ตัวนำส่งยาชนิดใหม่ฐานอีดีทีเอ

นายณัฐพงศ์ ปางคำ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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NEW EDTA-BASED DRUG DELIVERY CARRIER

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
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Thesis Title	NEW EDTA-BASED DRUG DELIVERY CARRIER
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ณัฐพงศ์ ปางคำ : ตัวนำส่งยาชนิดใหม่ฐานอีดีทีเอ (NEW EDTA-BASED DRUG DELIVERY CARRIER) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. นงนุช เหมืองสิน, 52 หน้า.

ยาต้านมะเร็งที่รับประทานเข้าไปผ่านระบบทางเดินอาหารส่วนใหญ่มักละลายน้ำได้น้อย ไม่เฉพาะเจาะจงกับอวัยวะเป้าหมาย อีกทั้งยังอาจสูญสลายในสภาวะกรดเมื่อผ่านไปยังกระเพาะอาหาร จึงส่งผลให้ลดประสิทธิภาพของตัวยาลงและเพิ่มผลข้างเคียง ระบบนำส่งยาจึงถูกนำมาใช้เพื่อแก้ปัญหาเหล่านี้ ระบบนำส่งยาส่วนใหญ่จะถูกทำในรูปแบบของระบบพอลิเมอร์ไมเซล แต่ระบบพอลิเมอร์ไมเซลเป็นโมเลกุลขนาดใหญ่และการควบคุมการสังเคราะห์ให้ได้คุณภาพสม่ำเสมอนั้นทำได้ค่อนข้างยาก เนื่องจากผลิตภัณฑ์พอลิเมอร์ที่สังเคราะห์ได้มีปริมาณสัมพัทธ์ของหมู่แทนที่ไม่สม่ำเสมอ จึงอาจส่งผลต่อคุณสมบัติทางเคมีกายภาพ ชีวภาพ และเคมีทางเภสัชวิทยา พอลิเมอร์ไม่สามารถกักเก็บตัวยาได้ดีเท่าที่ควรโดยเฉพาะยาที่ละลายน้ำได้น้อย และอาจเป็นพิษต่อตับได้เนื่องจากพอลิเมอร์ถูกเผาผลาญในร่างกายได้ค่อนข้างช้า ดังนั้นงานวิจัยนี้จึงได้คิดค้นตัวนำส่งยาชนิดใหม่ที่ง่ายต่อการดัดแปลงโครงสร้างโดยสามารถกำหนดปริมาณสัมพัทธ์ของการสังเคราะห์ที่แน่นอนได้ มีความปลอดภัย และไม่เป็นพิษต่อร่างกาย โดยทำการดัดแปลงโครงสร้างของเอทิลลีนไดเอมีนเททระอะซิติดเอซิด (อีดีทีเอ) ให้มีหมู่ที่ไม่ชอบน้ำคือออกตะเดซิลโบรไมด์ (Oc) และหมู่ฟังก์ชันที่มีความเฉพาะเจาะจงกับมะเร็งคือโฟลิคเอซิด (FA) จนได้ Oc-EDTA-FA รูปแบบไมเซลเพื่อกักเก็บตัวยาต้านมะเร็งที่ละลายน้ำได้น้อย โดยการพิสูจน์เอกลักษณ์ การหาความเข้มข้นที่น้อยที่สุดที่สามารถเป็นไมเซล และรูปร่างลักษณะไมเซลของอนุพันธ์ Oc-EDTA-FA ถูกศึกษาโดยเทคนิคทางสเปกโตรสโคปีและสแกนนิงอิเล็กตรอนไมโครสโคปี (SEM) การศึกษาประสิทธิภาพของยาต้านมะเร็งเมื่อใช้ Oc-EDTA-FA เป็นตัวนำส่งยาและการศึกษาความเป็นพิษต่อเซลล์ปกติถูกศึกษาโดยเอ็มทีที (MTT assay) ผลการทดลองพบว่า สามารถสังเคราะห์ Oc-EDTA-FA ได้สำเร็จ Oc-EDTA-FA รูปแบบไมเซลสามารถเตรียมได้ง่ายในน้ำและพบว่ามีตอบสนองต่อ pH โดยไม่ละลายในกรด นอกจากนี้ Oc-EDTA-FA ยังมีความเป็นพิษต่อเซลล์มะเร็งซึ่งมีประสิทธิภาพสูงสุดในเซลล์มะเร็ง SW620 และตัวนำส่งยา Oc-EDTA-FA ที่กักเก็บเคอร์คิวมิน (Oc-EDTA-FA@Cur) ยังมีความเฉพาะเจาะจงกับเซลล์มะเร็งมากกว่าเซลล์ร่างกายอย่างมีนัยสำคัญ ดังนั้น Oc-EDTA-FA รูปแบบไมเซลนี้จึงมีความเหมาสมที่จะนำไปประยุกต์ใช้ในการเป็นตัวนำส่งยา

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560

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KEYWORDS: EDTA / PH-SENSITIVE / MICELLES / SELF-ASSEMBLY MICELLE / DRUG DELIVERY CARRIER

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Most of the anticancer drugs are insoluble in water, unspecified to target site and degradable under acidic conditions in the stomach, resulting in decreasing their activities and increasing the side effects. To overcome these problems, drug delivery carriers were used to overcome these problems. Most of the drug delivery carriers were prepared by polymeric micelle systems. However, that of polymeric micelle systems are difficult to synthesize into the random polymers, where different units are aligned on a polymer chain in a random manner, immature drug-incorporation technology and possible chronic liver toxicity due to slow metabolic process. Therefore, the enhancement of anticancer drugs solubility, the pH-sensitive with safe, inexpensive condition and improvement of the selectivity were studied. Ethylenediaminetetraacetic acid (EDTA) derivative self-assembly micelles have been proposed and investigated for their potential as drug carriers. In this work, EDTA was synthesized to present both hydrophobic moiety and cancer targeting moiety in order to create self-assembly micelles and increase selectivity to the cancer cell. The EDTA was modified with hydrophobic part (octadecyl bromide: Oc) and cancer targeting (folic acid: FA) to Oc-EDTA-FA. The structure, critical micelles concentration and the morphologies of Oc-EDTA-FA were characterized by spectroscopic methods and scanning electron microscopy (SEM). The cytotoxicity of Oc-EDTA-FA self-assembly micelles as cancer drug delivery carriers was investigated by MTT assay. The results showed that Oc-EDTA-FA was successfully synthesized, self-assembled Oc-EDTA-FA micelles could be easily prepared by re-dispersion in distilled water and they were pH-sensitive and acid resistance. In addition, Oc-EDTA-FA carrier exhibited an effectiveness of cytotoxicity against cancer cells which showed the highest efficiency to the SW620 cancer cells over other cancer cells. The Oc-EDTA-FA@Cur carrier gave more specific to cancer cells than normal cells significantly. Therefore, the Oc-EDTA-FA self-assembled micelles were suitable to use in the application of drug delivery carrier.

Department: Chemistry Student's Signature .....

Field of Study: Chemistry Advisor's Signature .....

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Finally, I would like to thank my parents and my best friend for understanding encouragement and their love.

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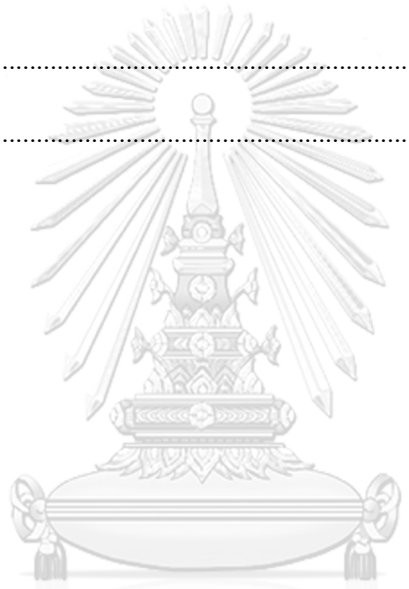

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List of Abbreviations

μL	microliter
Cur	curcumin
CMC	critical micelle concentration
°C	degrees celsius
DCC	N, N'-dicyclohexyl carbodiimide
DMSO-d_6	deuterated dimethylsulfoxide
D_2O	deuterium oxide
EDTA	ethylenediaminetetraacetic acid
Et_3N	triethylamine
FA	folic acid
FR	folate receptor
Free Cur	pure curcumin
h	hour
kV	kilovolt
MeOH	methanol
MHz	megahertz
mmol	milimol
mL	milliliter
mg	milligram
mm	millimeter
MALDI TOF	matrix-assisted laser desorption/ionization-time of flight
nm	nanometer
m/z	mass divided by charge number
NHS	N-hydroxysuccinimide
OcBr	octadecyl bromide

Oc	octadecyl
Oc-EDTA-FA	octadecyl-ethylenediaminetetraacetic-folic
Oc-EDTA-FA@Cur	Oc-EDTA-FA carrier encapsulated curcumin
ppm	part per million
TFA	trifluoroacetic acid

CHAPTER I

Introduction

1.1 Introduction

Cancer is a main cause of people death at the present [1]. Chemotherapy is one of the methods to treat cancer. However, this method is less specific to the targets site, resulting in drug deactivation and increasing side effects. Therefore, drug delivery carriers were used to overcome these problems. Most of the drug delivery carriers were prepared by polymeric micelle systems. However, that of polymeric micelle systems are difficult to synthesize into the random polymers, where different units are aligned on a polymer chain in a random manner, immature drug-incorporation technology and possible chronic liver toxicity due to slow metabolic process [2]. In this research, we have paid attention to ethylenediaminetetraacetic acid (EDTA) as a new drug delivery platform because EDTA molecule consisted of carboxyl groups that are easy to modify [3] EDTA is used as a food additive, in pharmaceuticals, in a variety of consumer products and a blood preservative by complexing free calcium ion to promote blood clotting [4]. EDTA has been used in chelation treatment for lead poisoning [5]. Furthermore, it is often used to treat various cardiovascular diseases [6].

Recently, EDTA has been developed for drug delivery system. Song and coworkers [7] adopted an NH_4EDTA gradient method to load doxorubicin into liposomes with the goal of increasing therapeutic effects and decreasing drug- related cytotoxicity. The results showed that the NH_4EDTA gradient method could significantly reduce drug toxicity without influencing antitumor activity. Gubernator and coworkers [8] used EDTA disodium as an agent to form low solubility complexes between the idarubicin drug and EDTA molecules inside the liposomes. The results showed that the efficiency of idarubicin encapsulation is close to 98% at a drug to lipid molar ratio of

1: 5. An *in vitro* long- term storage experiment confirmed the high stability of the liposomes.

In the past decade, self-assembly micelles have been developed for encapsulation of poorly water-soluble drugs. Self-assembly micelles are created from hydrophobic part that can be aggregated spontaneously when individual hydrophobic part is directly dissolved in aqueous solution [9], [10].

Yang and coworkers [11] developed injectable formulation and improved the stability of curcumin (Cur). Cur was encapsulated into monomethyl poly (ethylene glycol)-poly(**ε**-caprolactone)-poly (trim-ethylene carbonate) (MPEG-P(CL-co-TMC)) micelles through a single-step solid dispersion method. They found that Cur micelles could increase apoptosis and cellular uptake was enhanced to on CT26 cells comparing with free Cur. In addition, Cur micelles were stable under aqueous condition that could be applied for intravenous application to improve antitumor activity.

Furthermore, modification of drug delivery carriers with targeting ligand such as folic acid can also help to improve specific targeting. Folic acid (FA) is cancer targeting molecule which can bind specifically to folate receptor (FR) that over expressing on cancer cell membrane [12].

Song and coworkers [13] prepared folic acid-chitosan conjugated nanoparticles (FA-CS NPs) for evaluation of target specificity on tumor cells. Chitosan (CS) NPs were prepared by ionic cross-linking method, and folic acid (FA) was conjugated with CS NPs by electrostatic interaction. The properties of NPs were investigated and doxorubicin hydrochloride (Dox) as a model drug was encapsulated for investigation of drug release pattern in vitro. The cytotoxicity and cellular uptake of FA- CSNPs were also investigated. The results showed that the cellular uptake was increased when FA- CS NPs was used as Dox carrier. This provided the potential way for delivering drug to the target site due to the fact that folic acid can bind folate-receptor specifically.

In this study, EDTA derivative was modified with hydrophobic part (octadecyl bromide) and cancer targeting (folic acid) in order to create self-assembly micelles for encapsulation of water-insoluble anticancer drug and improvement of selectivity.

1.2 Scope of research

Most of the anticancer drugs are insoluble in water, unspecified to target site and degrade under acidic conditions in the stomach, resulting in decreasing their activities and increasing the side effects. To overcome these problems, we focused on increasing soluble of anticancer drugs, improves the selectivity, seeking a pH-sensitive and acid resistance with safe, inexpensive and easy preparation of drug carrier. EDTA derivative self-assembly micelles have been proposed and investigated for their potential as drug carriers. In this work, EDTA derivative was modified with hydrophobic part (octadecyl bromide) and cancer targeting (folic acid) in order to create self-assembly micelles and increase selectivity. Our proposed synthetic scheme of EDTA derivative self-assembly micelles is shown in Figure 1. The structure, critical micelles concentration and the morphologies of EDTA derivative were characterized by NMR, IR, MS, and SEM techniques. The quantity encapsulation and controlled release of anticancer drug was determined by UV-VIS technique. The cytotoxicity of EDTA derivative self-assembly micelles as cancer drug delivery carriers were investigated by MTT assay.

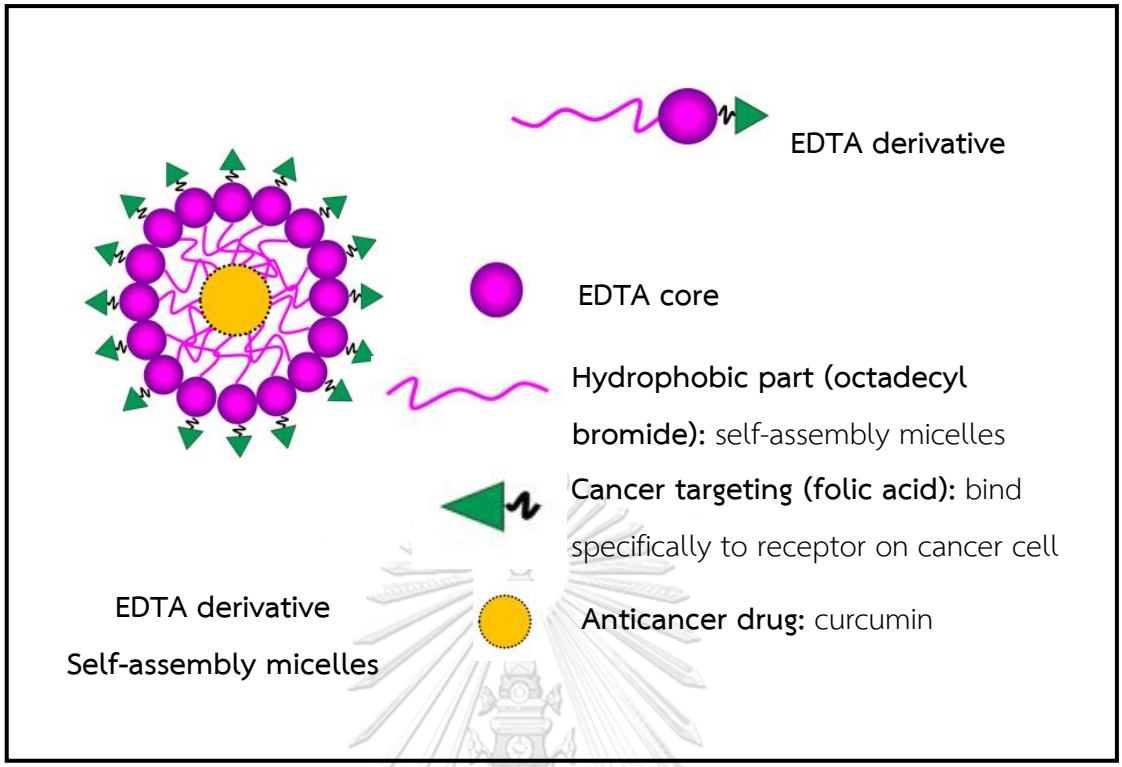


Figure 1 The proposed synthetic scheme of EDTA derivative self-assembled micelles

1.3 Objectives

1. To modify ethylenediaminetetraacetic acid (EDTA) with hydrophobic part (octadecyl bromide) and cancer targeting ligand (folic acid).
2. To study self-assembled EDTA derivative micelles by critical micelles concentration using fluorescence technique.
3. To study pH sensitive and morphology of self-assembled EDTA derivative micelles by SEM techniques.
4. To test the cytotoxicity of EDTA derivative by MTT assay to use in the application of drug delivery carrier.

