C-*6*-azido-*N*-phthaloylchitosan (N_3-PhCS) and (d) Pyr-CS-NH_2……………………………………………………………………... 45

7.5 FT-IR spectra of the product after (a) bromination, (b) azidation, (c) Click reaction between pyrene and C-*6*-azido-*N*-phthaloylchitosan (N_3-PhCS) and (d) Pyr-CS-NH_2……………………………………………………………………... 46

7.6 ^{1}H NMR spectra of (a) Pyr-CS-NH_2 and (b) Pyr-CS-HTAP……………………. 48

7.7 FT-IR spectra of (a) Pyr-CS-NH_2 and (b) Pyr-CS-HTAP………………………. 49

7.8 SEM micrograph of Pyr-CS-HTAP particles……………………………………. 49

7.9 ^{1}H NMR spectra of (a) mPEG-COOH, (b) Pyr-CS-NH_2 and (c) Pyr-CS-mPEG.. 50

7.10 CLSM micrographs of (a) มurcumin, (b) Pyr-CS-HTAP and (c) encapsulated curcumin of Pyr-CS-HTAP particles…………………………………………… 51

LIST OF SCHEMES

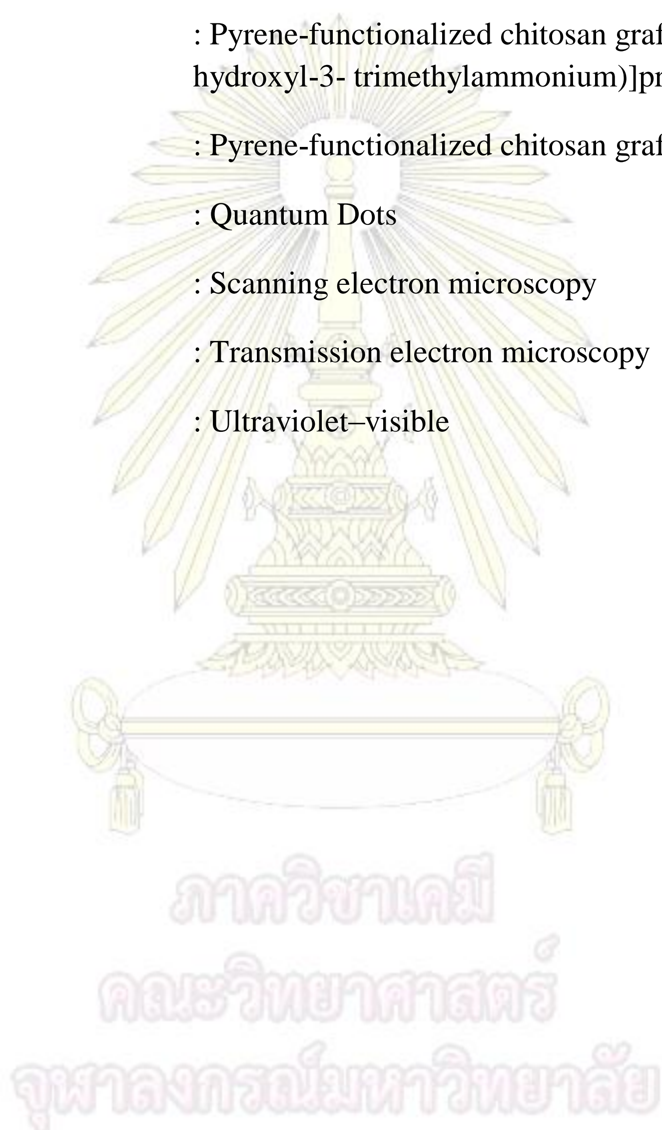
Schem e		Pag e
2.1	Preparation of QDs-PMAMPC-FA…………………………………………..	9
5.1	Chemical structure of chitin and chitosan [2]…………………………………	30
5.2	Synthesis of chitosan labeled with fluorescein isothiocyanate (f-CS) [3]…….	31
7.1	Synthesis of PhCS [1]………………………………………………………	38
7.2	Mechanism of opening PhA ring by amino groups of chitosan that yields *N*-phthaloylchitosan.[1]…………………………………………………....	39
7.3	Mechanism of opening PhA ring by hydroxyl groups of chitosan that yields *O*-phthaloylchitosan.[1]…………………………………………………….	39
7.4	Synthesize of mPEG-COOH……………………………………………….	41
7.5	Synthesis of Pyr-CS-NH_2……………………………………………………	43
7.6	Mechanism of click reaction between 1-ethynylpyrene and N_3-PhCS. [1]…...	44
7.7	Mechanism of GTMAC ring opening by hydroxyl groups of amino group on chitosan………………………………………………………………….......	47
7.8	Synthesis of Pyr-CS-mPEG-COOH………………………………………...	50

ภาควิชาเคมี
คณะวิทยาศาสตร์
จุฬาลงกรณ์มหาวิทยาลัย

List of abbreviation

APP	: 1-Aminopropanol
CS	: Chitosan
CLSM	: Confocal Laser Scanning Microscopy
DD	: Degree of deacetylation
DIEA	: *N,N*-diisopropyl ethylamine
DMF	: *N,N*-dimethylformamide
DS	: Degree of substitution
EDC	: 1-ethyl-3-(3´-dimethylaminopropyl)carbodiimide
FA	: Folic Acid
NBS	: *N*-hydroxysuccinimide
NHS	: *N*-bromosuccinimide
FT-IR	: Fourier transform infrared spectroscopy
GTMAC	: Glycidyltrimethylammonium chloride
HTAP	: *N*-[(2-hydroxyl-3- trimethylammonium)]propyl
mPEG	: poly(ethylene glycol) methyl ether
mPEG-COOH	: Poly(ethylene glycol)methyl ether terminated with carboxyl groups (mPEG-COOH)
nm	: nanometer
NMR	: Nuclear magnetic resonance spectroscopy
NMR	: Nuclear magnetic resonance spectroscopy
N3-PhCS	: Azide-functionalized phthaloylchitosan
PCS	: Photon correlation spectroscopy
PMAMPC	: Poly(methacrylic acid)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)
ppb	: part per million

PhA	: Phthalic anhydride
PhCS	: Phthaloylchitosan
Pyr-PhCS	: Pyrene-functionalized phthaloylchitosan
Pyr-CS-HTAP	: Pyrene-functionalized chitosan grafted with poly(ethylene glycol) methyl ether
Pyr-CS-mPEG	: Pyrene-functionalized chitosan grafted with *N*-[(2-hydroxyl-3- trimethylammonium)]propyl
Pyr-CS-NH_2	: Pyrene-functionalized chitosan grafted with amino groyup
QDs	: Quantum Dots
SEM	: Scanning electron microscopy
TEM	: Transmission electron microscopy
UV-vis	: Ultraviolet–visible


ภาควิชาเคมี
คณะวิทยาศาสตร์
จุฬาลงกรณ์มหาวิทยาลัย

Part I

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CHAPTER I

INTRODUCTION

Currently, fluorescence bioimaging technologies play an important role in biomedical applications because of providing the easy and convenient way to monitor and label the bio-targets such as tumor or cancer for early diagnosis. To obtain the efficient bioimaging technique, several researches have focused on the development of fluorescent probes. Organic dyes act as common and inexpensive fluorescent molecule for imaging. However, organic dyes limit cell labeling in culture for a long time because of photo-bleaching effect over time.[1] From limitation of organic dyes, inorganic fluorophores, quantum dots (QDs), have attracted great interest as alternative fluorescent probes to organic dyes. QDs are semiconductor nanoparticles consisted of atoms in periodic groups (II-VI, III-V or IV-VI), such as CdSe, CdS, CdTe, PbS, PbSe and lnP. Among them, CdSe is widely used for synthesis of core-QDs.[2] As compared with organic dyes, QDs provide the superior properties: size-tunable emissions, high quantum yields, long fluorescent lifetime, broad absorption spectrum, excellent resistance to photo-bleaching and large surface area for functionalization.[3-5] In general method for QDs synthesis, hexadecylamine (HDA) and trioctylphosphine oxide (TOPO) are used as hydrophobic ligands for stabilizing to produce highly stable QDs, controlling nanometer-sized and preventing particles aggregation. Also, the as-synthesized QDs are insoluble in water which limits application of QDs in biological fields. Moreover, QDs are consisted of toxic cadmium compound.[1,6] To increase water solubility and reduce toxicity of QDs, several studies have focused on the coating of water-soluble polymer having excellent biocompatibility and low toxicity on QDs surface.[7-11] The using of polymer as stabilizer not only increases the biocompatibility of QDs, but also introduces functional groups by selecting the right choice of comonomer for immobilizing of specific molecule and then specific binding with targeted cell. Generally, there are two well-known strategies used for surface modification of QDs with water-soluble polymer including encapsulation with amphiphilic polymers[7-9] and ligand exchange with hydrophilic polymer. [10-11] In the way of encapsulating with amphiphilic polymers, hydrophobic parts of polymer will physically bind with original ligands of QDs, while hydrophilic parts are located outer to be

hydrated with water molecule promoting the dispersion of QDs in aqueous media. Many studies[7-9] have revealed the using of amphiphilic polymer encapsulation for improving water solubility of QDs.

In 2004, Gao, *et al.*[7] reported multifunctional QDs probe for cancer targeting and imaging in living animals. For in vivo protection, this new class of water-soluble QDs was encapsulated with amphiphilic triblock copolymer having polybutylacrylate and polyethylacrylate as hydrophobic sides and polymethacrylic acid as hydrophilic side. Targeting-ligands for tumor antigen recognition and multiple PEG molecules for improving biocompatibility and circulation were then linked onto amphiphilic triblock copolymer. For *in vivo* study, the QD probes could accumulate at tumors with sensitive and multicolor fluorescence imaging.

In 2011, Chen *et al.*[8] prepared water-soluble CdSe/ZnS QDs by overcoating oil soluble CdSe/ZnS-TOPO QDs with poly(acrylamide-co-acrylic acid)-ethanolamine (PSMA-EA). Photoluminescence (PL) efficiency and lifetime of photoexcited carriers of the modified QDs were studied. The results showed that PL efficiency was increased 5–30% after introducing PSMA-EA polymers to encapsulate CdSe/ZnS-TOPO QDs. Moreover, photoexcited carriers' lifetime was elongated 2–17 ns indicating that PL quenching defects were effectively removed by coating CdSe/ZnS QDs with hybrid TOPO-PSMA-EA shell.

In 2013, Yang *et al.*[9] constructed the targeting, imaging and pH-responsive drug release system by encapsulating QDs with amphiphilic and pH-responsive poly(ethylene glycol) and dodecylamine-modified poly(itaconic acid) (PEG-PIA-DDA), incorporating doxorubicin (DOX) within the polymer shell, and binding anti-vascular endothelial growth factor (anti-VEGF). PIA, PEG, and DDA in amphiphilic polymer's structure provided water solubility, non-specific resistance, and hydrophobic side, respectively. DOX acted as anti-cancer drug, while anti-VEGF served as targeting material. The results indicated that this system could target the liver cancer cell and release drug at pH 5.5, 37°C within 24 h.

By using amphiphilic polymer, although the water-soluble QDs having high stability and high photoluminescence quantum yields are obtained, this method also provides large hydrodynamic size of amphiphilic polymer-coated QDs on the level of 30-40 nm which is often much larger than the cellular receptorsof specific target limiting bioimaging application of these polymer-capped QDs.[10] However, preparation of highly stable and water-soluble QDs by maintenance of QDs size can be prepared via ligand exchange method.[2,10-11] In this method, functional anchor groups, such as thiol, amine and

carboxyl group, are required in the polymer's structure to more strongly bind with QDs than original hydrophobic resulting in replacing of original stabilizer by water-soluble polymer. Thiol group is usually used as functional anchor groups due to its highest affinity with metallic surface.[12]

In 2010, Liu *et al.*[10] synthesized multidentate ligand for strong anchoring onto the QD surface by grafting several thiol groups to a linear polymer chain through the reaction between poly(acrylic acid) (PAA) and mercaptoethylamine (MEA) and used this ligand for capping hydrophobic QDs to obtain the water-soluble QDs. Carboxyl groups on the PAA chain confer hydrophilicity and biocompatibility. The obtaining PAA-g-MEA capped water-soluble QDs with relatively small hydrodynamic diameters possessed superior brightness, high stability over extended periods of time, over a broad pH range (3-14), in salt concentrations, and on thermal treatment at 100 ºC.

In 2010, Zhao *et al.*[11] reported the synthesis of folate-poly(ethylene glycol)-polyamidoamine (FPP) dendrimer-functionalized CdSe/ZnS QDs through direct ligand-exchange reactions. These dendrimer ligands had multivalent amino groups, which can react with Zn^{2+} on the surfaces of CdSe/ZnS QDs resulting in replacing of original ODA ligands by FPP dendrimer and obtaining the highly hydrophilic and biocompatible QDs targeted to folate receptors in tumor cells. Cellular uptake and imaging studies were evaluated and found that FPP-coated QDs provided more significant cellutar uptake in HeLa cells than QDs without folate suggesting the occurrence of folate receptor recognition on cell surface.

Poly(2-methacryloyloxyethyl phosphorylcholine (PMPC) is a well-known hydrophilic polymer for improving the biocompatibility and water solubility of the QDs' surfaces[1,13] and other nanocolloids' surfaces, such as gold nanoparticles[14-15] and silver nanoparticles,[16] in biomedical applications. In addition, the PMPC provides the reduction of nonspecific adsorption to protect against nonselective cellular uptake resulting in precise evaluation of bio-labeling.[1,17-20] Because PMPC has zwitterionic structures with electrically neutral, this polymer shows the similar properties, such as biocompatibility and resistance of nonspecific interaction, as compared with poly(ethylene glycol) (PEG), outstanding biocompatible polymer.[7,9,11] Previously, few researches have reported on the grafting of PMPC on QDs' surface to obtain biocompatibility, water solubility and nonspecific interaction resistance.

In 2008, Goto *et al.*[1] prepared polymer nanoparticles embedding QDs by using amphiphilic poly(MPC-*co*-*n*-butyl methacrylate (BMA)-*co*-*ω*-methacryloyloxy

poly(ethylene oxide) oxycarbonyl 4-nitrophenol (MEONP)) (PMBN). MPC, BMA, and MEONP in PMBN copolymer provided hydrophilicity and biocompatibility, hydrophobicity, and probe immobilization, respectively. QDs and water-insoluble poly(L-lactic acid) (PLA), as a core material in various biological fields, were embedded inside the polymer nanoparticles to obtained the artificial cell membrane-covered nanoparticles for fluorescence bioimaging. The polymer nanoparticles showed biocompatibility and resistance to nonspecific cellular uptake from HeLa cells owing to the nature of the phosphorylcholine groups. For specific tracking, octaarginine (R8), specific molecule, was immobilized on active ester groups in MEONP units, and it was found that they were able to penetrate the membrane of HeLa cells effectively.

In 2009, Matsuno *et al.*[13] synthesized sodium 2-dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionate (DMP-Na) as double functional reversible addition–fragmentation chain transfer (RAFT) agent. As the first function, the amphiphilic DMP-Na could form micelle in aqueous solution to solubilize the TOPO-coated QD into the aqueous solution. For the second function, the DMP-Na possessed chain transfer agent ability to polymerized poly(MPC) initiated from QDs' surface. The synthesized poly(MPC)-modified showed good biocompatibility and suppressed uptake by HeLa cells due to biocompatibility and nonspecific reduction of the poly(MPC).

To obtain the specific affinity to the targeted cell, other monomers having active sites for conjugation of biomolecules are incorporated. As our previous work, methacrylic acid (MA) was copolymerized with MPC monomer to achieve both functionalizability and nonspecific adsorption resistance for modifying gold substrate in surface plasmon resonance (SPR) sensors.[21] The MA units provided active sites for specific probe binding whereas the MPC units enhanced biocompatibility and reduced non-specific adsorption of non-targeted molecules. When biotin was immobilized on MA units, the PMAMPC-grafted surface gave the best sensing performance for avidin detection. Furthermore, incorporation of MPC unit in the copolymer truly helped in suppressing the nonspecific adsorption of the non-targeted components and allowed for the effective detection in diluted blood plasma.

From the previous success of using PMAMPC copolymer for surface modification, herein, it is anticipated that this PMAMPC copolymer can be applied as polymeric stabilizer for QDs to prepare water-soluble, biocompatible and functionalisable QDs preventing nonspecific interaction for specific fluorescence bioimaging. To the best of our knowledge, there are few reports of grafting polymers containing MPC on QDs, and it has not yet been

reported for this copolymer, PMAMPC. Reversible addition-fragmentation chain transfer (RAFT) polymerization, living radical polymerization, will be used for PMAMPC synthesis due to providing terminal dithioester group at the polymer chain end which can then be converted to thiol group for strongly binding onto the surface of QDs via ligand exchange method. To achieve the specific labeling to cancer cells which folate receptor are overexpressed, folic acid (FA) will be immobilized on MA units in PMAMPC via EDC/NHS coupling chemistry, and then FA-functionalized PMAMPC will be grafted on QDs via ligand exchange method obtaining QDs stabilized with PMAMPC-FA (QDs-PMAMPC-FA).

