

รายงานวิจัยฉบับสมบูรณ์

เรื่อง

การสังเคราะห์สาร monoacylglycerol และศึกษาวิธีการที่เหมาะสม
ในการนำไปใช้เป็นองค์ประกอบของอนุภาคนาโน

SYNTHESIS OF MONOACYLGLYCEROL AND USE FOR
ELABORATION OF NANOPARTICLE

โดย

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บทคัดย่อ

การศึกษานี้ได้เลือก monomyristin ซึ่งอยู่ในกลุ่มของโมโนเอซิลกลีเซอรอล ซึ่งมีโครงสร้างไม่ซับซ้อน และมีฤทธิ์ด้านเชื้อแบคทีเรียเป็นตัวศึกษา โดยทำการสังเคราะห์ monomyristin ด้วยวิธีทางเคมี และใช้เป็นต้นแบบของยาในการศึกษาสภาวะที่เหมาะสมในการเตรียมอนุภาคนาโน โดยใช้วิธีเตรียมอนุภาค 2 วิธี คือ emulsion/solvent evaporation และ nanoprecipitation ผลการทดลองพบว่า สภาวะที่ดีที่สุดในการเตรียมอนุภาคนาโนที่บรรจุ monomyristin โดยมี dextran ห่อหุ้มและมี DexC10₁₆₄ เป็นองค์ประกอบภายในแกนของอนุภาคนาโนคือ ภาวะเมื่อใช้ DexC10₁₆₄ และ DexP₂₀ ซึ่งทำหน้าที่เป็น stabilizer ในความเข้มข้นที่สูง โดยทำให้เกิดอนุภาคนาโนที่มีขนาดประมาณ 200 นาโนเมตรและไม่มีการเกาะกลุ่มกัน นอกจากนี้ยังได้ทำการทดสอบความเป็นพิษของ monomyristin ต่อเซลล์ปกติ ซึ่งผลการทดลองที่ได้พบว่า monomyristin ไม่มีความเป็นพิษต่อเซลล์ปกติ



Abstract

In this study, monomyristin (myristyl glycerol) which was reported on exhibiting antibacterial activity was chosen as a monoacylglycerol model to be chemically synthesized and used as drug to be encapsulated into a nanoparticle. Two preparation methods; emulsion/solvent evaporation and nanoprecipitation, were used to encapsulate monomyristin in dextran-coated nanoparticles with DexC10₁₆₄ as a core. The best condition was obtained when high concentration of DexC10₁₆₄ and DexP₂₀ were used as a stabilizer. The nanoparticle obtained was in 200 nm particle size without macroscopic aggregation. Moreover, the cytotoxicity of monomyristin and the results showed no cytotoxicity with normal cells.



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CHAPTER I

INTRODUCTION

Monoacylglycerols (MAGs) are amphiphilic molecules which exhibit the surfactant activity and belong to glycolipid biosurfactants. In 2004, Thanomsab and colleagues isolated *Candida ishiwadae*, a thermotolerant yeast from plant material in Thailand. This microorganism produced biosurfactants which exhibited surfactant activity higher than chemical surfactants such as SDS and Tween 80. Their structures were elucidated as oleoylglycerol and linoleoylglycerol and classified as monoacylglycerols.⁽¹⁾ Moreover, there are many reports which show the biological activity of monoacylglycerol.⁽²⁾ As a biosurfactant, MAGs is potentially applicable in drug delivery system by its low toxicity and biodegradability properties.

The amphipathic compound renders the self assembly and development of nanoparticles. Therefore nanoparticles can be used in drug delivery system as a vehicle to encapsulate drugs, polypeptides, proteins, vaccines, nucleic acids, genes and others which aim to deliver the substance to the target site.⁽³⁾

So far, liposome is a main encapsulated drug carrier.⁽⁴⁻⁶⁾ Surfactants or non-ionic surfactants named as niosome have also been used to develop as carriers.⁽⁷⁾ Nevertheless, very few of biosurfactant have been used as carrier. A report on mannosylerythritol lipids A (MEL-A), a biosurfactant produced by *Candida antarctica* was used as plasmid DNA carrier, namely MEL-liposome. MEL-liposome (MEL-L) comprised 3 β -[N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), dioleoyl phosphatidylethanolamine (DOPE) and mannosylerythritol lipids A (MEL-A). The MEL-L/plasmid DNA complex (MEL-lipoplex) was investigated in the efficiency of transfection in human cervix carcinoma Hela cells. The result showed that MEL-A induced a significantly higher level of gene expression.⁽⁸⁾

Cancer is an important disease which encounter with difficulty in treatment due to the problem of drug efficacy and side effect to the normal cell. Therefore, the drug delivery system is the advantage to use for cancer treatment by enhancing the specificity to the cancer cells and prolong drug half life.

In conclusion, not only the property of monoacylglycerols as amphiphilic structure to encapsulate the drug in drug delivery system application but also their biological activity make them very interesting to be applied in pharmaceuticals.

CHAPTER II

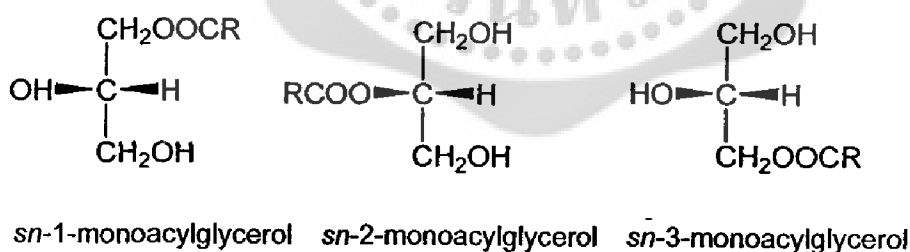
REVIEW LITERATURE

1. Monoacylglycerol

Monoacylglycerols (MAGs) (or monoglyceride) are mono- fatty acid esterified with only one of the hydroxyl group glycerol, and they can exist in three stereochemical forms (Figure 1). The terms of 1- and 3-isomers are alike, as α -monoacylglycerols, because they are not distinguished from each other, while the 2-isomers are β -monoacylglycerols.

1- and 3-Monoacylglycerols tend to be minor components only of most plant and animal tissues. They show strong surfactant properties as detergent which would not be expected to accumulate and would have a disruptive effect on membranes. In commercial, this monoacylglycerol are synthesized and used as constituents of detergent which are recently important..⁽⁸⁾

2-Monoacylglycerols are usually as a major end product of the intestinal digestion of dietary fats in animals by pancreatic lipase enzyme. Before being transported in lymph to the liver, they can be taken up directly by the intestinal cells and converted to triacylglycerols by monoacylglycerol pathway..⁽⁸⁾



(R = hydrocarbon chains of fatty acids)

Figure 1 Structure of monoacylglycerol⁽⁸⁾

1.1 Biological activity of monoacylglycerol

Monoacylglycerols (MAGs) are used in food and pharmaceutical industries. In addition to the emulsifying properties, they exhibit inhibitory effects against some types of microorganisms. This property makes them able to be used in various fields of food industry.⁽²⁾

Kabara and colleague, 1977 reported that the number of carbon atom and double bond in their hydrocarbon chains presenting on MAGs, are somewhat potent to the antimicrobial activity of them.⁽⁹⁾

In many research works, it was proved that monoacylglycerols have a broad spectrum of microbicidal activity. They inhibited various bacteria such as group A *Streptococci* (GAS), group B *Streptococci* (GBS),⁽¹⁰⁾ *Staphylococcus aureus*,⁽¹¹⁾ *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Helicobacter pylori*.⁽¹²⁾ In addition, monoacylglycerols were reported to inhibit growth of yeast, *Candida albicans*,⁽¹³⁾ and herpes simplex virus (HSV).^(14,15) The antimicrobial effects of these monoacylglycerols are summarized in table 1.

Table 1 The antimicrobial effects of monoacylglycerols

Monoacylglycerol	Structure	Inhibited microbes
Monocaprin (C10:0)	Capric acid esterified with glycerol	- group A <i>Streptococci</i> (GAS), group B <i>Streptococci</i> (GBS), <i>Candida albicans</i> ^(10,13) - <i>Neisseria gonorrhoeae</i> ⁽¹⁶⁾ , <i>Chlamydia trachomatis</i> ⁽¹⁷⁾ - <i>Helicobacter pylori</i> ⁽¹⁸⁾ -Herpes simplex virus (HSV), vesicular stomatitis virus ⁽¹⁴⁾ , human immunodeficiency virus (HIV) ⁽¹⁵⁾
Monolaurin (C12:0)	Monolauric acid esterified with glycerol	- <i>Staphylococcus aureus</i> ⁽¹¹⁾ - <i>Neisseria gonorrhoeae</i> ⁽¹⁶⁾ - <i>Chlamydia trachomatis</i> ⁽¹⁷⁾ - <i>Helicobacter pylori</i> ⁽¹⁸⁾ - <i>Candida albicans</i> ⁽¹³⁾
Monomyristin (C14:0)	Myristic acid esterified with glycerol	- <i>Helicobacter pylori</i> ^(12,17)

In addition to antimicrobial activity, monoacylglycerols have been reported on antileukemic property by showing ability to induce apoptosis (programmed cell death) of murine leukemic cell.⁽¹⁹⁾ The unsaturated MAGs, 1-C16:1, 1-C18:1, and 1-C18:2 MAG were the best

inducers of murine leukemic cell death. However, some saturated MAGs such as 1-C14:0 MAG and 1-C16:0 MAGs were also able to induce cell death which the others (1-C10:0 MAG, 1-C12:0 MAG, 1-C18:0, and 1-C8:0 MAG) were inactive.⁽¹⁹⁾

In 2003, Philippoussis and colleagues found that monoacylglycerols had cytotoxicity effect against malignant but not normal human cells. 1-monooleoylglycerol (1-C18:1), 1-monopalmitoleyl glycerol (1-C16:1) and 3-monopalmitoyl glycerol (3-C16:0) were able to induce cell death in human leukemic cell lines by which 1-monooleoylglycerol is the most effective.⁽²⁰⁾

In 2008, Barta et al. studied the effect of monoacylglycerol on activity and expression of P-glycoprotein (P-gp) in Caco-2 cells, a human colon adenocarcinoma cell line. The results from this study suggested that non-cytotoxic concentrations of 1-monoolein and 1-monostearin are inhibitors of P-gp in Caco-2 cells, a human colon adenocarcinoma cell line.⁽²¹⁾

2. Drug delivery systems

Drug delivery system means the process bringing drug to the target site by a vehicle, including the method of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals. The goal of drug delivery system is to carry the medication intact to the specific target in the body to avoid toxicity to normal cells. To achieve this goal, researchers are turning to advance in micro- and nanotechnology,⁽²²⁾ especially, the nanotechnology which is trying to adapt the system of drug vehicle (drug delivery system) in the form of particle in nanometer size which is called as nanoparticle. The drug encapsulated nanoparticle is advantage for drug targeting because of the alteration in the drug's biodistribution favors drug accumulation at the desired site. The improvement to pass of the drug across various barriers may allow alternative administration routes.⁽²³⁾

For the drug release, some kinds of nanoparticle are gastric-resistant dosage forms which drug release occurred mainly in the colon. They cannot release in the gastric part.⁽²⁴⁾

The development of drug vehicles had begun many years ago. The vehicles were classified into three generations.⁽²⁵⁾ The first generation vectors are liposomes and nanoparticles (nanospheres and nanocapsules).

The liposome was the first drug vehicle which has been developed forty years ago.⁽²⁶⁾ It is small spherical vesicle constituted by phospholipids bilayers and aqueous phase in central cavity. They can carry the active compound by dissolving the hydrophilic compound in the aqueous phase and the hydrophobic in the bilayer. The liposomes have a good biocompatibility because of their raw materials composition such as natural phospholipids, sterols, glycerolipid.

However, these vesicular structures are unstable in chemical (oxidation and hydrolysis of phospholipids) and physical (aggregation, fusion and loss of contents) environments.⁽²⁶⁾

The nanospheres are spherical polymeric matrix which were first developed in 1976 by Birrenbach and Speiser⁽²⁷⁾ using the cross-linked polyacrylamide, a non-biodegradable polymer. Thereafter, the system (bio) degradable based PACA, were developed by Couvreur and colleague, 1979.⁽²⁸⁾ The active ingredient is either incorporated within the polymer network but not chemically bonded to the matrix during the formation of nanospheres, or adsorbed on their surface through hydrophobic-type bonds, electrostatic or covalent. It is released by simple diffusion or as a result of the (bio) degradation of the polymer in the body.

The nanocapsules are spherical structures generally consisting of an oil core surrounded by a thin wall polymer. The active ingredient is usually dissolved in the oil core, but can also be adsorbed on the surface of the nanocapsules.⁽²⁹⁾

Hydrophobic polymer such as aliphatic polyesters, are generally hydrophobic and a negative surface charge. After intravenous administration, the opsonins, cationic plasma proteins, adsorb rapidly to the surface of these particles which facilitates their capture by the organs of mononuclear phagocyte system (MPS) such as the liver, spleen, kidney and bone marrow.⁽³⁰⁻³¹⁾ The particles are then internalized by endocytosis to reach in lysosomes where they can potentially be degraded by the lysosomal enzyme. The drug is released primarily in the cells of the liver, spleen and kidney. This accumulation, mainly liver, can be an advantage to improve the treatment of certain infections of MPS.⁽³²⁻³³⁾ The degree of hydrophobicity of the particles influence the quantity and composition of plasma proteins adsorbed on the surface of particles.⁽³⁴⁻³⁵⁾ So, the modification of the hydrophobic surface of the particles for reducing their accumulation in the liver and extend their time in the bloodstream. Surface hydrophilisation is one effective way to minimize both the hydrophobic and electrostatic interactions between proteins and particle. The hydrophilic layer can act as a steric barrier that hides the hydrophobicity and surface charge,⁽³⁶⁾ and renders the particles hydrophobic "invisible" MPS ("stealth" effect).⁽³⁷⁾ This development of particles hydrophobic "invisible" MPS is the second generation which can extend the time of drugs movement in blood, and control their release. However, specific targeting of drugs remains elusive; so, the third generation vectors are occurred. This is an advantage especially for cancer treatment which is necessary to use the relatively toxic drugs and to monitor the quantity administered. The supramolecular structure-ligands (antibodies, peptides, sugars, folic acid) are attached to the surface of vectors⁽³⁸⁾ which bind selectively the specific markers (antigens or receptors) that are hyperexpressed on the surface of target cells (cancer cells, infected cells, etc.).

Recently, Suzawa et al., 2002 reported of using an adriamycin-conjugated poly(ethylene glycol) linked with enzymatically cleavable peptide sequences (alanyl-valine, alanyl-proline, and glycyl-proline). The result showed a greater selectivity cleavage at tumor cells.⁽³⁹⁾

2.1 Type of drug delivery vehicle⁽⁴⁰⁾

2.1.1 The liposome group

2.1.1.1 Liposome

The structure of liposome is a phospholipids bilayer and cholesterol. The cholesterol is used for increasing stability because of raising bilayer rigidity. They sizes are about 50 nm to several micrometers.

2.1.1.2 Virosomes

Virosomes consist viral phospholipids and viral membrane proteins (Hemagglutinin, HA). HA acts as a targeting device.

2.1.1.3 Transfersomes

The components of this vehicle are phospholipids (phosphatidylcholine, PC) and cholate (9:2 molar ratios). This type of vehicle performs better than the classical liposome after epicutaneous administration.

2.1.1.4 Archaeosomes

The compositions of this vehicle type are the lipid parts in the archaeobacteria membrane. The archaeosomes show the physical and chemical stability properties.

2.1.1.5 Niosomes

They are non-phospholipids liposomes which are the combination of non-ionic surfactants and cholesterol forming as small unilamellar vesicle. The chemical stability of this vehicle is the same as archaeosomes but is higher than phospholipids liposome.

2.1.1.6 Cochleate

The structure is phospholipids (phosphatidylethanolamine), negatively charged phosphatidylserine and cholesterol, intercalating with calcium ions between bilayers. Its appearance is non-vesicular bilayer sheets.

2.1.1.7 Proteosomes

The main structures of proteosomes consist of protein from outer membrane of bacteria. The average size of the vesicles is about 100 nm.

2.1.2 ISCOMs (Immunostimulating complex)

The components of this vehicle are saponin (Quil A), cholesterol and phospholipids (PE) by ratio 4:1:1 (Quil A:cholesterol: phospholipids(PE)) . Its shape is spherical, micellar assemblies of about 40 nm.

2.1.3 Emulsions

The examples of this vehicle are MF59 (o/w), montanide (w/o), Freund's adjuvant (w/o) and emulsomes.

