LIPASE-CATALYZED HYDROPHOBIC MODIFICATION OF DEXTRAN. PREPARATION OF POLYSACCHARIDE-BASED NANOPARTICLES FOR DRUG DELIVERY APPLICATIONS.

KULTHIDA KAEWPRAPAN

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (BIOTECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2012**

Thesis
entitled

LIPASE-CATALYZED HYDROPHOBIC MODIFICATION OF DEXTRAN. PREPARATION OF POLYSACCHARIDE-BASED NANOPARTICLES FOR DRUG DELIVERY APPLICATIONS.

Kulthida K.
.....
Miss Kulthida Kaewprapan
Candidate

.....
Asst. Prof. Pranee Inprakhon, Ph.D.
Major advisor

Pranee Phinyocheep
.....
Assoc. Prof. Pranee Phinyocheep, Ph.D.
Co-advisor

.....
Prof. Alain Durand, Ph.D.
Co-advisor

(Go Abroad)
.....
Mrs. Emmanuelle Marie, Ph.D.
Co-advisor

B. Mahaisavariya
.....
Prof. Banchong Mahaisavariya,
M.D., Dip Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

Chuenchit Boonchird
.....
Assoc. Prof. Chuenchit Boonchird, Ph.D.
Program Director
Doctor of Philosophy Program in
Biotechnology
Faculty of Science, Mahidol University

Thesis
entitled

LIPASE-CATALYZED HYDROPHOBIC MODIFICATION OF DEXTRAN. PREPARATION OF POLYSACCHARIDE-BASED NANOPARTICLES FOR DRUG DELIVERY APPLICATIONS.

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Doctor of Philosophy (Biotechnology)
on
January 30, 2012

Miss Kulthida Kaewprapan
Candidate

Asst. Prof. Somchai Chauvacharin, Ph.D.
Chair

Asst. Prof. Pranee Inprakhon, Ph.D.
Member

Assoc. Prof. Pranee Phinyocheep, Ph.D.
Member

Prof. Alain Durand, Ph.D.
Member

Miss Nilubon Jong-Anurakkun, Ph.D.
Member

Prof. Banchong Mahaisavariya,
M.D., Dip Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

Prof. Skorn Mongkolsuk, Ph.D.
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude and appreciation to my advisors, Asst. Prof. Pranee Inprakhon and Prof. Alain Durand for their invaluable guidance, constant encouragement and kind support through my study both at Department of Biotechnology, Faculty of Science, Mahidol University, Thailand and at Laboratoire de Chimie-Physique Macromoléculaire (LCPM), Ecole Nationale Supérieure des Industries Chimiques (ENSIC), Institut National Polytechnique de Lorraine (INPL), France.

I also deeply express my gratitude to my co-advisors Assoc. Prof. Pranee Phinyocheep and Dr. Emmanuelle Marie for their kindness and helpful guidance during my study. I wish to thank Dr. Nilubon Jong-Anurakkun, my external examiner from Science and Innovation Center of PTT Chemical Co. Ltd., for her kindness and useful suggestion.

This thesis is partially supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office (PERDO), Commission on Higher Education, Ministry of Education and also by Research Assistant scholarship from the Faculty of Graduate Studies, Mahidol University.

I would like to thank the Franco-Thai cooperation in Higher Education and Research and the Eiffel scholarship from French Center of Higher Education, French Ministry of Foreign and European Affairs for the partial financial support during my stay in Nancy, France.

I gratefully thank to all staffs in Faculty of Science, Mahidol University and in ENSIC who help me to complete experiments and document works for this thesis. I am also thankful to all of my friends for sharing experiences during the period of my study.

Finally, I would like to express my deepest gratitude and appreciation to my family for their love, consultation and encouragement throughout my study.

Kulthida Kaewprapan

LIPASE-CATALYZED HYDROPHOBIC MODIFICATION OF DEXTRAN. PREPARATION OF POLYSACCHARIDE-BASED NANOPARTICLES FOR DRUG DELIVERY APPLICATIONS.

KULTHIDA KAEWPRAPAN 5038125 SCBT/D

Ph.D.(BIOTECHNOLOGY)

THESIS ADVISORY COMMITTEE: PRANEE INPRAKHON, Ph.D., PRANEE PHINYOCHEEP, Ph.D., ALAIN DURAND, Ph.D., EMMANUELLE MARIE, Ph.D.

ABSTRACT

Amphiphilic dextran derivatives were synthesized by lipase-catalyzed transesterification between dextran and vinyl decanoate in dimethyl sulfoxide. Several techniques were used to enhance catalytic activity of lipase AY from *Candida rugosa* in dimethyl sulfoxide. Use of lipase pretreated with 18-crown-6 together with stepwise addition of enzyme and vinyl decanoate was found to be the most effective strategy to increase the degree of substitution of dextran. Solvent fractionation of modified dextrans obtained from different reaction conditions showed that macromolecules with very different degrees of substitution were formed. Physico-chemical properties of fractionated modified dextrans were investigated (including oil/water interfacial tension, surface pressure isotherm and capillary viscometry in dimethyl sulfoxide). Dextranase and pancreatic lipase were used to evaluate their biodegradability. Nanoparticles of dextran decanoate samples with degrees of substitution between 25 and 150 % were fabricated by nanoprecipitation. Their size and colloidal stability then were studied as a function of degree of substitution. The use of a low-modified dextran decanoate (~15%) as stabilizer during nanoprecipitation appeared necessary to ensure colloidal stability at ionic strengths exceeding 0.01 M. The encapsulation capacity of nanoparticles was demonstrated using lidocaine as the model drug. Nanoparticles of dextran decanoate were found to be potentially useful for drug delivery applications.