CHAPTER II

EXPERIMENTAL SECTION

2.1 Materials

Cadmium 2,4-pentanedionate, tri-n-octylphosphine oxide (TOPO), tri-octylphosphine (TOP), 1-hexadecylamine (HAD), trioctylamine, zinc acetate dihydrate ($Zn(OAC)_2.2H_2O$), sulfur powder, phosphate buffered saline pH 7.4 (PBS), folic acid (FA), hydrazine monohydrate, methacrylic acid (MA), mercaptopropanoic acid (MPA), and dialysis bag (cut-off molecular weight of 3500 g/mol) were purchased from Sigma-Aldrich (USA). 1,2-Hexadecanediol (HDDO) was obtained from Tokyo Chemical Industry (Japan). Selenium powder was bought from Riedel-de Haen (Germany). Hexane and ethanol were purchased from Merck (Germany). Chlorofrom was obtained from RCI Labscan (Thailand). Epidermoid cervical carcinoma (CaSki) cell lines were purchased from ATCC. 2-Methacryloyloxyethyl phosphorylcholine (MPC) was provided by Prof. Yasuhiko Iwasaki (Kansai University, Japan). 4,4′-Azobis(4-cyanovaleric acid) (ACVA) and 4-cyano-4-(thiobenzoylthio)-pentanoic acid (CPD) were obtained from Aldrich. 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and 2-ethanolamine were bought from Fluka (Switzerland). Centrifuge tube containing membrane with a cut-off molecular weight of 30,000 g/mol was obtained from Millipore (USA). The above chemicals and solvents were analytical grade and used as received without further purification except MA which was distilled under reduced pressure prior to use. Moreover, all solutions were prepared using ultrapure distilled water that was obtained after purification using a Millipore Milli-Q system (USA) that involves reverse osmosis, ion exchange, and a filtration step (18.2 MΩ cm resistance).

2.2 Synthesis of Quantum Dots (QDs)

Quantum dots were synthesized via 2 steps. The first step was the synthesis of QDs core (CdSe). In the second step, the synthesized QDs core-were then coated with ZnS shell to increase stability in terms of fluorescence emission and optimize optical properties.[25] For the synthesis of core, CdSe (orange emission) was performed via pyrolysis of the organometallic precursors according to a method modified from that of Dabbousi *et al.*[22]

by using cadmium 2,4-pentanedionate and trioctylphosphine selenide (TOPSe) as precursors and TOPO and HDA as stabilizers to prevent the aggregation and control the particle size. TOPSe was first prepared by mixing selenium powder (3.55 g, 0.05 mol) and TOP (30 mL, 0.067 mol) under N_2 gas at room temperature for 24 h to obtain 1.5 mol/mL of TOPSe. Then, TOPO (6.25 g, 0.016 mol) and HDA (5.75 g, 0.02 mol) were added to a three neck flask (reaction flask) at room temperature. The reaction flask was stirred and heated by using temperature controller ranging from 140 to 360°C under nitrogen atmosphere for 2 h. After that, the precursors, cadmium 2,4-pentanedionate (0.7608 g, 2.45×10^{-3} mol) and TOPSe (6 mL, 1.5 M), were added at 360°C. After the mixture was stirred for 30 min, the reaction's temperature was decreased to 60°C, and hexane (40 mL) was then injected. Finally, the synthesized QDs core (CdSe) were purified by precipitation with the excess amount of ethanol and then re-dispersion with hexane. The final QDs core (CdSe) were kept at room temperature. For the synthesis of ZnS shell, the modified method from Yang *et al.*[23] was used. Briefly, $Zn(OAC)_2.2H_2O$ (0.21 g, 1×10^{-3} mol), sulfur powder (64.1 mg, 2.00×10^{-3}mol), and the synthesized QDs core solution (8 mL) were mixed and heated at temperature ranging from 100 to 260°C under nitrogen atmosphere for 2.5 h. The obtained CdSe/ZnS were kept in the dark at room temperature.

2.3 Preparation of QDs Stabilized by PMAMPC-SH (QDs-PMAMPC) via Ligand Exchange Method

As shown in Scheme 2.1, QDs-PMAMPC were prepared through 2 steps. Firstly, thiol-terminated PMAMPC (PMAMPC-SH) was synthesized. The synthesized PMAMPC-SH was then grafted onto QDs' surface by ligand exchange method.

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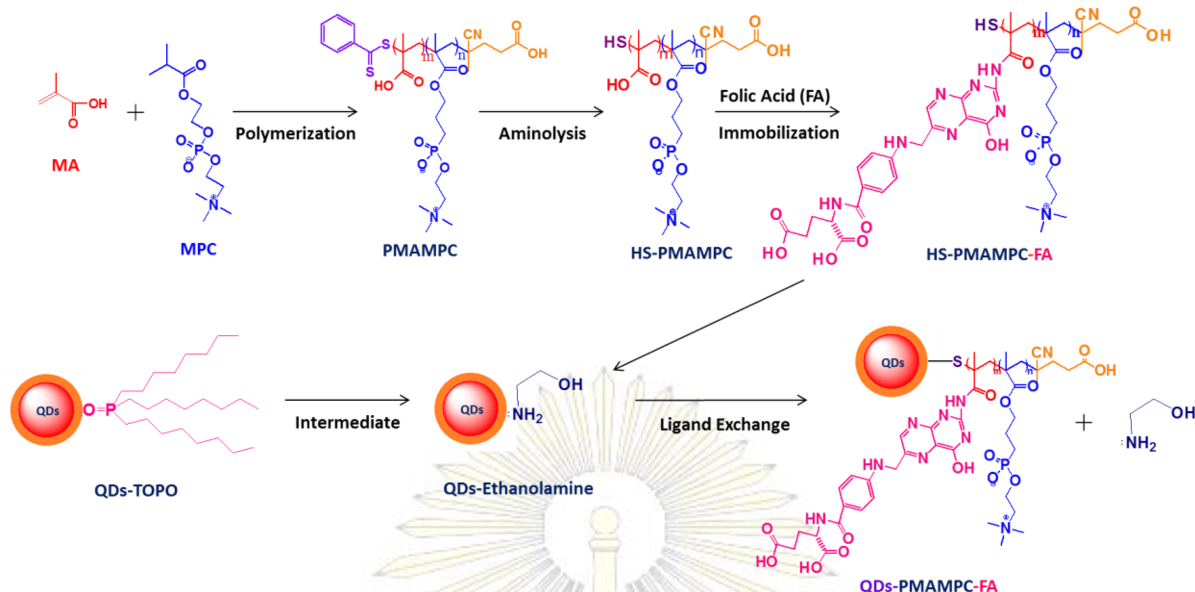

Scheme 2.1 Preparation of QDs-PMAMPC-FA

2.3.1 Synthesis of Thiol-terminated PMAMPC (PMAMPC-SH)

Poly(methacrylic acid)-*ran*-(2-methacryloyloxyethyl phosphorylcholine) (PMAMPC) having degree of polymerization (DP) of 100 and comonomer ratio (MA:MPC) of 60:40 was prepared by RAFT polymerization. According to a method modified from that of Akkahat *et al.*,[21] MPC monomer (591.36 mg, $2.00x10^{-3}$mol) and MA monomer (254.35 mg, $3.00x10^{-3}$mol) were dissolved in 8 mL of mixed solvent (1:1 v/v, EtOH:PBS). Thereafter, ACVA (3.51 mg, $12.5x10^{-6}$mol) and CPD (13.97 mg, $4.70x10^{-5}$mol) were added in the comonomer solution. Before the solution was placed in an oil bath at 70°C for 6 h, it was degassed by purging with N_2 gas for 30 min. After reacting for 6 h, the solution was cooled down at room temperature and was purified by dialysis (cut-off molecular weight of 3,500 g/mol) in deionized water for 3 days. The final product was obtained after lyophilization and kept at -20°C. In order to generate thiol group at polymer chain end for strong binding with QDs' surface, the terminal dithiobenzoate groups were aminolyzed with hydrazine monohydrate. In brief, the solution of the copolymer in MilliQ (5mM) was treated with hydrazine monohydrate (30 mol equivalents of PMAMPC) at 25 °C for 6 h, and the mixture was then dropped in HCl solution (1.2 M, 10 mL). For purification, the aminolyzed polymer was dialyzed in HCl solution (pH 3) for 2 days and deionized water for 2 days, respectively. Finally, the PMAMPC-SH was obtained after lyophilization and kept at -20°C.

2.3.2 Preparation of QDs Stabilized by PMAMPC (QDs-PMAMPC) by Ligand Exchange Method

Ligand exchange process was modified from that of Cao *et al.*[24] by using ethanolamine as phase transfer agent. To transfer the as-synthesized QDs dispersed in hexane to aqueous solution, ethanolamine (1 mL, 16.56×10^{-3} mol) was added to 2 mL of the as-synthesized QDs under vigorous stirring at room temperature and 2 mL of Milli Q water was immediately added afterwards. After stirring for 10 min, the QDs in water phase at lower layer was transferred to the aqueous solution of PMAMPC-SH (1.0% w/v, 2 mL), and the mixture was vigorously stirred at room temperature for 24 h for ligand exchange. Finally, to remove the excess PMAMPC-SH, aqueous solution of QDs-PMAMPC was placed into a centrifuge tube containing membrane with a cut-off molecular weight of 30,000 g/mol and centrifugally washed with Milli Q water by centrifuge (model 7780, Kubota Corporation, Japan) at 4,000 rpm for 10 min. The purified QDs-PMAMPC solution was obtained after redispersion in Milli Q water and kept at room temperature.

2.4 Preparation of QDs stabilized by Folic Acid-modified PMAMPC-SH (QDs-PMAMPC-FA) via Ligand Exchange Method

QDs-PMAMPC-FA were prepared via 2 steps. In the first step, amino group of folic acid (FA) was attached to carboxyl groups of methacrylic (MA) unit in PMAMPC-SH through amidation by using EDC/NHS as coupling agent, and then the FA-modified PMAMPC-SH was grafted onto QDs' surface to prepare QDs-PMAMPC immobilized with FA.

2.4.1 Immobilization of FA on PMAMPC-SH (FA-PMAMPC)

PMAMPC-SH in 10mM PBS, pH 7.4 (0.75% w/v, 4 mL) was mixed with EDC (700 μL, 1.17 M) and NHS (300 μL, 0.67 M),the final concentration of EDC and NHS of 0.2 M and 0.05 M, respectively, and stirred for 3 h to activate carboxyl groups in PMAMPC. Then, FA (10 mol equivalents of PMAMPC) was added to the mixture and stirred for 24 h at room temperature in the dark. After finishing the reaction, the prepared FA-PMAMPC was purified by dialysis (cut-off molecular weight of 3,500 g/mol) in deionized water for 3 days, and the final product was obtained after lyophilization and kept at -20°C in the dark.

2.4.2 Preparation of QDs-PMAMPC-FA by Ligand Exchange Method

The synthesis of QDs-PMAMPC-FA was similar to the synthesis of QDs-PMAMPC, except that PMAMPC-FA was used instead of PMAMPC.

2.5 Preparation of QDs Stabilized by Mercaptopropanoic Acid (QDs-MPA) via Ligand Exchange Method

QDs-MPA were synthesized by using the same method as the preparation of QDs-PMAMPC, except that the QDs to be transferred to water phase were added to the aqueous solution of MPA (0.3% v/v, 2 mL) instead of PMAMPC-SH solution in the ligand exchange process.

2.6 Cell Study

To test cytotoxicity and specific cellular uptake and imaging, QDs-PMAMPC, QDs-PMAMPC-FA, and QDs-MPA, as control water-soluble QDs without polymer modification, were comparatively investigated on human epidermoid cervical carcinoma (CaSki) cell having overexpressed folate receptor. MTT [3-(4,5-dimethylthiazlo-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay was performed to evaluate toxicity and confirm the biocompatibility of QDs grafted with PMAMPC. In addition, to study cell-targeting specificity, QDs-PMAMPC-FA was incubated with CaSki cells and compared with QDs-PMAMPC-FA and QDs-MPA by using a confocal laser-scanning fluorescence microscope (Nikon Digital Eclipse C1-Si, Japan).

2.7 Characterizations

Fluorescence signals of the modified QDs were recorded by Perkin-Elmer precisely (LS 45) luminescence spectrometer (PerkinElmer Inc., UK) with 350 nm excitation in a scanning wavelength range of 400-800 nm. QDs' concentrations and quantum yields were determined by fluorescence spectrometer, CARY 100 Bio UV-visible spectrophotometer (Varian Ltd., USA). QDs' concentrations were calculated by using the maximum emission wavelength form fluorescence spectrometer and absorbance at 350 nm analyzed by UV-visible spectrophotometer according to a published procedure.[26] In the measurement of quantum yields, fluorescence spectra under 350 nm excitation and absorbance at 350 nm were used, and as-synthesized QDs (CdSe/ZnS) was chosen as the reference standard (quantum yield 100%). Molecular weight and comonomer ratio of PMAMPC were

determined by Varian, model Mercury-400 nuclear magnetic resonance (NMR) spectrometer (USA) operating at 400 MHz. To confirm the successful polymerization of PMAMPC and the attachment of FA on PMAMPC, the structural information was analyzed by using a Nicolet 6700 Fourier transform -Infrared (FT-IR) spectrometer with 32 scans at resolution of 4 cm^{-1}. Size, size distribution and morphology of the QDs were characterized with JEOL JEM-2010 transmission electron microscope (TEM) (Japan) operating at 80 keV. The presence of the PMAMPC and PMAMPC-FA around the QDs and morphology were confirmed by a Seiko SPA 400 atomic force microscope (AFM) (SII Nanotechnology Inc., Japan). The samples were prepared by dropping the QDs solution on a freshly cleaved mica plate and dried in a dessicator for 24 h prior to analysis. Measurements were performed in air at ambient temperature using tapping mode and silicon tips with a resonance frequency of 138 kHz. Cellular uptake of the modified QDs were analyzed by confocal laser scanning fluorescence microscope (CLSFM, Nikon Digital Eclipse C1-Si equipped with Plan Apochromat VC 100×, Melles Griot Diode Laser and 85 YCA-series Laser at 405 and 561 nm, a Nikon TE2000-U microscope, a 32-channel-PMT-spectral-detector and Nikon-EZ-C1 Gold Version 3.80 software).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Synthesis and Characterization of PMAMPC-SH

Polymerization of PMAMPC copolymer via RAFT polymerization was performed according to our published procedure.[1] The method was capable of controling molecular weight and ratio of comonomer. It can also provide thiol groups at polymer chain end after the copolymer was subjected to aminolysis by hydrazine ^{1}H NMR spectrum of the synthesized PMAMPC shown in Figure 3.1 demonstrates a characteristic signals of aromatic protons of dithiobenzoate group of the copolymer chain end at 2.8-3.2 ppm (position e). From ^{1}H NMR data, Mn of PMAMPC was found to be 20069.4 g/mol for targeted DP of 100 (expected Mn =16990.8 g/mol with ratio of the co-monomer was MA:MPC of 68:48 (comonomer ratio in the feed = 60:40. Polymerization of PMAMPC via RAFT polymerization, as shown in Figure 3.1, was characterization by ^{1}H-NMR. The signals of protons at 1.6-2.2 (H_a and $H_{a'}$), 0.6-1.2 (H_b and $H_{b'}$), 2.8-3.2 (H_e), 3.8-4.2 (H_c), and 3.4-3.6 (H_d) ppm assigned to CH_2 in main structure, CH_3, $N^+(CH_3)_3$ and the signals of dithiobenzoate protons at 7.4-8.2 (H_f) ppm. In addition to Mn of PMAMPC and ratio of MA:MPC in copolymer, these can calculate by equation 3.1c of ratio of MA:MPC in PMAMPC copolymer. To confirm the success of dithiobenzoate group at the copolymer chain end being converted to thiol group, UV-vis spectroscopy was studied. As shown in Figure 3.2, it was found that the absorption band around 300 nm corresponding to the dithiobenzoate group disappeared suggesting the formation of thiol group. Moreover, the appearance of PMAMPC shown as inset of Figure 1), which changed from pink to white as a result of dithiobenzoate group removal, also confirmed the successful aminolysis. In addition FT-IR spectrum shows the characteristic peaks of PMAMPC at 1708 cm^{-1}, 1160 cm^{-1}, 1223 cm^{-1}, and 1050 cm^{-1} which correspond to C=O stretching of carboxyl group in polymer chain, C-O stretching carboxyl group in polymer chain, C-O stretching carboxyl group of ester group, and P-O stretching in polymer chain, respectively, as shown in Figure 3.3.