- MF59 emulsion consists of Tween 80, SPAN 85 and squalene.
- Montanide emulsion is a mixture of oil and water which combined with a specific antigen to boost the immune response to that antigen.⁽⁴¹⁾
- Freund's adjuvant is a water-in-oil emulsion consisting of a mineral oil, an antibody stimulator such as *tubercle bacilli*, and an emulsifying agent such as lanolin or Arlacel A.⁽¹³⁵⁾

- Emulsome's structure is a solid fat core surrounded by phospholipids bilayers. They are nanoemulsions and physically considered as between oil in water emulsion and liposome.⁽⁴¹⁾

2.1.4 Microparticle and Nanoparticle

The examples of this carrier are poly(lactic-co-glycolic acid)(PLGA), chitosan, alginate, etc..⁽⁴¹⁾

Due to the small size, nanoparticles are suitable for intravenous administration. On the contrary, the microparticles which are larger size are used via alternative routes.⁽²³⁾

2.1.5 Inorganic insoluble salt

Aluminium phosphate, Aluminium hydroxide and calcium phosphate are classified in this group.⁽⁴¹⁾

2.2 Advantages of using nanoparticle in drug delivery system

2.2.1 To prolong the action of drugs or biomolecules: The particle size is ultra fine, so they can pass through the smallest capillary vessels and these particles promote avoidance the rapid clearance by phagocytes, therefore their duration in blood stream is greatly prolonged.

2.2.2 To arrive at the specific target: They can penetrate the gap of the cells and tissue to arrive target organs such as liver, spleen, lung, spinal cord and lymph.

2.2.3 To be able of controlled-release properties: The biodegradability, pH, ion and/or temperature sensitivity of materials are properties of these particles which are the advantages to control the release of the inner molecule.

2.2.4 To reduce drug toxicity: They can affect cell and organ specifically, so the utility of the drugs can be improved and reduce toxic side effects.⁽³⁾

As drug delivery system, the drugs or biomolecules can be entrapped into their interior structures and/or absorbs drugs or biomolecules onto their exterior surfaces of the nanoparticle. Recently, nanoparticles have been widely used to deliver drugs, genes, vaccines, polypeptides, proteins, nucleic acids, etc..⁽⁴²⁾

2.3 Nanoparticle delivery system in pharmaceuticals

Over the years, nanoparticle drug delivery systems are very potential in biological, medical and pharmaceutical applications.⁽⁴¹⁾ Currently, the direction to study nanoparticle drug delivery is

- To find and combine of carrier which produce the best drug release rate profile.
- To modify the surface of nanoparticle in order to improve their targeting ability
- To optimize the preparation of nanoparticle to receive the better drug delivery capacity
- To develop the nanoparticle for application in clinics and the possibility of industrial production
- Investigate *in vivo* dynamic process to reveal the nanoparticle interaction with blood and targeting tissues and organs, etc..⁽⁴²⁾

In 1998, a nanoparticle formulation of ciprofloxacin-loaded polyethylbutylcyanoacrylate (PEBCA) was investigated and optimized. Ciprofloxacin with nanoparticles was performed by emulsion polymerization. The ciprofloxacin nanoparticle suspension was stable when preparing only with acetone in the polymerization medium.⁽⁴³⁾

Chan and colleague in 2009 prepared core-shell nanoparticle which consists of a poly (D,L-lactide-co-glycolide) hydrophobic core, a soybean lectin monolayer and a poly(ethylene glycol) shell. They tried to use this vehicle to carry a model chemotherapy drug, docetaxel. The process to prepare nanoparticle is a modified nanoprecipitation method and self-assembly.⁽⁴⁴⁾

Tacrine was the first acetylcholinesterase inhibitor licensed for treatment of Alzheimer's disease. It was encapsulated by using magnetic chitosan microparticles which were prepared by emulsion cross-linking. *In vivo* study on evaluating the concentration of the tacrine at the target and non-target organ was investigated. The result is the tacrine concentration was significantly increased in brain with magnetic chitosan microparticle comparing with free drug.⁽⁴⁵⁾

The delivery of insulin was investigated by Singh and Chauhan in 2009 by preparing psyllium polysaccharide and methacrylamide based hydrogels with *N, N'*-methylenebisacrylamide as crosslinker and evaluated the mechanism and drug release.⁽⁴⁶⁾

For antibacterial therapy, drug carrier was used to control antibiotic drugs efficacy by hiding and protecting the molecule from degradation and delivering it to accessible target cells. Liposomes and nanoparticles are the main carriers developed for these logistic targeting strategies.⁽⁴⁶⁾ So, there are many researchers who studied the potential of liposomes and nanoparticles in treatment of bacterial infections. Vladimirovsky and coworkers studied on streptomycin liposomes treated mice infected with *Mycobacterium tuberculosis*. The result showed a significant inhibition of bacterial growth in the liver and spleen and prolongation the survival of mice.⁽⁴⁶⁾

For vaccine delivery, combination of heat-inactivated influenza virus with LTR192G adjuvant (a whole virus influenza vaccine) was incorporated in Amioca[®] starch / poly (acrylic acid) powder and nasally administered to rabbits. Different ratio of powder formulations based on spray-dried mixtures of Amioca[®] / poly(acrylic acid) (Carbopol[®] 974P) was investigated and tested *in vivo* with a rabbit model. Individual rabbit sera were tested against hemagglutinin (HA), the major surface antigen of influenza. From this study, the carriers facilitated the induction of systemic anti-HA antibody response after intranasal vaccination with a whole virus influenza vaccine.⁽⁴⁷⁾

In gene delivery, chitosan is used but the chitosan-based complexes may lead to difficulties in DNA release when they arrive at the target site. Therefore in 2009, Peng and colleague, reported to use a negatively charged poly(γ -glutamic acid) incorporated in chitosan/DNA complex nanoparticles. They found a significant increase in its transfection efficiency and enhanced the cellular uptake. The nanoparticles containing chitosan, DNA and poly(γ -glutamic acid) were examined the characteristics of the compound by dynamic light scattering (DLS), transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS). Fluorescence and luminance spectrometry and flow cytometry were used to evaluate the potential of gene expression and transfection efficiency of tested nanoparticles. The internalization efficiency was examined using a confocal laser scanning microscope (CLSM) and a flow cytometer.⁽⁴⁸⁾

For the pharmaceuticals, glycolipid biosurfactant, mannosylerythritol lipids-A (MELs-A) produced by yeast, *Candida antarctica* was demonstrated to dramatically increase the efficiency of gene transfection which mediated by liposomes with a cationic cholesterol derivative.⁽⁴⁹⁾ Recently, the cationic liposome bearing MEL has been shown to increase the efficiency of gene transfection into mammalian cells dramatically.⁽⁸⁾

3. Nanoparticle preparation

3.1 Methods of nanoparticle preparation

The techniques used to prepare nanoparticles are numerous. They are generally classified into two groups. In the first group, the nanoparticles are formed by polymerization of monomers in emulsion. The drug loaded nanoparticles can be obtained by incorporation of active ingredients during or after the preparation of the polymer dispersion. In the other group, nanoparticles are prepared from preformed polymers. These polymers can be soluble or insoluble in water, and they may be synthetic, semi-synthetic or natural origin.

The choice of preparation method of polymer particles and the constituents depend on the physicochemical properties of the active ingredient, the desired characteristics of drug release, purpose of therapy, and route of administration.⁽³³⁾ The method chosen should allow the proper encapsulation with excellent performance and should be potentially applicable to an industrial point of view.

3.1.1 Preparation of nanoparticles by polymerization of monomers

Emulsion polymerization has been developed to limit the use of toxic organic solvents that are used during production of the polymer. The method is to disperse monomer in an aqueous medium containing a surfactant with a high shear. The monomer droplets are formed by hydrophilic initiator and stabilized by the surfactant.

3.1.2.1 Emulsion/solvent evaporation

In 1981, the emulsion/solvent evaporation method was begun by Gurny et al.⁽⁵⁰⁾ by preparing of PLA nanospheres. At first, the chosen polymer to form the nanoparticles is dissolved in an organic solvent immiscible in water (dichloromethane, chloroform, or ethyl acetate).⁽³⁵⁾ Then, this mixture is emulsified in an aqueous phase containing a stabilizer (sodium cholate, poly (vinyl alcohol) (PVA)) by strong shearing or sonication to form a stable O/W emulsion.⁽⁵¹⁻⁵²⁾ The organic solvent is then evaporated by raising the temperature or pressure, or simply shaking at room temperature. This method allows the encapsulation of hydrophobic active ingredient by simple addition of the compound in the polymer solution. After evaporation of organic solvent, the drug is encapsulated within the polymer matrix.⁽⁵³⁾

In the case of a hydrophilic active ingredient, this active compound must be dispersed in an aqueous solution which is inside the organic phase containing the polymer (W/O). Moreover, this emulsion is itself dispersed in an external aqueous phase to form a double emulsion W/O/W.⁽⁵²⁾ After evaporation of the organic solvent, nanospheres containing

the hydrosoluble active ingredient are formed. This technique has been used to encapsulate bovine serum albumin (BSA)⁽⁵²⁾ or erythropoietin.⁽⁵⁴⁾

3.1.2.2. Nanoprecipitation

The process of nanoprecipitation was first described by Fessi et al. in 1986.⁽⁵⁵⁾ This requires the use of a polar organic phase containing the polymer and the organosoluble active ingredient. The organic phase must be an organic solvent miscible with water such as acetone.⁽⁵⁶⁾ This polymer solution is emulsified in an aqueous phase (the surfactant is optional) to form droplets. The spontaneous diffusion of the solvent in the aqueous phase leads to precipitation of the polymer and the formation of solid nanoparticles.

This process allows an excellent incorporation of a large quantity of medication, however, it is limited to the hydrophobic active ingredients, which are soluble in polar solvents but insoluble in water, for example, isradipine (antihypertensive drug).⁽⁵⁶⁾

3.2 Materials used in nanoparticle preparation

The materials used for preparing the nanoparticle are made from hydrophobic polymers such as poly (lactic acid) (PLA), poly (ϵ -caprolactone) (PCL), poly (alkyl cyanoacrylate) (PACA). They are biodegradable and/or bioeliminable to avoid accumulation in the body.^(57-58,28)

Poly(lactic acid) (PLA) has been widely applied as carriers for drug delivery by emulsion evaporation method.⁽⁵⁹⁾ The evaluation of PLA nanoparticles by stabilizing with water-soluble α -methoxy- ω -hydroxyl polyethylene (MPEO) was reported.⁽⁶⁰⁾ However, these hydrophobic nanoparticles can be easily caught and eliminated by the organs of the mononuclear phagocyte system (MPS). So, it is necessary to achieve a hydrophilisation the surface of nanoparticles to reduce catching from MPS by modification of nanoparticle surface with hydrophilic layer and neutral.⁽³⁶⁾ This modified nanoparticle consists of amphiphilic polymers, i.e. dextran-based or hydrophilic polymers such as poly (ethylene oxide) (PEO), a linear polysaccharide as pullulan and pullulan acetate (PA).

At the beginning of the 1980s, the first study of polymer surfactants based on polysaccharides started. Many reports studied on emulsan; a kind of biosurfactant, which is a polymer of polysaccharide backbone carrying esterified fatty acids and proteins. Their properties in emulsification and characteristics of hydrocarbon-in-water emulsions were investigated. However, the identification of emulsan structure is lacking, especially, the protein part which is potentially surface-active molecules. So, it is difficult to deduce the clear information about the effect of polymer structure. In 1982, Landoll characterized viscometric, solubility and surface-active properties of cellulose modified with alkyl chains. They used

different hydrophilic backbones and varied bearing hydrophobic groups.⁽⁶¹⁾ Subsequently, there were many researchers studied on hydrophobically modified polysaccharides, but their surface-active properties were not mentioned. The first preparation of hydrophobic derivative dextran in aqueous phase system by partial esterification with benzoic acid was investigated.⁽⁶²⁾

For several years, Durand et al in LCPM laboratory, Nancy-France, have been studying the properties of "modified dextran"; the neutral polymeric surfactants which consisted of the polysaccharide backbone consisting mainly of α -1,6-glucosidic linkages (dextran) and hydrophobic groups (aromatic rings, aliphatic or cyclic hydrocarbons) attached to dextran by covalent bonding. They varied the number of grafted hydrophobic group to define the best conditions for using this polymer as the stabilizer of nanoparticle. The method used to prepare the nanoparticle was miniemulsion polymerization.⁽⁶³⁾

In 2003, Rouzes et al, synthesized the dextran derivative containing various grafted hydrophobic groups (phenoxy, P; decyl, C₁₀) and used this product to be the stabilizer for preparing of poly(lactic acid) (PLA) nanospheres by an oil in water with emulsion evaporation technique. The effect of dextran modification on particle size and stability was evaluated. Moreover, they examined encapsulation of lidocaine into the PLA nanosphere.⁽⁶⁴⁾

In order to modify the polarity of the nanoparticle core, two conjugated compounds such as modified dextran and PLA were used to prepare nanoparticle for encapsulating various polarity active molecule.⁽⁶⁴⁾ The examples of conjugated nanoparticle are PLA and poly(ethyleneglycol) conjugated nanoparticles encapsulating with lactoferrin. There was the novel brain delivery system reported in 2009.⁽⁶⁵⁾ Methoxy poly(ethylene glycol) (MPEG) and poly(lactide) (PLA) were used to prepare the nanoparticle to deliver anticancer drug.⁽⁶⁶⁾ Chitosan and cyclodextrin were used in nasal delivery.⁽⁶⁷⁾ For chemotherapy drug delivery, Poly(DL-lactide-co-glycolide)-grafted pullulan nanospheres was used to control release of Adriamycin⁽⁶⁸⁾ and hydrogel nanoparticles of pullulan acetate/oligo-sulfonamide was used with doxorubicin, anticancer drug.⁽⁶⁹⁾

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Materials

1.1 DexP₂₀ (see Methods and Appendix).

1.2 DexC10₁₆₄ obtained from Laboratoire de Chimie Physique Macromoléculaire, Nancy, France; preparations followed as Rouzes et al., 2003 (see Appendix).⁽⁶⁴⁾

1.3 polylactide (PLA), Aldrich[®], France (see Appendix).

1.4 silica gel 60 for column chromatography (0.0036-0.200 nm) Merck, Germany

1.5 TLC aluminum sheets of silica gel 60 F₂₅₄, pre-coated, 20x20 cm, Merck, Germany

1.6 glass columns with inner diameter 2, 2.5, 3, 3.5, and 10 cm

2. Chemicals

2.1 tetrahydrofuran: THF (C₄H₈O) HPLC grade VWR Prolabo[®], France

2.2 diethyl ether (CH₃CH₂OCH₂CH₃) HPLC grade Merck[®], Germany

2.3 trifluoroacetic acid, 99% (CF₃CO₂H) AR grade Merck[®], Germany

2.4 dichloromethane (CH₂CL₂) HPLC grade Merck[®], Germany

2.5 phosphomolybdic acid, Aldrich[®], France

2.6 solketal, 97%, Alfa Aesar[®], USA

2.7 myristol chloride, Aldrich[®], France

2.8 dextran T40 Pharmacia[®], Sweden

2.9 1,2-epoxy-3-phenoxypropane, Aldrich[®], France

2.10 sodium hydroxide (NaOH), Aldrich[®], France

2.11 ethanol (C₂H₅OH), Aldrich[®], France

2.12 sodium dodecyl sulphate (SDS), Bio-Rad[®], USA

3. Reagent

3.1 phosphomolybdic acid (PMA) reagent (see Appendix)

METHODS

1. Synthesis of monoacylglycerol (monomyristin)

For the study on the encapsulation of the biosurfactant in nanoparticles, the synthetic monomyristin, myristic acid (a saturated long-chain fatty acid (C₁₄)) esterified with glycerol, a kind of monoacylglycerol, was used. The structure of monomyristin was shown in Figure 2.

The structure of synthetic monoacylglycerol was based on myristic acid (which is a saturated long-chain fatty acid (C₁₄)) esterified with glycerol. The molecular formula was C₁₇H₃₄O₄. For the study of the encapsulation of the biosurfactant in nanoparticles, this model monoacylglycerol was synthesized. The schematic of synthesis process was shown in Figure 3.