CHAPTER II

Background and literature reviews

2.1 Cancer

Cancer is a disease caused by cellular disorders of the body cells in the DNA or the genetic material that causes the cells to grow rapidly and irregularly (Figure 2). Cellular disorders are caused by carcinogens such as tobacco, smoke, radiation and other chemicals. Genes that are involved in cancer can occur not specifically during inheritance. Each cancer is severely different, depending on the specific relationship between cancer and the patient. There are many methods to treat cancer, such as chemotherapy, radiation therapy, immunotherapy, targeted therapy and hormone therapy [14].

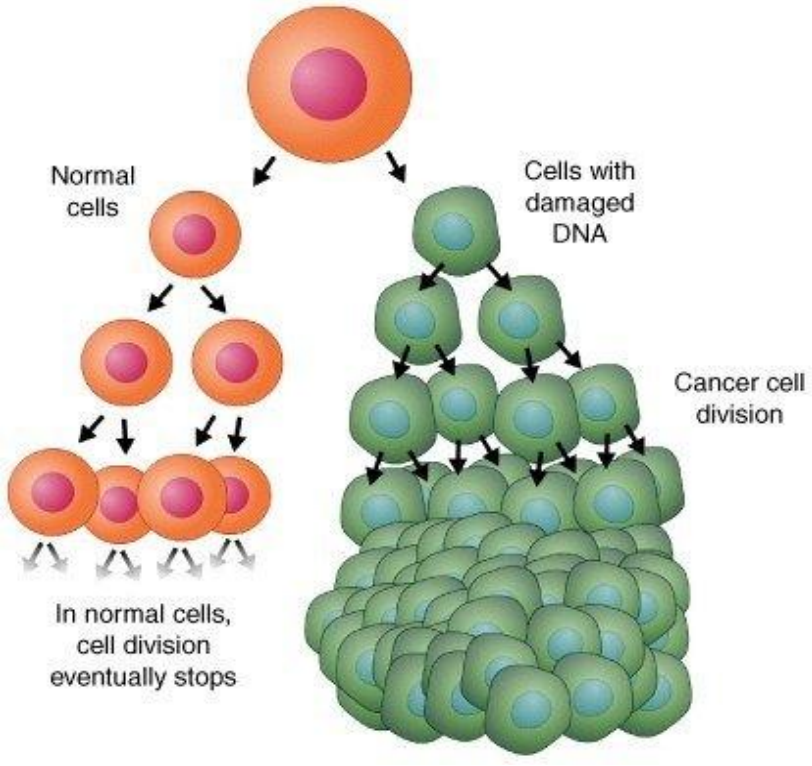


Figure 2 Normal cell and cancer cells division [14]

2.2 Chemotherapy

Chemotherapy is a treatment for cancer by ingestion or injection into the body. The effects of chemotherapy on the cell cycle are divided into two types. First, cell cycle phase-specific chemotherapy and second, cell cycle phase-nonspecific chemotherapy [15]. Chemotherapy is divided into 5 groups based on chemical properties or mechanism of action.

2.2.1 Alkylating Agents

Alkylating Agents are grouped by cell cycle phase-specific chemotherapy. The mechanism of action by binding to the DNA of the cell, resulting in the separation of the DNA do not divide and eventually die [16].

2.2.2 Antimetabolites

The drugs in this group have a chemical structure similar to those used in cell division, which may be used to generate DNA or RNA. As a result, enzyme cannot be used to build DNA during cell division which is cell cycle phase-specific chemotherapy [17].

2.2.3 Vinca Alkaloids

The drugs in this group interacts with tubulin, inhibiting the formation of microtubule and spindle fibers necessary to separate the chromosome in the cell cycle. which is cell cycle phase-specific chemotherapy [18].

2.2.4 Antibiotics

These drugs are derived from fungi that destroy cancer cells. There are two types of anthracyclines and non-anthracyclines which is cell cycle phase-nonspecific chemotherapy. The mechanism of action such as interrelation, membrane binding, free radical formation, metal ion chelation and alkylation [19].

2.2.5 Miscellaneous

The drugs in this group are divided in to 6 subgroups [20].

1. Taxanes: Taxanes will inhibit the formation of spindle fibers used to isolate DNA strands, such as paclitaxel and docetaxel.
2. NON-classical alkylating agents: The drugs act as an alkylation agent and inhibits intrastand cross-link and interstand cross-linking, including cisplatin carboplatin and eloxatin.
3. Anthracenediones: Anthracenediones are a similar structure formula of anthracycline. The action against the DNA, but less radicals, cause less effect to heart than anthracyclines such as mitoxantrone.
4. Epipodophyllotoxins: They are synthesized from podophyllotoxin which inhibit the enzyme topoisomerase II that acts to separate the double stranded DNA during cell division. Example of drug is etoposide.
5. Topoisomease I Inhibitor: The drugs inhibit the topoisomerse I enzyme, which cleaves and cleaves single stranded DNA during cell division, including irinotecan and topotecan.

In addition, medical science has been researching and modifying the extracts from natural products to enhance its bioavailability and inhibit cancer cells[21]. Many herbs are extracted and reported to treat cancer as shown in Table 1.

Table 1 Herbs used to treat cancer [22, 23].

Name of Plant	Active ingredient	Treatment
Garlic	dially sulphide	gastric cancer
Ginger	gingerol	general cancer
Curcuma longa	curcumin	ovarian Cancer skin Cancer colorectal Cancer
Tumeric	curcuminoids	colorectal Cancer
Anona muricata	acetogenin	general cancer
Centella asiatica	asiaticoside	endometrial Cancer
Taxus brevifolia Nutt	Taxol	general cancer
Madagascar periwinkle	vinblastine -VLB	leukemia
Andrographis paniculata	andrographolide	general cancer
Indian gooseberry	Homoharringtonine	leukemia
Figs	benzaldehyde	general cancer
Tripterygium wilfordii Hook	Triptolide	leukemia
Aloevera	aloe-emodin	general cancer
Heliotropium indicum	Indicine-N-oxide	leukemia

At the present, most of the anticancer drugs are insoluble in water, unspecified to target site and degrade under acidic and basic conditions in the body, resulting in decreasing their activities and increasing the side effects. Scientists have been interested in developing another way to increase the effectiveness of anticancer drugs.

2.3 EDTA

Ethylene diamine tetra-acetic acid (EDTA), an ethylene diamine tetra-acetic acetate salt of EDTA, is a food additive that is a chelating agent [3, 24]. EDTA can form the bond 4-6 bonds with metal which is chelate that contains metal in the molecule. EDTA is used as a food additive, in pharmaceuticals, in a variety of consumer products and a blood preservative by complexing free calcium ion to promote blood clotting [4]. EDTA has been used in chelation treatment for lead poisoning [5]. Furthermore, it is often used to treat various cardiovascular diseases[6]. The structure of EDTA as shown in Figure 3.

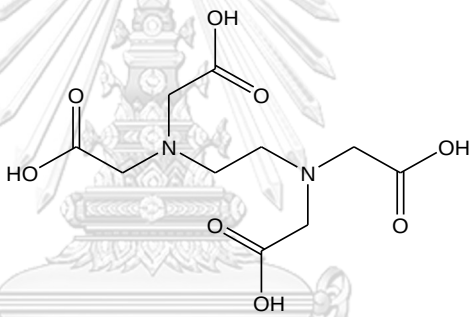


Figure 3 Structure of EDTA

In Pharmacology and pharmacokinetics, Calcium disodium EDTA ($CaNa_2EDTA$) is used as a lead poisoning drug in the body. Calcium in $CaNa_2EDTA$ is replaced by divalent or trivalent metals. The bond between the metal and EDTA is higher than the affinity between the metal and the enzyme or tissue, so it can pull metal chelate out of the enzyme or tissue [25].

Recently, EDTA has been developed for drug delivery system. Song and coworkers [7] adopted an NH_4EDTA gradient method to load doxorubicin into liposomes with the goal of increasing therapeutic effects and decreasing drug-related cytotoxicity. The results showed that the NH_4EDTA gradient method could significantly

reduce drug toxicity without influencing antitumor activity. Gubernator and coworkers [8] used EDTA disodium as an agent to form low solubility complexes between the idarubicin drug and EDTA molecules inside the liposomes. The results showed that the efficiency of idarubicin encapsulation is close to 98% at a drug to lipid molar ratio of 1: 5. An *in vitro* long- term storage experiment confirmed the high stability of the liposomes.

2.4 Drug delivery carrier

Drug delivery system is a method to achieve a therapeutic effect in humans or animals by using nanocarriers [26]. Drug delivery system has many benefits to treatment of diseases, including increased efficacy of the drugs and convenience, site specific delivery and decreased toxicity and side effects. Examples of drug delivery carriers are micelles, liposome, hydrogel, dendrimer and nanoparticle.

2.4.1 Micelles

Micelles can be formed self-assembly of hydrophobic and hydrophilic block copolymers in the aqueous solution. The hydrophobic drugs can be physically encapsulated in the polymer micelles and transported to the target sites which can exceed their intrinsic water- solubility [27].

2.4.2 Liposome

Liposome can be a carrier for both water-soluble drug and water-insoluble drug inside lipid bilayer membrane. To deliver drugs at the target sites, lipid bilayer of liposome membrane can fuse with cell membrane of target site [28].

2.4.3 Hydrogel

Hydrogel-based drug delivery carriers can encapsulate drugs and protected drugs from hostile environments such as enzymes and low pH in the stomach. The control drug release from hydrogels can also be controlled by volume transition of

gels in response to environmental stimuli such as temperature, pH, ionic strength and electric field [29].

2.4.4 Dendrimer

The dendrimer is high potential carrier for delivering pharmaceutical compounds. The characteristic features of dendrimer are water solubility, encapsulation ability, and large number of functionalize peripheral groups for evaluation as drug delivery vehicles [30].

2.4.5 Nanoparticle

The nanoparticles as drugs delivery systems have been advanced in the bioavailability, solubility, *in vivo* stability, intestinal absorption, sustained and targeted delivery, and therapeutic effectiveness of several anticancer agents. Particle size and surface properties of nanoparticles and release rate of pharmacologically active agents can be controlled in order to achieve the site-specific action at the therapeutically optimal rate and dose regimen [31].

Most of the drug delivery carriers were prepared by polymer systems. However, that of polymer systems are difficult to synthesize into the random polymers, where different units are aligned on a polymer chain in a random manner, immature drug-incorporation technology and possible chronic liver toxicity due to slow metabolic process [2].

2.5 Self-assembly micelles

self-assembly micelles have been developed for encapsulation of poorly water-soluble drugs [32]. Self-assembly micelles are created from hydrophobic part that can be aggregated spontaneously when individual hydrophobic part is directly dissolved in aqueous solution [9], [10] (Figure 4).

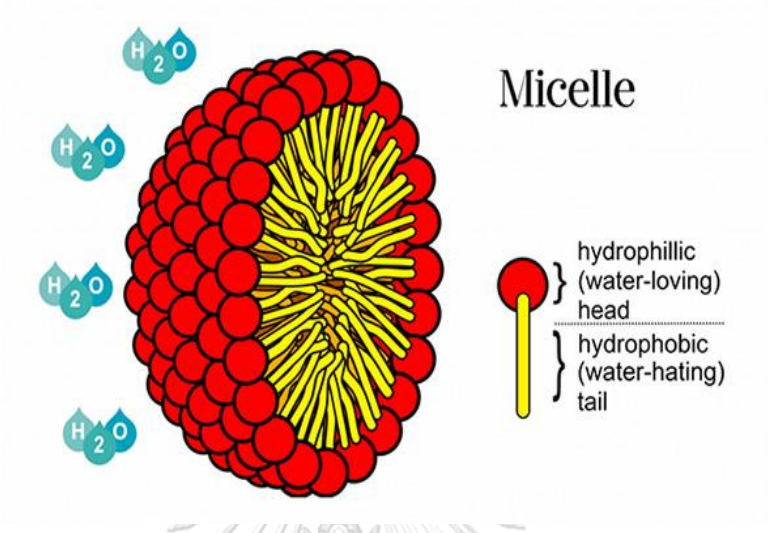


Figure 4 Schematic representation of micelles in water [10]

Yang and coworkers [11] developed injectable formulation and improved the stability of curcumin (Cur). Cur was encapsulated into monomethyl poly (ethylene glycol)-poly(**ε**-caprolactone)-poly (trim-ethylene carbonate) (MPEG-P(CL-co-TMC)) micelles through a single-step solid dispersion method. They found that Cur micelles could increase apoptosis and cellular uptake was enhanced to on CT26 cells comparing with free Cur. In addition, Cur micelles were stable under aqueous condition that could be applied for intravenous application to improve antitumor activity.

2.6 Cancer targeting molecule

Folic acid (FA) is cancer targeting molecule which can bind specifically to folate receptor (FR) that over expressing on cancer cell membrane [12]. The general model for the cellular uptake of drug conjugates targeted to the FR is illustrated in Figure 5.