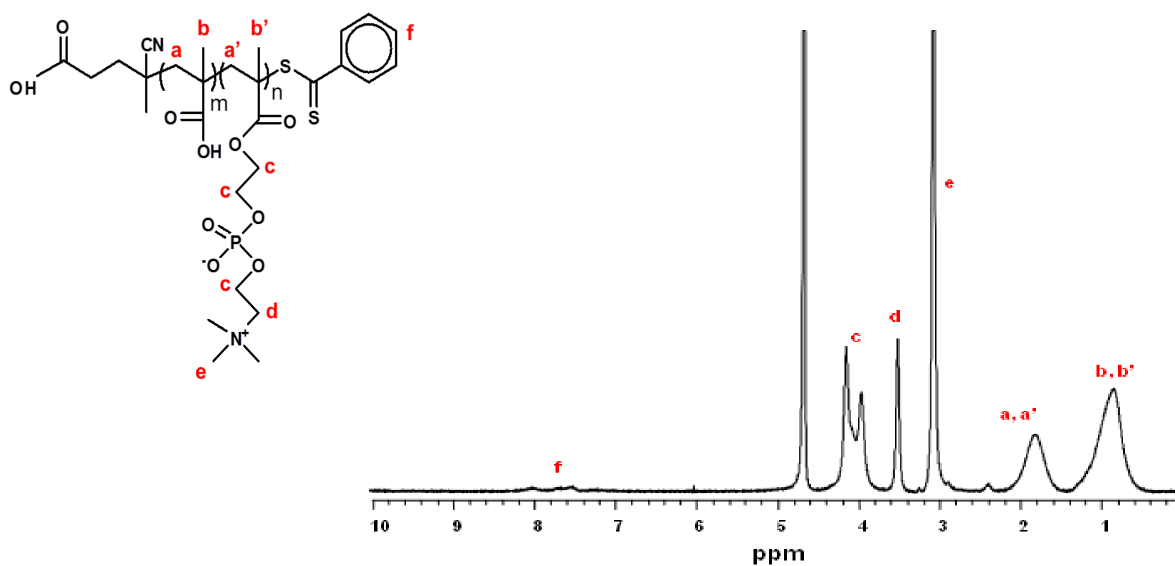

Figure 3.1 ^{1}H NMR of PMAMPC copolymer.

$$\text{Total repeating units} = \left\{\frac{\text{Integral of the Hb and Hb}'/6}{\text{Integral of the Hf}/5}\right\} \times 2 \quad \text{——— Equation 3.1a}$$

$$\text{Mn} = [\text{Repeating units of MA} \times \text{MW of MA (86.06 g/mol)} +$$

$$\text{Repeating units of MPC} \times \text{MW of MPC (295.68 g/mol)}] \quad \text{——— Equation 3.1b}$$

$$\text{Ratio of MA in copolymer (\%)} = \left\{\frac{\text{Repeating units of MA}}{\text{Total repeating units}}\right\} \times 100$$

$$\text{Ratio of MPC in copolymer (\%)} = \left\{\frac{\text{Repeating units of MPC}}{\text{Total repeating units}}\right\} \times 100 \quad \text{——— Equation 3.1c}$$

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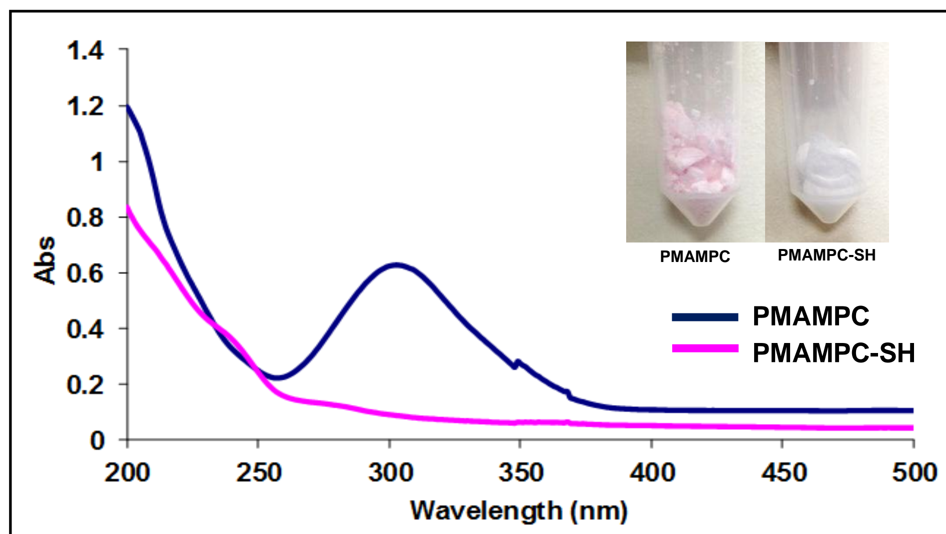

Figure 3.2 UV-vis spectra of PMAMPC and PMAMPC-SH

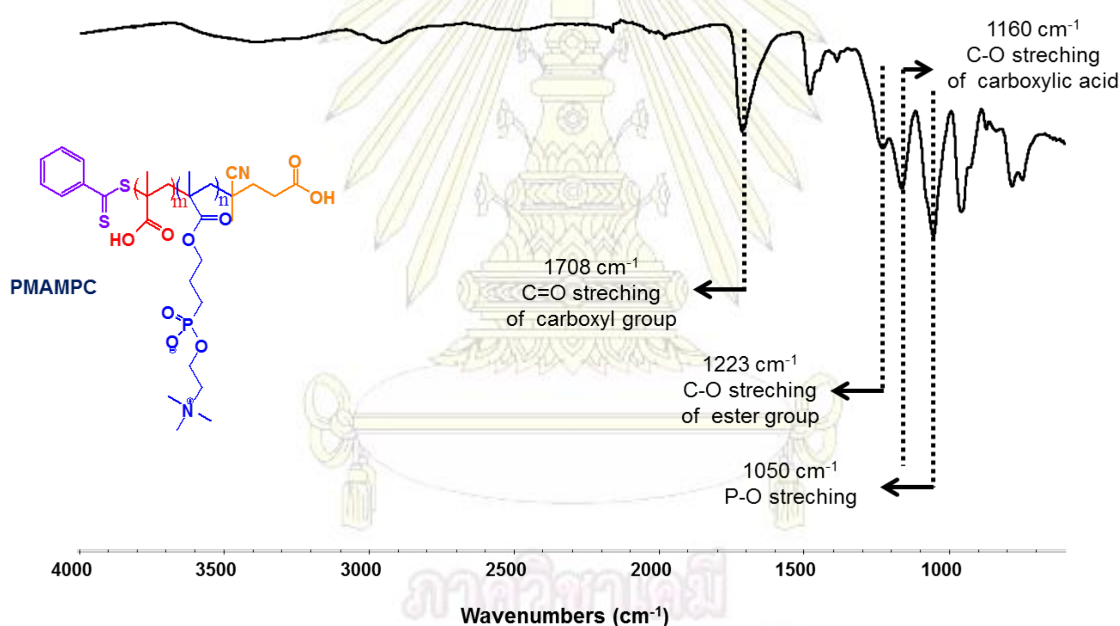

Figure 3.3 IR spectra of PMAMPC.

Synthesis and Characterization of QDs-PMAMPC via Ligand Exchange Method

The success of QDs preparation was verified by fluorescence spectroscopy. As shown in Figure 3.4a, emission spectrum of the as-synthesized CdSe/ZnS quantum dots in hexane (purple line) exhibits a maximum wavelength at 597 nm which is in good agreement with its physical appearance of in orange solution (Figure 3.5). As determined by UV-Vis spectroscopy (Figure 3.4b) following a published procedure[26], the concentration of QDs was found to be 23.2 μM.

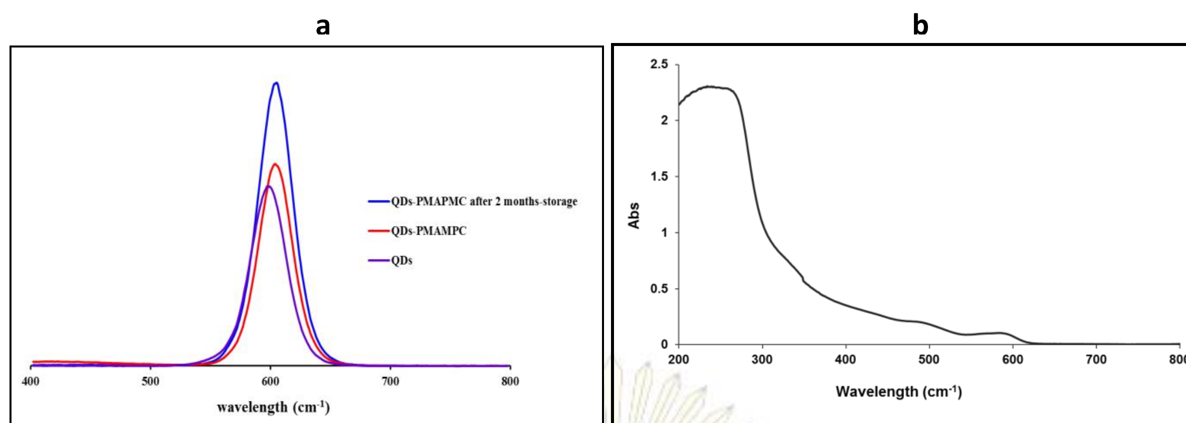

Figure 3.4 (a) Fluorescence spectra of QDs and QDs-PMAMPC, and QDs-PMAMPC_after storage for 2 months in the dark, (b) UV-Vis spectrum of QDs.

Figure 3.5 Physical appearance of the as-synthesized QDs under ambient light (left) and black light (right).

In this research, the method of ligand exchange by using a phase transfer agent was applied in order to transfer the QDs which are dispersed in hexane to aqueous phase prior to PMAMPC immobilization. A first attempt was done by using 1-aminopropanol (APP) as a phase transfer agent following a published procedure by Cao *et al.*[24] The method relies on the fact that APP is a soft binding ligand having lone paired electrons of amino groups that can bind to the surface of QDs. It was anticipated that the QDs can be transferred from hexane phase to APP phase. Upon mixing aqueous solution of PMAMPC with APP solution containing QDs, it was believed that the PMAMPC immobilization on the surface of QDs should take place as a result of stronger binding forces between the thiol groups of PMAMPC and the surface of QDs. The first attempt was not successful because APP cannot dissolve in water although it can dissolve in a number of solvents with high polarity like DMSO, DMF, or THF. The failure of this attempt is demonstrated as the turn-off of fluorescence emission of the QDs as shown in Figure 3.6a. The second attempt was done

using a more polar compound having similar functionalities, ethanolamine which is water soluble. As revealed in Figure 3.6b, the bright fluorescence emission of the QDs under the black light after ligand exchange using ethanolamine and mixing with PMAMPC aqueous solution implied that the PMAMPC immobilization was successful. The fluorescence emission maximum wavelength of the QDs-PMAMPC red shifted from 597 nm of the as-synthesized QDs to 608 nm (Figure 3.4a, red line). The QDs-PMAMPC was quite stable. Its fluorescence emission maximum wavelength was not altered upon storage at ambient temperature in the dark for 2 months.

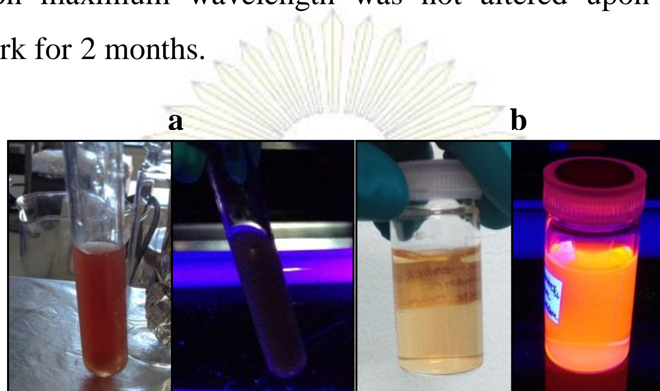

Figure 3.6 Physical appearance of QDs-PMAMPC after ligand exchange process and mixing with PMAMPC aqueous solution using (a) aminopropanol and (b) ethanolamine as phase transfer agents (left: under ambient light and right: under black light).

The success of PMAMPC immobilization on the QDs surface via ligand exchange using ethanolamine as phase transfer agent was further characterized by IR analysis. As shown in Figure 3.7c, the characteristic band of carboxyl group (C=O stretching) in the copolymer chain (MA units) appears at 1733 cm^{-1} which did not exist in Figure 3.7a and shifted from free polymer in Figure 3.7b because the QDs surfaces obstructed bonds' vibration of polymer chain. As determined by UV-Vis spectroscopy, the concentration of the QDs-PMAMPC was 1.57 µM.

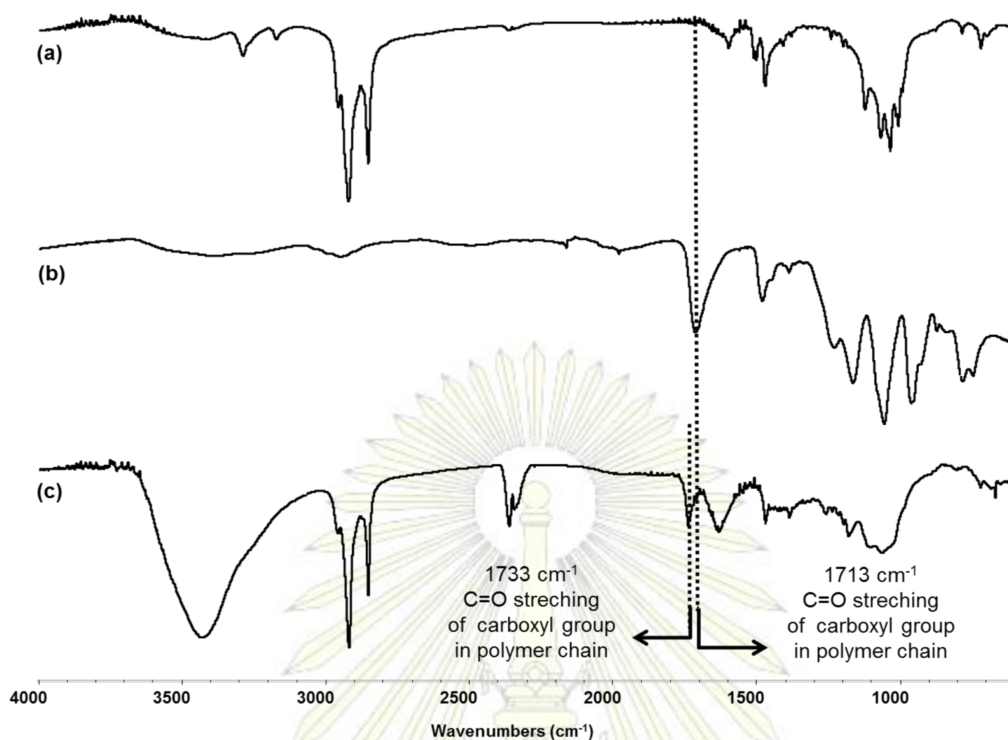

Figure 3.7 IR spectra of (a) QDs, (b) PMAMPC, and (c) QDs-PMAMPC.

3.2 Synthesis and Characterization of QDs-PMAMPC-FA

Attachment of FA to PMAMPC was done via amide bond formation by using EDC/NHS as coupling agents. The success of PMAMPC-FA preparation was proven by FT-IR analysis, as shown in Figure 3.8. The characteristic bands of amide linkage; C=O stretching (Amide I) and N-H bending (Amide II) emerge at 1606 and 1563 cm^{-1}, respectively.

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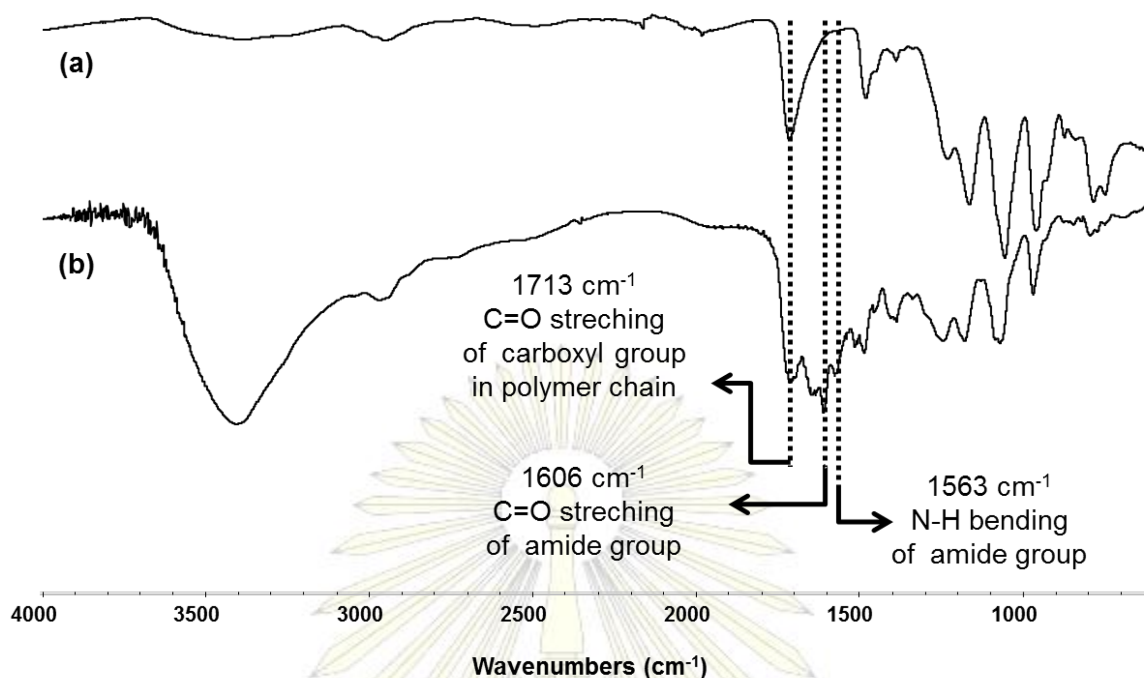

Figure 3.8 IR spectra of (a) PMAMPC, and (b) PMAMPC-FA.

The immobilization of PMAMPC-FA on QDs surface was carried out using the same method as the preparation of QDs-PMAMPC via ligand exchange using ethanolamine as the phase transfer agent. Typically, folic acid has an emission band around 458 nm (A. Tyagi and A. Penzkofer (2010)[27]). As shown in Figure 3.9, the fluorescence emission spectrum of QDs-PMAMPC-FA has two emissions at 452 and 606 nm, respectively. The 452 nm emission band can be assigned to folic acid whereas that at the 606 nm can be assigned to the signal of QDs. The co-existence of the two peaks indicated that the PMAMPC-FA were successfully conjugated to the QDs and it is the reason why the solution of QDs-PMAMPC-FA appeared in different color (Figure 3.9b) under the black light when compared with those of the QDs and the QDs-PMAMPC As determined by UV-Vis spectroscopy, the concentration of QDs-PMAMPC-FA was 0.2 μM.