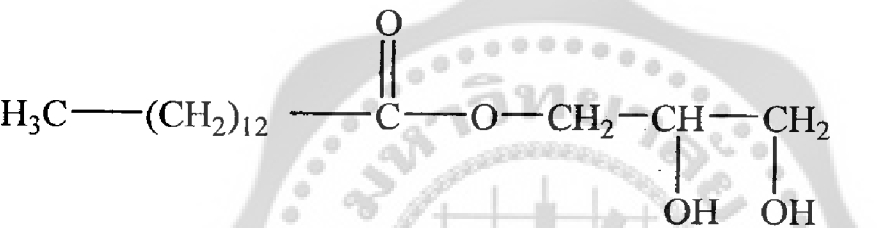


Figure 2 Structure of monomyristin (glycerol ester of myristic acid)

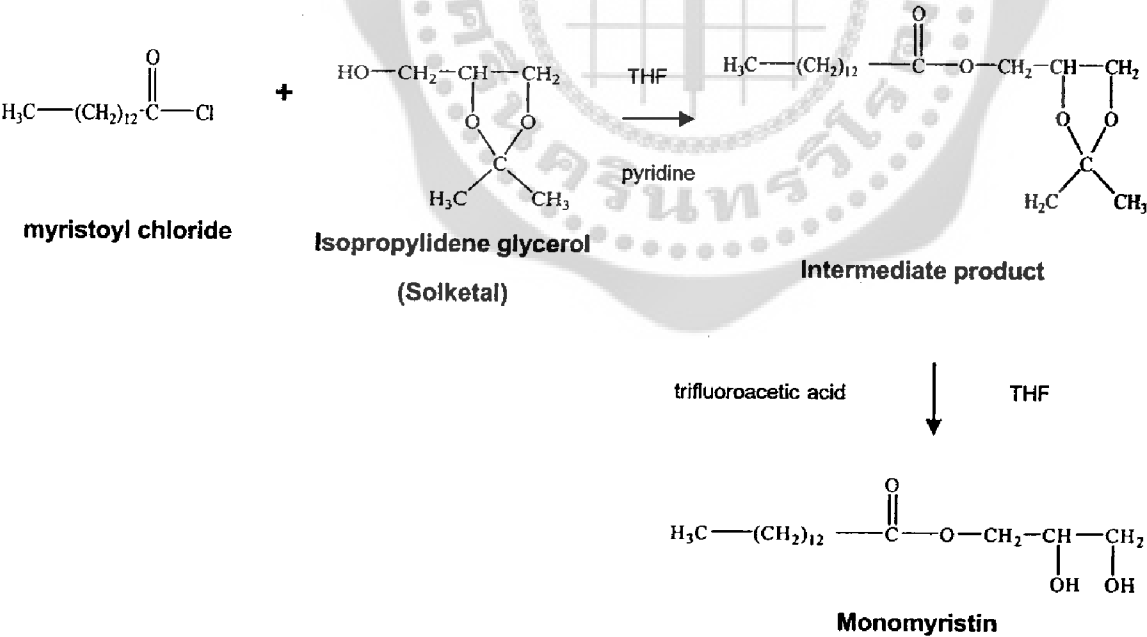


Figure 3 The synthetic process of monomyristin

1.1 The synthetic process method

1.1.1 1.32 g solketal was mixed with 10 ml THF (tetrahydrofuran) and 4.94 g myristyl chloride was added dropwise. Then 1.6 g pyridine was added into the reaction as a catalyst.

1.1.2 The mixture was extracted by diethyl ether and MilliQ water by ratio 1:1, three times. The organic phase which dissolved in diethyl ether was collected and dried over MgSO_4 to absorb water. MgSO_4 was removed by filtration. The intermediate solution was evaporated and the obtained product was purified by silica gel adsorption chromatography, checking the purity by thin layer chromatography, sprayed with a phosphomolybdic acid (PMA) reagent (see appendix). The chemical structure of the intermediate product was analyzed by ^1H NMR in CDCl_3 .

1.1.3 The intermediate product was dissolved in 8ml THF and 4 ml trifluoroacetic acid was added dropwisely. The reaction was neutralized to pH7 with NaHCO_3 .

1.1.4 The mixture was extracted by diethyl ether and MilliQ water by ratio 1:1, three times. The organic phase which dissolved in diethyl ether was collected and evaporated. The product was purified further by silica gel column chromatography. The product pattern was checked by thin layer chromatography and sprayed with PMA reagent. The structure was analyzed by ^1H NMR in CDCl_3 .

2. Cytotoxicity Assay

Cytotoxicity Assay of monomyrin was investigated as follows. 90 μl of cell suspension containing 1.0×10^4 cells of cell line suspensions (Vero cells) were seeded into the wells of 96-well plates. After 24 h of incubation, 10 μl of monomyristin dissolved in 0.01% ethanol was added to the final concentrations of 500 or 1,000 $\mu\text{g/ml}$, and incubated for a further 48 h. The cytotoxicity test was conducted through the use of an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay⁽⁷⁰⁾, which detected the formazan dye at 595 nm using a microplate spectrophotometer (Multiskan, Finland). Each treatment of monomyristin was assayed in triplicate. As a vehicle control, 0.01% ethanol was used as the negative control. The cytotoxicity of the monomyristin was expressed as a value of IC_{50} (where concentrations exhibited 50% cytotoxicity).

3. Synthesis of the hydrophobic derivatives of dextran, DexP₂₀

The hydrophobic derivatives of dextran (DexP) were prepared for nanoparticle preparation from dextran T40 (manufacturer data: $M_n = 13,750$ g/mol, $M_w = 37,500$ g/mol). 10 g of dextran T40 in 1M aqueous NaOH was added with 1,2-epoxy-3-phenoxypropane and stirring at ambient temperature for 48 h. Then, the crude product was precipitated twice with ethanol

and dialyzed with deionized water for 24 h and then freeze-dried. The chemical structure of the hydrophobically modified dextran was presented in Appendix

4. Preparation of nanoparticles

Two methods were used for preparing nanoparticles: emulsion/solvent evaporation and nanoprecipitation methods.

The size, stability of nanoparticles, the amount of coagulum and ¹H-NMR of coagulum were used to analyze the best conditions for nanoparticles preparation.

4.1 Emulsion/solvent evaporation method⁽⁶⁴⁾

Types of material and solvent used for preparing the nanoparticles by emulsion/solvent evaporation method were shown in Table 2 and the methods of preparation were described in section 3.1.1-3.1.5.

Table 2 Types of material and solvent used in nanoparticles preparation by emulsion/solvent evaporation method

Aqueous phase	Organic phase
DexP ₂₀ in milliQ water	PLA in dichloromethane
DexP ₂₀ in milliQ water	MAG in dichloromethane
DexP ₂₀ in milliQ water	DexC10 ₁₆₄ in dichloromethane
DexP ₂₀ in milliQ water	PLA and MAG in dichloromethane
DexP ₂₀ in milliQ water	DexC10 ₁₆₄ and MAG in dichloromethane

4.1.1 Preparation of aqueous phase: 10 ml of a solution of the modified dextran (DexP₂₀) in milliQ water at various concentration; 0, 0.5, 1, 2, 5, 8, 10 g/L was prepared the day before.

4.1.2. Preparation of organic phase: 0.5, 1, 1.5, 2 ml of dichloromethane solution containing 0.5, 1, 1.5, 2 mg/ml of Polylactide (PLA) and/or monoacylglycerol (MAG) and/or modified dextran (DexC10₁₆₄) was prepared. The proportion of monoacylglycerol, PLA and DexC10₁₆₄ was optimized by varying the ratio of monoacylglycerol and PLA or DexC10₁₆₄ by 0:100, 75:25, 50:50, 25:75, 100:0.

4.1.3 The organic and aqueous phases were mixed vigorously with vortex for 1 minute and the mixture was quickly sonicated with a Vibracell model 600 W (Sonics & Materials Inc., Danbury,CT) with pulsed mode, 10 W, 5 power, 50% active cycle, 1 min in an ice bath.

4.1.4 The mixture was left at room temperature to allow the solvent to evaporate for 24 h.

4.1.5 The size of nanoparticles was measured by High Performance Particle Sizer (Malvern Instrument Ltd., Worcestershire, UK). After 24 h, nanoparticles size was determined again to check the stability of the nanoparticles.

In case of the macroscopic aggregation occurred, the large particles were separated by centrifugation at 1500xg for 15 min. The upper suspension was taken and measured for the particle size by High Performance Particle Sizer and removed water using freeze-drying process. The amount of dried polymer in upper suspension was measured by balance (ABT 220-5DM, KERN®, Germany) and the structure of the polymer was determined by ¹H-NMR.

In the aggregated part, the water was removed by freeze-drying process. The amount of dried coagulum was measured and the structure was determined by ¹H-NMR.

4.2 Nanoprecipitation method⁽⁷¹⁾

Types of material and solvent used for preparing the nanoparticles by nanoprecipitation method were shown in Table 3 and the methods of preparation were described in section 3.2.1-3.2.5.

Table 3 Types of material and solvent used in nanoparticles preparation by nanoprecipitation method

Aqueous phase	Organic phase
DexP ₂₀ in milliQ water	PLA in THF
DexP ₂₀ in milliQ water	MAG in THF
DexP ₂₀ in milliQ water	DexC10 ₁₆₄ in THF
DexP ₂₀ in milliQ water	DexC10 ₁₆₄ and MAG in THF
DexP ₂₀ in milliQ water	PLA in Acetone
DexP ₂₀ in milliQ water	MAG in Acetone

4.2.1 Preparation of aqueous phase: 9 ml of a solution of the modified dextran (DexP₂₀) in milliQ water at various concentrations; 0.5, 1, 2, 4, 5, 8, 10 g/L was prepared the day before.

4.2.2 Preparation of organic phase: 4.5 ml of acetone or THF solution containing 10, 25 mg/ml of PLA I and/or monoacylglycero and/or modified dextran (DexC10₁₆₄)

was prepared. The proportion of monoacylglycerol, PLA and DexC10₁₆₄ was optimized by varying the ratio of monoacylglycerol and PLA or DexC10₁₆₄ by 0:100, 75:25, 50:50, 25:75, 0:100.

4.2.3 4.5 ml of the organic phase was added dropwise to the aqueous dextran derivative solution. The suspension was stirred with magnetic bar, 300 rotations per minute during the addition of the organic phase and 10 minutes further.

4.2.4 The mixture was left at room temperature to allow the solvent to evaporate for 24 h.

4.2.5 The size of nanoparticles was measured by High performance particle sizer (Malvern Instrument Ltd., Worcestershire, UK). After 24 h the size of nanoparticles was determined again to check the stability of the nanoparticles.

In case of the macroscopic aggregation occurred, the large particles were separated by centrifugation at 1500xg for 15 min. The upper suspension was taken and measured for the particle size by High Performance Particle Sizer and removed water using freeze-drying process. The amount of dried polymer in upper suspension was measured by balance (ABT 220-5DM, KERN[®], Germany) and the structure of the polymer was determined by ¹H-NMR.

In the aggregated part, the water was removed by freeze-drying process. The amount of dried coagulum was measured and the structure was determined by ¹H-NMR.

CHAPTER IV

RESULTS

1. Synthesis of monoacylglycerol

The monoacylglycerol (monomyristin) was synthesized by mixture of solketal and myristyl chloride using pyridine as a catalyst and adding trifluoroacetic acid to continue the second synthetic process.

The intermediate product which was obtained after mixing myristoyl chloride and solketal was evaporated and purified by adsorption chromatography on silica gel column. The solvent system for eluting of this intermediate product by column chromatography was ethyl acetate:petroleum ether (5:95).

The purity was checked with thin layer chromatography (Figure 4). The purified intermediate product was collected from fraction no. 11-14 and the structure was analyzed by ^1H NMR (Figure 5).

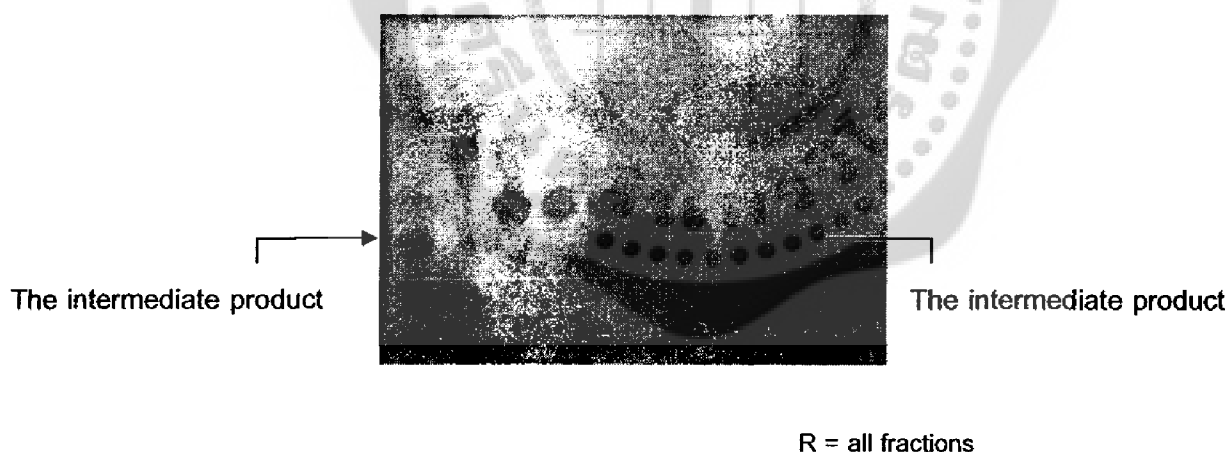


Figure 4 Thin layer chromatography of product from the first synthetic reaction of monomyristin, spraying with phosphomolybdic acid (PMA) reagent. Mobile phase; ethyl acetate:petroleum ether, 1/9(v/v).

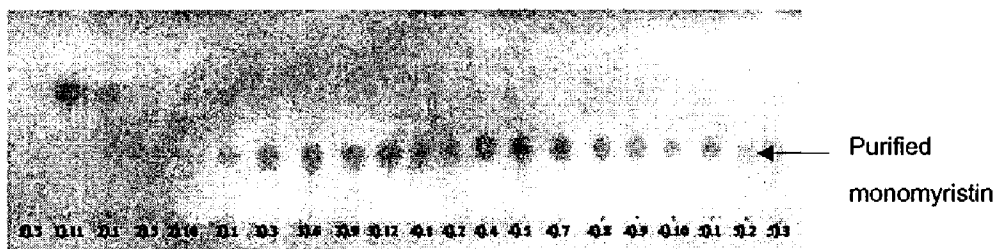


Figure 6 Thin layer chromatography of product from the second synthetic reaction of monomyristin, spraying with phosphomolybdic acid (PMA) reagent. Mobile phase; ethyl acetate:petroleum ether, 1/9(v/v).

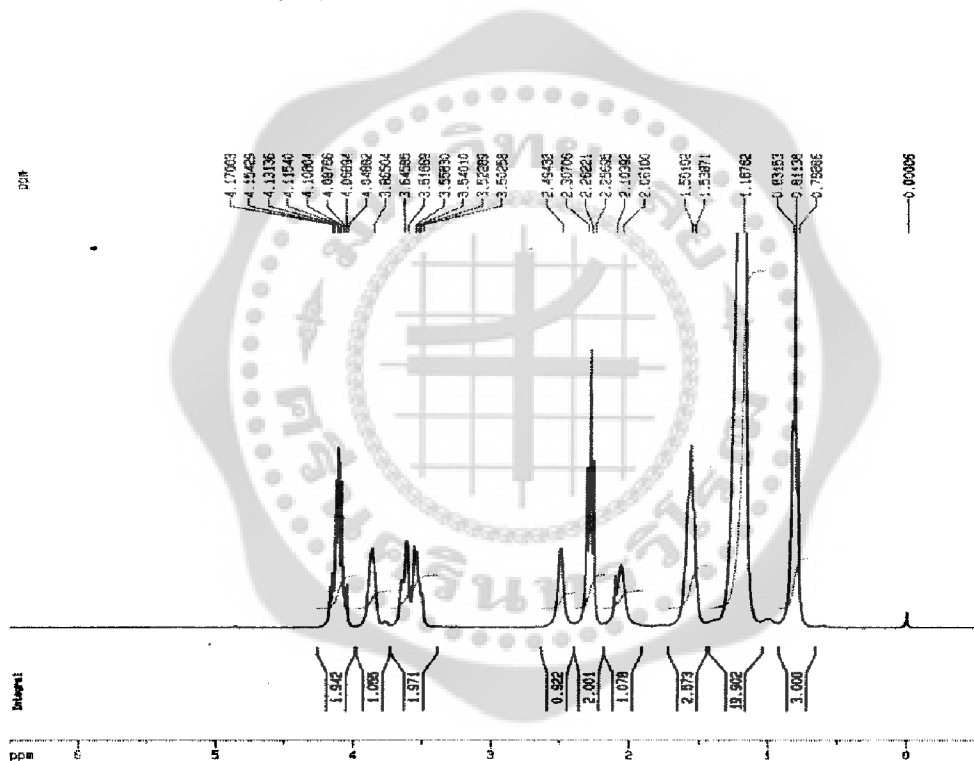


Figure 7 ¹H NMR spectrum of purified monomyristin, in CDCl₃, 300 MHz

2. Cytotoxicity Assay

The cytotoxicity assay of monomyristin comparing with medium control and 0.05% ethanol by using vero cell was shown in value of IC₅₀ (Table 5). The data demonstrated that this monoacylglycerol gave the non cytotoxicity result.