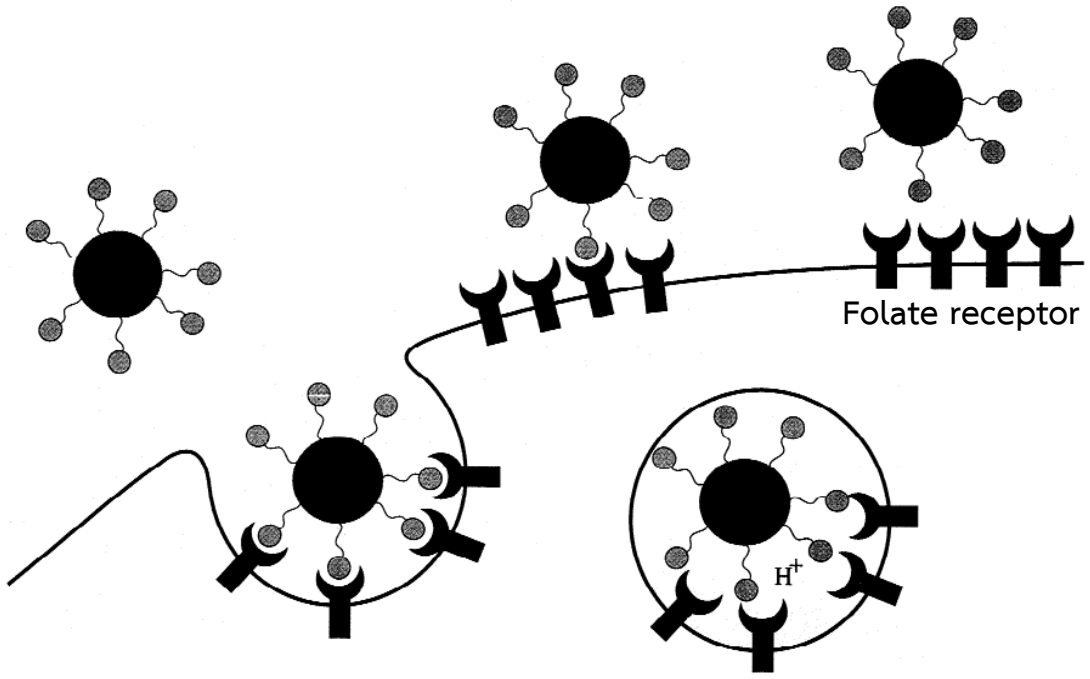


Figure 5 Schematic diagram of the folate receptor-mediated endocytosis pathway [12]

Song and coworkers [13] prepared folic acid-chitosan conjugated nanoparticles (FA-CS NPs) for evaluation of target specificity on tumor cells. Chitosan (CS) NPs were prepared by ionic cross-linking method, and folic acid (FA) was conjugated with CS NPs by electrostatic interaction (Figure 6). The properties of NPs were investigated and doxorubicin hydrochloride (Dox) as a model drug was encapsulated for investigation of drug release pattern in vitro. The cytotoxicity and cellular uptake of FA-CSNPs were also investigated. The results showed that the cellular uptake was increased when FA-CS NPs was used as Dox carrier. This provided the potential way for delivering drug to the target site due to the fact that folic acid can bind folate-receptor specifically.

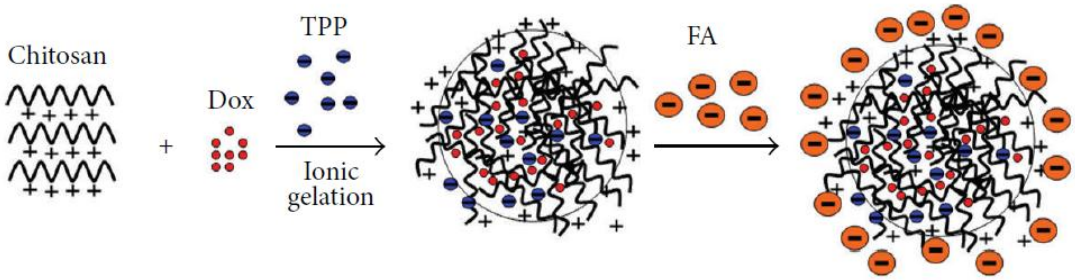


Figure 6 Schematic formation of FA conjugated chitosan Dox-loaded nanoparticles [13]

2.7 pH-sensitive

pH sensitive have specific property that show physical properties transition by the change in environmental pH [33]. Usually, pH sensitive compound contains acidic or basic side groups (carboxyl groups or amino groups) which can accept or donate the proton with respect to the change in pH [34]. The compound which contains anionic/acidic groups can form anion in the solution at higher pH than pKa because acidic group donates the proton into the solution. On the other hand, the compound that containing cationic/basic groups can form cation in the solution at low pH [35].

2.8 Cytotoxicity assay by MTT assay

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is tetrazolium salt which uses in biological testing in many fields. The MTT principal is only metabolically active can be used succinate dehydrogenase enzyme change MTT to generate purple formazan products via reduction as shown in Figure 7. The formazan product formed is directly proportional to the amount of succinate dehydrogenase enzyme, which depends on the number of living cells. Therefore, it is possible to measure the quantity of living cell by measuring the formazan product produced [36-38].

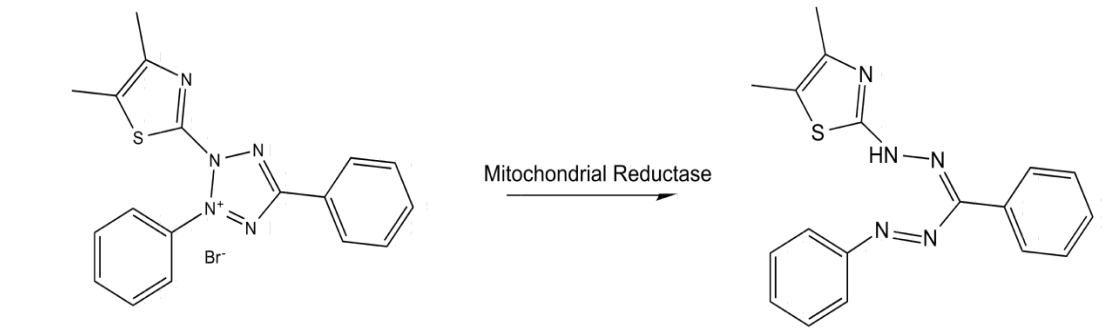


Figure 7 The reduction of MTT to formazan product [37]

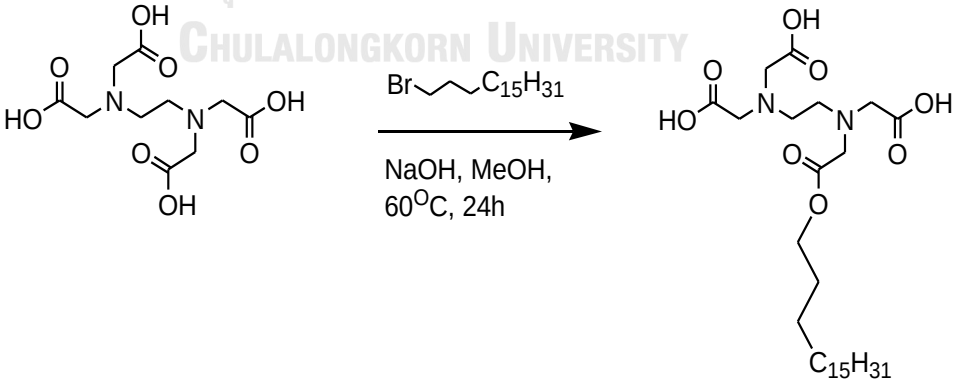
CHAPTER III

Experimental

3.1 Synthesis of Oc-EDTA-FA

3.1.1 Synthesis of Oc-EDTA

EDTA was synthesized with octadecyl bromide via substitution reaction by 1:1 mole ratio of carboxylic group of EDTA: octadecyl bromide. EDTA (AR grade, Sigma Chemical Co) 100 mg (0.2 mmol) was dissolved in 20 ml of methanol (reagent grade, Aldrich Chem. Co.). Adjusted the pH of the solution around 2.5 by 3 M of sodium hydroxide (AR grade, Sigma Chemical Co) to deprotonate one proton of carboxylic group of EDTA because the pka of EDTA was 2.0, 2.7, 6.2 and 10.3 after that stirred at 60 °C for 30 minutes. Then, 0.12 ml (0.2 mmol) of octadecyl bromide (AR grade, Sigma Chemical Co) was slowly added to the mixture solution and stirred at 60 °C for 24 h (Figure 8). The solution cooled down at room temperature and precipitated in acetone (reagent grade, Aldrich Chem. Co.). The precipitate was purified by washing with ethyl acetate (reagent grade, Aldrich Chem. Co.) and acetone. The percent yield of Oc-EDTA was 70%.

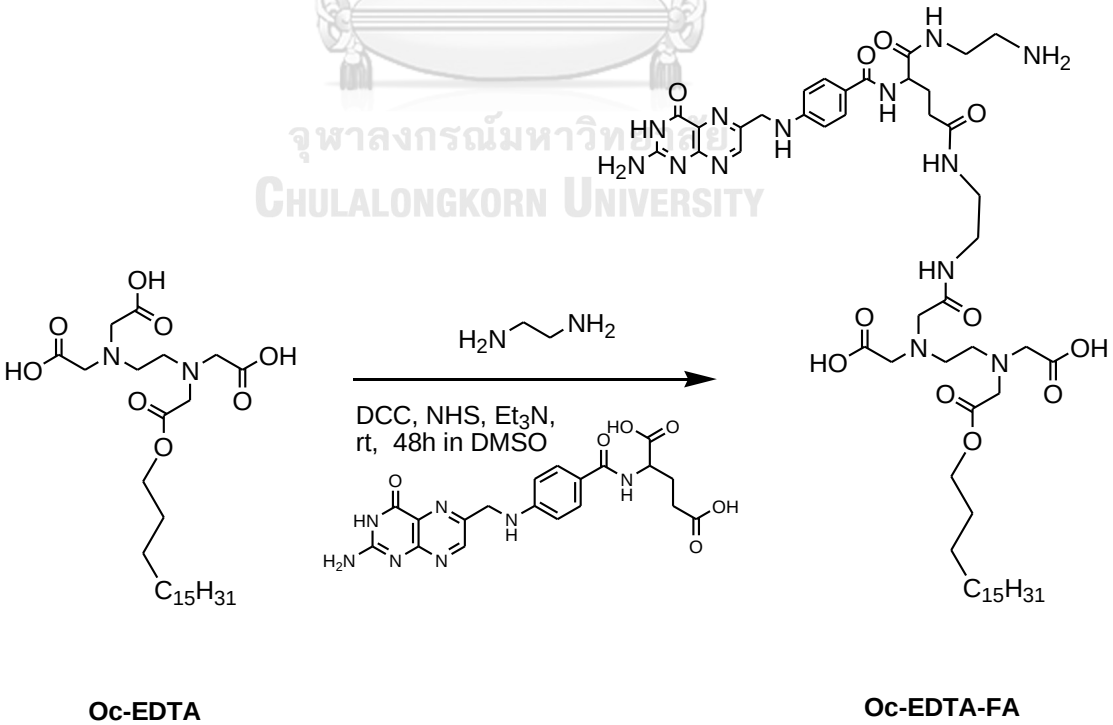


EDTA **Oc-EDTA**

Figure 8 Synthesis of Oc-EDTA

3.1.2 Synthesis of Oc-EDTA-FA

Folic acid (AR grade, Sigma Chemical Co) 100 mg (0.2 mmol) was dissolved in 20 ml of dimethyl sulfoxide (reagent grade, Aldrich Chem. Co.) after that 93 mg of N, N'-dicyclohexyl carbodiimide (0.4 mmol, AR grade, Sigma Chemical Co), 47 mg of N-hydroxysuccinimide (0.4 mmol, AR grade, Sigma Chemical Co) and 64 µL of triethylamine (reagent grade, Aldrich Chem. Co.) were added and stirred at room temperature for 30 minutes. Then, 30 µL (0.4 mmol) of ethylene diamine (reagent grade, Aldrich Chem. Co.) was slowly added to the mixture solution. The reaction was stirred at room temperature for 12h in the dark and then a mixture of 108 mg Oc-EDTA (0.2 mmol), 41 mg N, N'-dicyclohexyl carbodiimide (0.2 mmol), 21 mg of N-hydroxysuccinimide (0.2 mmol) in 20 ml of dimethyl sulfoxide was added to the solution. Adjusted the pH of the solution around 5 by 3 M of sodium hydroxide and then, the reaction was stirred at room temperature for 48 h in the dark (Figure 9). The solution was precipitated in water and purified by washing with ethyl acetate and acetone. The percent yield of Oc-EDTA-FA was 65%.



Oc-EDTA **Oc-EDTA-FA**

Figure 9 Synthesis of Oc-EDTA-FA

3.2 Characterization of Oc-EDTA-FA

3.2.1 ^{1}H Nuclear magnetic resonance spectroscopy (^{1}H NMR)

^{1}H NMR spectra of EDTA, Oc-EDTA, folic acid and Oc-EDTA-FA in DMSO were recorded from Varian NMR spectrometer operating at 400 MHz at room temperature.

3.2 2 ^{13}C Nuclear magnetic resonance spectroscopy (^{13}C NMR)

^{13}C NMR spectra of Oc-EDTA and Oc-EDTA-FA in DMSO were recorded from Burger NMR spectrometer operating at 400 MHz at room temperature.

3.2.3 Fourier transformed infrared spectroscopy (FTIR)

The EDTA, Oc-EDTA, folic acid and Oc-EDTA-FA were mixed with potassium bromide (KBr) and analyzed by FTIR (Nicolet 6700) in the region from 4000 to 400 cm^{-1}.

3.2.4 Mass spectroscopy (MS)

The excess of Oc-EDTA-FA was dissolve in 1 mL of water and added sodium hydroxide (3 M, 10 µL) to improve the solubility. Then, the saturated solution of Oc-EDTA-FA (10 µL) was mixed with 100 µL of metric solution (2:1 of 10% TFA:CH_3CN). The mixture was analyzed by MALDI TOF mass spectrometer (Bruker Microflex MALDI-TOF MS).

3.3 Critical micelles concentration of Oc-EDTA-FA

The critical micelles concentration was used to measure the surfactant amphiphilic molecule contains both the hydrophilic part (water soluble) hydrophobic part (water insoluble) component [39] . Oc-EDTA-FA (5 mL) was prepared with various concentrations and added pyrene (0.1 mM, 50 µL) (AR grade, Sigma Chemical Co) into each concentration. All of concentrations were determined the excitation (391 nm) and emission (371 nm) of pyrene by fluorescence technique (Perkin Elmer LS45 luminescence spectrometer). Use these results to plot graph between concentration

of Oc-EDTA-FA and intensity of excitation/emission. The intersection of graph was the critical micelles concentration.

3.4 The quantity encapsulation

This work, Curcumin was used as a model drug for study of quantity encapsulation in Oc-EDTA-FA. Curcumin is a hydrophobic polyphenol derived from the rhizome of the herb *Curcuma longa* (Figure 10) which has a wide spectrum of biological and pharmacological activities [40].

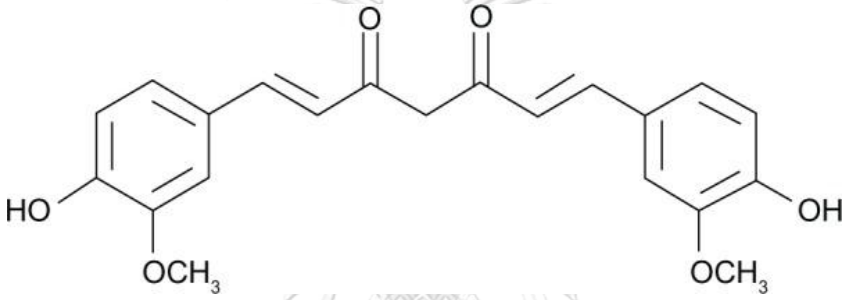


Figure 10 Structure of curcumin [40]

The solution of Oc-EDTA-FA was prepared with concentration 12 mg/ 3 mL in water after that added curcumin with various mass at 1, 2, 3, 4, 5 and 6 mg and stirred at room temperature for 1 h. Then, separated only solution to freeze dry and using UV-Vis technique by scanning spectrophotometer (HP 8435, Varian Ltd.) with a 1-cm path length determine the quantity encapsulation curcumin of Oc-EDTA-FA.