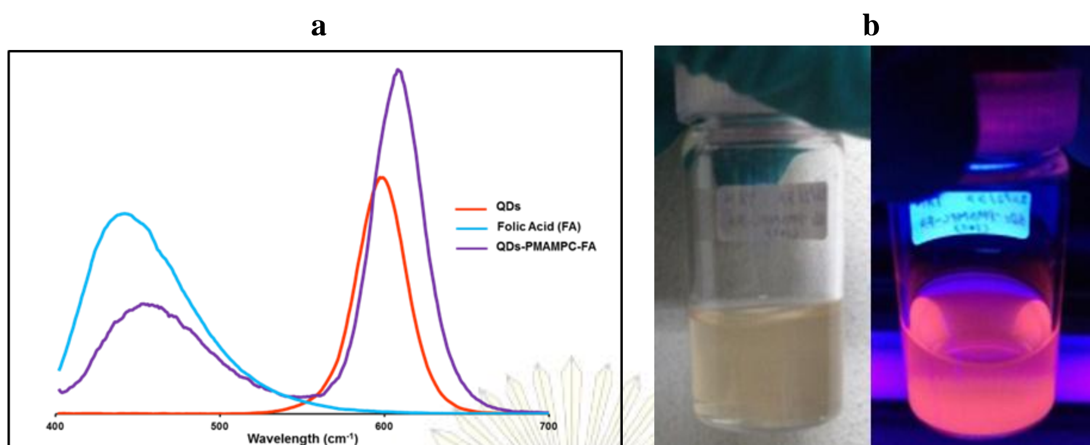

Figure 3.9 (a) Fluorescence emission spectra of QDs, FA, and QDs-PMAMPC-FA and (b) physical appearance of QDs-PMAMPC-FA (left: under ambient light and right: under black light).

The success of PMAMPC-FA immobilization was also verified by IR analysis. As shown in Figure 3.9, the characteristic bands of amide which consisted of C=O stretching at 1640 cm^{-1} and N-H bending at 1553 cm^{-1} was found on the IR spectrum of QDs-PMAMPC-FA. Size and morphology of the QDs-PMAMPC-FA were characterized by TEM and AFM analyses. As reviewed by TEM (Figure 3.11), they were highly stable, spherical in shape and had a monodispersed size of 5.2±0.8 nm. This dimension was slightly larger than the as-synthesized QDs (3.8±0.6 nm) and QDs-PMAMPC (3.93±0.65 nm). From AFM analysis (Figure 3.12), the inorganic core of QDs and organic polymeric shell can be observed for both QDs-PMAMPC and QDs-PMAMPC-FA. The greater size of the latter than the former is in good agreement with the results obtained from TEM analysis.

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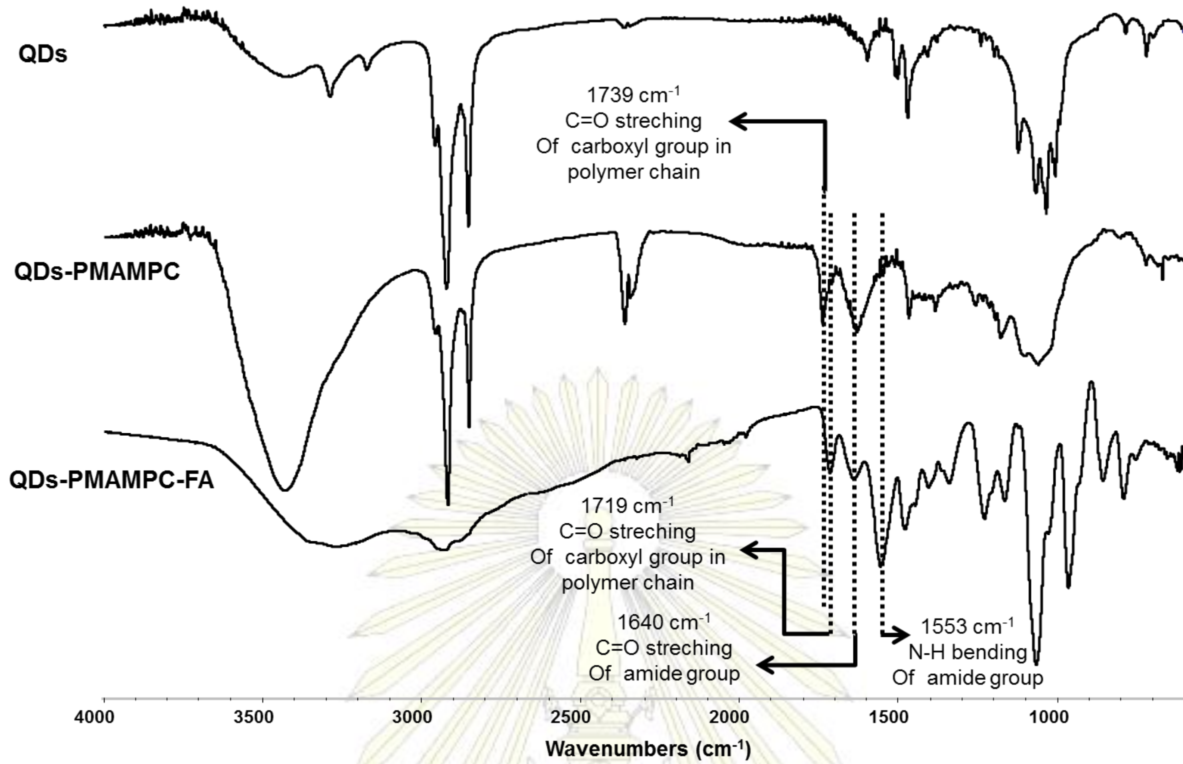

Figure 3.10 IR spectra of (a) QDs, (b) QDs-PMAMPC, and (c) QDs-PMAMPC-FA.

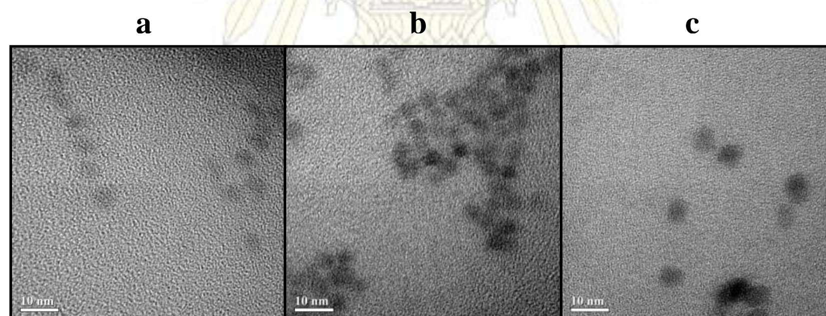

Figure 3.11 TEM images of (a) QDs, (b) QDs-PMAMPC, and (c) QDs-PMAMPC-FA

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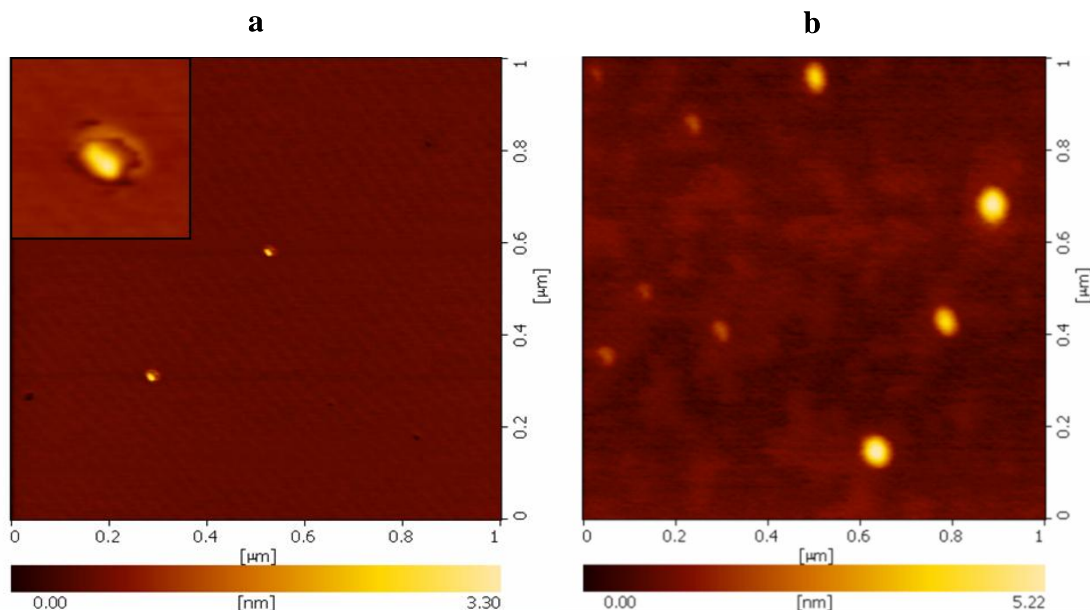

Figure 3.12 AFM images of (a) QDs-PMAMPC (Inset: zoom of QDs-PMAMPC) and (b) QDs-PMAMPC-FA.

To determine the efficiency of fluorescence emission, quantum yields of QDs-PMAMPC and QDs-PMAMPC-FA were measured and calculated by equation 3.2.[25] As compared with QDs stabilized by mercaptopropanoic acid (QDs-MPA), water-soluble model QDs without polymer modification, the QDs stabilized by PMAMPC and PMAMPC-FA provided higher quantum yield than that of the QDs-MPA. This may be explained as a result of the polymeric coating being capable of protecting the QDs from photo bleaching.

$$QY_x = QY_s \times \frac{A_x \times F_s}{A_s \times F_x} \times \left[\frac{n_x}{n_s}\right]^2 \qquad \text{Equation 3.2}$$

A = integrated area under the emission spectrum

F = the fraction of exciting light absorbed at the excitation wavelength

n = the refractive index of the solvent

Subscript x = sample

Subscript s = reference standard

Table 3.1 Fluorescence emission, UV-Vis absorbance data and quantum yield of QDs-MPA, QDs-PMAMPC, and QDs-PMAMPC-FA.

Quantum dots	Absorbance at 350 nm	Peak area of fluorescence (excitation wavelength of 350 nm)	Quantum Yield (%QY)	Relative Quantum Yield (au.)
QDs shell	0.069146	37,692.0686		545,108.45
QDs-MPA	0.689264	1,181.6782	0.30	1,714.41
QDs-PMAMPC	0.985230	3,293.0278	0.60	3,342.39
QDs-PMAMPC-FA	0.652312	5,468.2336	1.45	8382.85

3.4 Study of Cytotoxicity

The results of QDs toxicity were observed for Ca SKi cervical cancer cells as shown in Figure 3.13. Apparently, the cells cannot survive in the presence of QDs-MPA at a concentration as high as 100 nM indicating its extremely high toxicity. On the other hand, at the same concentration, cell viability remained relatively high at almost 80% in the case of QDs-PMAMPC-FA and 60% for QDs-PMAMPC. These results strongly suggested that coating the QDs with PMAMPC and PMAMPC-FA can protect and reduce QDs toxicity.

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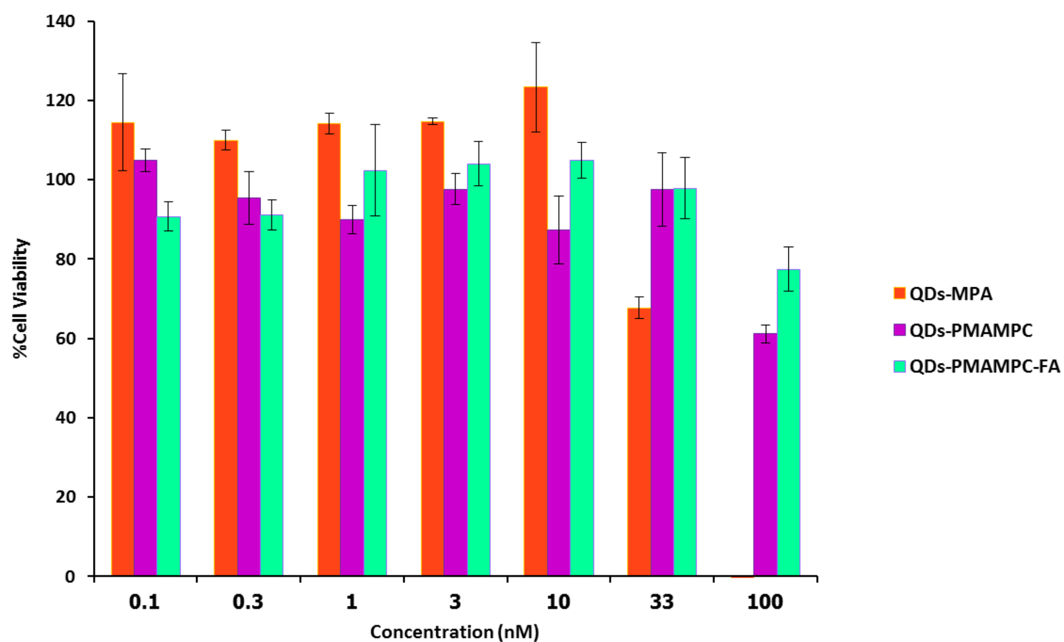

Figure 3.13 Viability with Ca SKi cervical cancer cells in the presence of QDs-MPA, QDs-PMAMPC, and QDs-PMAMPC-FA.

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CHAPTER IV

CONCLUSION

QDs stabilized by FA-modified PMAMPC (QDs-PMAMPC-FA) were prepared to obtain biocompatible and water-soluble QDs having specific affinity to the targeted cancer cell. FA was first attached to PMAMPC-SH, polymerized via RAFT polymerization, via EDC/NHS coupling chemistry. Then, thiol groups at chain end of PMAMPC-FA were grafted onto QDs' surface through ligand exchange process by using ethanolamine as a novel phase transfer agent. The immobilizing of FA on PMAMPC and grafting of PMAMPC-FA on QDs were confirmed by FT-IR analysis. As revealed by fluorescence spectroscopy, the fluorescence emission spectrum of QDs-PMAMPC-FA had two emissions at 452 and 606 nm. The 452 nm emission band could be assigned to folic acid also confirming the success of FA attachment whereas that at the 606 nm could be assigned to the signal of QDs which was slightly red shift with as-synthesized QDs (597 nm) indicating the high stability of QDs after surface modification. In addition, as analyzed by TEM, the stability of particles was in good agreement with fluorescence spectroscopy. The QDs-PMAMPC-FA were spherical in shape and had a monodispersed size of 5.2±0.8 nm, slightly larger than the as-synthesized QDs (3.8±0.6 nm) and QDs-PMAMPC (3.93±0.65 nm). From AFM analysis, the core-shell structure of QDs-PMAMPC-FA was observed. Quantum yield of QDs stabilized by PMAMPC and PMAMPC-FA was studied and found that these polymer-modified QDs provided higher quantum yield than QDs stabilized by mercaptopropanoic acid (QDs-MPA), as water-soluble model QDs, demonstrating the improvement of photoluminescence of QDs by polymer modification. It is anticipated that the synthesized QDs-PMAMPC immobilized with FA can be used for specific tracking with epidermoid cervical carcinoma (CaSki) cell having overexpressed folate receptor.

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Part II

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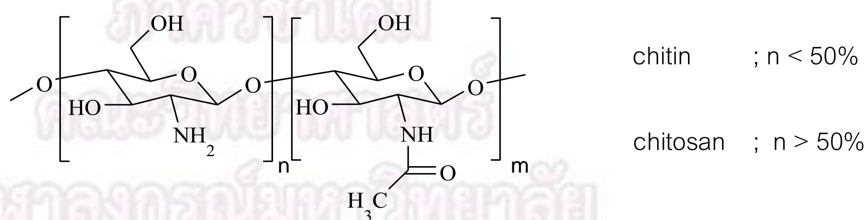
CHAPTER V

INTRODUCTION

Many biopolymers have been used to prepare nanoparticle for various applications as drug delivery carriers or bioactive compounds. Due to its biocompatible and non-toxicity, chitosan was chosen for development of drug carriers extensively. To follow the mechanism of chitosan nanoparticles uptake by cells, chitosan nanoparticles were labelled with fluorescent dyes which allowed them to be detected by fluorescence microscopy. According to Taboonpong's thesis [1], blackberry-like chitosan nanoparticles could be prepared from self-assembly of amphiphilic chitosan with *N*-[(2-hydroxyl-3-trimethyl ammonium)]propyl (HTAP) groups as hydrophilic part and pyrene (pyr), which is a hydrophobic fluorescent dye, as hydrophobic part. Aforementioned nanoparticle was found to be stable and could be detected by fluorescence microscope easily. For the second research, we are interested in studying the application of the synthesized particles for drugs or bioactive compounds delivery to cells, which would be detected by fluorescence microscopy.