Table 5 IC₅₀ values to vero cell line of monomyristin, medium control and 0.05% ethanol were used as negative control

Treatment	IC ₅₀ (µg/ml)
medium control	98.16±0.34
0.05% ethanol	96.23±1.78
monomyristin	69.48±1.38

3. Synthesis of the hydrophobic derivatives of dextran, DexP₂₀

The result of ¹H NMR spectrum in DMSO (Figure 8) was relevant to the structure of modified dextran as described in Appendix. The percentage of the modified molecule compared with non modified molecule was τ, calculated from the surface area of the peak (A₁, A₂) from ¹H NMR spectrum result (Figure 8) as the formula below. The polymer obtained is called as DexP_τ.

$$\tau = 100 \times \frac{\text{number of attached phenoxy groups}}{\text{number of glucose units in the chains}}$$

$$\tau = 4 \times \frac{A_2/5}{A_1 + (A_2/5)} \times 100$$

A₁ = surface area of the peak between 4.2 and 5.2

A₂ = surface area of the peak between 6.5 and 7.5

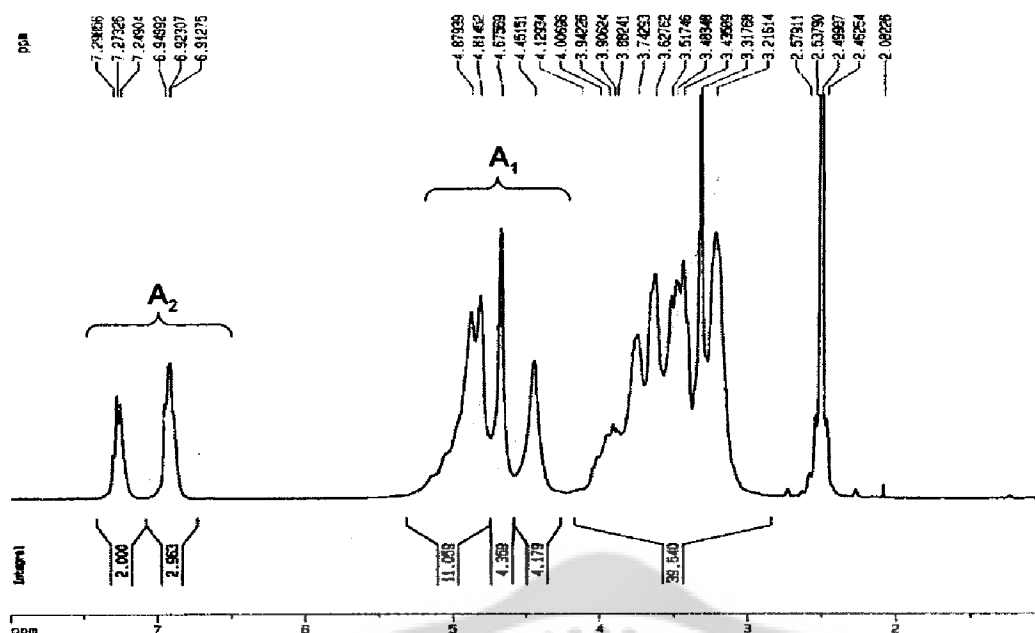


Figure 8 ^1H NMR spectrum of synthesized DexP₂₀ in DMSO, 300 MHz

$$\tau = 4 \times \frac{(11.059+4.359+4.179)/5}{(11.059+4.359+4.179)+(2.000+2.963)/5} \times 100$$

$$= 19.29\%$$

The result showed the percentage of the modified molecules compared with non modified molecules approximately is 20%.

4. Preparation of nanoparticles

4.1 Emulsion/solvent evaporation method

The nanoparticles were prepared by emulsion/solvent evaporation method in varying conditions. The compositions used for nanoparticles preparation in each condition were described in 4.1.1-4.1.7.

4.1.1 aqueous phase: 10 ml of a solution of the modified dextran (DexP₂₀) in milliQ water at various concentration; 0.5, 1, 2, 3, 4, 6, 8 g/L

organic phase: 1 ml of dichloromethane solution containing 25 mg/ml Polylactide (PLA)

The first experiment was displayed by using only PLA as a material in organic phase. The result of nanoparticles size in various concentration of DexP₂₀, measuring at 0 and

24 h was shown in Figure 9. 2 g/L DexP₂₀ led to the smallest PLA nanoparticles. Between 0 and 24 h, only 8 g/L DexP₂₀ showed small difference of nanoparticles size.

2 g/L DexP₂₀ was defined as good concentration for using in further preparation of PLA nanoparticles.

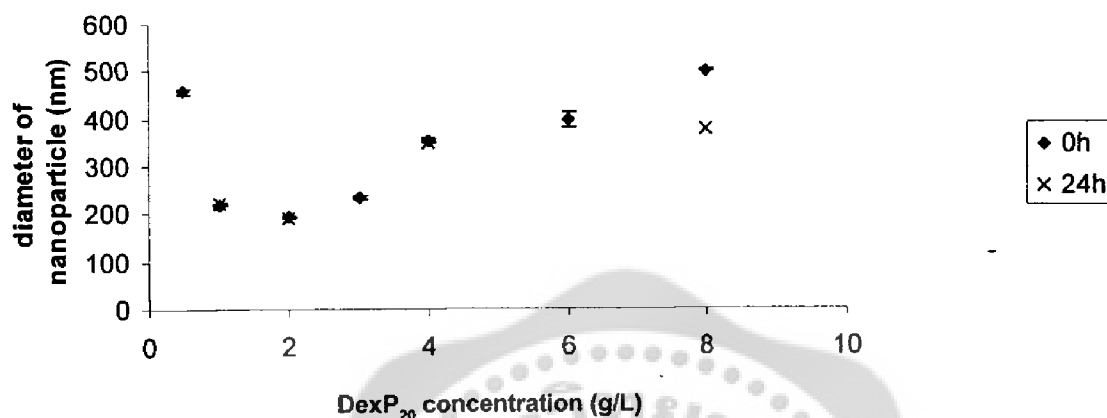


Figure 9 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of DexP₂₀ concentration in the aqueous phase at 0 h (♦), 24 h (×). The PLA concentration in the organic phase was 25 mg/ml.

4.1.2 aqueous phase: 10 ml of a solution at 2 g/L of modified dextran (DexP₂₀) in milliQ water

organic phase: 0.5, 1, 1.5, 2 ml of Polylactide (PLA) in dichloromethane at various concentration; 5, 10, 25, 50 mg/ml

This experiment was conducted for determination of the conditions which influenced the nanoparticles diameter. The nanoparticles size were determined at 0 h with various concentration of PLA and volume of organic phase (Figure 10) and weight% of (PLA)/(PLA+DexP₂₀) (Figure 11). The result showed that the ratio between DexP₂₀ and PLA had a great influence on the nanoparticles size but the volume of organic phase did not affect the size of nanoparticles.

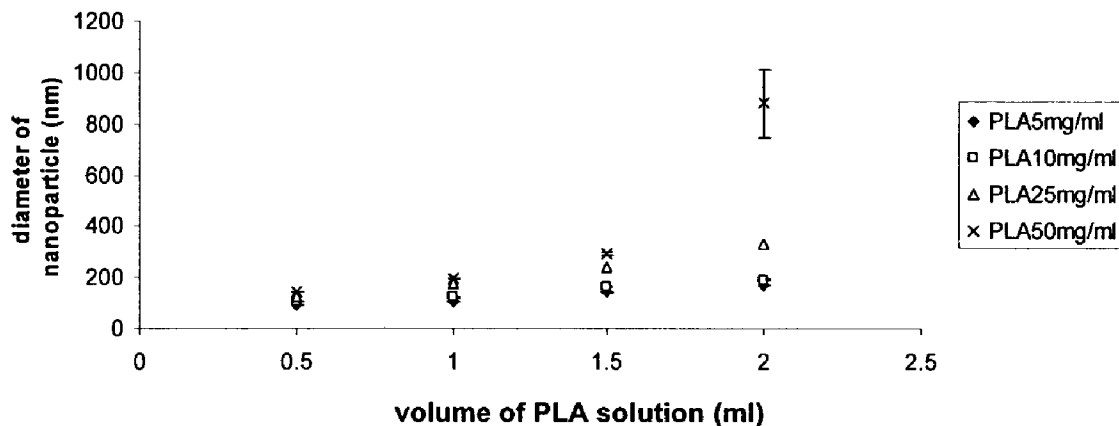


Figure 10 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of volume of PLA solution. The DexP₂₀ concentration in the aqueous phase was 2 g/L. The PLA concentration was varied: 5 mg/ml (♦), 10 mg/ml (□), 25 mg/ml (Δ), 50 mg/ml (×).

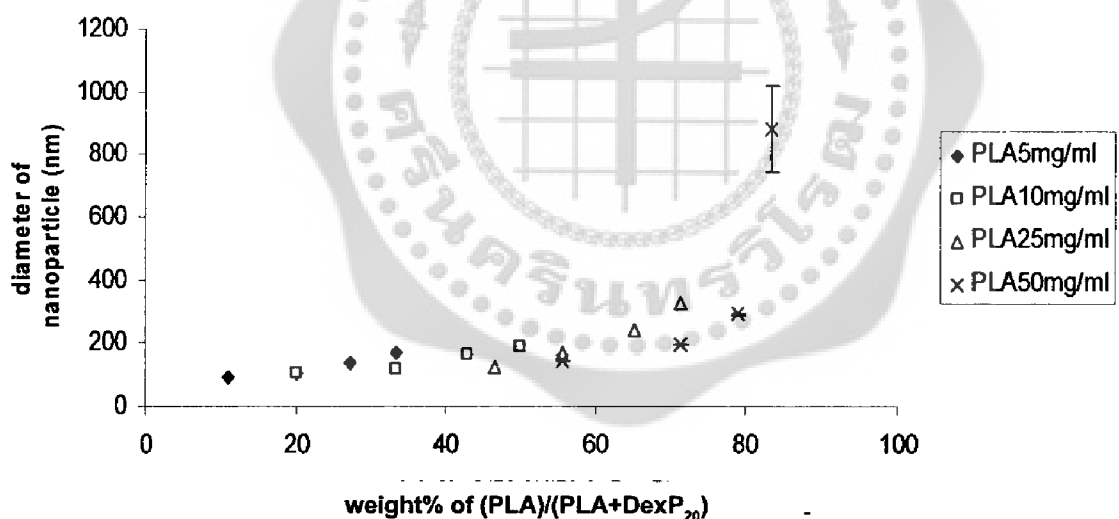


Figure 11 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of weight% of (PLA)/(PLA+DexP₂₀). The DexP₂₀ concentration in the aqueous phase was 2 g/L. The PLA concentration was varied: 5 mg/ml (♦), 10 mg/ml (□), 25 mg/ml (Δ), 50 mg/ml (×).

4.1.3 aqueous phase: 10 ml of a solution of the modified dextran (DexP₂₀) in milliQ water at various concentration; 0, 1, 2, 5, 10 g/L

organic phase: 1 ml of dichloromethane solution containing 25 mg/mL DexC10₁₆₄

Only DexC10₁₆₄ was used as a material in organic phase in this experiment. The size of DexC10₁₆₄ nanoparticles in various concentration of DexP₂₀, measuring at 0h was shown in Figure 12. The diameter of particles was decreased when increasing of DexP₂₀ concentration. The submicronic particles were obtained when DexP₂₀ at concentrations 5 g/L and higher were used. The nanoparticles showed the smallest size at 10 g/L DexP₂₀. So, this concentration of DexP₂₀ was used for further preparation of DexC10₁₆₄ nanoparticles.

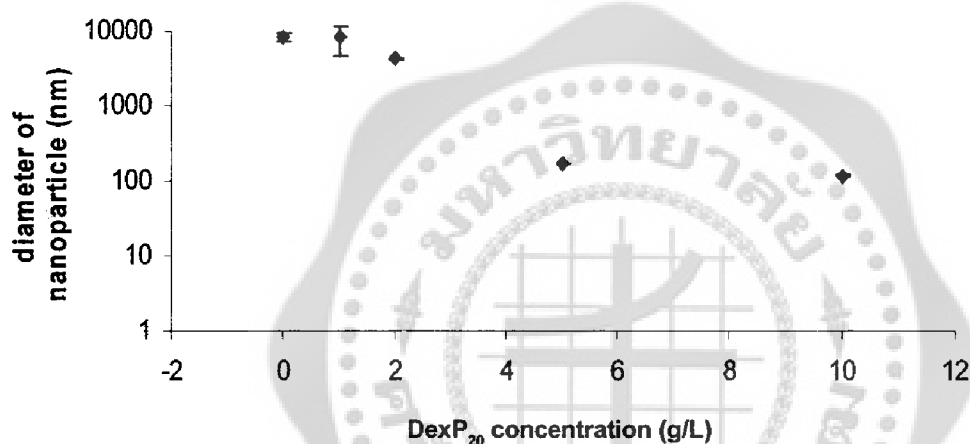


Figure 12 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of DexP₂₀ concentration in the aqueous phase. The DexC10₁₆₄ concentration in the organic phase was 25 mg/ml.

4.1.4 aqueous phase: 10 ml of a solution at 2 g/L of modified dextran (DexP₂₀) in milliQ water

organic phase: 0.5, 1, 1.5, 2 ml of a solution of monomyristin in dichloromethane at various concentration; 5, 25 mg/ml monomyristin

This experiment was the preparation of nanoparticles by using only monomyristin as a material in organic phase. The result of nanoparticles size in various concentration of monomyristin and volume of organic phase when measuring at 0 h was shown in Figure 13. The results showed that using monomyristin alone gave large particles size which were bigger than 1000 nm.

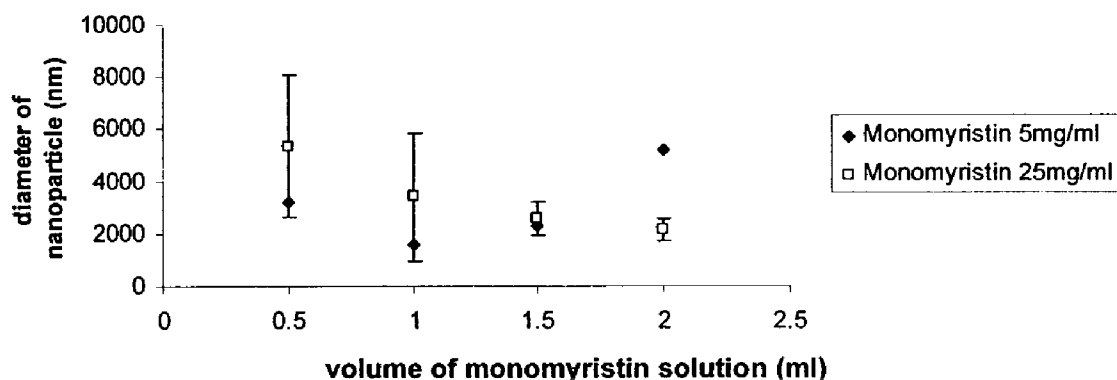


Figure 13 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of volume of monomyristin solution. The DexP₂₀ concentration in the aqueous phase was 2 g/L. The monomyristin concentration was varied: 5 mg/ml (♦), 25 mg/ml (□).

4.1.5 aqueous phase: 10 ml of a solution at 2 g/L of the modified dextran (DexP₂₀) in milliQ water

organic phase: 1 ml of a solution in dichloromethane at 25 mg/ml of a mixture of monomyristin/PLA in various proportions

In this experiment, PLA and monomyristin was used the organic phase. The proportion of both materials was varied. The particle size according to weight% of (monomyristin)/(monomyristin+PLA), measuring at 0 and 24 h was showed in Figure 14. Either micronic or macroscopic aggregation was occurred in the suspension of all condition. At proportion of monomyristin higher than 50 weight%, the diameter of nanoparticles at 0 and 24 h was consistent.