3.5 pH sensitive and morphology

The Oc-EDTA-FA carrier encapsulated curcumin (Oc-EDTA-FA@Cur) (4 mg) was dissolved in 1 mL of buffer solution pH 1.2, 4.4, 6.8 and 8.4. Then, stirred at room temperature for 30 min. All the solutions were analyzed size and shape by using scanning electron microscopy (SEM). Each solution was prepared on microscope slides (0.5 mm x 0.5 mm) and dried at room temperature for 1 day. Then, the samples were

mounted onto aluminum stub and coated with gold-palladium. Using 15kV of beam voltage by under high vacuum and at ambient temperature.

3.6 Cytotoxicity study

The cytotoxicity of pure curcumin (Free Cur), Oc-EDTA-FA carrier and Oc-EDTA-FA carrier encapsulated curcumin (Oc-EDTA-FA@Cur) were measured via reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye which viable cells to generate purple formazan products. Cancer cells (5×10^3 cells/well, 200 µL) were seeded into 96-well plates and incubated at 37 °C for 24 h. Each sample (2 µL) was added into triplicate wells and incubated at 37 °C for 3 days. The MTT solution (5 mg/mL, 10 µL) was added and incubated for 4h. Then, the solution was removed from well after that wells which had formazan crystals were solubilized in DMSO (150 µL). All the solution was measured absorbance at 540 nm (MCC//340 MK II Serial RS-232C reader). The cytotoxicity activity was measured as % cell death by using Equations 1 and 2. The IC_{50} values calculated by using the software GraphPad Prism 5 (GraphPad Software, Inc.) using a nonlinear regression of "log(inhibitor) versus response": Y = Bottom + (Top-Bottom)/ (1 + 10^((X-LogIC_{50}))).

$$\%\ \text{Cell viability} = \frac{\text{OD value of treated well}}{\text{OD value of untreated well}} \text{ x } 100 \quad \textbf{(1)}$$

$$\%\ \text{Cell deat} = 100 - \%\ \text{Cell viability} \quad \textbf{(2)}$$

CHAPTER IV
Results and Discussion

4.1 Synthesis and characterization of Oc-EDTA-FA

The Oc-EDTA was hydrophobic part which modified by introducing the octadecyl bromide onto the carboxylic group of EDTA via substitution reaction. Synthesis of Oc-EDTA-FA, folic acid was activated by N, N'-dicyclohexyl carbodiimide (DCC) to the reactive form. The reaction was added N-hydroxysuccinimide (NHS) to improve the efficiency of DCC coupling reactions with ethylene diamine as the conjugation via an amide bond between folic acid and ethylene diamine. Besides, the Oc-EDTA was modified with folic-ethylene diamine by Oc-EDTA was activated with DCC and improve the efficiency by NHS via an amide bond between Oc-EDTA and folic-ethylene diamine, the resulting for synthesis of Oc-EDTA-FA was shown in Figure 11. The percent yield of Oc-EDTA and Oc-EDTA-FA as 70% and 65% respectively. The modification of EDTA was characterized by using ^{1}H NMR, ^{13}C NMR, FTIR and MS.

^{1}H-NMR spectrum of Oc-EDTA (Figure 12c) showed the proton of long chain hydrocarbon at 2.1 (H_4), 1.4 (H_5), 1.2 (H_6) and 0.9 (H_7) ppm. They were shifted to downfield shift compared with the spectrum of octadecyl bromide (Figure 12b) besides, the proton at 3.6 ppm (H_3) of octadecyl bromine disappeared in the spectrum of Oc-EDTA because it was downfield shift to 5.4 ppm (H_8) due to deshielding proton from substitution reaction. The proton of EDTA in Oc-EDTA spectrum disappeared because it was possible to overlap with peak of solvent. However, when we changed NMR solvent as DMSO-d_6: D_2O (6:1) the spectrum showed in Figure 13, It found that proton of EDTA in Oc-EDTA (Figure 13c) showed at 3.5 (H_1) and 3.0 (H_2) ppm and proton of long chain hydrocarbon (H_{3-7}) showed at 1.2 ppm. Oc-EDTA-FA (Figure 12e) showed the proton of long chain hydrocarbon at 2.75 ppm (H_5). It was shifted to downfield

shift compared with the spectrum of octadecyl bromide (Figure 12b). In addition to the proton of Oc-EDTA-FA at 1.03 and 1.21 ppm ($H_{16\text{-}17}$) upfield shift compared with the spectrum of folic acid (Figure 12d) but the proton at 4.45 and 5.55 ppm downfield shift. However, the proton in the spectrum of EDTA (Figure 12a) at 1.90 and 2.75 ppm ($H_{1\text{-}2}$) disappeared in the spectrum of Oc-EDTA-FA because it was possible to overlap with peak of solvent.

^{13}C-NMR spectrum of Oc-EDTA-FA (Figure 14b) showed the carbon of EDTA at 47.5 ppm (C_2). In addition, at 24.50-25.02 and 35.70 ppm ($C_{5\text{-}6}$, C_4) showed the carbon of folic and ethylene diamine. The carbon of long chain hydrocarbon C_1 showed at 54.95 ppm and another position of long chain hydrocarbon might be possible to overlap with peak of solvent.

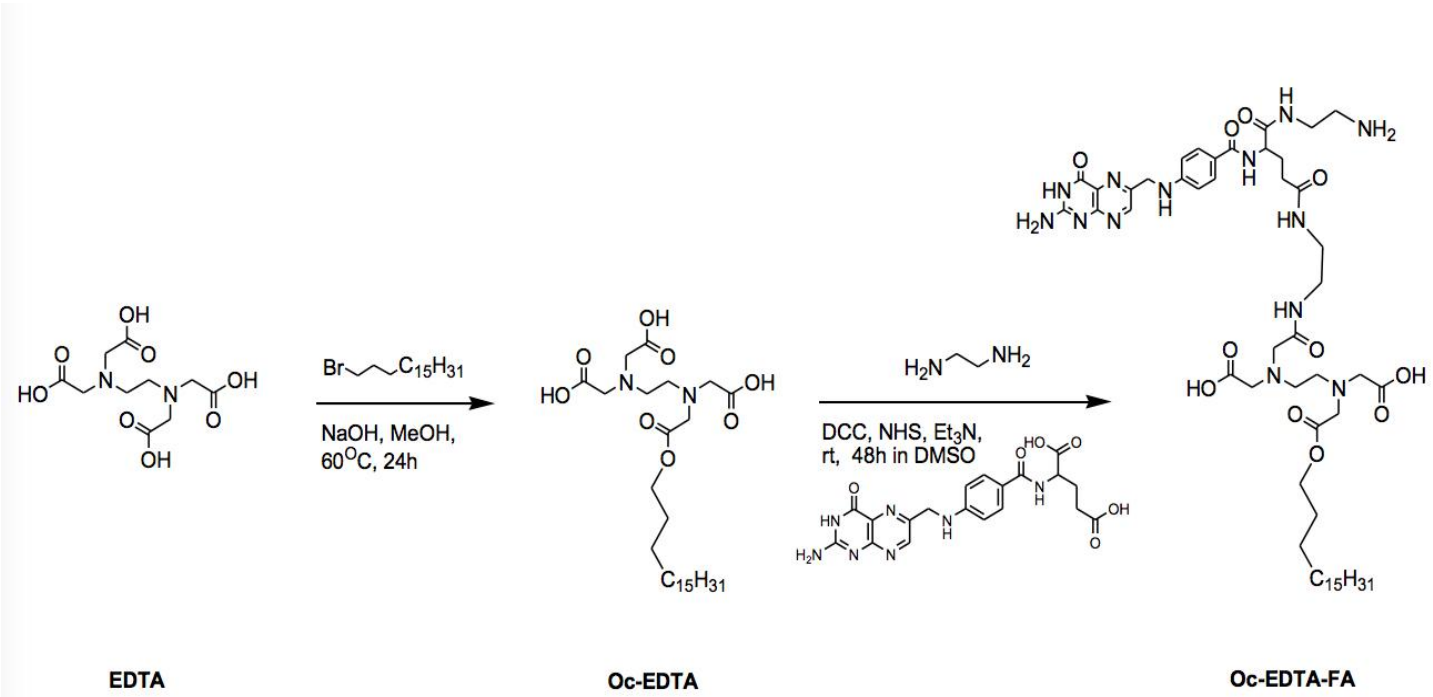


Figure 11 Synthetic pathway of Oc-EDTA-FA

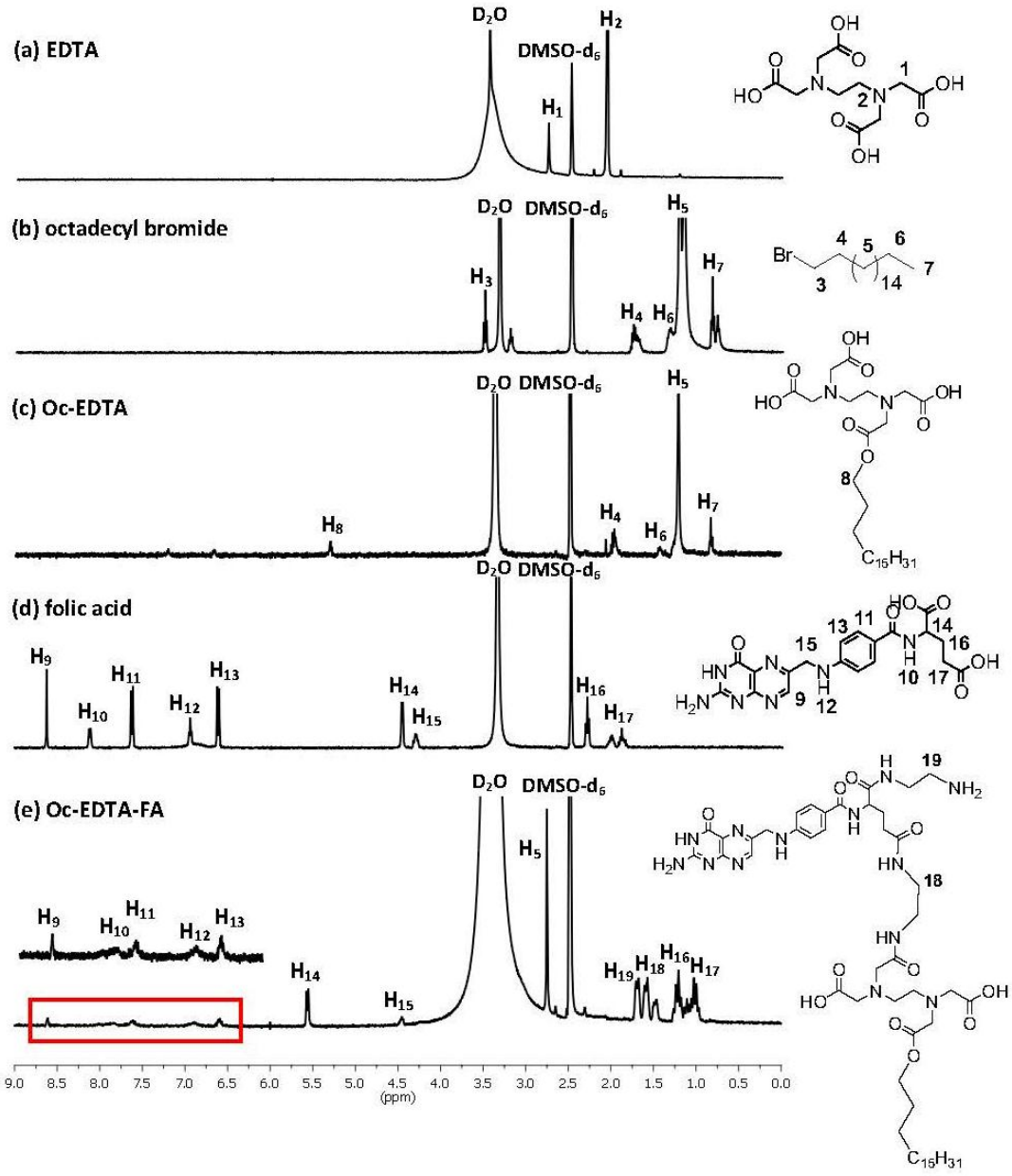


Figure 12 ^{1}H NMR spectra of (a) EDTA, (b) octadecyl bromide, (c) Oc-EDTA, (d) folic acid and (e) Oc-EDTA-FA

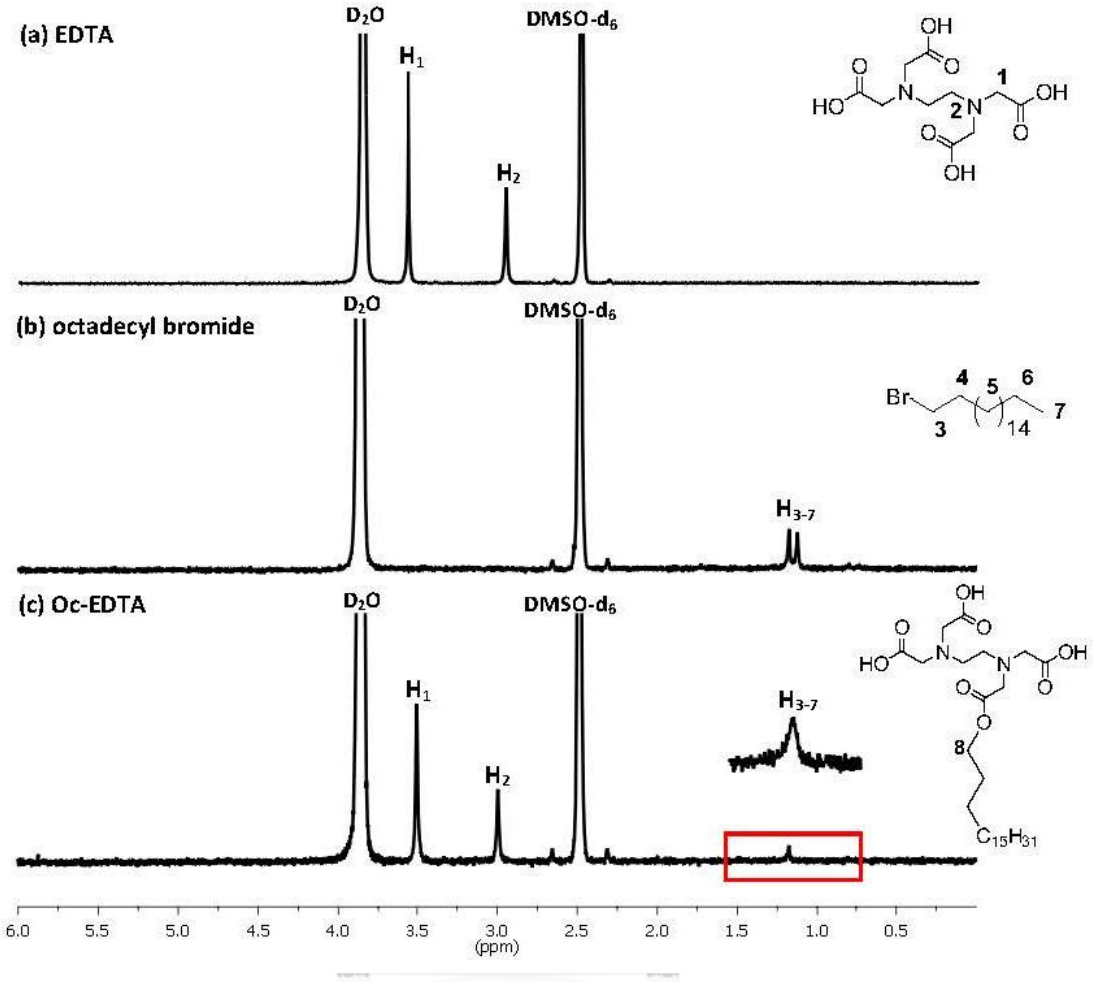


Figure 13 ^{1}H NMR spectra of (a) EDTA, (b) octadecyl bromide and (c) Oc-EDTA in the mixture of solvent DMSO-d_6: D_2O (6:1)