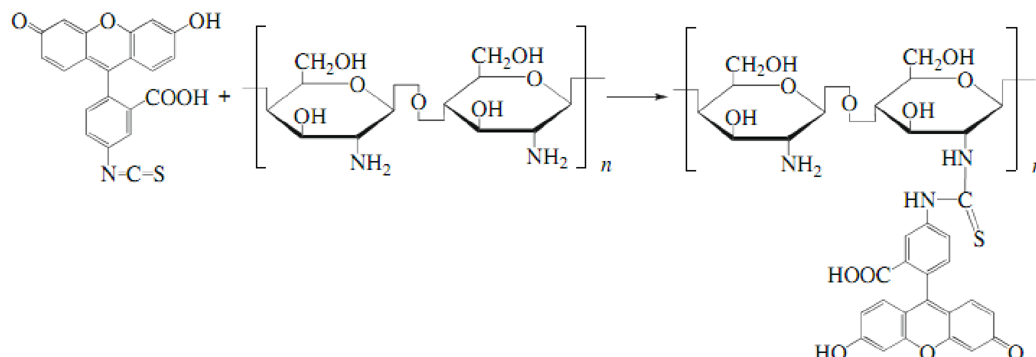
Chitosan is a partially deacetylated form of chitin. A common method for the synthesis of chitosan is the deacetylation of chitin using concentrated sodium hydroxide in excess as a reagent and water as a solvent. The degree of deacetylation (%DD) of chitosan is higher than 50% while that of chitin is lower than 50%.



Scheme 5.1 Chemical structure of chitin and chitosan [2]

In 2009, Jia *et al.*[3] synthesized chitosan labeled with fluorescein isothiocyanate (f-CS), which was used to prepare fluorescein isothiocyanate-labeled chitosan nanoparticle (f-CNP) via ionic cross-linking with tripolyphosphate sodium (TPP). The f-CNP exhibited an average size of 58.04 nm and ζ-potential of +41.63 mV. Fluorescence micrograph revealed

that the f-CNP could be transported into Caco-2 cells across the cell membrane. The intercellular fluorescence signal depended on concentration of the particles.



Scheme 5.2 Synthesis of chitosan labeled with fluorescein isothiocyanate (f-CS). [3]

In 2011, Cui *et al.* [4] prepared fluorescent nanoparticles for drug delivery system. The fluorescent nanoparticles (FNPs) were prepared via ionic self-assembly of anthracene derivative and chitosan for applications as drug delivery carriers with real-time monitoring of drug release. While FNP was loaded with drug, the strong blue fluorescence of FNPs was quenched due to electron transfer and fluorescence resonance energy transfer (FRET). With release of drug *in vitro*, the fluorescence was recovered again. Thus, drug release of FNPs could be followed by real-time monitoring via signal change.

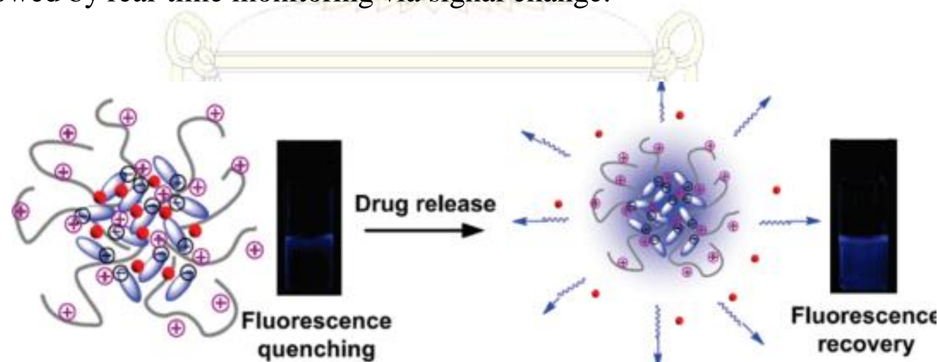
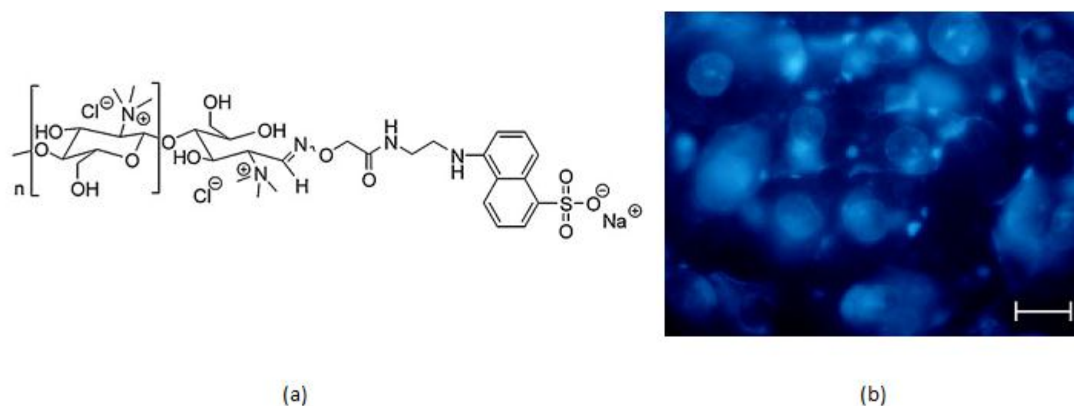


Figure 5.1 Signal change of loading drug and releasing drug. [4]

In 2012, Benediktsdóttir *et al.* [5] prepared derivative of chitosan that is *N,N,N*-trimethylated chitosan (TMC) labeled with 5-(2-((aminooxyacetyl)amino)ethylamino)naphthalene-1-sulfonic acid (EDANS-O-NH_2) via oxime formation. Average molecular weight of the TMC-oxime-EDANS was ~7.7 kDa as characterized by ^{1}H NMR and fluorescence spectroscopy. Then, the TMC-oxime-EDANS

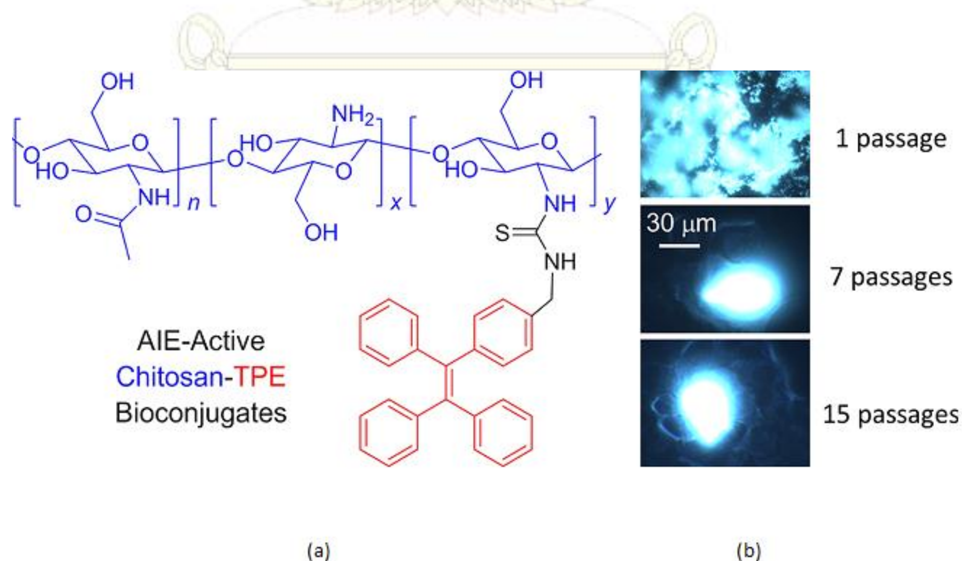
was used to detect bronchial epithelial cells via fluorescence microscope. It was found that the TMC-oxime-EDANS could be attached to cell membrane and absorbed into the epithelial cell.



(a) (b)

Figure 5.2 (a) Structure of f-TMC and (b) Result from fluorescent microscope in bronchial epithelial labeling f-TMC. [5]

In 2013, Wang *et al.*[6] synthesized chitosan labeled with tetraphenylethene (TPE-CS) via thiourea formation. The synthesized chitosan showed fluorescent signal when TPE-CS was absorbed into HeLa cell and aggregated because pH of the HeLa cells was 7.2-7.4, in which TPE-CS could not dissolve. Fluorescence micrograph revealed that fluorescence of TPE-CS would remain noticeable in HeLa cells for up to 15 passaging of cell culture.



(a) (b)

Figure 5.3 (a) Structure of TPE-CS and (b) Result of fluorescent microscope of HeLa cell with TPE-CS. [6]

From previously literature review, there have not been researches preparing fluorescent-labeled chitosan particles via self-assembly of amphiphilic chitosan having fluorescent dye, pyrene as hydrophobic part. This research aims to investigate the application of the self-assembled particles as carriers for bioactive compound. In this case, curcumin was used as a model bioactive compound.

Objectives

1. To prepare and characterize fluorescent-labeled chitosan particles.
2. To encapsulate curcumin into the fluorescent-labeled chitosan particles.

CHAPTER VI

EXPERIMANTAL SECTION

6.1 Materials

Chitosan flakes (DD 95%, Mw 100 kDa) was purchased from Seafresh Chitosan (Lab) Co., Ltd. (Thailand). 1-Ethynylpyrene was purchased from Alfa Aesar (USA). *N*-bromosuccinimide (NBS), copper(I)acetate, glycidyltrimethylammonium chloride (GTMAC) and succinic anhydride were purchased from Aldrich (USA). *N,N*-diisopropylethylamine (DIEA), hydrazine solution ($H_2N\text{-}NH_2.H_2O$), phthalic anhydride (PhA), sodium azide (NaN_3) and triphenylphosphine (TPP) were obtained from Sigma-Aldrich. 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), poly(ethylene glycol) methyl ether (mPEG; MW = 5000) and Pyridine were purchased from Fluka (Switzerland). Chloroform ($CHCl_3$) and N,N-dimethylformamide (DMF) was obtained from RCI Labscan (Poland). Ethanol (EtOH) and diethyl ether were purchased from Merck (Germany).

6.2 Equipments

6.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H NMR spectra were recorded in solution of CF_3COOH/D_2O or D_2O and DMSO-d_6 using a Varian, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz. Chemical shifts were reported in part per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference.

6.2.2 Fourier Transform -Infrared Spectroscopy (FT-IR)

IR spectra were collected using a Nicolet 6700 FT-IR spectrometer with 128 scans at resolution 4 cm^{-1}. A frequency of 400-4000 cm^{-1} was collected by using TGS detector. All samples were prepared as KBr pellets.

6.2.3 Scanning Electron Microscopy (SEM)

The size and the morphology of particles were determined by scanning electron microscope (SEM, Model JEOL JSM-6480LV). The average diameter of particles was calculated by measurement of 100 random particles using Semafore software.

6.2.4 Transmission Electron Microscopy (TEM)

The size and the morphology of particles were examined by transmission electron microscope (TEM, Model JEM-2100, Japan).

6.2.5 Confocal Laser Scanning Microscopy (CLSM)

Encapsulated particles were examined by CLSM system of Nikon Digital Eclipse C1-Si (Tokyo, Japan) equipped with Plan Apochromat VC 100×, BDLaser (405 nm, Melles Griot, Carlsbad, CA, USA), a Nikon TE2000-U microscope, a 32-channel-PMT-spectral-detector and Nikon-EZ-C1 Gold Version 3.80 software was used to capture the fluorescence signals of the samples.

6.3 Methods

6.3.1 Synthesis of Phthaloylchitosan (PhCS)

Chitosan (3.0 g, 17.4 mmol of $–NH_2$ group) was reacted with PhA (5 mole equivalent to amino groups of chitosan) in 20 mL DMF at 110°C for 6 h under N_2 atmosphere. Then, the temperature was reduced to 80°C and left overnight under N_2 atmosphere. The product was purified by dialysis against DMF for 3 days and deionized water for 3 days. The light yellow powder was obtained after lyophilization.

6.3.2 Synthesis of Poly(ethylene glycol)methyl ether Terminated with Carboxyl Groups (mPEG-COOH)

mPEG (3.0 g, 0.6 mmol) was reacted with SA (1 mole equivalent to mPEG) in 10 mL DMF using pyridine as catalyst at 60°C overnight. White powder was obtained as a product after precipitation in diethyl ether and drying *in vacuo*.

6.3.3 Preparation of Amphiphilic Chitosan Particles

6.3.3.1 Particles Having Pyrene as Hydrophobic Entity and Amino Group as Hydrophilic Entity. (Pyr-CS-NH_2)

NBS (10 mole equivalent to amino groups of chitosan) and TPP (10 mole equivalent to amino groups of chitosan) were added to a solution of PhCS (0.5 g, 2.9 mmol of $–NH_2$ group) in 50 mL DMF. The mixture was stirred at 80°C for 2 h under N_2 atmosphere. After precipitated in ethanol and dried *in vacuo*, the product appeared as dark brown powder. Then, 0.4 g of product was reacted with NaN_3 (1.51 g, 10 mole equivalent to amino groups

of chitosan) in 40 mL DMF at 80°C for 4 h under N_2 atmosphere. After precipitated in ethanol and dried *in vacuo*, the C-*6*-azido-*N*-phthaloylchitosan (N_3-PhCS) appeared as brown powder. Click reaction between the N_3-PhCS (0.1 g) and 1-ethynylpyrene (39.3 mg, 0.25 mole equivalent to amino groups of chitosan) was reacted by using Cu(I) (3.19 mg, 0.15 mole equivalent to 1-ethynylpyrene) as catalyst and DIEA (0.5 mole equivalent to amino groups of chitosan) as base for 24 h under N_2 atmosphere. Then, the product was dialyzed against deionized water and lyophilization to yield pyrene functionalized PhCS (Pyr-PhCS) as yellow powder. Subtraction of phthaloyl groups by hydrolysis using hydrazine solution (64-65% H_2N-NH_2.H_2O) was performed by stirring 1 g of Pyr-PhCS in 100 mL of hydrazine solution at 80°C for 4 h under N_2 atmosphere before being dialyzed against deionized water for 4 days and lyophilization. The yellow powder product was obtained.

6.3.3.2 Particles Having Pyrene as Hydrophobic Entity and HTAP Group as Hydrophilic Entity. (Pyr-CS-HTAP)

Chitosan having pyrene, hydrophobic entity, obtained from section 2.3.3.1 (Pyr-CS-NH_2) 15.0 mg was reacted with GTMAC (23.5 µL, 2 mole equivalent to amino groups of chitosan) at 70°C for 24 h. The particles were formed after dialysis the solution against deionized water for 4 days and lyophilization. The amphiphilic chitosan particles having GTMAC as the hydrophilic entity and pyrene as the hydrophobic entity are defined as Pyr-CS-HTAP.

6.3.3.3 Particles Having Pyrene as Hydrophobic Entity and mPEG-COOH as Hydrophilic Entity. (Pyr-CS-mPEG)

Chitosan having pyrene as hydrophobic entity obtained from section 2.3.3.1 (Pyr-CS-NH_2) 15 mg was reacted with mPEG-COOH from section 2.3.2 (3 mole equivalent to amino groups on chitosan) in 10 mL of DMF. NHS (1 mole equivalent to amino groups of chitosan) was added and stirred until the solution became clear. Then EDC (1 mole equivalent to amino groups of chitosan) was added and the solution was stirred for 24 h. Finally, the particles which are designated Pyr-CS-mPEG were obtained after dialysis against deionized water for 4 days and lyophilization.

6.3.4 Encapsulation of Curcumin by Amphiphilic Chitosan Particles

The Pyr-CS-HTAP particles (2 mg, 0.01 mmol) was dissolved in 1 mL DMF and stirred for 10 minutes. Then, curcumin (2 mole equivalents to pyrene groups of chitosan) was dissolved in 1 mL DMF and added dropwise to the dispersed particles. After stirred for 3 h, the solution/dispersion was dialyzed against deionized water until the yellow solution turned to colorless to eliminate the non-encapsulated curcumin adsorbed on the particle surface followed by lyophilization.

To determine encapsulation efficiency of particles, combined solution that obtained after lyophilization were analyzed by Confocal Laser Scanning Microscopy. The excitation and emission wavelength of pyrene were 330 and 393 nm, respectively, and the excitation and emission wavelength of curcumin were 420 and 470 nm, respectively.

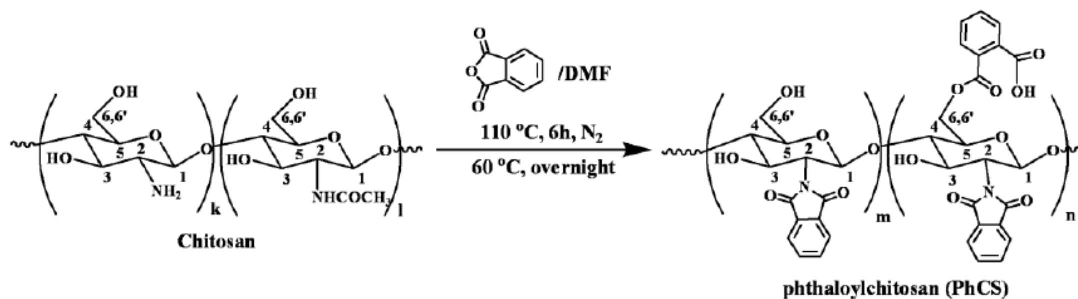
CHAPTER VII

RESULTS AND DISCUSSION

This chapter is divided into 2 parts. The first part (section 7.1-7.3) concentrates on the synthesis, preparation and characterization of amphiphilic chitosan particles. The second part (section 7.4) reveals the encapsulation of curcumin.

7.1 Synthesis of Phthaloylchitosan (PhCS)

To protect amino group of chitosan, a reaction between chitosan and phthalic anhydride (PhA) was employed. Because the electronegativity of nitrogen is less than oxygen, nucleophilicity of amino group is higher than that of hydroxyl groups. Phthaloyl groups were attached to chitosan backbone through ring opening of PhA by amino groups and hydroxyl groups of chitosan (Scheme 7.1). When amino groups react with PhA as nucleophiles, the product is a phthalimide or *N*-phthaloylchitosan (Scheme 7.2). Hydroxyl groups can also act as nucleophiles, in which the reaction results in phthalic acid ester or *O*-phthaloylchitosan (Scheme 7.3).