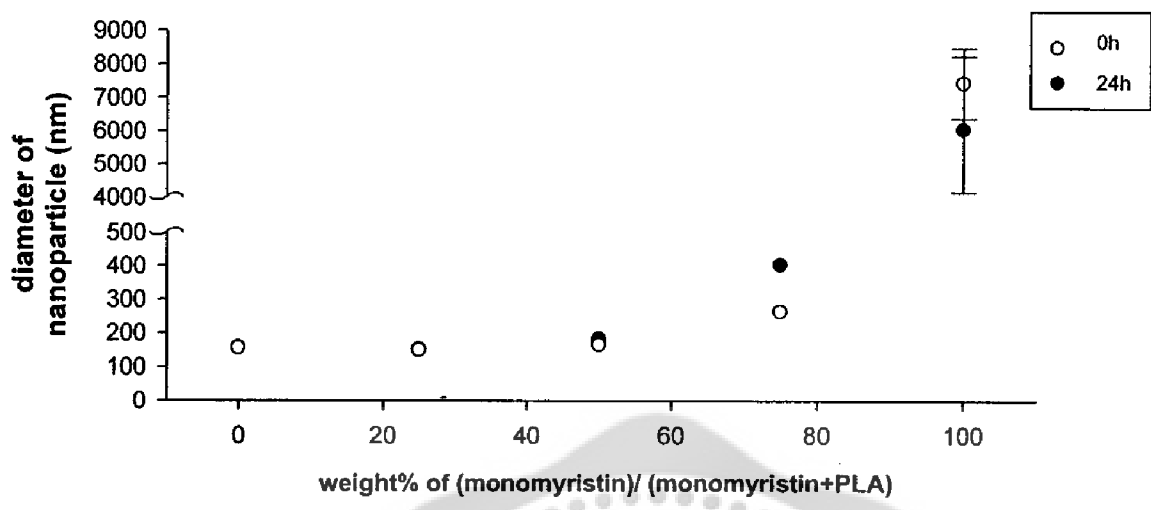


Figure 14 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of weight% of (monomyristin)/(monomyristin+PLA) with a total concentration kept at 25 mg/ml, 0 h (●) and 24 h (○). The DexP₂₀ concentration in the aqueous phase was 2 g/L.

After centrifugation, the diameter of particle in supernatant was measured comparing with the condition before centrifugation as shown in Figure 15. At higher than 50 weight% of monomyristin, before and after centrifugation gave the different size of nanoparticles by the diameter obtained after centrifugation decreased.

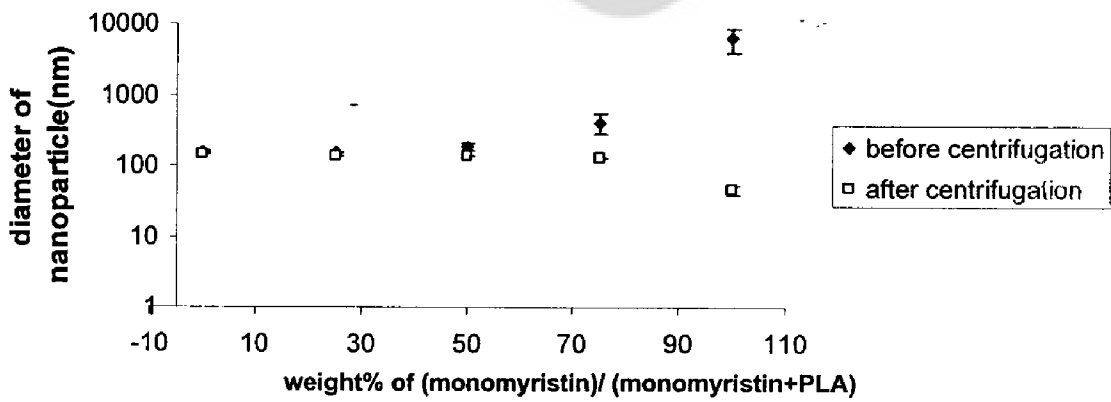


Figure 15 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of weight% of (monomyristin)/monomyristin+PLA) with a total

concentration kept at 25 mg/ml, (♦) before centrifugation and (◻) after centrifugation.

The DexP₂₀ concentration in the aqueous phase was 2 g/L.

The suspension was centrifuged to separate the large aggregation from submicronic nanoparticles. After freeze-drying, the amount of large aggregated polymer was measured (Table 6) and defined the structure by ¹H-NMR (Figure 16) for calculating the ratio of monomyristin/PLA in all conditions. The dried polymer in upper solution was also defined the structure by ¹H-NMR and calculated at 50 weight% of monomyristin (Table 7).

When the weight% of monomyristin raising, the diameter of nanoparticles after centrifugation was decreased (Figure 15) but the large aggregation was increased (Table 7). It was correlated with ¹H NMR result of the large aggregate which showed the enhancement of monomyristin when increasing of weight% of monomyristin.

Table 6 The weight of large aggregated polymer at 2 g/L DexP₂₀ concentrations used

Weight% of (monomyristin)/ (monomyristin+PLA)	Weight (mg)
0	4.4
25	5.8
50	7.4
75	8.2
100	9.0

-The total amount of hydrophobic polymer was 25 mg.

The ratio of monomyristin/PLA was χ , calculated from the surface area of the peak (B_1 , B_2) from ¹H NMR spectrum result (Figure 16) as the formula below.

$$\chi = \frac{(B_2/3) \times 356}{(B_1 \times 72) + [(B_2/3) \times 356]}$$

B_1 = surface area of the peak between 5.0 and 5.5

B_2 = surface area of the peak between 0.5 and 1.0

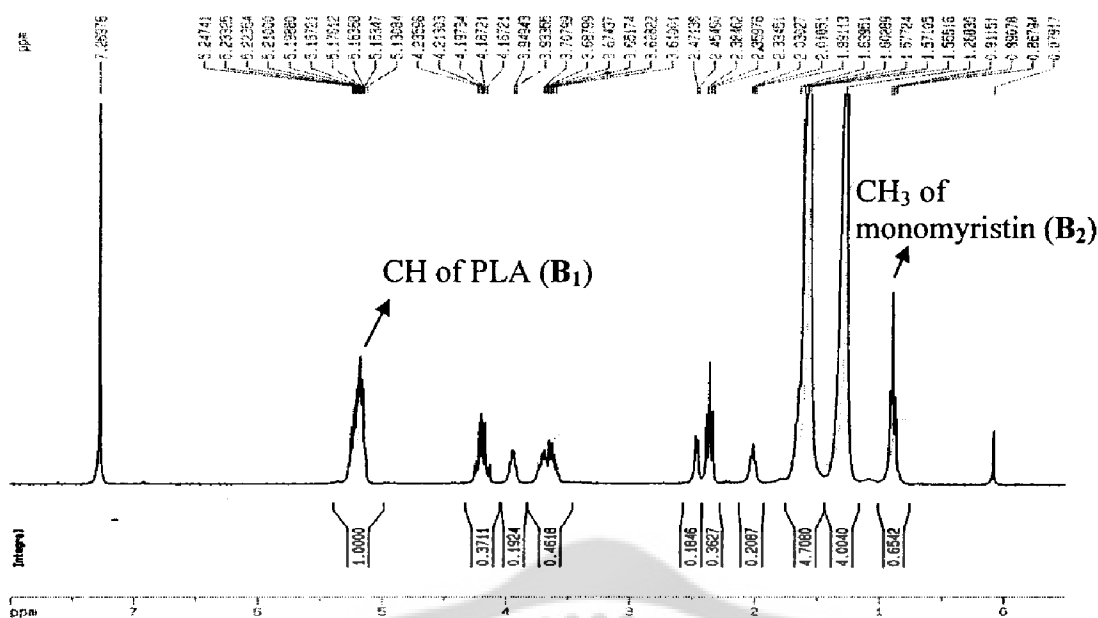


Figure 16 ¹H NMR in CDCl₃ of coagulum at 25 weight% of (monomyristin)/(monomyristin+PLA). The DexP₂₀ concentration in the aqueous phase was 2 g/L.

Table 7 The ratio of monomyristin/PLA at 2 g/L DexP₂₀ concentrations used

Weight% of (monomyristin)/ (monomyristin+PLA)	Ratio of monomyristin/PLA (χ)	
	Aggregated part	Upper solution part
0	0	ND*
25	0.51	ND
50	0.71	0.23
75	0.85	ND
100	0.84	ND

* ND = Not determined

4.1.6 aqueous phase: 10 ml of a solution of the modified dextran (DexP₂₀) in milliQ water at various concentration; 2, 5, 10 g/L

organic phase: 1 ml of a solution in dichloromethane at 25 mg/ml of a mixture of monomyristin/PLA, 50/50 (w/w)

When increasing of DexP₂₀ concentration, the tendency of particles size was decreased either before or after centrifugation (Table 8) but the large aggregation was

increased (Table 9). The DexP₂₀ concentration also effected to the chemical composition of aggregation which proving by ¹H NMR. They showed enrichment of monomyristin in the aggregation (Table 10) while nanoparticles in supernatant had low amount of monomyristin (Table 10). The PLA content in the aggregation part was deducted when high concentration of DexP₂₀ which was showed by ¹H NMR (Figure 17).

Table 8 Average diameter of nanoparticles obtained by emulsion/solvent evaporation process, as a function of DexP₂₀concentration, before centrifugation and after centrifugation

DexP ₂₀ concentration (g/L)	Average particle diameter (nm)	
	Before centrifugation	After centrifugation
2	220	134
5	281	106
10	167	102

Table 9 The weight of large aggregated polymer at various DexP₂₀ concentrations used

DexP ₂₀ concentration (g/L)	Weight (mg)
2	1.0
5	8.6
10	13.5

-The total amount of hydrophobic polymer was 25 mg.

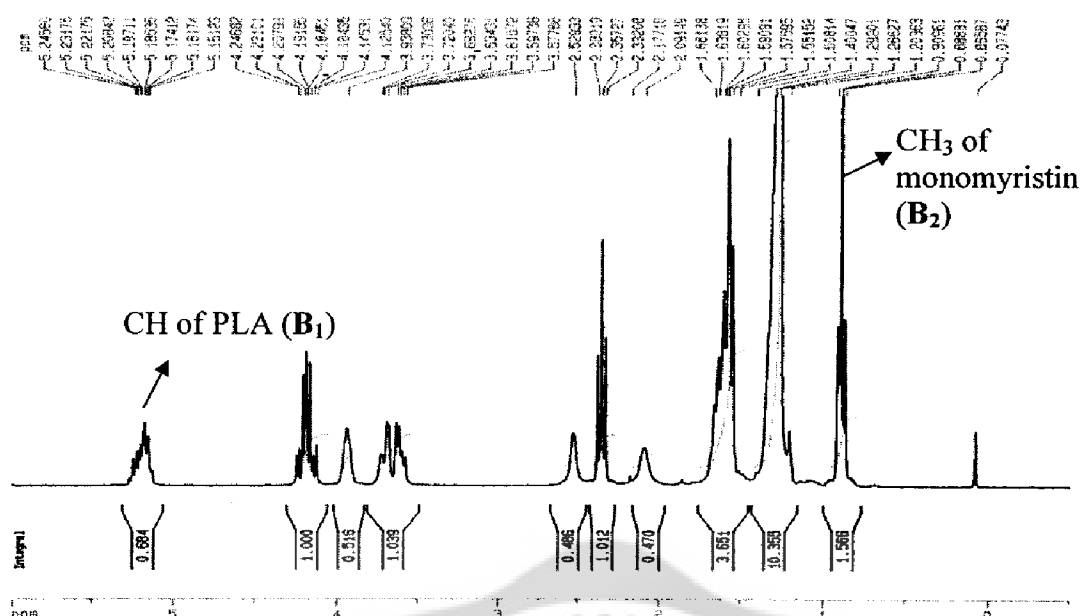


Figure 17 ^1H NMR in CDCl_3 of coagulum at 50 weight% of (monomyristin)/(monomyristin+PLA). The DexP_{20} concentration in the aqueous phase was 2 g/L.

Table 10 The ratio of monomyristin/PLA at 50 weight% of (monomyristin)/(monomyristin+PLA) used

DexP ₂₀ concentration (g/L)	Ratio of monomyristin/PLA (χ)	
	Aggregated part	Upper solution part
2	0.79	0.23
5	0.93	ND*
10	0.98	ND

* ND = Not determined

4.1.7 aqueous phase: 10 ml of a solution at 10 g/L of the modified dextran (DexP_{20}) in milliQ water

organic phase: 1 ml of a solution in dichloromethane at 25 g/L of a mixture of $\text{DexC}_{10_{164}}$ /monomyristin in various proportions

The organic phase was composed of $\text{DexC}_{10_{164}}$ and monomyristin in varying proportion. The effect of weight% of (monomyristin)/(monomyristin+ $\text{DexC}_{10_{164}}$) on particle size was shown in Figure 18. There were no visually large particles inside the suspension at the proportion of monomyristin below 40 weight%. Between 10-20 weight% of monomyristin, the

diameter of particle in suspension before centrifugation (initial suspension) and after centrifugation (supernatant) were similar (Figure 18 and Table 11).

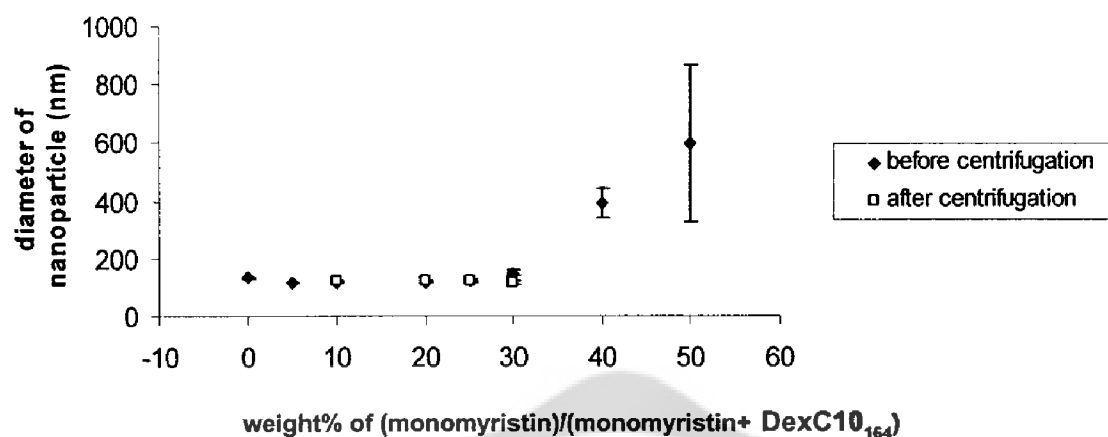


Figure 18 Average diameter of nanoparticles was obtained by emulsion/solvent evaporation process, as a function of weight% of (monomyristin)/(monomyristin+ DexC10₁₆₄) with a total concentration kept at 25 mg/ml, (♦) before centrifugation and (□) after centrifugation. The DexP₂₀ concentration in the aqueous phase was 10 g/L.

Table 11 Average particle diameter for nanoparticles suspensions prepared by emulsion/solvent evaporation using mixtures of DexC10₁₆₄ and monomyristin (MM): The suspension samples were taken for particle size determination from initial suspension, supernatant recovered after centrifugation and suspension obtained after re-dispersion of the centrifuged solid in water.

DexC10 ₁₆₄ :MM (wt:wt)	Average particle diameter (nm)		
	Initial suspension	Supernatant	Re-dispersed solid
90:10	116	114	150
80:20	114	116	247
70:30	116	115	253

The tendency of large aggregated polymer weight was increased when weight% of monomyristin was higher (Table 12). ¹H NMR results of the composition of the aggregates obtained from centrifugation showed mainly monomyristin (Figure 19). There was less or no DexC10₁₆₄ in the aggregates although the concentration of DexC10₁₆₄ increased from 60-95

weight%. From ¹H NMR results, DexP₂₀ was found in both aggregates and supernatant (Figure 20).

Table 12 The weight of large aggregated polymer when increasing of weight% of (monomyristin)/(monomyristin+ DexC10₁₆₄).

Weight% of (monomyristin)/(monomyristin+ DexC10 ₁₆₄)	Weight (mg)
5	0.7
10	2.1
20	2.3
30	7.8
40	7.6

-The total amount of hydrophobic polymer was 25 mg.