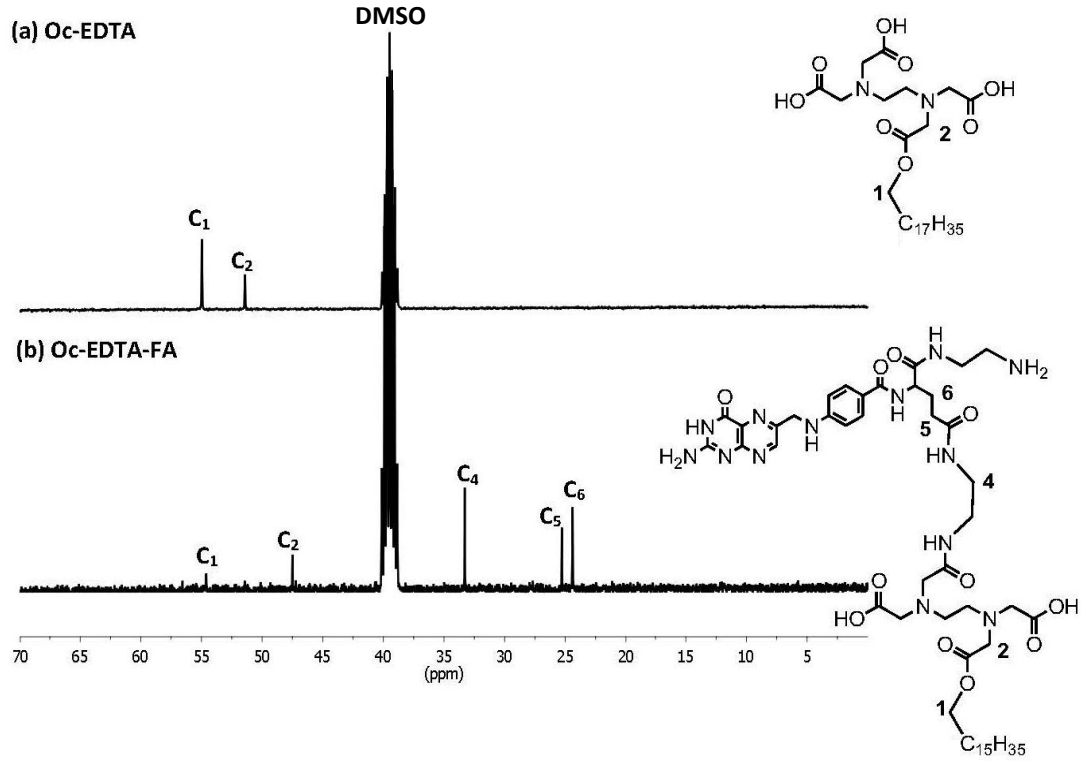


Figure 14 ^{13}C NMR spectra of (a) Oc-EDTA and (b) Oc-EDTA-FA in solvent DMSO

FT-IR spectra of Oc-EDTA (Figure 15b) showed the characteristic absorption band of the residue C=O stretching of carboxylic acid peak, C=O stretching of ester group of Oc-EDTA conjugate and C-H rock of long chain hydrocarbon of octadecyl at 1678, 1614 and 811 cm^{-1}, respectively. FT-IR spectrum of Oc-EDTA-FA (Figure 15d) showed the characteristic absorption band of the residue C=O stretching of ester group of Oc-EDTA conjugate and C-H stretching of long chain hydrocarbon of octadecyl at 1620 and 2848 cm^{-1}, respectively. The absorption peak of Oc-EDTA-FA at 3322 and 3411 cm^{-1}, which assigned to primary and secondary of N-H stretching. In addition, O-H stretching of EDTA in Oc-EDTA-FA showed at 2930 cm^{-1}.

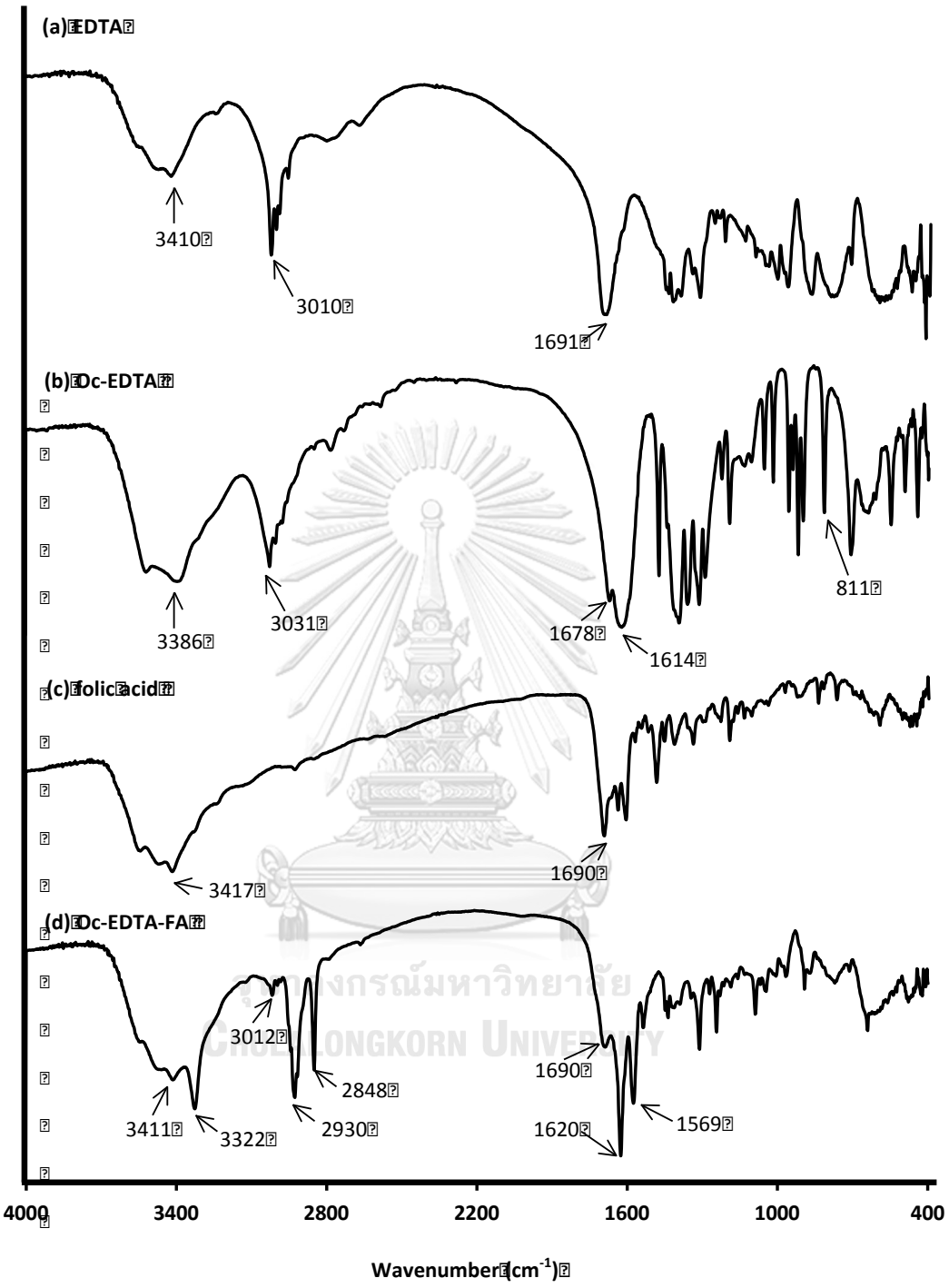


Figure 15 FTIR spectra of (a) EDTA, (b) Oc-EDTA, (c) folic acid and (d) Oc-EDTA-FA

Mass spectrum of Oc-EDTA-FA (Figure 16) showed the highest relative abundance at 1077.88 m/z which the molecular weight of Oc-EDTA-FA by calculation (Mw) is 1053.62 m/z. It is possible to mass that showed in the spectrum is form $[M+Na]^+$ which has value equal to 1076.61 m/z. MS could be supported that the Oc-EDTA-FA were successfully synthesized.

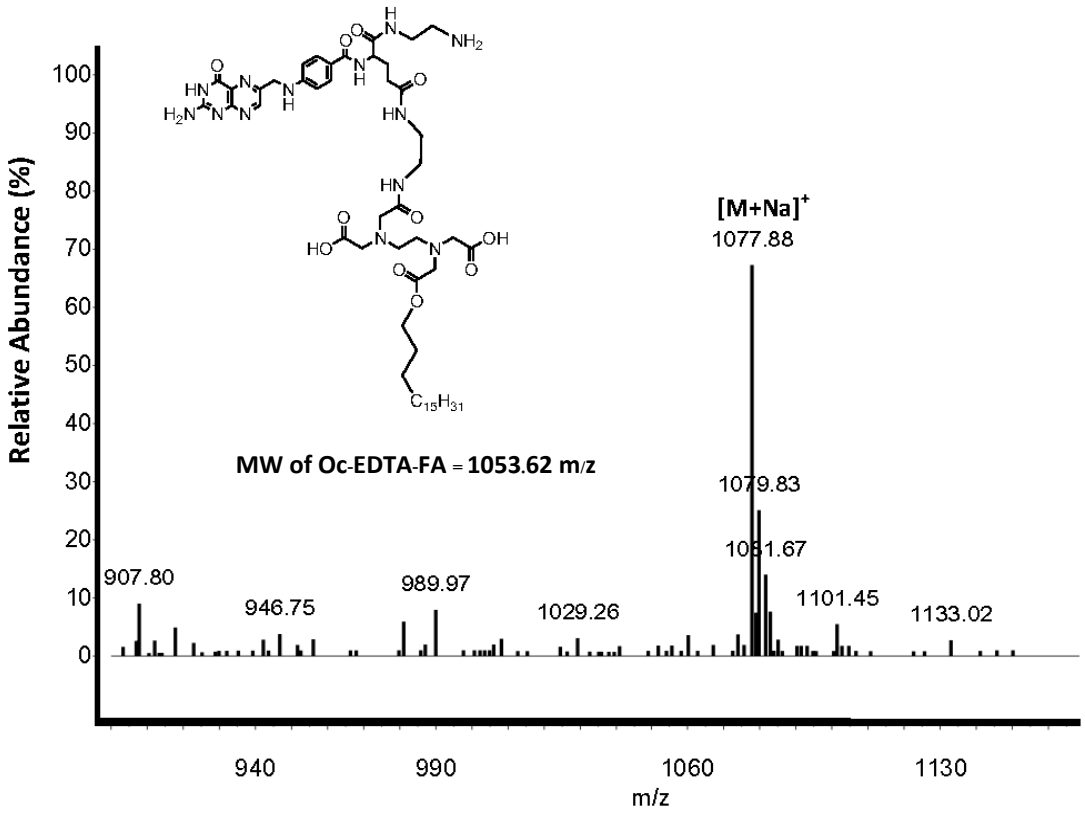


Figure 16 Mass spectrum of Oc-EDTA-FA

4.2 Self-assembly micelles of Oc-EDTA-FA

The Oc-EDTA-FA that contained hydrophobic part in the long chain hydrocarbon of octadecyl and hydrophilic part in the carboxylic group of EDTA could be self-assembled micelles in distilled water. The critical micelle concentration based on the forming of pyrene in the hydrophobic regions and results in the pyrene fluorescence intensity will be changed. The critical micelle concentration was determined at 0.06 mg/mL as shown in Figure 17.

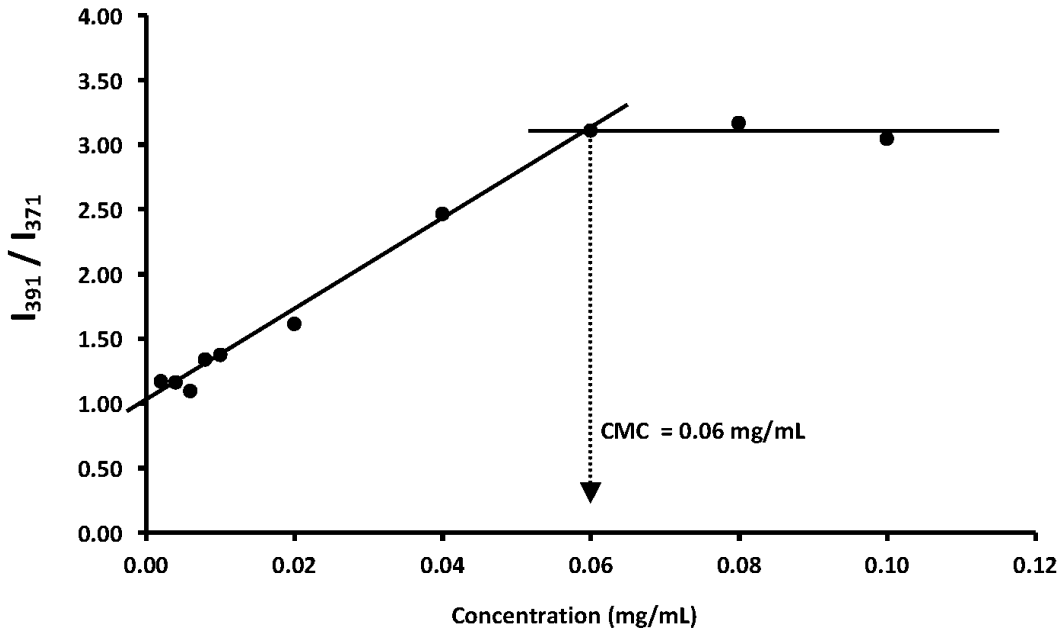


Figure 17 Critical micelles concentration of Oc-EDTA-FA

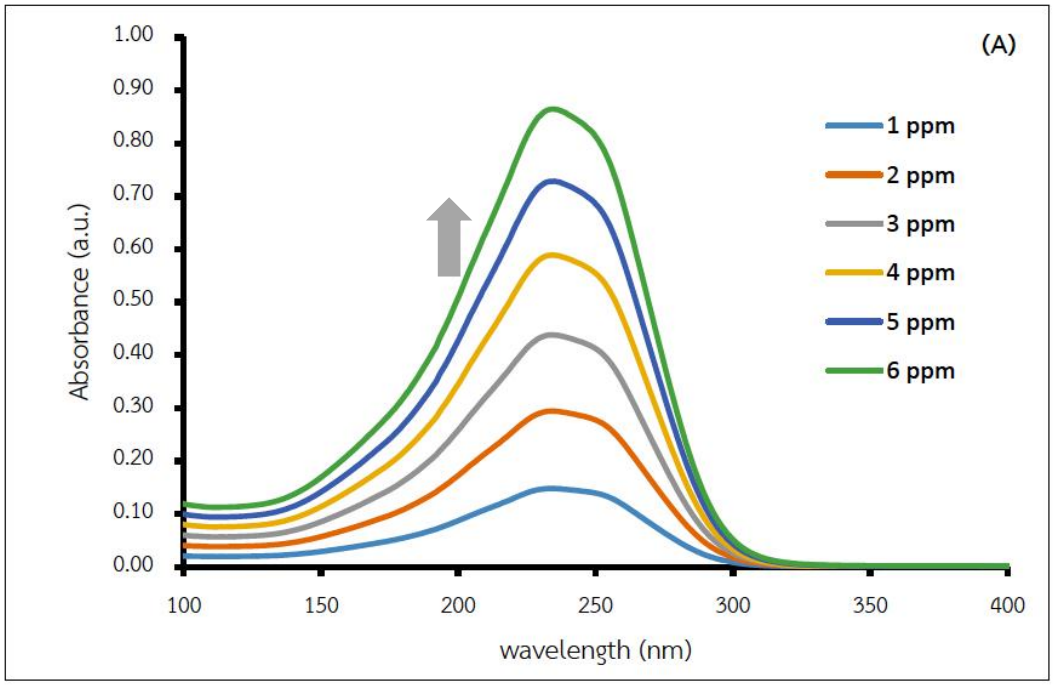
4.3 Encapsulation curcumin of Oc-EDTA-FA

Encapsulation efficacy of curcumin into Oc-EDTA-FA was calculated by using calibration curve of curcumin (Figure 18). The percent encapsulation of curcumin (model drug) into Oc-EDTA-FA as shown in Table 2.