Scheme 7.1 Synthesis of PhCS [1]

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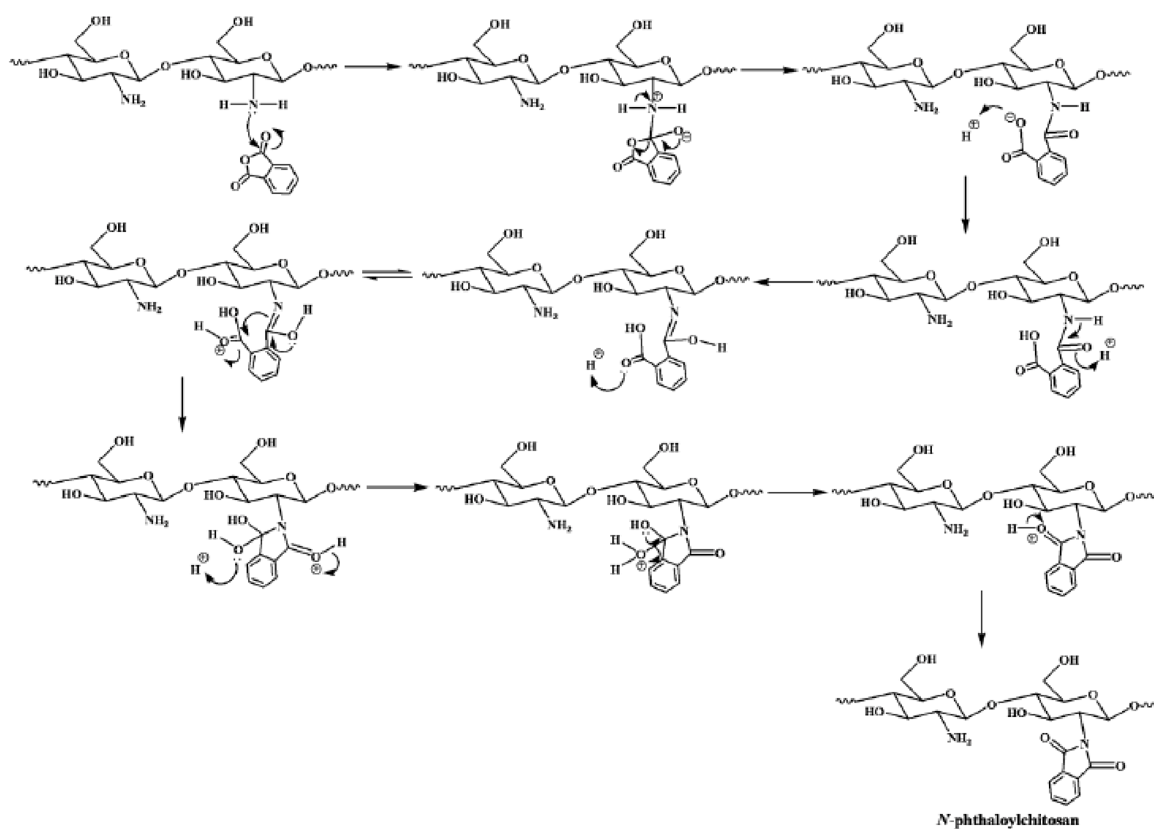
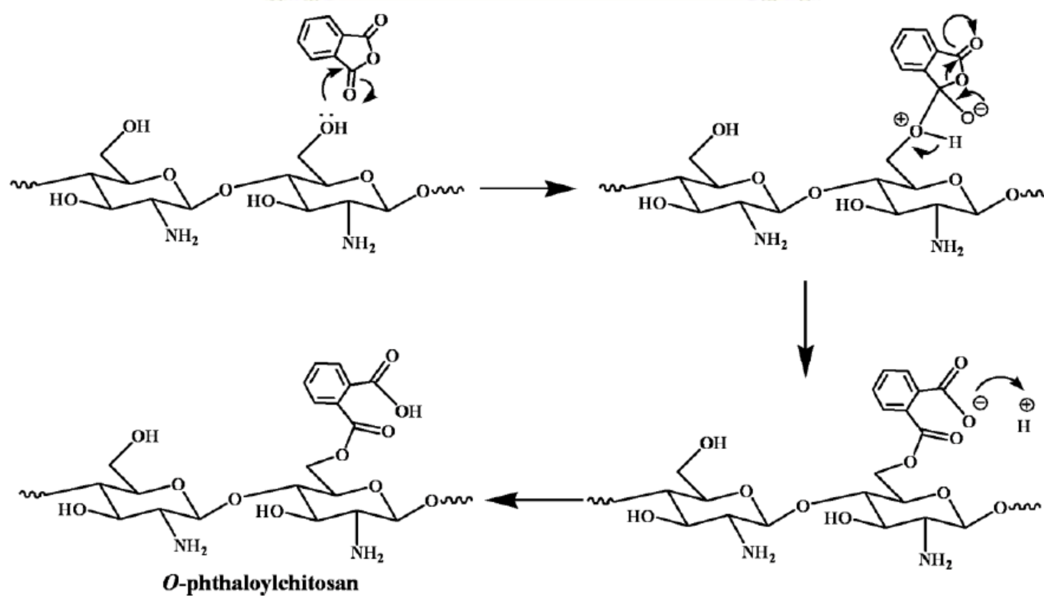


Scheme 7.2 Mechanism of opening PhA ring by amino groups of chitosan that yields *N*-phthaloylchitosan. [1]



Scheme 7.3 Mechanism of opening PhA ring by hydroxyl groups of chitosan that yields *O*-phthaloylchitosan.[1]

^{1}H NMR spectra of chitosan and PhCS are shown in Figure 7.1. The peak at 2.9 ppm was assigned to proton at C-2 on chitosan backbone. Figure 7.1b (PhCS) showed the peak at 7.2 – 8.5 cm^{-1}, that can be assigned to protons of phthaloyl groups, suggesting that the PhCS was successfully synthesized. Degree of phthaloyl group substitution (%DD_{Ph}) was calculated from integration of ^{1}H NMR spectra using the relative ratio between integration of the peak at 2.9 ppm, assigned to proton at C-2 on chitosan backbone, and the peak at 7.2 – 8.5 cm^{-1}, assigned to protons of phthaloyl groups using equation 7.1). The calculated %DD_{Ph} was 249 ± 5.6%, which was higher than 100%. The result implied that the phthaloyl groups were attached to both amino groups and hydroxyl groups.

$$\%DS_{Ph} = \left\{ \frac{\text{integral of } C_6H_4 /4}{\text{integral of H-2}/1} \right\} \times 100 \tag{7.1}$$

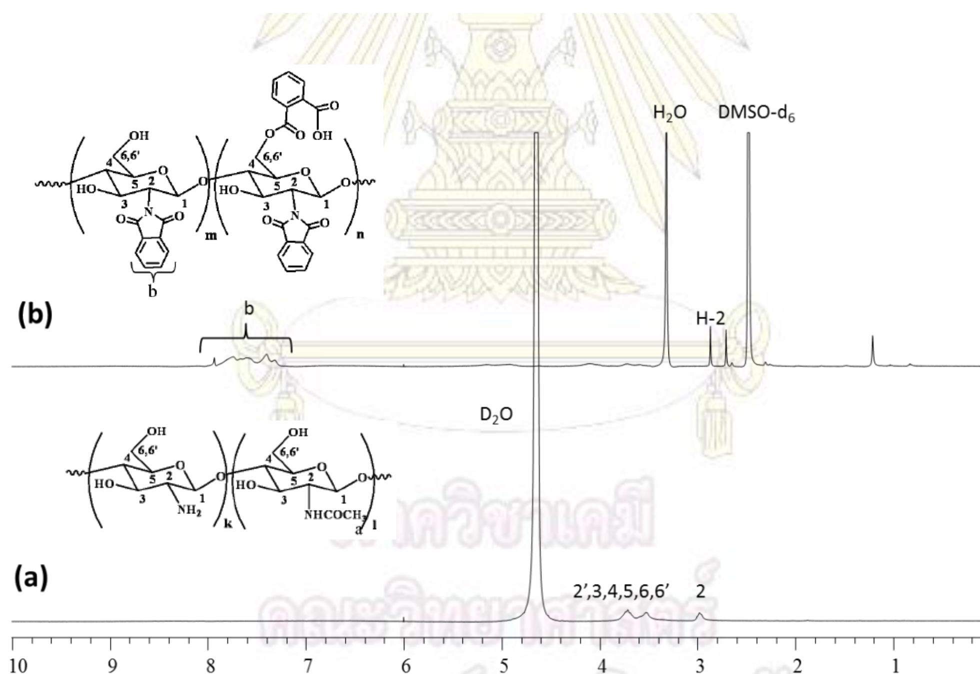


Figure 7.1 ^{1}H NMR spectra of (a) chitosan and (b) PhCS

FT-IR spectra of chitosan and PhCS are shown in Figure 7.2. Figure 7.2b (PhCS) showed the peak at 720 cm^{-1} which was assigned to C-H deformation of aromatic rings of phthaloyl groups. The peak at 1,710 cm^{-1} corresponded to C=O stretching in cyclic imide and anhydride and the peak at 1,770 cm^{-1} can be assigned to C=O stretching in cyclic imide. The characteristic signals of chitosan, N-H bending at 1600 cm^{-1} significantly decreased as a

result of the amino groups being modified by phthaloyl groups. These results, like ^{1}H NMR spectra, confirmed the success of PhCS synthesis.

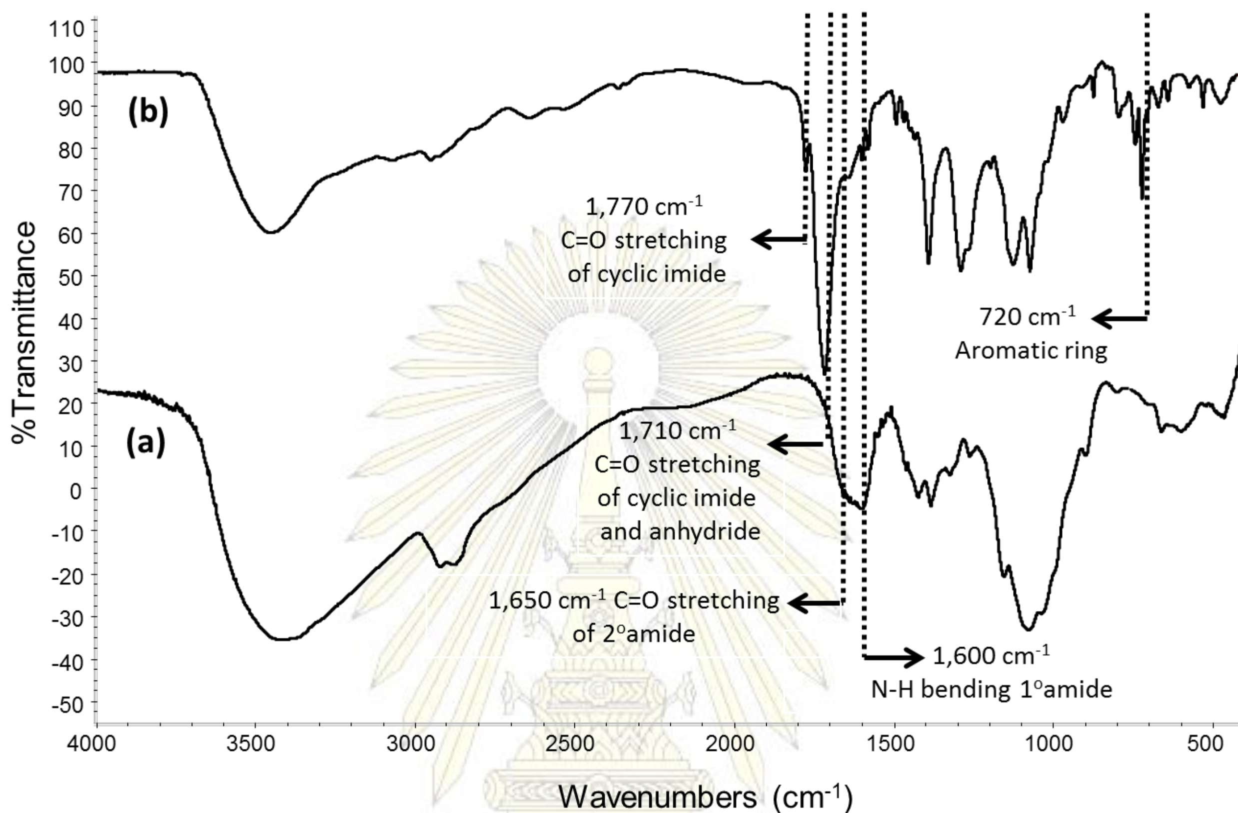
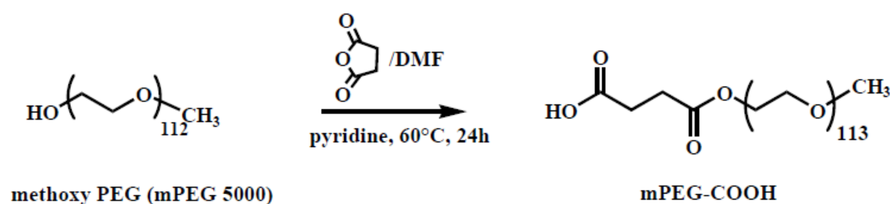


Figure 7.2 FT-IR spectra of (a) chitosan and (b) PhCS

7.2 Synthesis of Poly(ethylene glycol)methyl ether Terminated with Carboxyl Groups (mPEG-COOH)

Carboxyl-terminated poly(ethylene glycol)methyl ether (mPEG-COOH) was synthesized from poly(ethylene glycol)methyl ether (mPEG) by ring opening reaction of succinic anhydride. (Scheme 7.4).



Scheme 7.4 Synthesize of mPEG-COOH

^{1}H NMR spectra of poly(ethylene glycol)methyl ether (mPEG) and carboxyl-terminated poly(ethylene glycol)methyl ether (mPEG-COOH) are shown in Figure 7.3. Figure 7.3b (mPEG-COOH) shows the peak at 2.5 and 3.4 ppm which can be assigned to methylene protons of succinic anhydride. Thus, they could be used to confirm the successful mPEG-COOH synthesis.

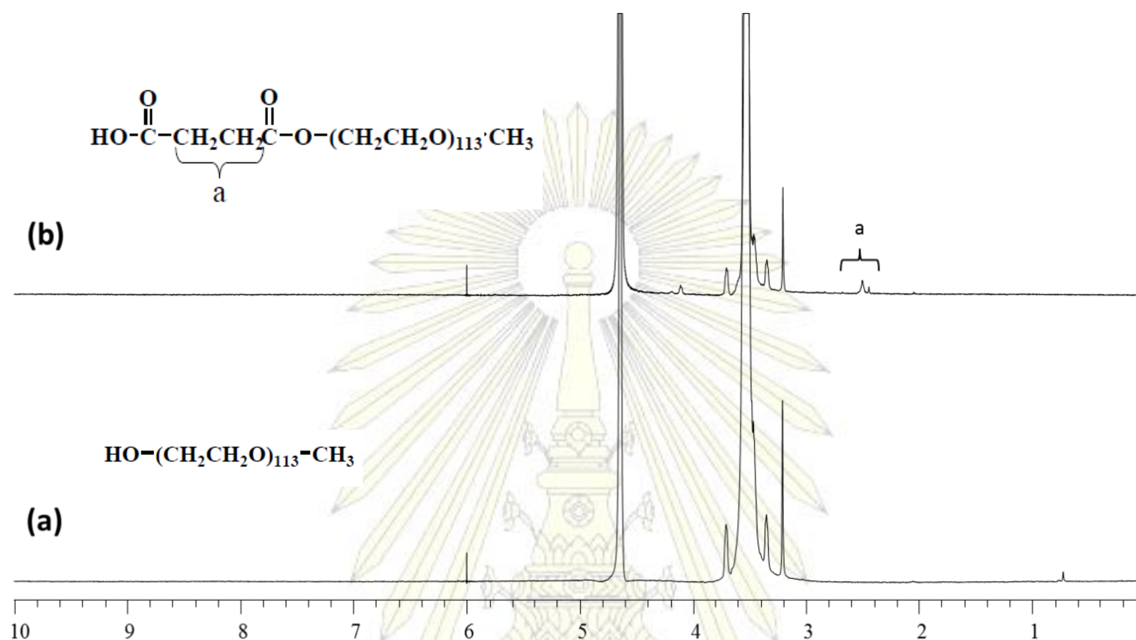
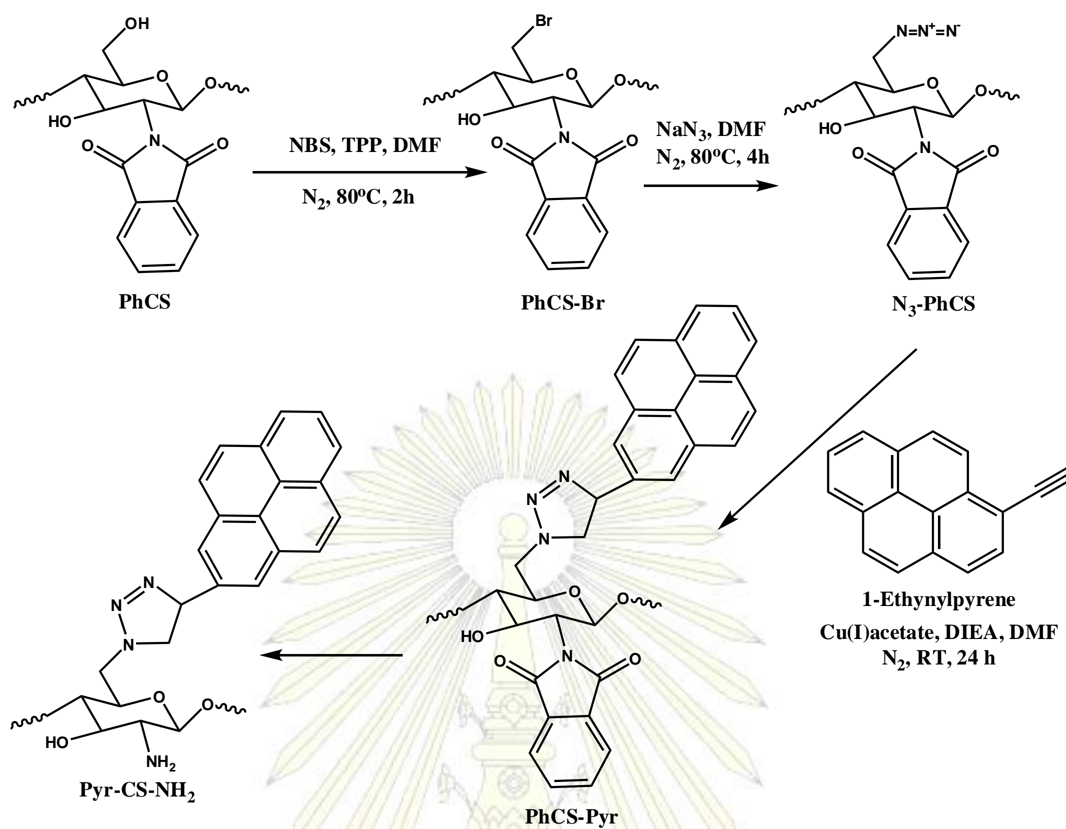


Figure 7.3 ^{1}H NMR spectra of (a) mPEG and (b) mPEG-COOH.