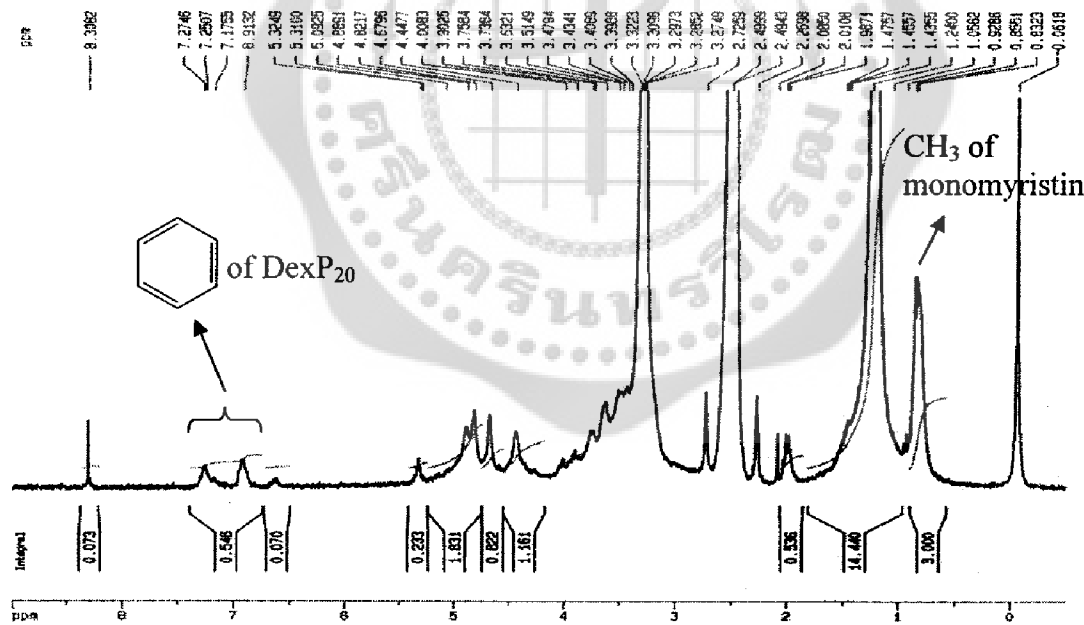


Figure 19 ¹H NMR in DMSO of coagulum at 5 weight% of (monomyristin)/(monomyristin+ DexC10₁₆₄). The DexP₂₀ concentration in the aqueous phase was 10 g/L.

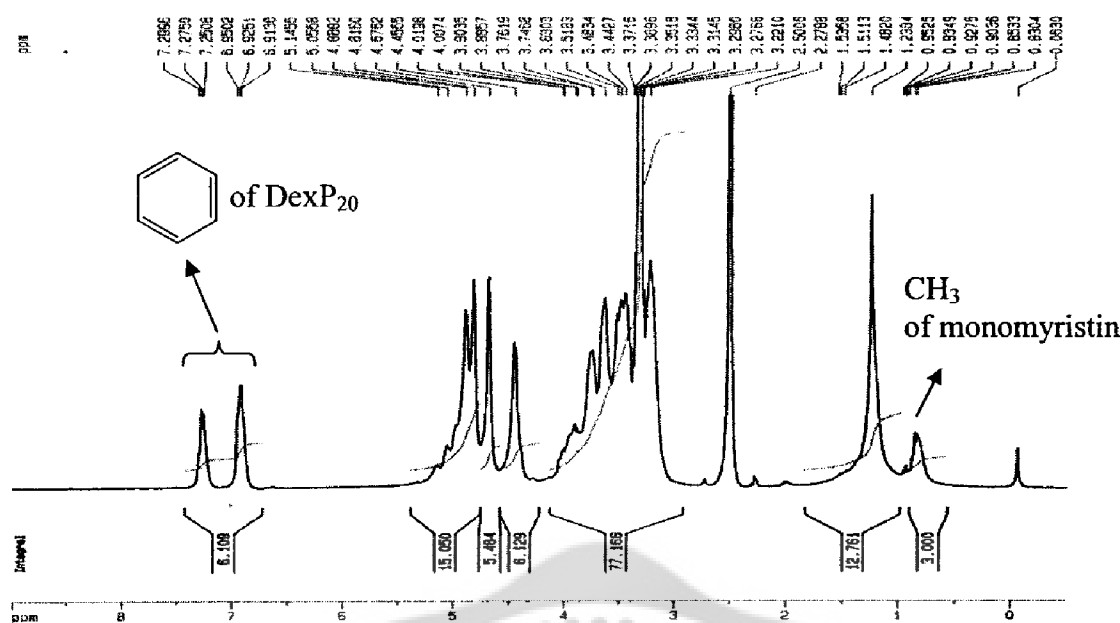


Figure 20 ¹H NMR in DMSO of the polymer in upper solution at 5 weight% of (monomyristin)/(monomyristin+ DexC10₁₆₄). The DexP₂₀ concentration in the aqueous phase was 10 g/L.

4.2 Nanoprecipitation method

The nanoparticles were prepared by nanoprecipitation method in varying conditions. The compositions used for nanoparticles preparation in each condition were described in 4.2.1-4.2.7.

4.2.1 aqueous phase: 9 ml of a solution of the modified dextran (DexP₂₀) in milliQ water at various concentration; 1, 2, 4, 8 g/L

organic phase: 4.5 ml of THF solution containing 25 mg/ml Polylactide (PLA)

This experiment used only PLA as a material in organic phase. The Figure 21 showed the nanoparticles size, measuring before and after centrifugation when various concentration of DexP₂₀ were used. 1 g/L DexP₂₀ led to the smallest PLA nanoparticles. The large particles (macroscopic aggregates) were in the all suspension. The large aggregated particle weight obtained after centrifugation was shown in Table 13. There was 70-80 weight% of PLA comparing with the initial PLA.

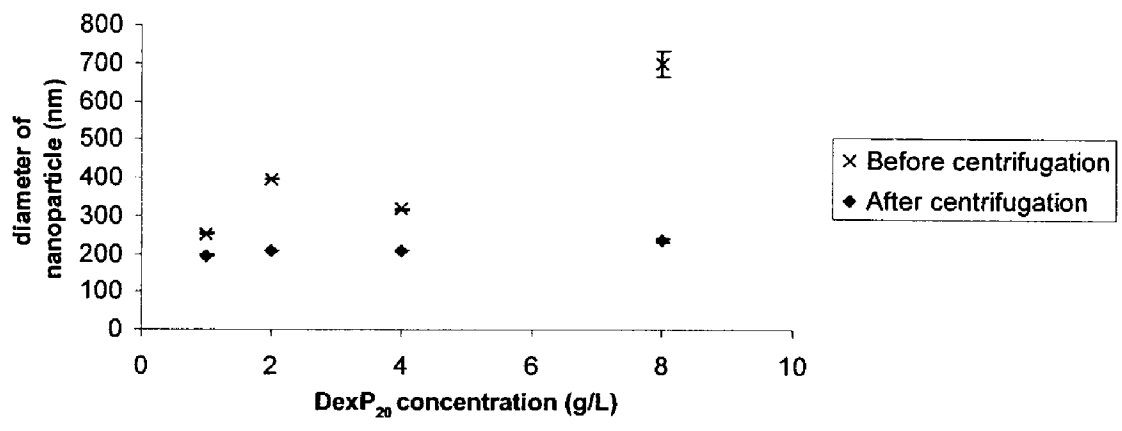


Figure 21 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of DexP₂₀ concentration, (x) before centrifugation and (♦) after centrifugation. The PLA concentration in the organic phase was 25 mg/ml.

Table 13 The weight of large aggregated polymer when increasing concentration of DexP₂₀

Concentration of DexP ₂₀ (g/L)	Weight (mg)
1	91.83
2	93.97
4	88.63
8	81.29

-The total amount of hydrophobic polymer is 112.5 mg.

The aggregated polymer occurred by nanoprecipitation method, therefore, the suspension was centrifuged and the size of particle was measured after centrifugation. All of ¹H NMR results showed that the composition of the aggregated part was only PLA as shown in Figure 22-25.

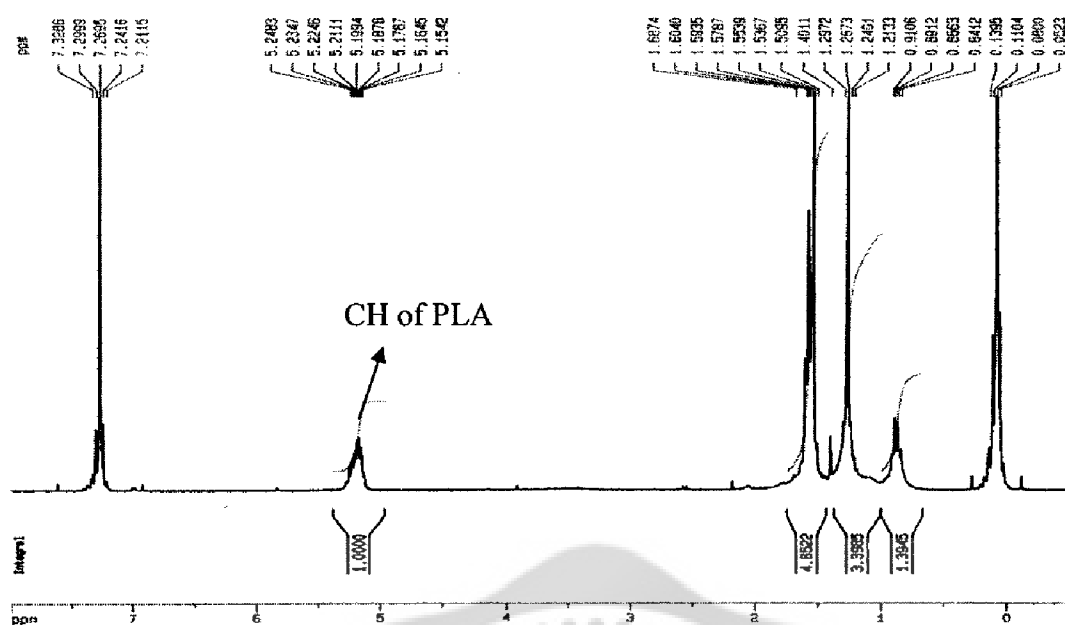


Figure 22 ¹H NMR in CDCl₃ of coagulum at DexP₂₀ concentration was 1 g/L. The PLA concentration in the organic phase was ~25 mg/ml.

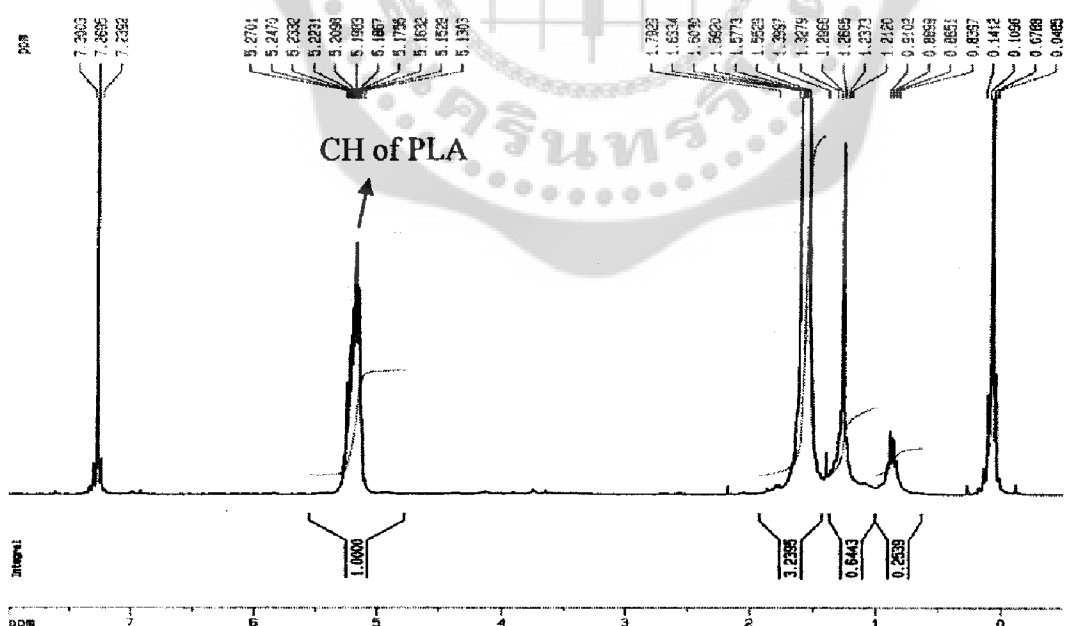


Figure 23 ¹H NMR in CDCl₃ of coagulum at DexP₂₀ concentration was 2 g/L. The PLA concentration in the organic phase was 25 mg/ml.

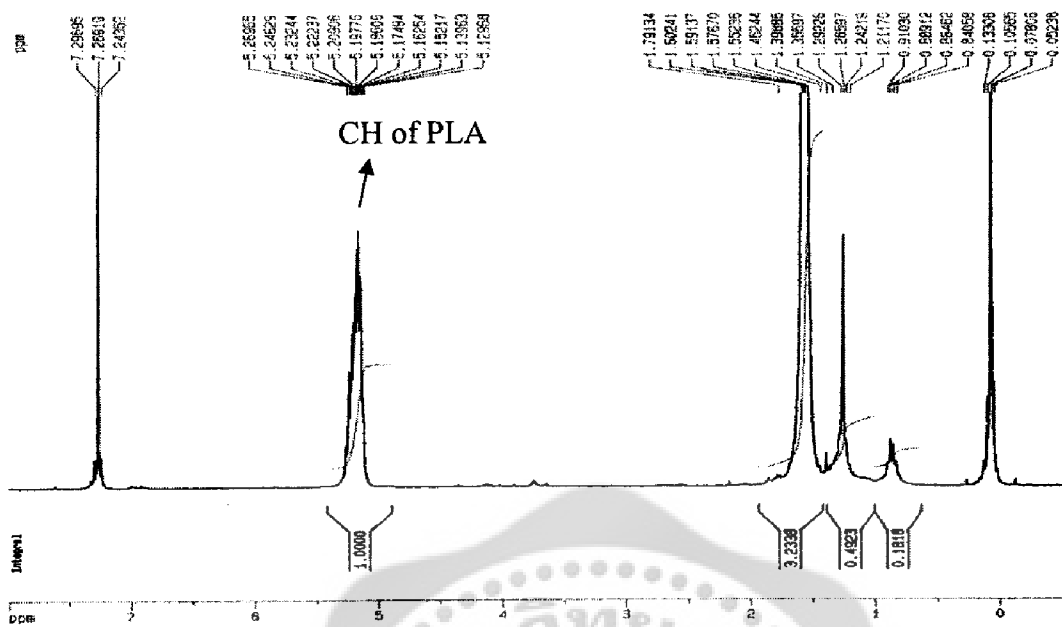


Figure 24 ^1H NMR in CDCl_3 of coagulum at DexP₂₀ concentration was 4 g/L. The PLA concentration in the organic phase was 25 mg/ml.

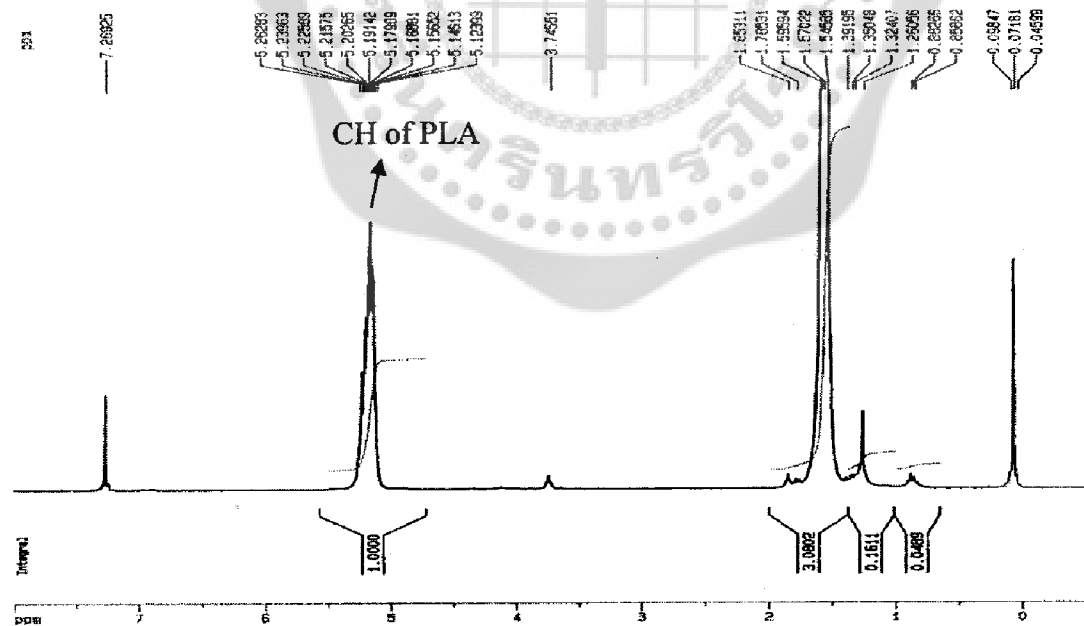


Figure 25 ^1H NMR in CDCl_3 of coagulum at DexP₂₀ concentration was 8 g/L. The PLA concentration in the organic phase was 25 mg/ml.