Table 2 % Encapsulation of curcumin into Oc-EDTA-FA

No.	OC-EDTA-FA (mg/ 3 mL)	Curcumin (mg)	% Encapsulation curcumin (w/w)
1	4	1	18.25±2.31
2	4	2	25.62±1.23
3	4	3	28.23±1.56
4	4	4	32.31±2.01
5	4	5	32.75±1.48
6	4	6	31.62±2.33

When increasing the amount of curcumin while the amount of Oc-EDTA-FA constant found that % Encapsulation curcumin was relatively stable when 4, 5, and 6 mg curcumin were added. The Oc-EDTA-FA 12 mg in 3 mL water can be encapsulated curcumin around 32% (w/w).



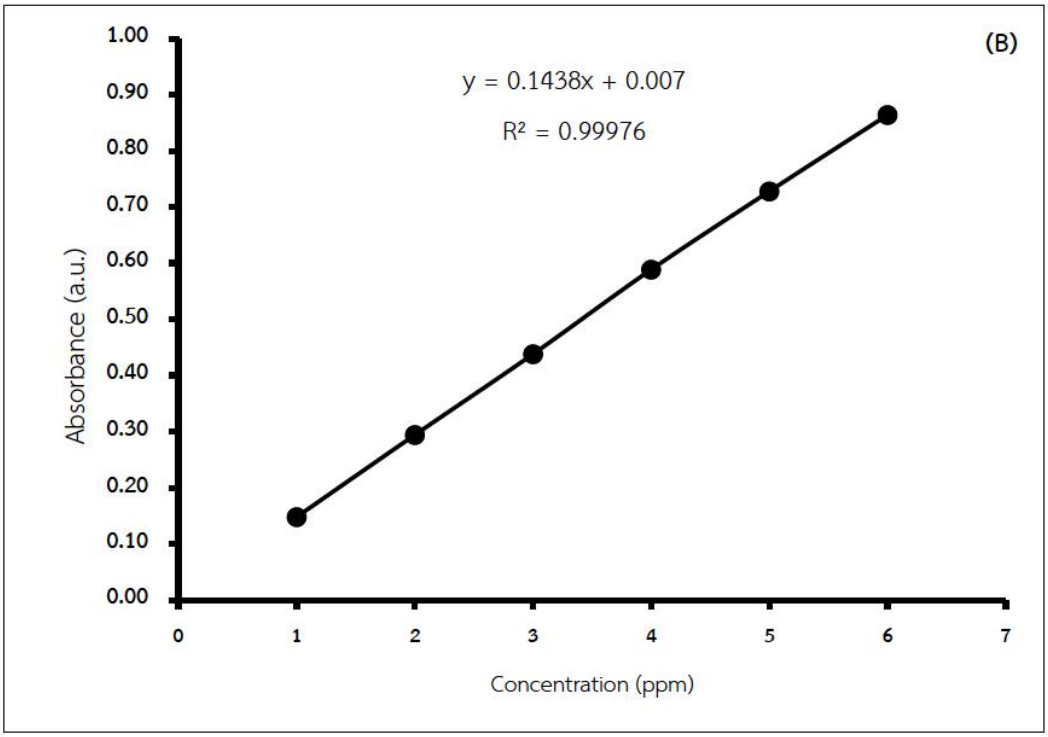


Figure 18 Calibration curve of curcumin *(A)* Spectra of curcumin with various concentration *(B)* Plot of absorbance with various of curcumin

4.4 pH sensitive and morphology

The solutions of pure Oc-EDTA-FA and Oc-EDTA-FA mixed with curcumin at pH 1.2, 4.4, 6.8 and 8.4 respectively as shown in (Figure 19). The morphologies of the Oc-EDTA-FA assembled micelles in distilled water were observed by SEM (Figure 21a). Oc-EDTA-FA contains hydrophobic regions to control the self-assembled of micelles by shielding the octadecyl as long chain hydrocarbon from distilled water to create the remained carboxylic acid residue of EDTA as hydrophilic region on the surface. In addition, the Oc-EDTA-FA could be suspension and soluble in neutral and basic conditions (Figure 19 D3-4) because at neutral and basic condition the structure of Oc-EDTA-FA donate proton and form negative charge of carboxylate (COO-) which inhibited hydrophobic interaction of long chain hydrocarbon of octadecyl. The proposed mechanism of pH dependent as shown in Figure 20. In acidic condition, Oc-EDTA-FA insoluble at pH = 1.2 (Figure 19 D1) because Oc-EDTA-FA accept proton and form positive charge of amino group which all the positive charge near the hydrophobic of folic part in Oc-EDTA-FA leading to the positive charge of amino group cannot inhibit hydrophobic interaction of long chain hydrocarbon of octadecyl. The morphologies of the Oc-EDTA-FA in acidic condition (pH=1.2) as shown in Figure 21b. Therefore, Oc-EDTA-FA can be shown pH-sensitive and acid resistance micelles.

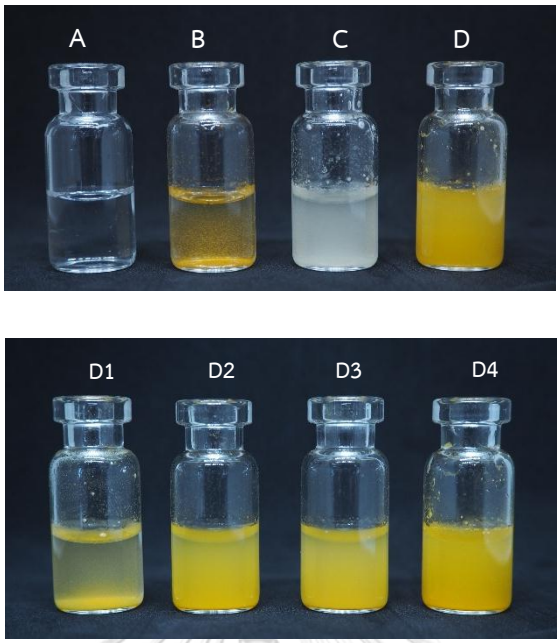


Figure 19 The solutions of (A) distilled water (B) Cur in distilled water (C) Oc-EDTA-FA in distilled water (D) Cur+Oc-EDTA-FA in distilled water and (D1-4) Oc-EDTA-FA@Cur in buffer solution pH 1.2, 4.4, 6.8 and 8.4 respectively.

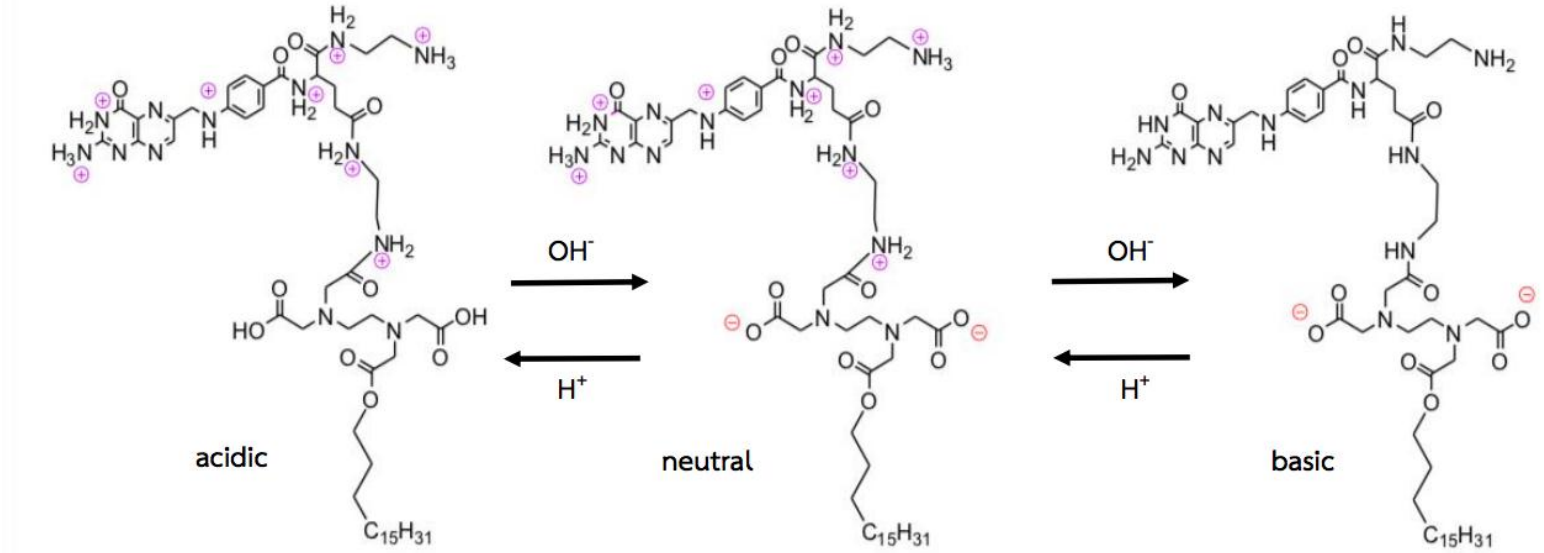


Figure 20 The structure of Oc-EDTA-FA in neutral acidic and basic conditions

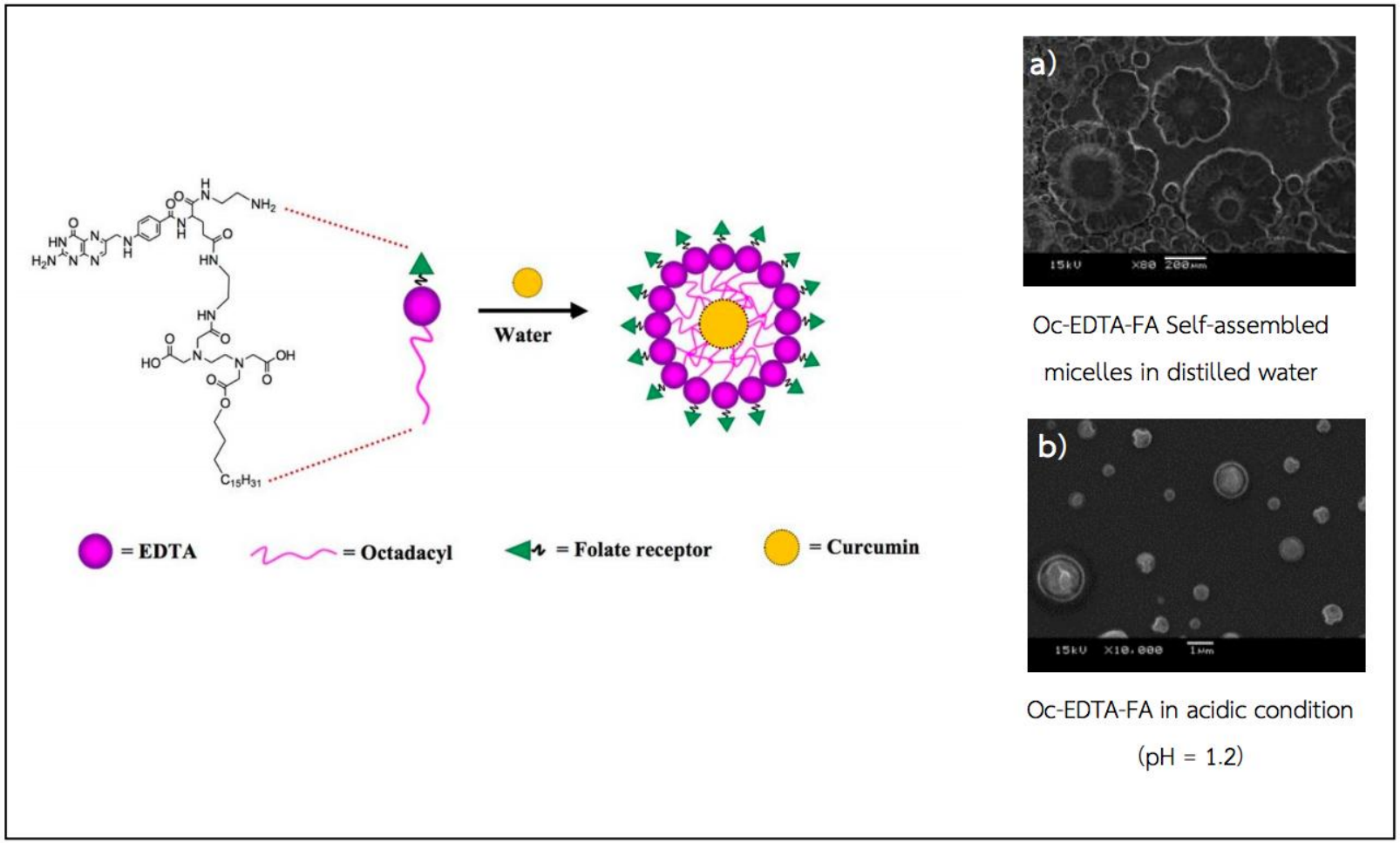


Figure 21 Design of Oc-EDTA-FA and the morphologies of Oc-EDTA-FA

4.5 Cytotoxicity assay

In this work, cytotoxicity of pure curcumin (Free Cur), Oc-EDTA-FA carrier and Oc-EDTA-FA carrier encapsulated curcumin (Oc-EDTA-FA@Cur) were studied by MTT assay. The cytotoxicity of Free Cur and synthesized products against cancer cells (KATO 3, BT 474, ChaGo, A549, KB and SW620) and the normal cells (WI-38) was shown in Table 3 and Figure 22.