7.3 Preparation of Amphiphilic Chitosan Particles

7.3.1 Particles Having Pyrene as Hydrophobic Entity and Amino Group as Hydrophilic Entity (Pyr-CS-NH_2)

To attach fluorescent pyrene to chitosan backbone, first, hydroxyl groups were replaced with azido group (-N_3) via bromination followed by nucleophilic substitution of azide ion (N_3^-) to give C-*6*-azido-*N*-phthaloylchitosan (N_3-PhCS). Then, N_3-PhCS was reacted with 1-ethynylpyrene via Click reaction to give chitosan having pyrene as hydrophobic entity (Scheme 7.6). After that, the product was hydrolyzed by hydrazine solution to remove phthaloyl group from amino group to give chitosan having pyrene as hydrophobic entity and amino group as hydrophilic entity (Pyr-CS-NH_2) as shown in Scheme 7.5.



Scheme 7.5 Synthesis of Pyr-CS-NH_2.

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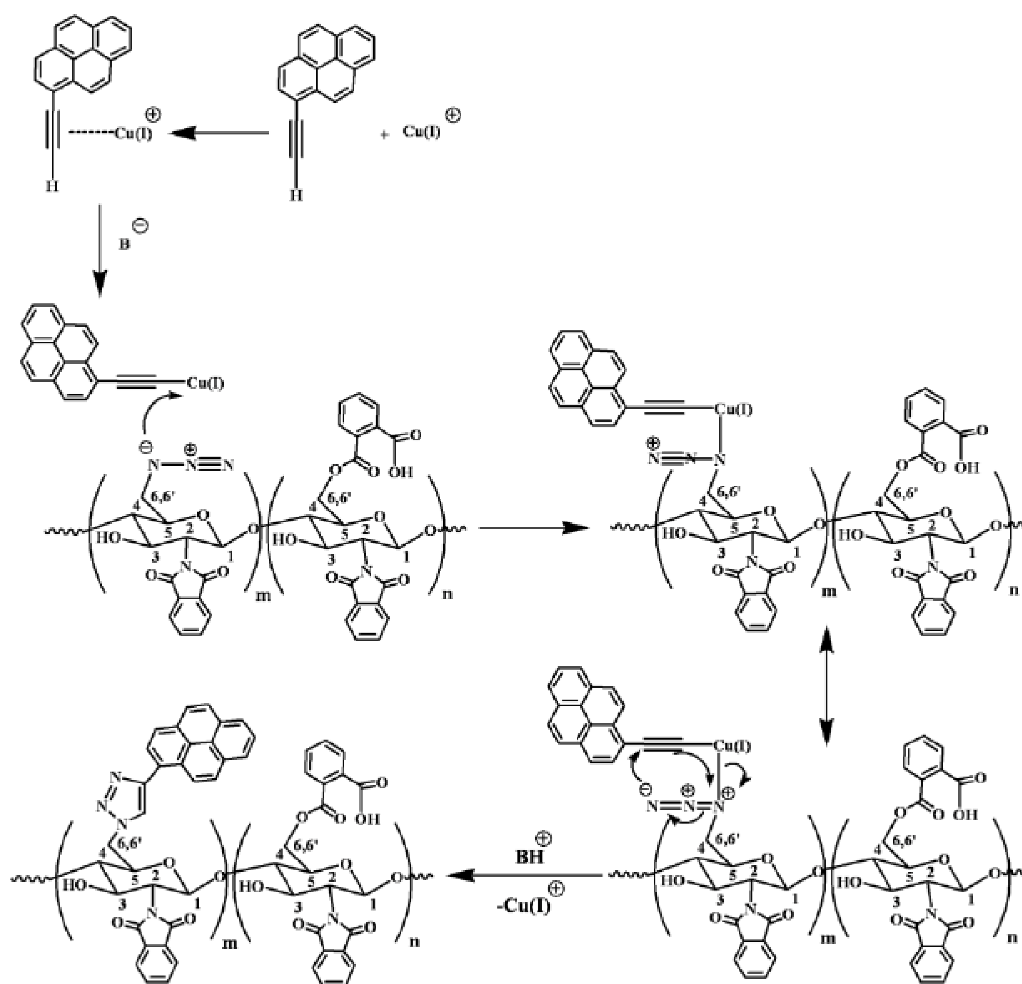

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Scheme 7.6 Mechanism of click reaction between 1-ethynylpyrene and N_3-PhCS. [1]

^{1}H NMR spectra of the product in each steps are shown in Figure 7.4. Prior to reaction with 1-ethynylpyrene (a, b), the peaks between 7.2-8.5 ppm are assigned to aromatic protons in phthaloyl groups. After attachment of pyrene (c), multiple peaks appeared in a range of 8.0 - 8.8 ppm, which could be assigned to aromatic protons of attached pyrene. After hydrolysis (d), disappearance of peaks in a range of 7.2-8.5 ppm which is corresponded to the removal of phthaloyl group was observed.

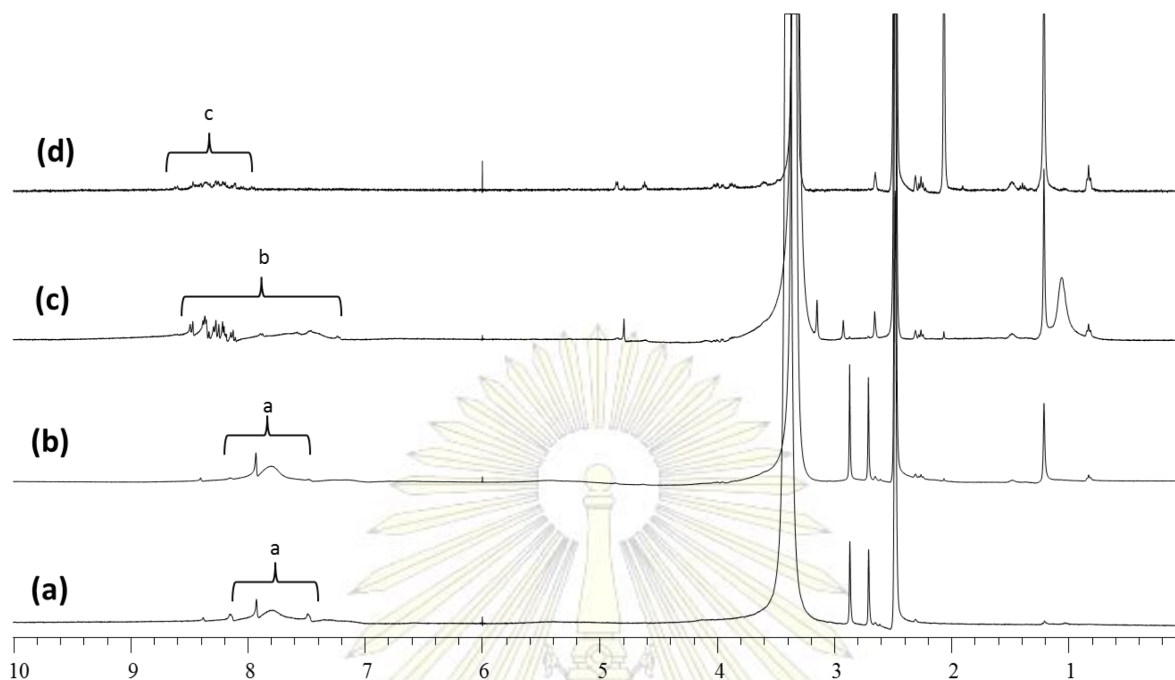

Figure 7.4 ^{1}H NMR spectra of the product after (a) bromination, (b) azidation, (c) click reaction between pyrene and C-*6*-azido-*N*-phthaloylchitosan (N_3-PhCS) and (d) Pyr-CS-NH_2.

Figure 7.5 shows FT-IR analysis results of the products after bromination, azidation, click reaction and hydrolysis. The peak at 2100 cm^{-1} which could be assigned to azide groups on chitosan backbone indicates the success of bromination followed by azidation. Figure 7.5c shows removal of the peak at 2,100 cm^{-1} indicaing that the reaction between alkyne-functionalized pyrene and azide group on chitosan backbone occurred. The appearance of the peaks at 1,710 and 1,770 cm^{-1} implied that the phthaolyl groups were not completely removed by hydrolysis.

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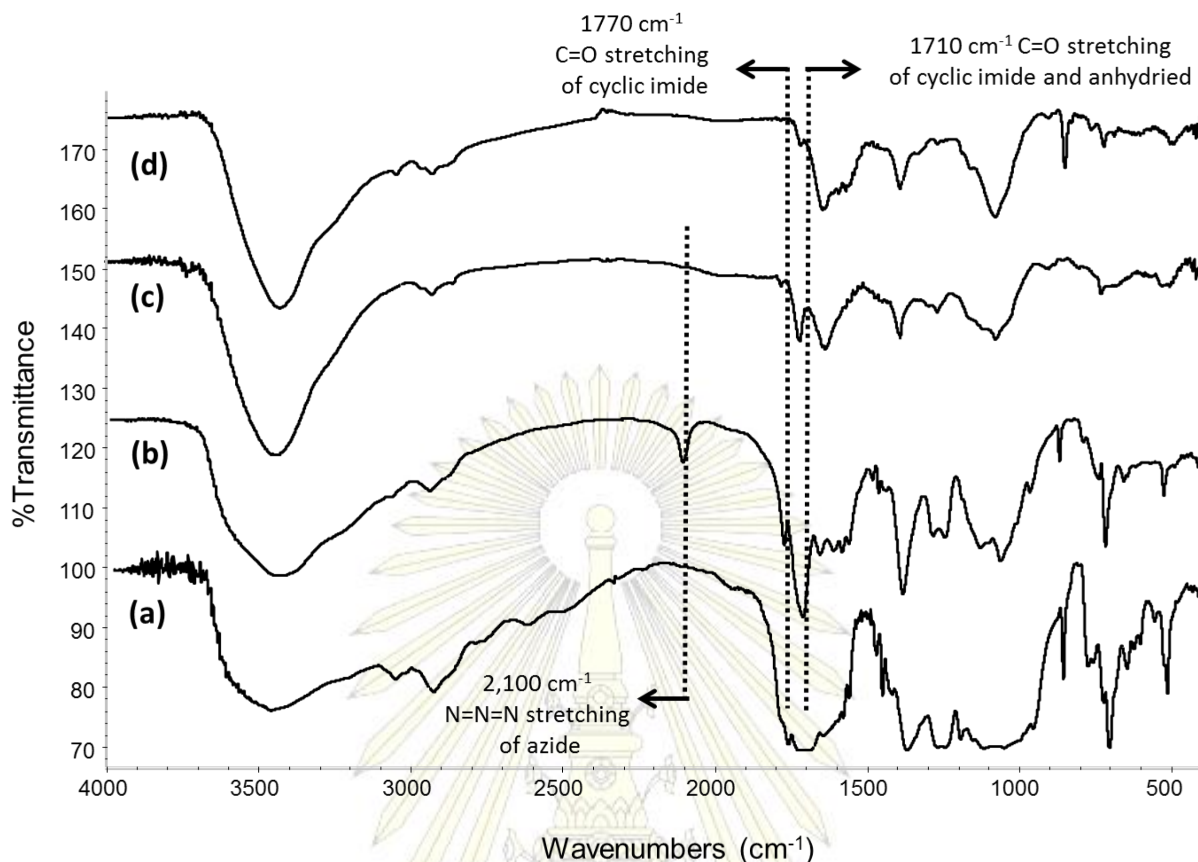
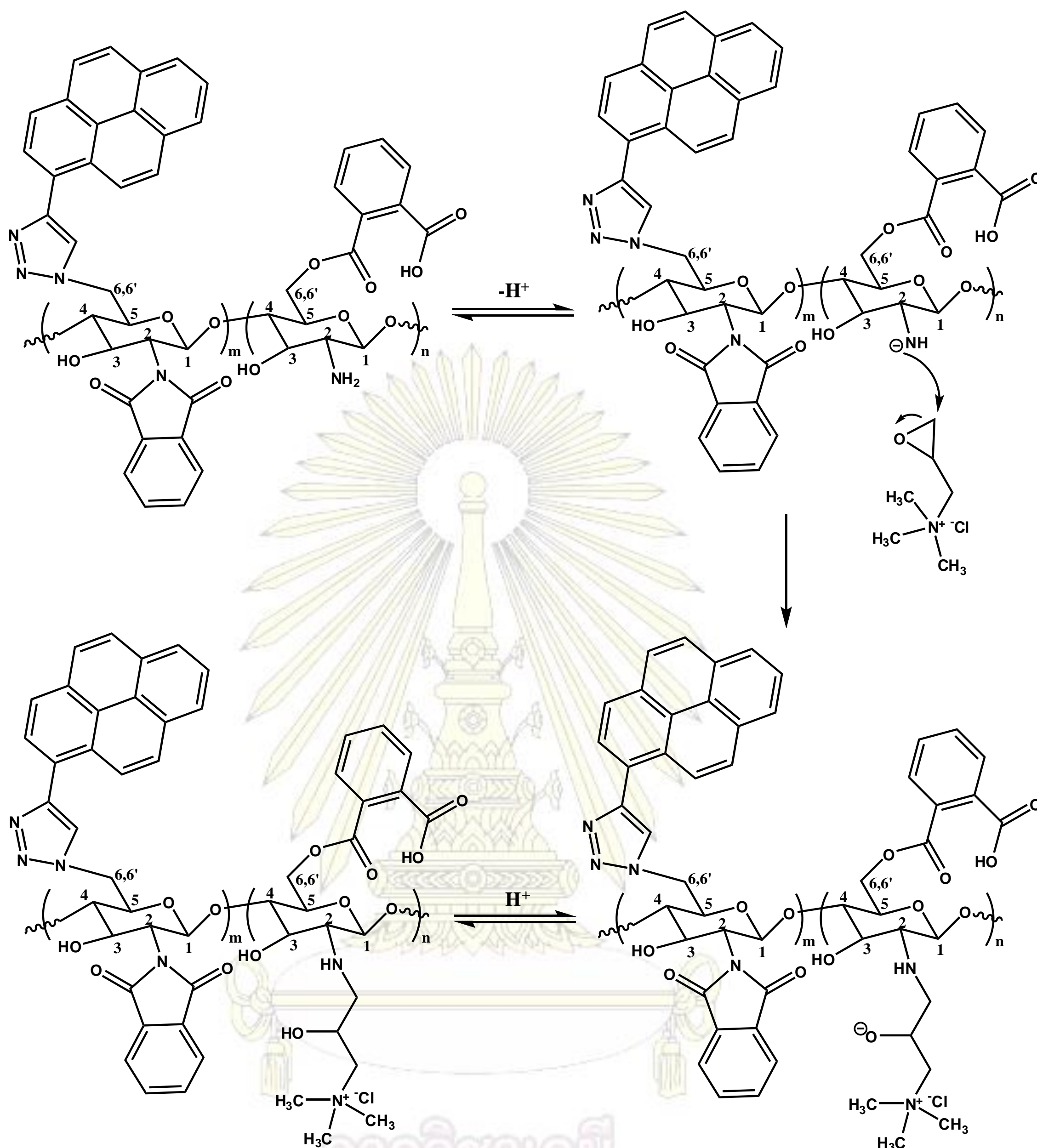


Figure 7.5 FT-IR spectra of the product after (a) bromination, (b) azidation, (c) Click reaction between pyrene and C-*6*-azido-*N*-phthaloylchitosan (N_3-PhCS) and (d) Pyr-CS-NH_2.