4.2.2 aqueous phase: 9 ml of a solution of the modified dextran (DexP₂₀) in milliQ water at various concentration; 0.5, 1, 2, 4, 8, 10 g/L

organic phase: 4.5 ml of THF solution containing 10 mg/ml Polylactide (PLA)

The condition used in this experiment was the same as describe in 9.2.1 but decreasing the concentration of PLA as 10 mg/ml to prepare the nanoparticles. The particle size smaller (Figure 26) and the amount of aggregates (Table 14) were lower than the condition of 25 mg/ml but there were still some large particles inside the suspension. The suspension was centrifuged and the size of particle was measured after centrifugation. The weight% of PLA aggregates was decreased as 6-20 weight% when comparing with the initial PLA. The size of nanoparticles before and after centrifugation was not different.

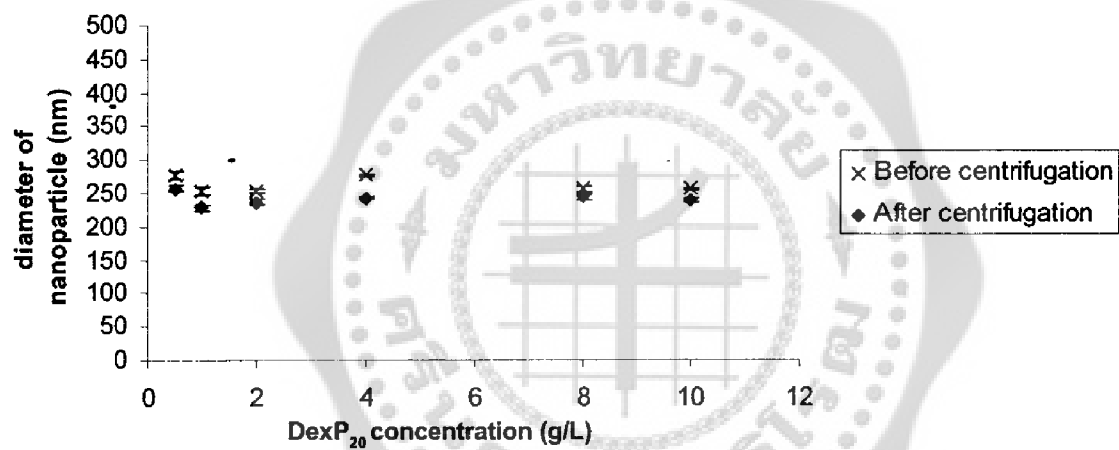


Figure 26 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of DexP₂₀ concentration, (x) before centrifugation and (♦) after centrifugation. The PLA concentration in the organic phase was 10 mg/ml.

Table 14 The weight of large aggregated polymer when using concentration of PLA as 10 mg/ml in THF

Concentration of DexP ₂₀ (g/L)	Weight (mg)
0.5	2.7
1	3.4
2	5.5
4	7.2
8	7.7
10	9.2

-The total amount of hydrophobic polymer is 45 mg.

4.2.3 aqueous phase: 9 ml of a solution of the modified dextran (DexP₂₀) in milliQ water at various concentration; 0.5, 1, 2, 4, 8, 10 g/L

organic phase: 4.5 ml of acetone solution containing 10 mg/ml Polylactide (PLA)

This experiment focused on the solvent used in nanoparticle preparation THF was replaced by acetone. The particle size was not different (Figure 27) but the aggregates size obtained after centrifugation was higher. The amount of aggregates was 70 weight% PLA when comparing the initial PLA (Table 15). ¹H NMR of aggregated polymer showed that this part was composed of PLA only (Figure 28-29).

Moreover, varying concentration of DexP₂₀ did not affect to size of nanoparticles.

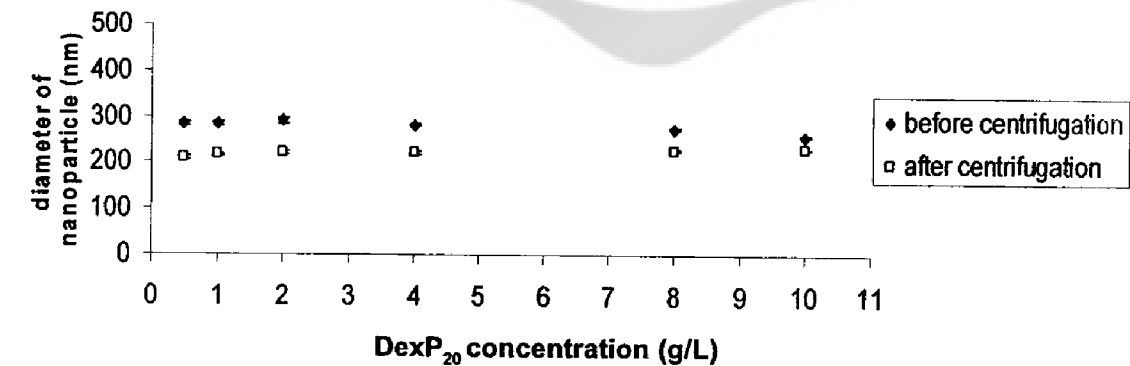


Figure 27 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of DexP₂₀ concentration, (♦) before centrifugation and (□) after centrifugation. The PLA concentration in the organic phase was 10 mg/ml.

Table 15 The weight of large aggregated polymer when using concentration of PLA as 10 mg/ml in acetone

Concentration of DexP ₂₀ (g/L)	Weight (mg)
0.5	31.49
1	31.87
2	31.35
4	31.37
8	24.26
10	32.90

-The total amount of hydrophobic polymer is 45 mg.

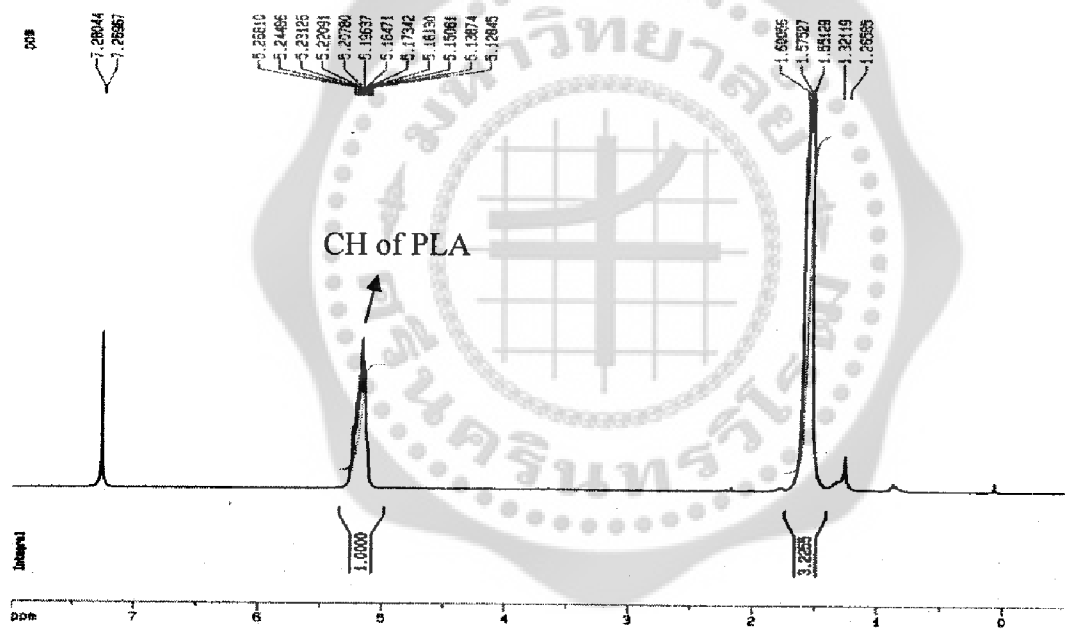


Figure 28 ¹H NMR in CDCl₃ of coagulum at DexP₂₀ concentration was 0.5 g/L. The PLA concentration in the organic phase was 10 mg/ml.

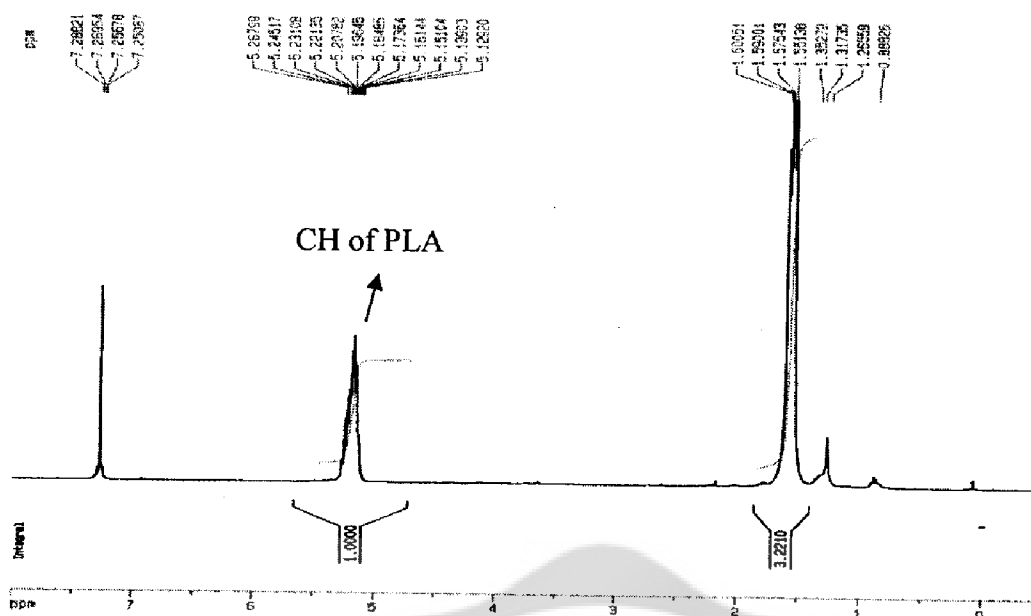


Figure 29 ^1H NMR in CDCl_3 of coagulum at DexP_{20} concentration was 10 g/L. The PLA concentration in the organic phase was 10 mg/ml.

4.2.4 aqueous phase: 9 ml of a solution of the modified dextran (DexP_{20}) in milliQ water at various concentration; 1, 5, 10 g/L

organic phase: 4.5 ml of THF solution containing 10 mg/ml $\text{DexC}_{10_{164}}$

$\text{DexC}_{10_{164}}$ was used to prepare nanoparticles in this experiment by varying the concentration of DexP_{20} and the result showed that 10g/L DexP_{20} led to the smallest nanoparticles (Figure 30).

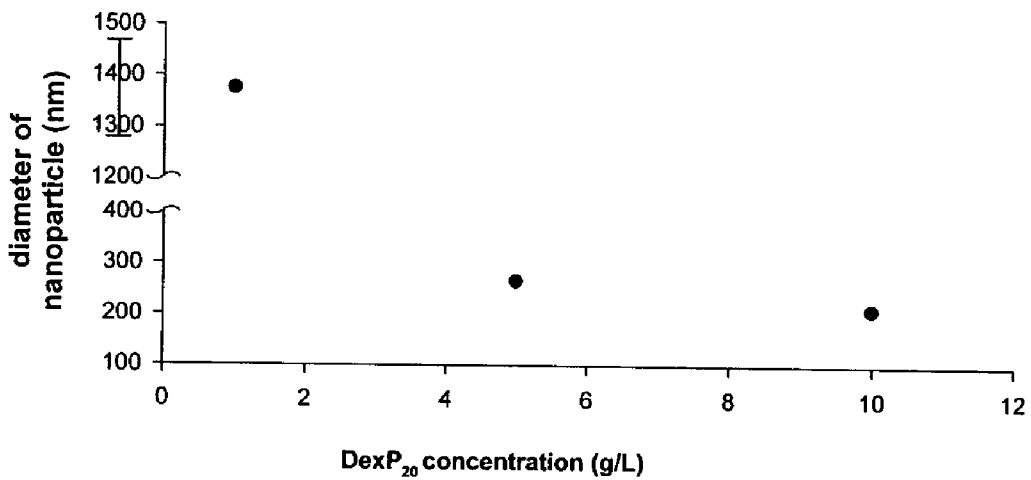


Figure 30 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of DexP₂₀ concentration. The DexC10₁₆₄ concentration in the organic phase was 10 mg/ml.

4.2.5 aqueous phase: 9 ml of a solution at 1 g/L of the modified dextran (DexP₂₀) in milliQ water

organic phase: 4.5 ml of a solution in THF at 10 mg/ml of a mixture DexC10₁₆₄/PLA in various proportions

The proportion of DexC10₁₆₄ and PLA in organic phase was varied but fixed concentration in organic phase as 10 mg/ml. The result shown in Figure 31 that the size obtained was around 200 nm and no macroscopic aggregation in the suspension.

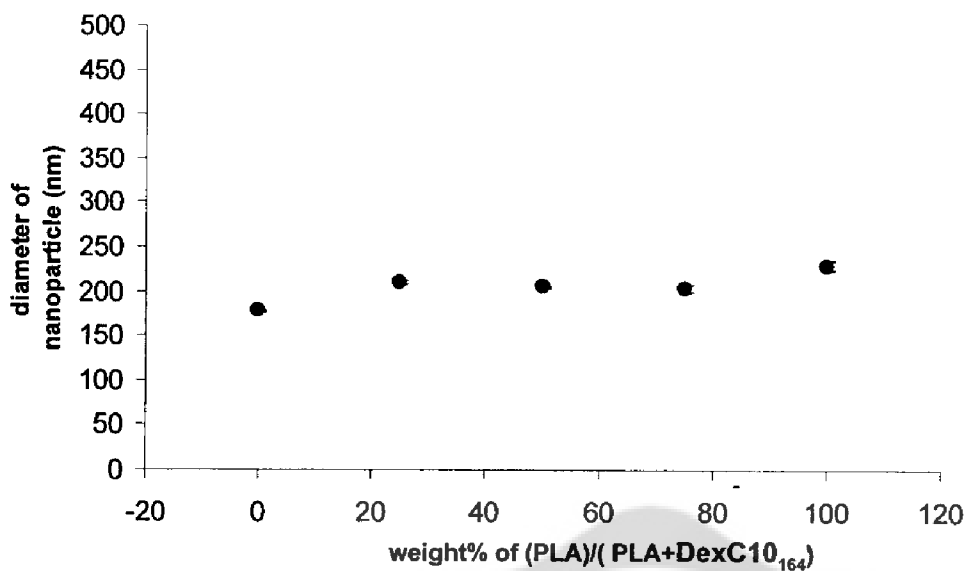


Figure 31 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of weight% of (PLA)/(PLA + DexC10₁₆₄) with a total concentration kept at 10 mg/ml. The DexP₂₀ concentration in the aqueous phase was 1 g/L.

4.2.6 aqueous phase: 9 ml of a solution at 1 g/L of the modified dextran (DexP₂₀) in milliQ water

organic phase: 4.5 ml of THF solution containing 10 mg/ml monomyristin
: 1 ml of acetone solution containing 10 mg/ml monomyristin

Only monomyristin was used in organic phase (either THF or acetone) and 1 g/L DexP₂₀ was used as aqueous phase. At the optimum amount of organic phase and monomyristin, the supernatant gave the microscopic particle around 300 to 600 nm after centrifugation (Table 16).

Table 16 Average particle diameter for nanoparticles suspensions prepared by nanoprecipitation process using monomyristin in THF and acetone, supernatant recovered after centrifugation

Material	Average particle diameter (nm)
-4.5 ml of THF solution (containing 10 mg/ml monomyristin)	369.67
-1 ml of acetone solution (containing 10 mg/ml monomyristin)	669.37

4.2.7 aqueous phase: 9 ml of a solution at 10 g/L of the modified dextran (DexP₂₀) in milliQ water

organic phase: 4.5 ml of a solution in THF at 10 mg/ml of a mixture DexC10₁₆₄/monomyristin in various proportions

The mixture of DexC10₁₆₄/monomyristin in various proportions was used as an organic phase to prepare the nanoparticles. The result shown in the Figure 32 that using the monomyristin lower than 20 weight% gave the nanoparticles around 200 nm without the macroscopic aggregation.