Table 3 The percentage of cell death of cancer cells and normal cells tested by Free Cur, Oc-EDTA-FA and Oc-EDTA-FA@Cur at 10 μg/mL

Cells	Percentage of cell death of cancer cells and normal cells		
	Free Cur 10 μg/mL	Oc-EDTA-FA 10 μg/mL	Oc-EDTA-FA@Cur 10 μg/mL
KATO 3 (gastric)	67.12±0.71	21.24±13.01	73.22±2.12
BT 474 (breast)	74.83±1.38	8.02±5.92	85.02±1.85
ChaGo (lung)	76.87±0.32	30.91±2.49	85.94±1.21
KB	72.42±0.11	35.31±4.99	90.55±0.63
SW 620 (colon)	82.62±0.58	43.72±7.84	94.95±0.27
A549	77.91±0.99	11.76±7.04	84.3±0.93
Hep-G2 (liver)	77.72±0.33	5.38±1.48	84.33±2.63
WI-38 (normal cell)	32.18±0.50	4.24±2.87	35.41±3.22

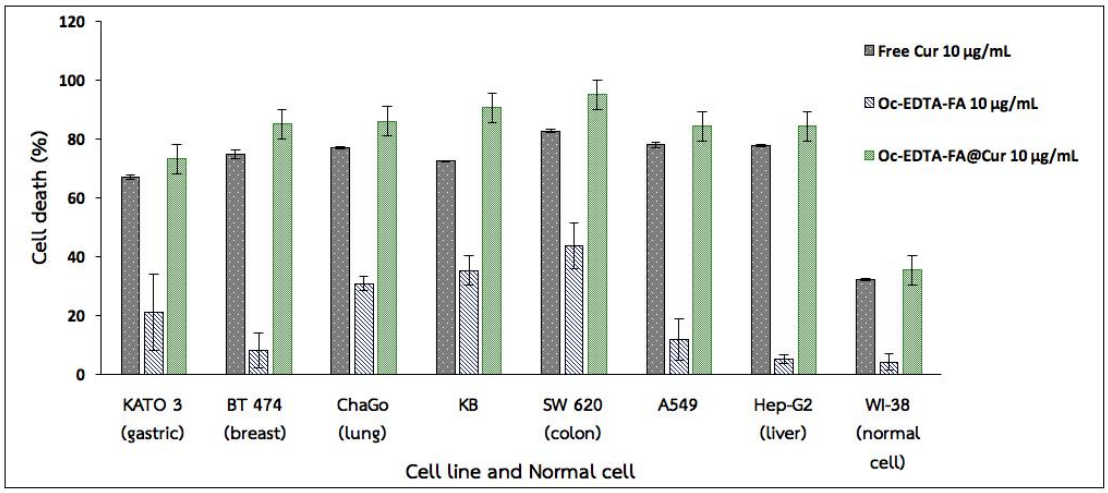


Figure 22 The percentage of cell death of cancer cells and normal cells tested by Free Cur, Oc-EDTA-FA and Oc-EDTA-FA@Cur at 10 µg/mL

The results showed that the percentage of cell death of cancer cells in the presence of Oc-EDTA-FA@Cur (green bar graph) was a little bit higher than Free Cur (gray bar graph) while Oc-EDTA-FA provided the lowest percentage of cell death which lower than 50% after 72 h incubation. In addition, Oc-EDTA-FA@Cur had the percentage of cell death of the normal cells (WI-38) lower than all cancer cell lines, suggested that Oc-EDTA-FA@Cur carrier gave more specific to cancer cells than normal cells significantly because Oc-EDTA-FA carrier contains cancer targeting molecule, FA which can bind specifically with folate receptors that overexpressed on the surface of many tumor types.

Among the cancer cell lines, Oc-EDTA-FA carrier showed the highest efficiency to the SW620 cancer cells over other cancer cells which suggested that the Oc-EDTA-FA carrier exhibited an effectiveness of cytotoxicity against cancer cells. However, it was still lower than the used of Oc-EDTA-FA@Cur. Comparing the % cells death of SW620 and WI-38, the cytotoxicity of SW620 was greater than WI-38 in the presence of Oc-EDTA-FA and Oc-EDTA-FA@Cur carrier, thus these two-synthesized materials can be used as a carrier for cancer treatment.

The study of Cur released after incubation at 6, 12, 24, 32, 48 and 72 h, showed in Table 4 and Figure 23. The results showed that, at 6, 12 and 24 h, percentage of cell death of SW620 by Free Cur was higher than Oc-EDTA-FA@Cur. The reason behind this might be due to an encapsulation of Cur in the Oc-EDTA-FA@Cur carrier which was slowly released from the carrier, resulting in low percentage of cell death when compared with Free cur. Moreover, at 32, 48 and 72 h, Cur that was encapsulated in the Oc-EDTA-FA@Cur carrier was released almost completely and showed an effectiveness a little bit higher than Free Cur. The schematic illustration of the Oc-EDTA-FA as drug delivery carrier as shown in Figure 24.

Table 4 The percentage of cell death of cancer cells (SW 620) by Free Cur, Oc-EDTA-FA and Oc-EDTA-FA@Cur at 10 μg/mL with various incubation time.

Incubation time (h)	Percentage of cell death of cancer cells and normal cells		
	Free Cur 5 μg/mL	Oc-EDTA-FA 5 μg/mL	Oc-EDTA-FA@Cur 5 μg/mL
6	39.53±0.01	4.62±0.93	22.29±1.02
12	44.70±0.87	3.01±0.86	32.51±0.53
24	64.31±0.02	6.93±1.01	51.77±0.96
32	69.08±0.70	11.20±2.59	72.18±0.59
48	75.03±0.61	11.90±2.86	79.43±1.22
72	76.10±0.43	8.24±1.23	81.07±0.48

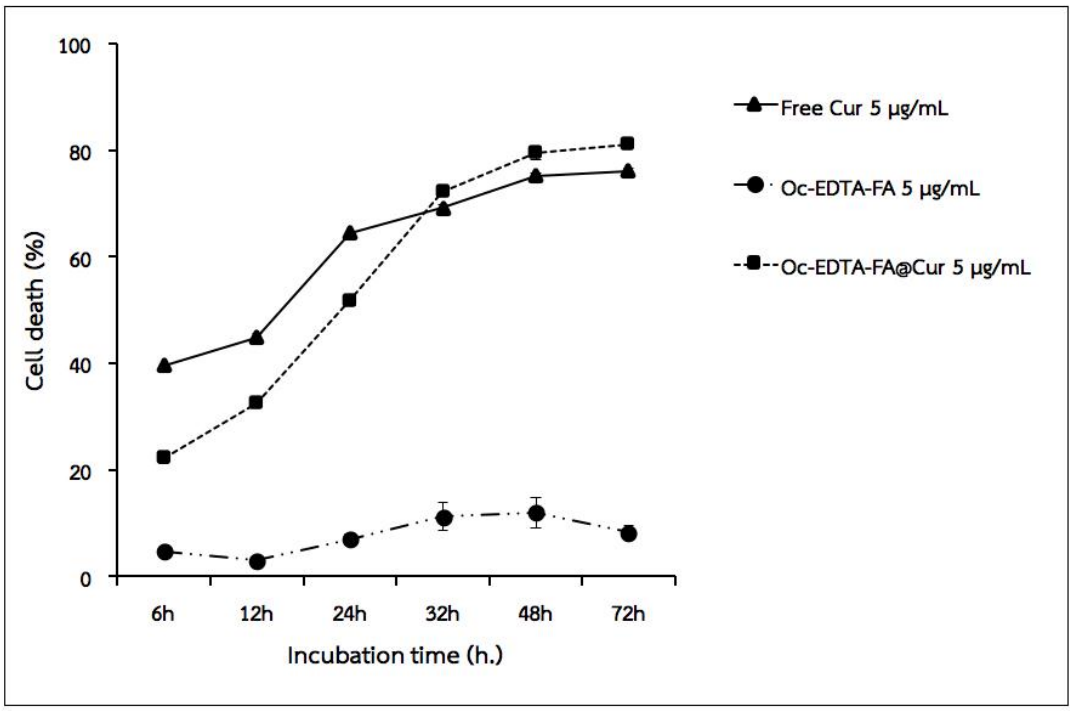


Figure 23 The percentage of cell death of cancer cells SW 620 of Free Cur, Oc-EDTA-FA and Oc-EDTA-FA@Cur with various incubation time at 5 µg/mL

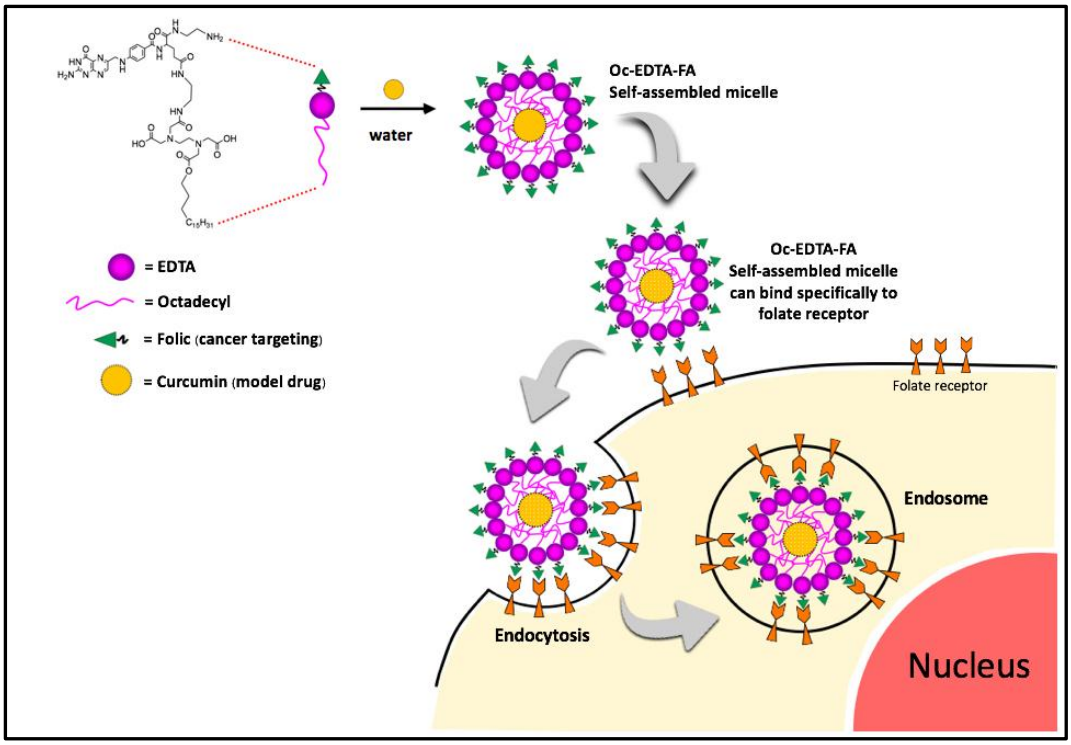


Figure 24 Schematic illustration of the Oc-EDTA-FA as drug delivery carrier.

CHAPTER V

Conclusions

The Oc-EDTA-FA was successfully synthesized by introducing the hydrophobic part (octadecyl bromide) and cancer targeting (folic acid) onto the carboxylic groups of the EDTA. ^{1}H-NMR, ^{13}C-NMR, FTIR and MS were used to confirm the chemical structure of the obtained Oc-EDTA-FA. Self-assembled Oc-EDTA-FA micelles could be easily preparing by re-dispersion in distilled water and they were pH-sensitive and acid resistance micelles. Oc-EDTA-FA carrier exhibited an effectiveness of cytotoxicity against cancer cells which showed the highest efficiency to the SW620 cancer cells over other cancer cells. The Oc-EDTA-FA@Cur carrier gave more specific to cancer cells than normal cells significantly. Therefore, the Oc-EDTA-FA self-assembled micelles were suitable to use in the application of drug delivery carrier.

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APPENDICES

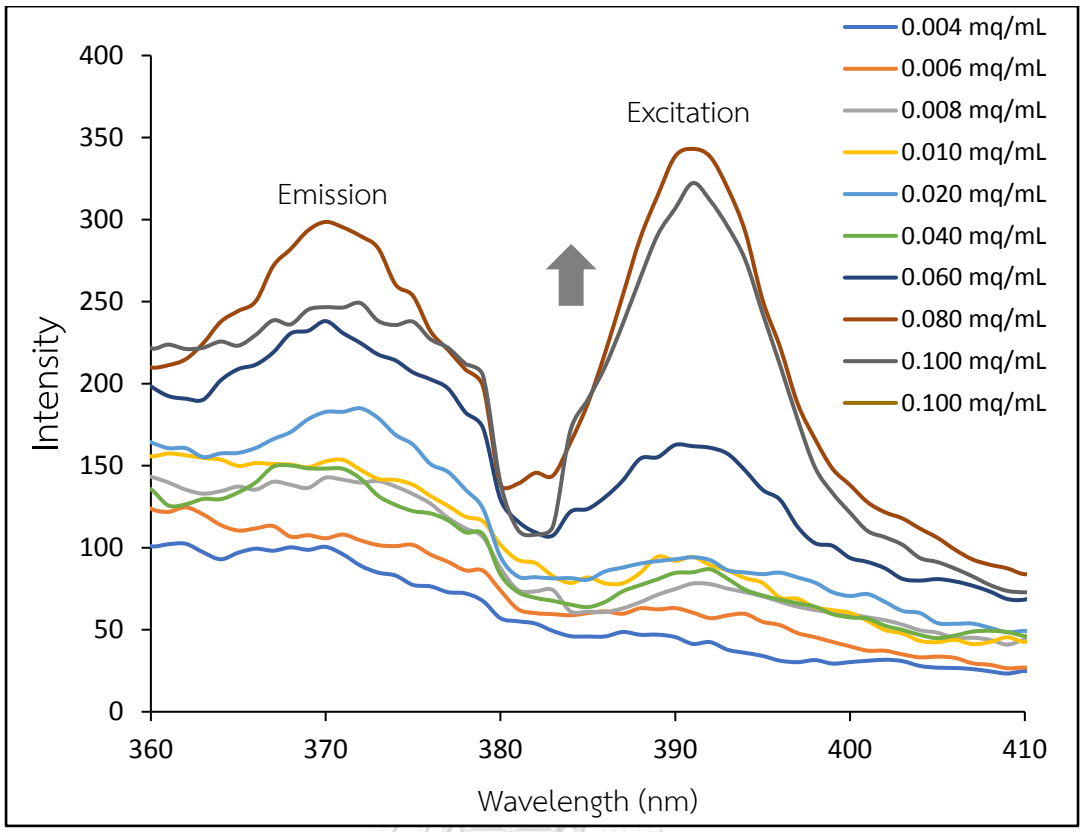

Figure A1 the excitation (391 nm) and emission (371 nm) of pyrene by fluorescence technique

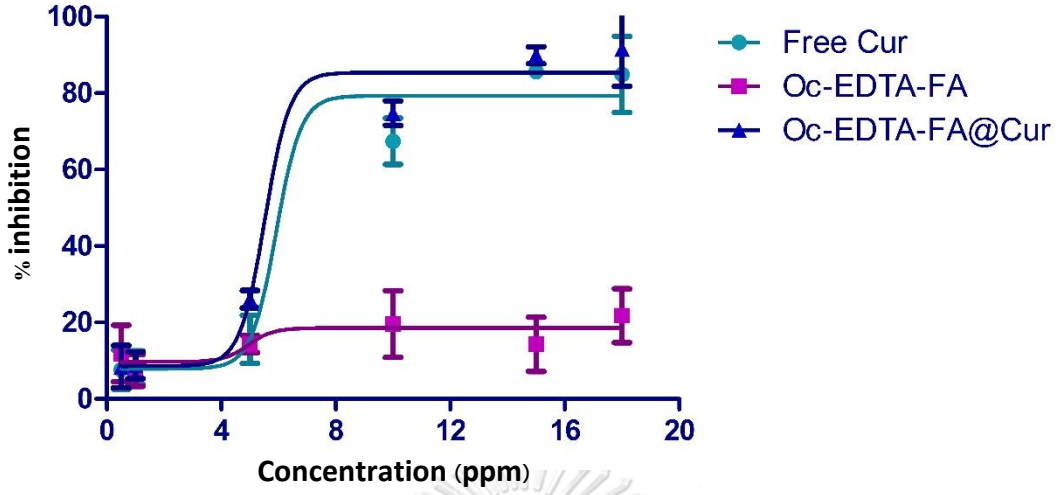

Figure A2 nonlinear regression of "log(inhibitor) versus response": Y = Bottom + (Top-Bottom)/ (1 + 10^((X-LogIC_{50}))) of A549 cancer cell.