KEY WORDS: LIPASE / TRANSESTERIFICATION / DEXTRAN / ESTER / NANOPARTICLES

124 pages

การดัดแปลงเดกซ์แทรนด้วยหมู่ไม่ชอบน้ำโดยใช้เอนไซม์ลิเพสเร่งปฏิกิริยา
การเตรียมอนุภาคนาโนของพอลิแซ็กคาไรด์เพื่อการประยุกต์ใช้ในการนำส่งยา
LIPASE-CATALYZED HYDROPHOBIC MODIFICATION OF DEXTRAN.
PREPARATION OF POLYSACCHARIDE-BASED NANOPARTICLES FOR DRUG DELIVERY APPLICATIONS.

กุลธิดา แก้วประพาฬ 5038125 SCBT/D

ปร.ด. (เทคโนโลยีชีวภาพ)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: ปราณี อินประโคน, Ph.D., ปราณี ภิญโญชีพ, Ph.D., ALAIN DURAND, Ph.D., EMMANUELLE MARIE, Ph.D.

บทคัดย่อ

อนุพันธ์แอมฟิฟิลิกของเดกซ์แทรนสังเคราะห์ได้จากปฏิกิริยาทรานเอสเทอริฟิเคชันระหว่างเดกซ์แทรนกับไวนิลเดคาโนเอตในไดเมทิลซัลฟอกไซด์ที่มีเอนไซม์ลิเพสเป็นตัวเร่งปฏิกิริยา หลายเทคนิคได้ถูกนำมาใช้ในการเพิ่มการเร่งปฏิกิริยาของเอนไซม์ลิเพส AY จาก *Candida rugosa* ในไดเมทิลซัลฟอกไซด์ พบว่า การใช้ลิเพสที่ผ่านการปรับสภาพด้วย 18-คราวน์-6 ควบคู่ไปกับเทคนิคการเติมเอนไซม์และไวนิลเดคาโนเอตอย่างเป็นลำดับเป็นวิธีที่มีประสิทธิภาพที่สุดที่ในการเพิ่มระดับการแทนที่ของไวนิลเดคาโนเอตในเดกซ์แทรน การแยกเดกซ์แทรนดัดแปลงที่ได้จากการสังเคราะห์ในสภาวะต่าง ๆ ด้วยตัวทำละลายต่างชนิดกันสามารถแยกเดกซ์แทรนที่มีระดับการแทนที่ของไวนิลเดคาโนเอตระดับต่าง ๆ ได้ งานวิจัยนี้ศึ กษาคุณสมบัติทางเคมีกายภาพของเดกซ์แทรนดัดแปลงที่ได้จากการแยกตัวยตัวทำละลาย (การศึกษาแรงตึงผิวระหว่างน้ำมัน/น้ำ แรงดันที่ผิวหน้า และการวัดความหนืดแบบค าปิลารีในไดเมทิลซัลฟอกไซด์) และยังศึกษาการย่อยสลายเดกซ์แทรนดัดแปลงด้วยเอนไซม์เดกซ์แทรนเนสและลิเพสจากตับอ่อน อนุภาคนาโนของเดกซ์แทรนเดคาโนเอตที่มีระดับการแทนที่อยู่ระหว่าง 25 และ 150 เปอร์เซ็นต์สังเคราะห์ได้โดยวิธีตกตะกอนแบบนาโน นอกจากนี้ยังศึกษาขนาดและความเสถียรของอนุภาคนาโนที่ได้จากเดกซ์แทรนที่มีระดับการถูกแทนที่ต่าง ๆ พบว่า เดกซ์แทรนที่มีระดับการถูกแทนที่ต่ำ (~15%) สามารถใช้เป็นสารเพิ่มความเสถียรของคอลลอยด์อนุภาคนาโนเมื่ออยู่ในสภาวะที่มีค่าความเข้มข้นของไอออน มากกว่า 0.01 โมลาร์ และยังศึกษาความสามารถในการบรรจุลิโดเคนซึ่งเป็นต้นแบบของยาลงในอนุภาคนาโน งานวิจัยนี้พบว่าอนุภาคนาโนของเดกซ์แทรนเดคาโนเอตนี้สามารถนำไปประยุกต์ใช้ในระบบการนำส่งยาในร่างกายได้อย่างมีประสิทธิภาพ