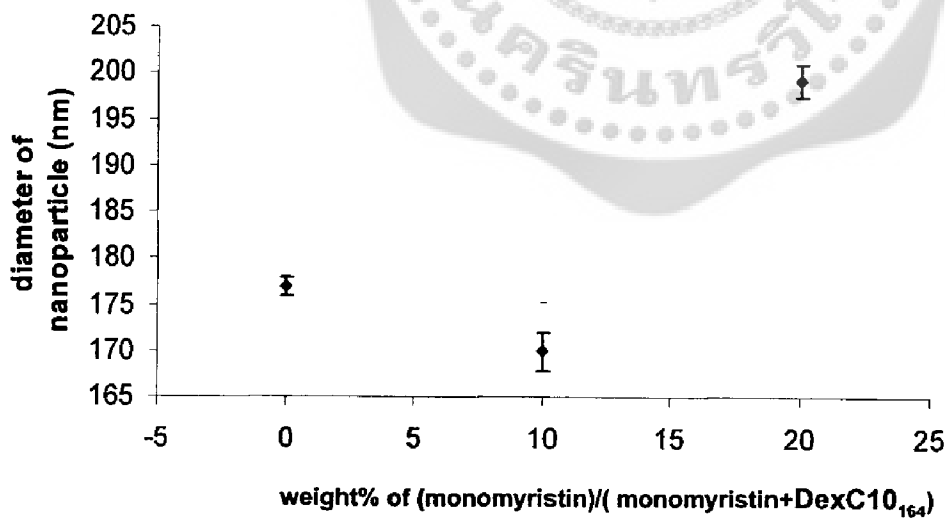


Figure 32 Average diameter of nanoparticles was obtained by nanoprecipitation process, as a function of weight% of (DexC10₁₆₄)/(DexC10₁₆₄+monomyristin) with a total concentration kept at 10 mg/ml. The DexP₂₀ concentration in the aqueous phase was 1 g/L.

CHAPTER V

DISCUSSION

The monoacylglycerol, either monoolein or monomyristin are widely used in many fields especially in pharmaceutic because not only the emulsifying property but also the biological activity.

Monoolein and monomyristin showed the antileukemic property by induction apoptosis of murine leukemic cell.⁽¹⁹⁾ Moreover, monoolein was able to induce cell death in human leukemic cell lines.⁽²⁰⁾ Only monomyristin was reported to show antimicrobial activity against *Helicobacter pylori*.^(12,18)

Monomyristin was synthesized by dehydrochlorination and deacetylation. After synthetic process, the silica gel column chromatography was used to separate the contaminants. Thin layer chromatography and ¹H NMR techniques were required to prove the purity of the synthesized product.

In this study, the emulsion/solvent evaporation process and nanoprecipitation processes were used for nanoparticle development. Either PLA or DexC10₁₆₄ was used as an inner core, DexP₂₀ as a stabilizer and monomyristin was encapsulated in the nanoparticle.

In emulsion/solvent evaporation process, DexP₂₀ was used as a stabilizer in aqueous phase and dichloromethane is the solvent in organic phase. Firstly, preparation of PLA in dichloromethane as organic phase was determined (see experimental 4.1.1 and 4.1.3). The parameter changes were evaluated by the nanoparticle size. The concentrations of organic and aqueous phases were varied and the mean diameter of nanoparticles at 0 h and compared with 24 h was evaluated for the particle stability. The results were shown by plotted graphs to determine the best condition and the parameter which influenced the particle size. The graph plotted by function of weight% of (PLA)/(PLA+DexP₂₀) from all conditions gave a single curve. The size of particle was controlled by weight ratio of PLA to DexP₂₀ as shown in Figure 34. This similar result was reported by Durand and colleague in 2006 which studied the polymeric nanoparticles using modified dextrans as stabilizer. They reported that the weight ratio of initial materials (polymer and oil) influenced the nanoparticle preparation.^(72,63)

The nanoparticles were stable at the concentration of DexP₂₀ as 6 g/L or lower and the best size of nanoparticle obtained when 2 g/L DexP₂₀ was used. So, this concentration of DexP₂₀ was used for further experiments.

When using DexC10₁₆₄ as organic phase instead of PLA and DexP₂₀ as the stabilizer at concentration from 5 g/L to higher (see experimental 4.1.3), the submicronic particles occurred. From the result showed that 10 g/L DexP₂₀ gave the best size of particles so, this concentration was proper for preparation of DexC10₁₆₄ nanoparticles and used for further study.

Monomyristin was used as encapsulated material in the polymeric nanoparticles by emulsion/solvent evaporation process. Only monomyristin was encapsulated in the nanoparticle (experimental 4.1.4). The experiment showed clearly that this material could not be encapsulated alone even using DexP₂₀ as a stabilizer. Moreover, the micronic and macroscopic aggregation was obtained when using monomyristin and PLA as organic phase (see experimental 4.1.5) so the centrifugation was used to separate the supernatant and aggregation parts. The proportion of monomyristin and PLA was varied. The average size of particle at 0 h and 24 h and before and after centrifugation was evaluated. The amount and chemical composition of aggregation part was compared with the initial material amount. When the weight% of monomyristin increased from 0 to 100, the average diameter of particle was decreased while the amount of aggregation part was increased. ¹H NMR used to analyze the chemical composition of aggregation obtained after centrifugation showed large amount of monomyristin comparing with the initial. On the contrary, the supernatant showed the lower proportion of monomyristin than the initial material. This result indicated that monomyristin is involved in the creation of aggregation. When chloroform evaporated, monomyristin which is composed of polar group (hydroxyl groups) from the part of glycerol may contact to water immediately and form the aggregation. So, the surface of particles could not absorb DexP₂₀ compound due to interruption from this part of monomyristin (hydroxyl groups). Thus, the evaluation of the influence in concentration of DexP₂₀ stabilizer was established (see experimental 4.1.6). DexP₂₀ concentration was increased and average size of particle was measured either before or after centrifugation. The amount of aggregation part and chemical composition of aggregate and supernatant were compared with the initial material. The result showed that when increasing of DexP₂₀ concentration, average size of particle after centrifugation and the PLA in aggregation was decreased. This showed that increment of DexP₂₀ concentration induced to increase the stability of only PLA nanoparticle. It did not improve the encapsulation of monomyristin.

When PLA was replaced by DexC10₁₆₄ in experimental 4.1.7, organic phase were presented both monomyristin and DexC10₁₆₄. The result showed that at weight% of monomyristin lower than 40 gave no macroscopic aggregation. Then the centrifugation was used to separate the bigger particles which superimposed with nanoparticles. This aggregation

part was re-dispersed in water and measured size of particles again. The result showed that the re-dispersed solid gave the biggest size of particles and the size of particles was higher when weight% of monomyristin increased. ^1H NMR result of aggregation part obtained after centrifugation showed that it mainly contained monomyristin, minority of DexP_{20} and almost no DexC10_{164} even the concentration of DexC10_{164} increasing. All results contributed that monomyristin is involved in the formation of bigger particles because of during the formation of particles the surface lacking of coverage by DexP_{20} stabilizer due to interruption of hydroxyl groups of monomyristin composition. This was the same mechanism as the report of ethyl cellulose nanoparticles.⁽⁷³⁾ Nevertheless, the increasing of DexP_{20} and using DexC10_{164} as the inner core of particles allowed suppressing the macroscopic and micronic aggregation.

The nanoprecipitation process was studied by using THF and acetone as an organic solvent, stabilizing with DexP_{20} . The first study using PLA alone in THF at concentration as 25 mg/ml (see experimental 4.2.1), the macroscopic and micronic aggregation was obtained in whatever DexP_{20} concentration. Only PLA was shown in ^1H NMR result of the aggregation part. Therefore, decreasing concentration of PLA to 10 mg/ml was used in next experiment (see experimental 4.2.2). However, the aggregation still occurred, but was lower than the first experiment when comparing weight% with the initial amount. Preparing the nanoparticles with acetone instead of THF at concentration of PLA as 10 mg/ml, the proportion of PLA in aggregation was higher than THF (see experimental 4.2.3). The average size of particles was similar to previous report by Legrand and colleagues. They prepared PLA nanoparticles by the same process with no surfactant.⁽⁷⁰⁾ The results showed that the aggregation which occurring when prepared PLA nanoparticles by nanoprecipitation process is depend on the solvent quality and polymer concentration, but not on DexP_{20} stabilizer. That was contrary to emulsion/solvent evaporation process in this study which the concentration of stabilizer in aqueous phase had direct effect to the final particle size. Nevertheless, when the ionic strength is increased, this surfactant is very useful to stabilize the suspension.⁽⁷⁴⁾

Changing the material from PLA to DexC10_{164} (see experimental 4.2.4), only THF was studied because DexC10_{164} was not soluble in acetone. The submicronic particles were obtained only at highest DexP_{20} stabilizer concentration in aqueous phase (10 g/L). This result was relevant to the observation of solvent/evaporation process. In experimental 4.2.5, the PLA and DexC10_{164} were prepared as an inner core of particle by varying proportion. The concentration of organic phase was kept at 10 mg/ml. The nanoparticles were obtained without macroscopic formation aggregation.

Monomyristin was studied on encapsulation in the polymeric nanoparticles with nanoprecipitation process. Preparing particle with monomyristin alone, the micrometric particle was obtained from supernatant after centrifugation (see experimental 4.2.6). The experiment on encapsulation monomyristin in DexC10₁₆₄ particle showed in experimental 4.2.7 by which the proportion of monomyristin and DexC10₁₆₄ was varied. At weight% of monomyristin lower than 20, the particle size around 200 nm could be obtained without macroscopic aggregation, similarly to emulsion/solvent evaporation method. The submicronic particle was occurred when using low weight% of monomyristin.

Monoglycerides are nonionic surfactants, allowing for stable emulsion, which facilitates their widespread use in a number of different fields, such as the food, cosmetic and pharmaceutical industries.⁽⁷⁵⁾ Interestingly, some monoglyceride exhibit antiviral and antibacterial activities.^(18,76,15) Treatment of normal cells (Vero) with 0.01% ethanol, as a vehicle control, and 1,000 µg/ml monomyristin showed no antiproliferative effect. (Table 5).

In summary, the emulsion/solvent evaporation and nanoprecipitation processes were used to prepare the dextran-cover nanoparticles for encapsulating monomyristin. Either PLA or DexC10₁₆₄ was prepared as a core of particle with variety of the polymers and monomyristin proportion. The results contributed that monomyristin induced the formation of macroscopic aggregation. However, the high concentration of stabilizer and/or using DexC10₁₆₄ as inner core of particle suppressed the macroscopic aggregation. This study is a model for encapsulation the monoacylglycerol in nanoparticles. Moreover, the monomyristin gave the non cytotoxicity result. The further study can be done to evaluate deeper the release of the encapsulated other monoacylglycerols. This study was an interested starter of using non cytotoxic monoacylglycerol as a drug in nanoparticle development.

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APPENDIX

1. Materials used in nanoparticle preparation

1.1 DexP₂₀ is dextran, bearing with phenoxy group which the percentage of the modified molecules compared with non modified molecules is 20% as the structure shown in Figure 33.

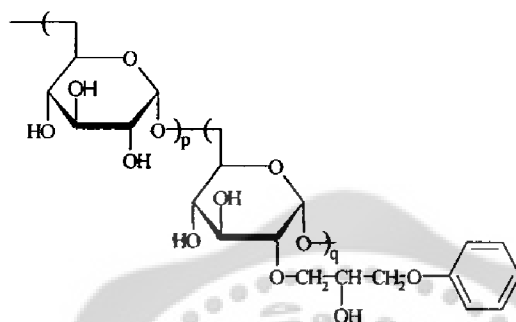


Figure 33 Structure of DexP₂₀ (Dextran bearing phenoxy group with $q/(p+q) = 20\%$)

1.2 Dex C10₁₆₄ is dextran, bearing with hydrophobic group ($n\text{-C}_{10}\text{H}_{21}$) which the percentage of the modified molecules compared with non modified molecules is 164% as the structure shown in Figure 34. The result of ¹H NMR spectrum in DMSO of DexC10₁₆₄ was shown in Figure 35.

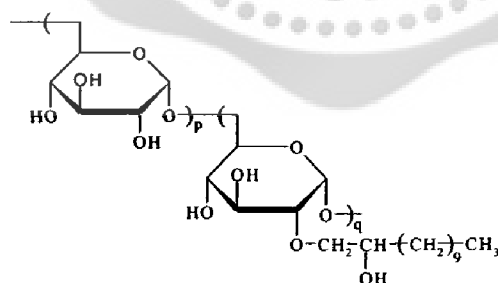


Figure 34 Structure of DexC10₁₆₄ (Dextran bearing hydrophobic group ($n\text{-C}_{10}\text{H}_{21}$) with $q/(p+q) = 164\%$)

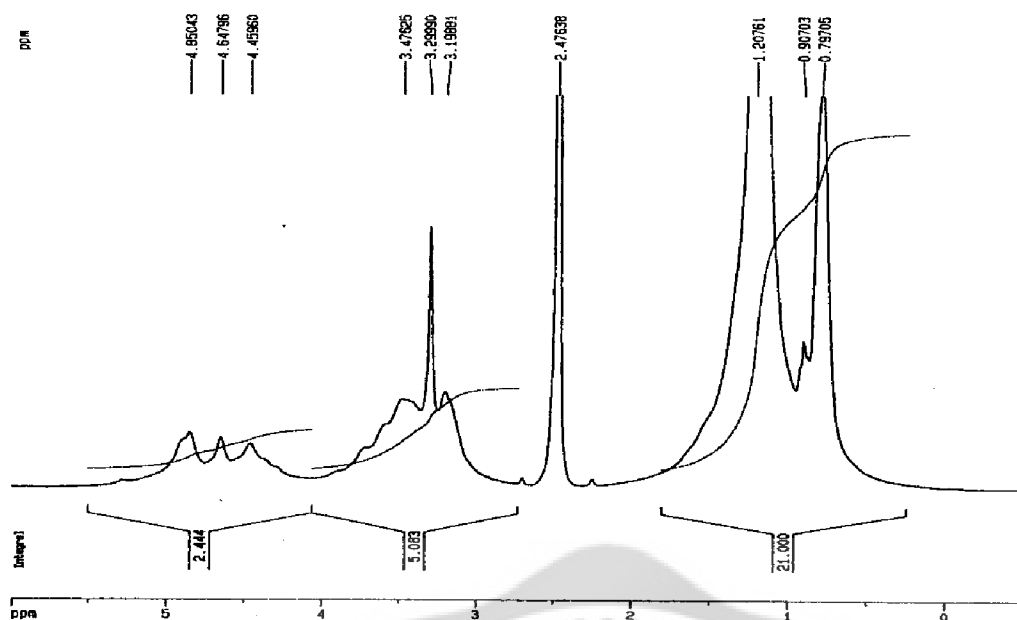


Figure 35 ^1H NMR spectrum of DexC10₁₆₄, in DMSO, 300 MHz

1.3 Polylactide (PLA) structure and ^1H NMR spectrum in CDCl_3 of PLA was shown in Figure 36 and 37, respectively.

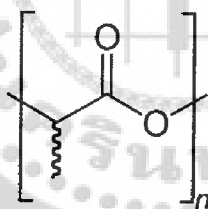


Figure 36 Structure of Polylactide (PLA)

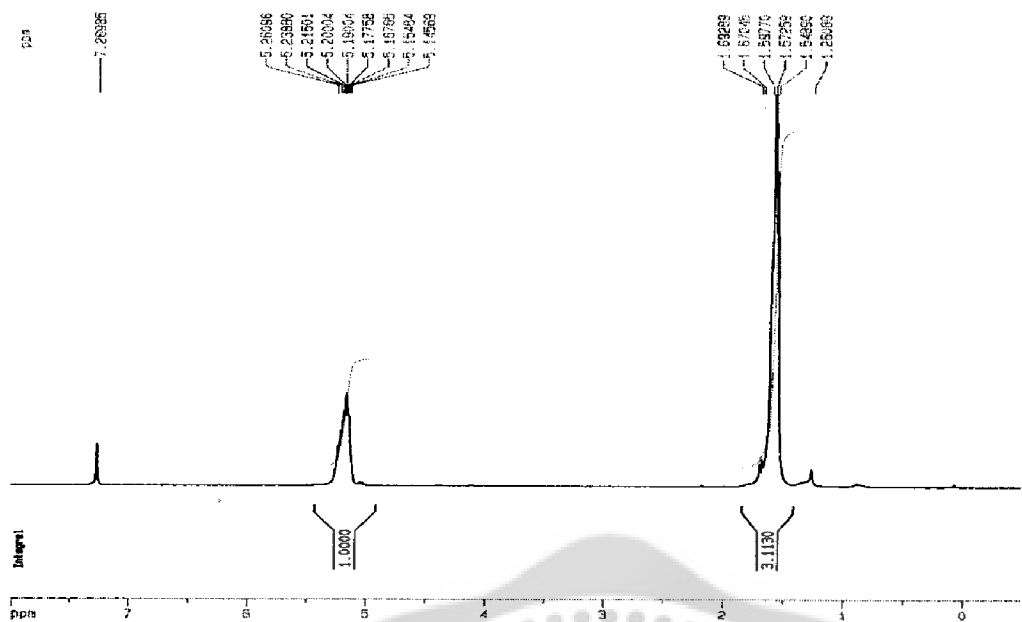


Figure 37 ¹H NMR spectrum of PLA, in CDCl₃, 300 MHz

2. Reagent

2.1 Phosphomolybdic acid (PMA) reagent

Phosphomolybdic acid	4	g
Ethanol	196	ml
H ₂ SO ₄	4-5	drops