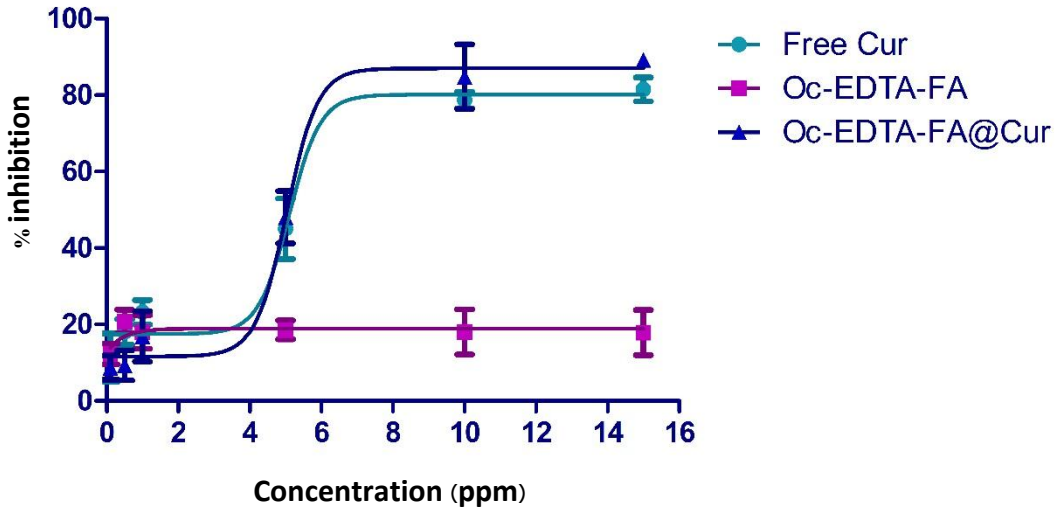

Figure A3 nonlinear regression of "log(inhibitor) versus response": Y = Bottom + (Top-Bottom)/ (1 + 10^((X-LogIC_{50}))) of BT474 cancer cell.

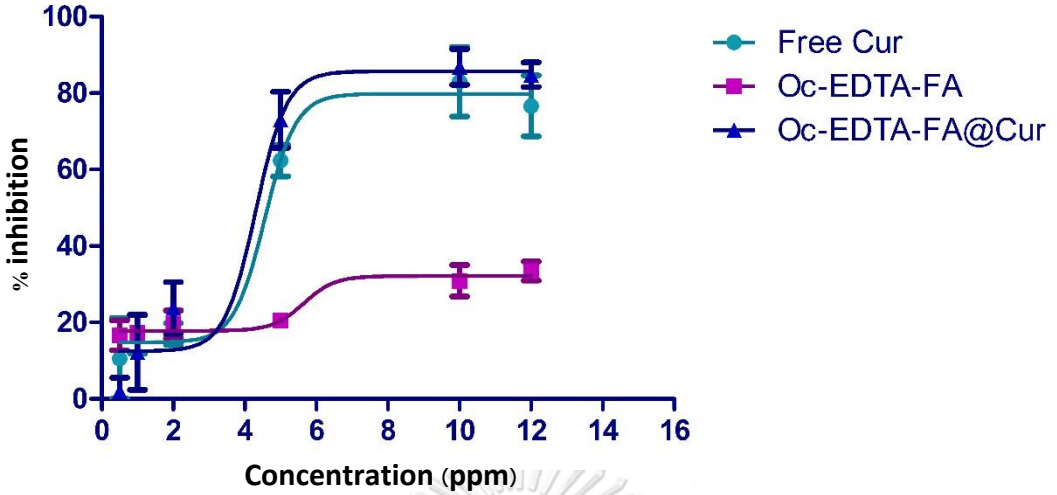

Figure A4 nonlinear regression of "log(inhibitor) versus response": Y = Bottom + (Top-Bottom)/ (1 + 10^((X-LogIC_{50}))) of ChaGo cancer cell.

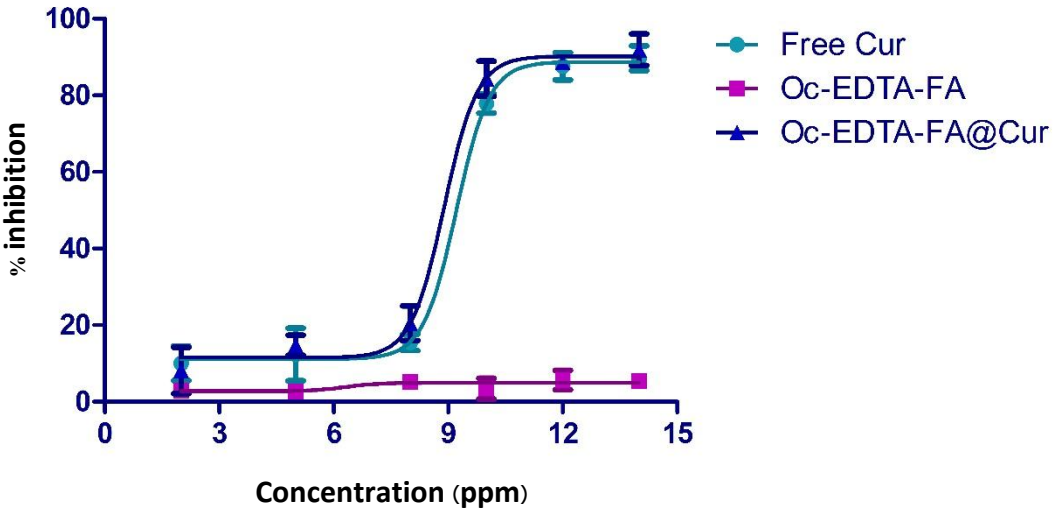

Figure A5 nonlinear regression of "log(inhibitor) versus response": Y = Bottom + (Top-Bottom)/ (1 + 10^((X-LogIC_{50}))) of Hep-G2 cancer cell.

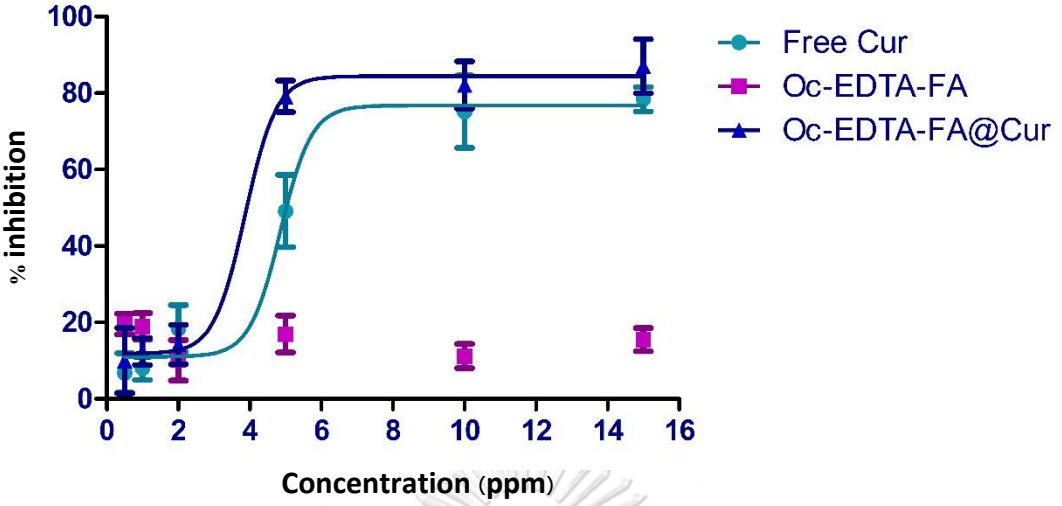

Figure A6 nonlinear regression of "log(inhibitor) versus response": Y = Bottom + (Top-Bottom)/ (1 + 10^((X-LogIC$_{50}$))) of KATO 3 cancer cell.

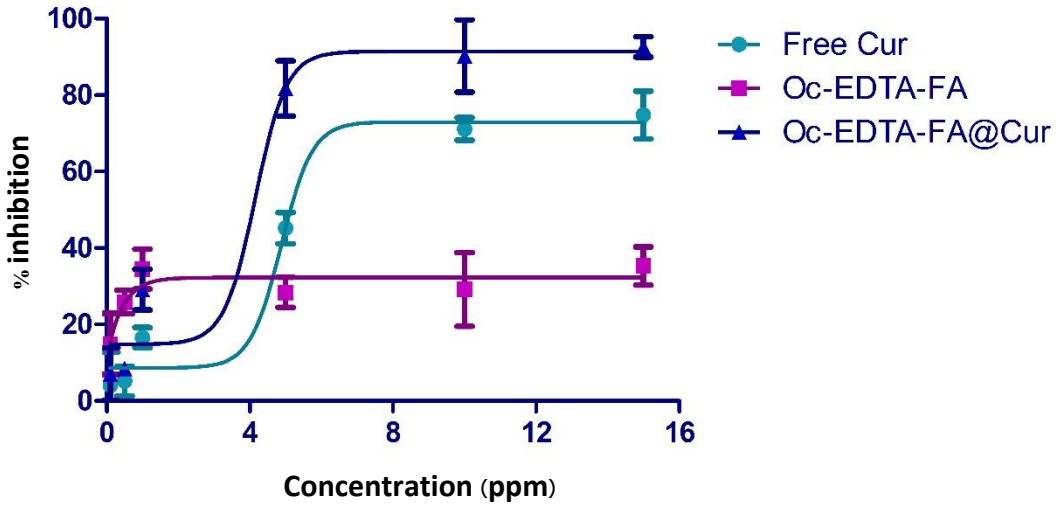

Figure A7 nonlinear regression of "log(inhibitor) versus response": Y = Bottom + (Top-Bottom)/ (1 + 10^((X-LogIC$_{50}$))) of KB cancer cell.

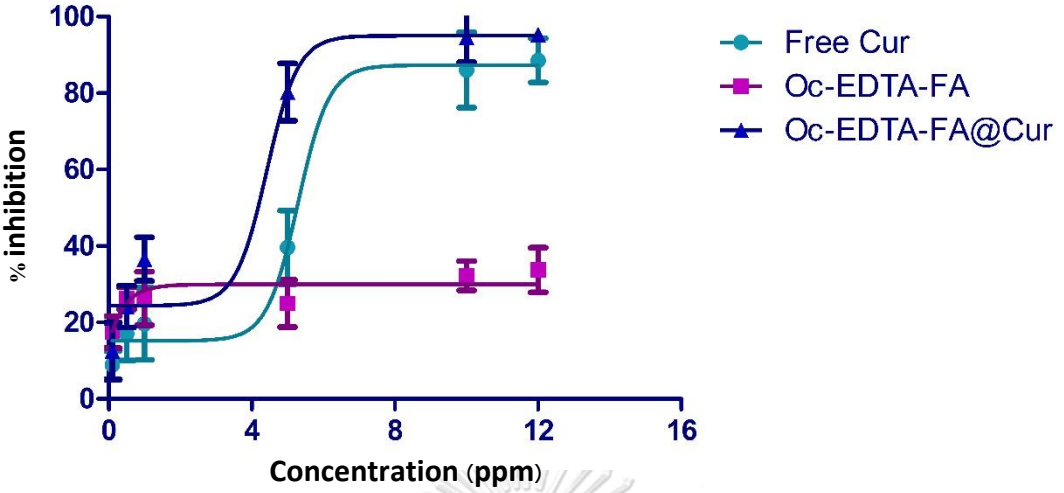

Figure A8 nonlinear regression of "log(inhibitor) versus response": Y = Bottom + (Top-Bottom)/ (1 + 10^((X-LogIC$_{50}$))) of SW620 cancer cell.

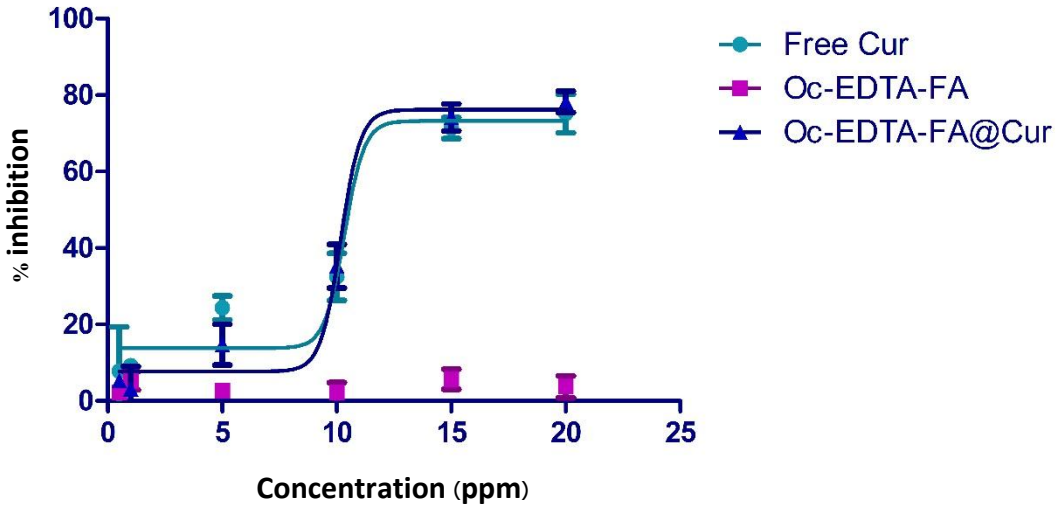

Figure A9 nonlinear regression of "log(inhibitor) versus response": Y = Bottom + (Top-Bottom)/ (1 + 10^((X-LogIC$_{50}$))) of WI-38 normal cell.

VITA

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