7.3.2 Particles Having Pyrene as Hydrophobic Entity and HTAP Group as Hydrophilic Entity (Pyr-CS-HTAP)

The product from 7.3.1 was attached by HTAP group via ring opening of glycidyltrimethylammonium chloride (GTMAC) by amino groups on chitosan backbone (Scheme 7.6). Because GTMAC could react with amino group following mechanism shown in Scheme 7.7.



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Scheme 7.7 Mechanism of GTMAC ring opening by hydroxyl groups of amino group on chitosan.

^{1}H NMR spectra (Figure 7.6) shows comparison between Pyr-CS-NH_2 and Pyr-CS-HTAP. Figure 7.6b showed the peak at 3.1 ppm which can be assigned to methyl protons of quaternary ammonium groups of HTAP attached to chitosan. Degree of pyrene substitution (%DS_{Pyr}) can be calculated from the relative ratio between peak integration of protons of pyrene and that of the proton at C-2 on chitosan backbone using equation 7.2. The %DS_{Pyr}

was found to be 118±1.2%. This over-estimated $\%DS_{Pyr}$ may be explained as a result of incomplete phthaloyl group removal. The phthaloyl groups also gave signals of aromatic protons in the same region as pyrene. Degree of HTAP substitution ($\%DS_{HTAP}$) can be calculated from the relative ratio between peak integration of methyl protons of quaternary ammonium groups of HTAP and that of the proton at C-2 on chitosan backbone using equation 7.3 which was found to be 15±2.3 %.

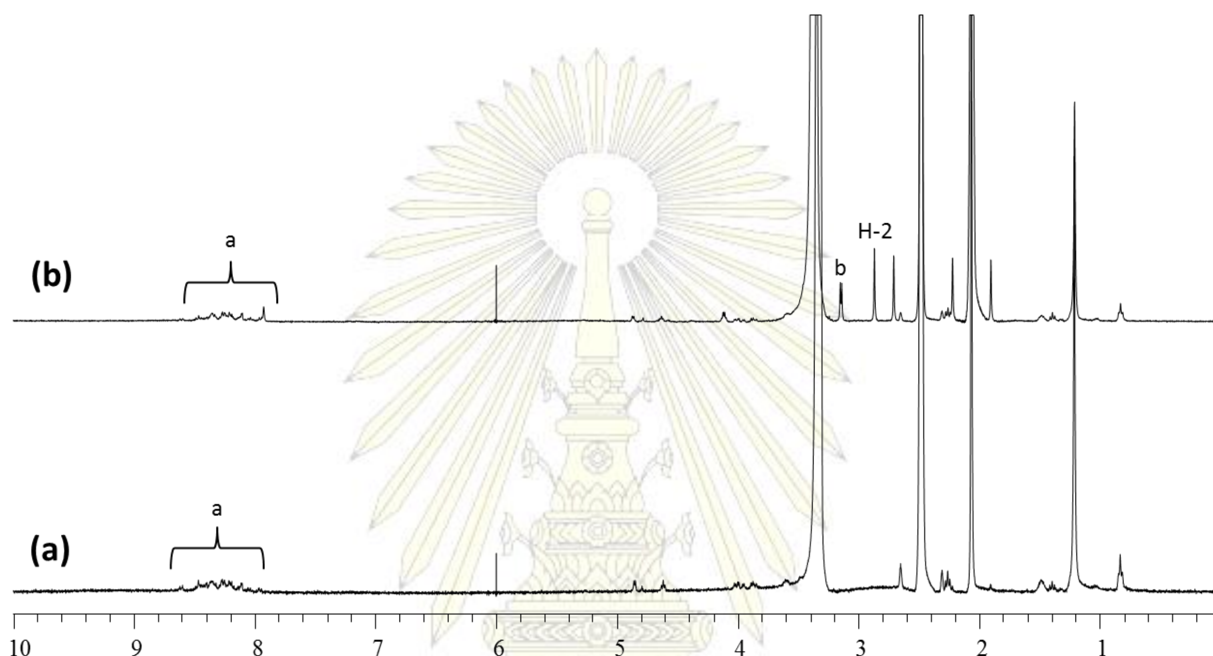


Figure 7.6 ^{1}H NMR spectra of (a) Pyr-CS-NH$_2$ and (b) Pyr-CS-HTAP.

$$\%DS_{Pyr} = \left\{ \frac{\text{integral of } C_{16}H_9 / 9}{\text{integral of H-2} / 1} \right\} \times 100 \qquad (7.2)$$

$$\%DS_{HTAP} = \left\{ \frac{\text{integral of } N^{+}(CH_3)_3 / 9}{\text{integral of H-2} / 1} \right\} \times 100 \qquad (7.3)$$

FT-IR analysis (Figure 7.7) shows comparison between chitosan and Pyr-CS-HTAP. Figure 7.7 shows the peak at 1,475 cm^{-1} which could be assigned to C-H deformation in HTAP groups. From SEM micrograph (Figure 7.8), particles having pyrene as hydrophobic entity and HTAP group as hydrophilic entity (Pyr-CS-HTAP) showed spherical shape. Size of the particles were 0.77± 0.186 μm. Unlike the work reported by Taboonpong [1], the

spherical shape of these particles were not blackberry-like. This may be explained as a result of low %DD_{HTAP} (15±2.3 %) as opposed to that of Taboonpong (67%).

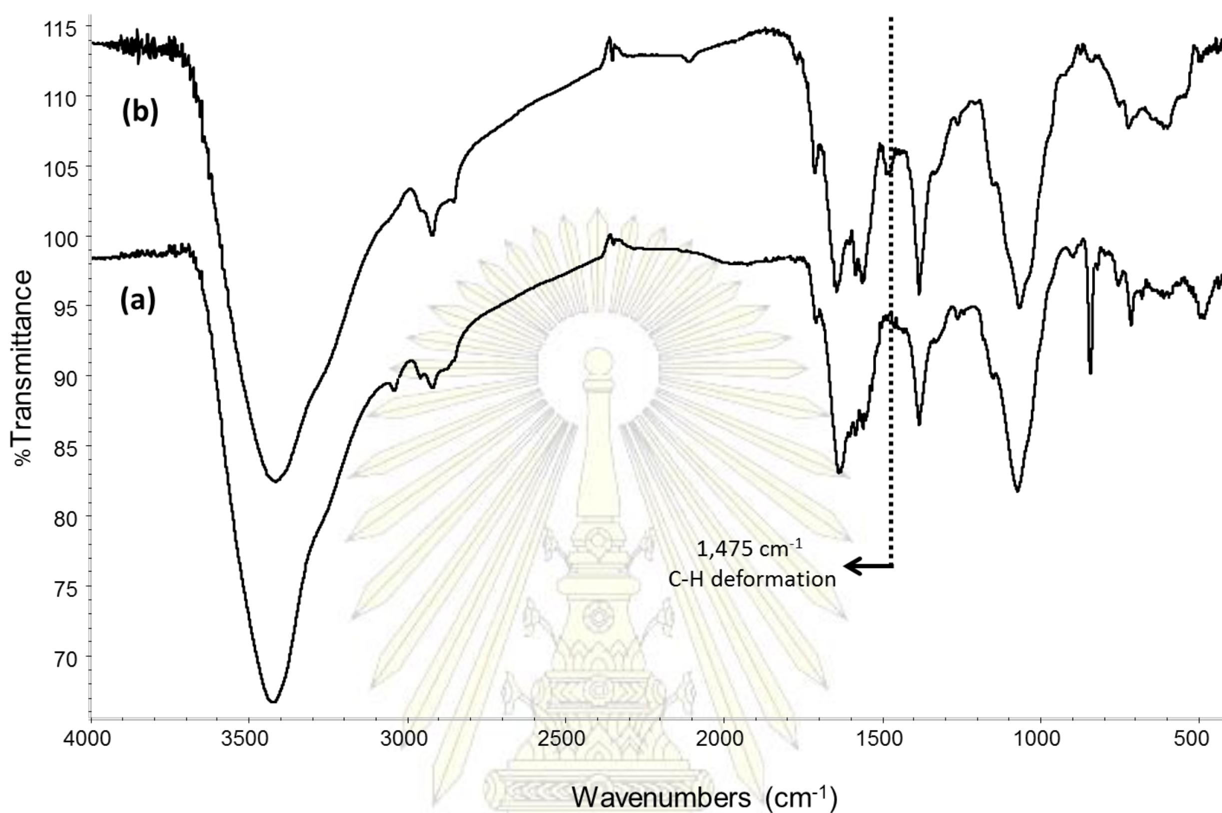


Figure 7.7 FT-IR spectra of (a) Pyr-CS-NH_2 and (b) Pyr-CS-HTAP.

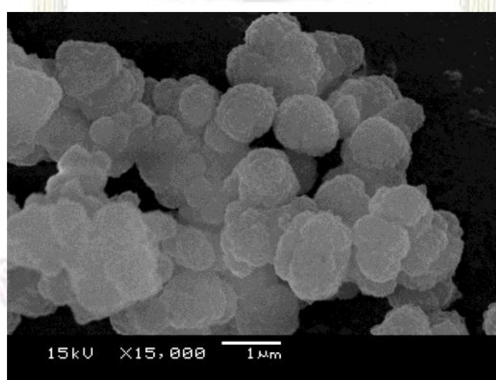
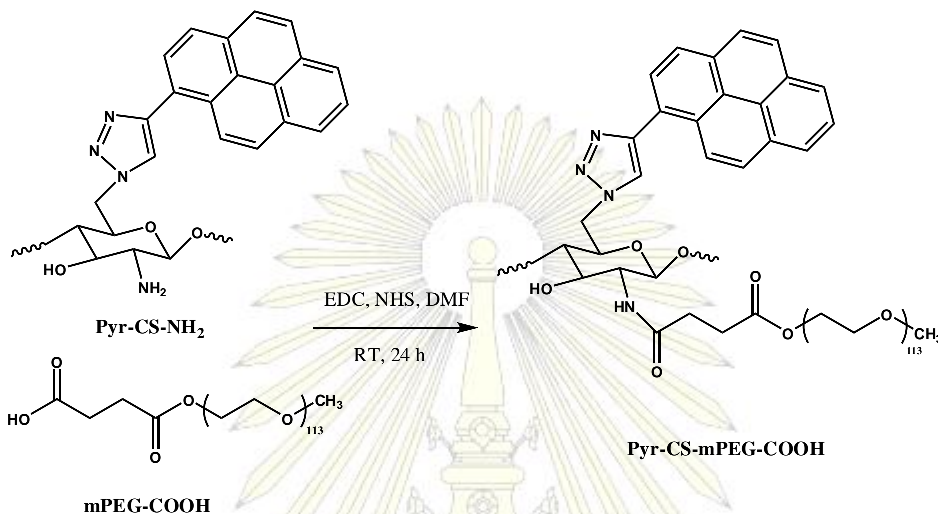


Figure 7.8 SEM micrograph of Pyr-CS-HTAP particles.

7.3.3 Particles Having Pyrene as Hydrophobic Entity and mPEG-COOH as Hydrophilic Entity

The product from section 7.3.1, Pyr-CS-NH_2, was attached by mPEG-COOH via EDC/NHS coupling agent to form amidelinkage at amino group on chitosan backbone (Scheme 7.8)



Scheme 7.8 Synthesis of Pyr-CS-mPEG-COOH.

^{1}H NMR spectra (Figure 7.9) shows comparison of mPEG-COOH, Pyr-CS-NH_2 and Pyr-CS-mPEG. The fact that there was no signals that correspond to characteristic peaks of chitosan appear in Figure 7.9c implying the mPEG-COOH attachment to chitosan was not successful.

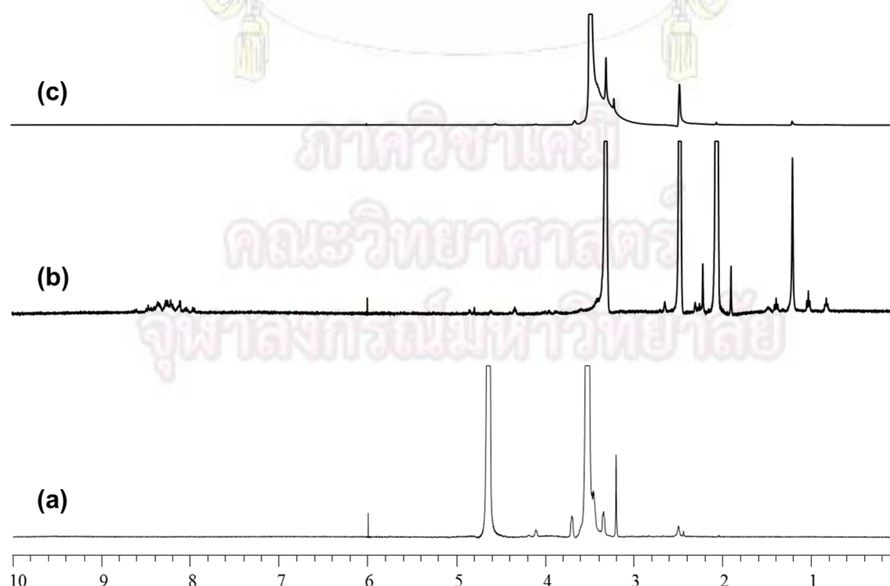


Figure 7.9 ^{1}H NMR spectra of (a) mPEG-COOH, (b) Pyr-CS-NH_2 and (c) Pyr-CS-mPEG.

7.4 Encapsulation of curcumin by amphiphilic chitosan particles (Pyr-CS-HTAP)

Curcumin was encapsulated with Pyr-CS-HTAP particles via hydrophobic/hydrophilic interaction and π-π interaction between the aromatic moieties of curcumin and pyrene. To confirm the success of curcumin encapsulation, the encapsulated particles were characterized by confocal laser scanning microscopy (Figure 7.10). Figure 7.10(c) showed 2 fluorescent colors of both Pyr-CS-HTAP particles (green) and curcumin (yellow). These result indicated that curcumin could be encapsulated in Pyr-CS-HTAP particles.

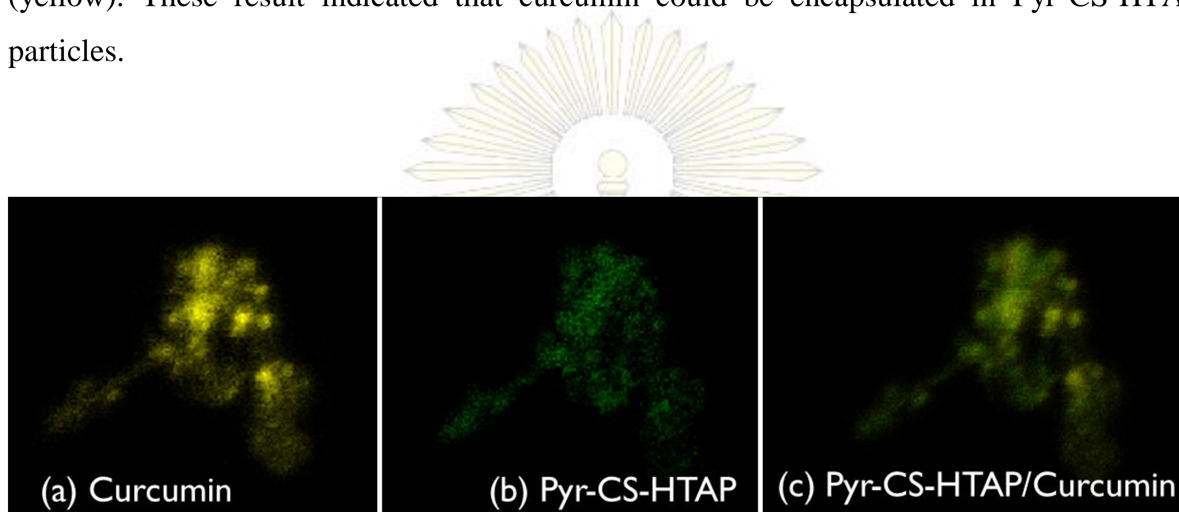


Figure 7.10 CLSM micrographs of (a) แurcumin, (b) Pyr-CS-HTAP and (c) encapsulated curcumin of Pyr-CS-HTAP particles.

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CHAPTER VIII

Conclusion

Two types of amphiphilic chitosan, Pyr-CS-HTAP and Pyr-CS-MPEG, were successfully prepared. Amino groups of chitosan were first protected by phthaloyl groups via reaction between chitosan and phthalic anhydride. Hydroxyl groups on chitosan backbone were replaced with azido groups by bromination followed by azidation before an attachment of alkyne-functionalized pyrene through click reaction. The phthaloyl groups were then removed by hydrolysis to recover back amino groups. Epoxide ring opening of GTMAC by the recovered amino groups yielded Pyr-CS-HTAP. Pyr-CS-HTAP particles having pyrene as hydrophobic entity and HTAP group as hydrophilic entity were formed upon dialysis. Amidation between the recovered amino groups with mPEG-COOH, on the other hand, gavePyr-CS-mPEG. Pyr-CS-mPEG particles having pyrene as hydrophobic entity and mPEG-COOH group as hydrophilic entity were formed upon dialysis. All derivatives were characterized by ^{1}H NMR and FT-IR. The results from SEM indicated that the Pyr-CS-HTAP particles having diameter of 0.77± 0.19 μm were spherical in shape. The encapsulation of curcumin by Pyr-CS-HTAP took place via hydrophobic interaction and π-π interaction between pyrene and aromatic groups in curcumin. The encapsulated product was characterized by CLSM, which confirmed the encapsulation of curcumin.

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