124 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF ABBREVIATION

cm^2	=	square centimeter
^oC	=	degree Celsius
DMSO	=	Dimethylsulfoxide
DMF	=	Dimethylformamide
DS	=	Degree of substitution
Da	=	dalton
DexT40	=	Dextran T40
g	=	gram
mN/m	=	milli Newton per meter
µm	=	micrometer
mg	=	milligram
mL	=	milliliter
µL	=	microliter
mM	=	millimole
µmol	=	micromole
M	=	molar
Mw	=	molecular weight
min	=	minute
nm	=	nanometer
h	=	hour
THF	=	Tetrahydrofuran
^1H NMR	=	Proton Nuclear Megnetic Resonance
^{13}C-NMR	=	Carbon Nuclear Megnetic Resonance
ppm	=	parts per million
MHz	=	megahertz
VD	=	Vinyl decanoate

CHAPTER I
INTRODUCTION

Polysaccharides are highly hydrophilic macromolecules obtained from renewable resources such as plants, animals or microorganisms. These biopolymers are increasingly used either in their native state or as starting materials for further modification because of their biocompatibility, biodegradability and commercial availability. Due to their inherent hydrophilicity linked to the large number of hydroxyl groups, the synthesis of amphiphilic derivatives involves the covalent attachment of hydrophobic groups, for instance hydrocarbon chains (aliphatic or aromatic), to sugar units. The amount of hydrophobic groups attached to polysaccharide main chain divided by the number of repeat units is called degree of substitution. Inter- or intra-molecular hydrophobic association of these polymers in aqueous solution provides valuable properties exploited in several practical applications including pharmaceuticals, coatings, adhesives, surfactants, emulsifiers, *etc.*

Among the different polysaccharides, dextran, a bacterial polysaccharide consisting essentially of α-1,6 linked D-glucopyranoside residues, is well known for its degradability by dextranase, biocompatibility and non-toxicity. Dextran chains bear a large number of secondary OH groups that can be modified into different types of functional groups, mainly ester or ether, or grafted with hydrophobic synthetic polymers. Amphiphilic dextrans have been reported for their application in medical and pharmaceutical fields. Recently, they have been used for the preparation of nanoparticles as drug delivery systems *via* several strategies, emulsion/solvent evaporation, nanoprecipitation, miniemulsion polymerization. Dextran derivatives were used either as stabilizers leading to the formation of a superficial hydrophilic shell around hydrophobic nanoparticles or to form the core of the nanoparticle, leading to the formation of hydrophobic polysaccharide-based nanoparticles. Nature of grafted functional groups and degree of substitution played important roles in nanoparticles

properties. Dextran derivatives densely grafted with short chain ester groups or with long chain ester groups but low modification degrees have been reported. Despite the number of studies, dextrans grafted with long chain esters at high modification degrees have not been exploited yet.

The synthesis of dextran esters is undertaken *via* esterification or transesterification reactions. Since dextran is only soluble in highly polar solvents such as dimethylsulfoxide (DMSO) or dimethylformamide containing LiCl (DMF + LiCl) in which are commonly used as reaction media. Even if dextran esters can be synthesized using chemical catalysts, the use of enzymatic catalysis allows the preparation of dextran esters in soft conditions. In addition, it is known that enzymatic catalysis can provide regioselective modifications of polymers that could be very difficult to obtain via chemical strategies. However, the drawback of enzymatic synthesis of dextran ester is the loss of enzyme activity in DMSO and DMF. Therefore, pretreatment of enzyme prior to use in the reaction is necessary for the synthesis of dextran esters with high degree of substitution.

In this study, strategies to synthesize dextran decanoate by enzymatic catalysis were developed. Pretreatment of enzyme prior to use in the reaction, the use of solvent mixtures and stepwise enzyme addition were investigated in order to obtain high degrees of substitution. Physico-chemical properties of the obtained derivatives were carefully characterized. Dextran decanoate derivatives could then be used to formulate novel promising dextran-based nanoparticles for drug delivery applications via the nanoprecipitation method. The influence of dextran derivatives characteristics and formulation conditions on nanoparticles size and stability, enzymatic degradation and encapsulation capacity of nanoparticles were depicted.

Objectives

The general objective:

The aim of this study was to develop the strategies to synthesize dextran decanoate by enzymatic catalysis and investigate their physico-chemical properties in order to formulate nanoparticle for the use as drug carrier.

The specific objectives:

1. To increase the degree of substitution (DS) of dextran decanoate by enhancing the enzymatic catalysis in DMSO.
2. To study physico-chemical properties of dextran decanoate at different DS.
3. To determine the degradability of dextran decanoate by dextranase and lipase from pancreatic.
4. To prepare nanoparticles of dextran decanoate by nanoprecipitation and evaluate the capacity of nanoparticles to encapsulate small hydrophobic molecule.

CHAPTER II
LITERATURE REVIEW

2.1 Dextran

2.1.1 Structure, physical properties and reactivity

Dextran is a neutral bacterial polysaccharide consisting of glucopyranose units mainly linked by α(1→6) glucosidic linkages with a small percentage of α-1,3 linked side chain (Figure 2.1). The degree of branching has been estimated as 5% by periodate oxidation [1] and methylation analyses [2].

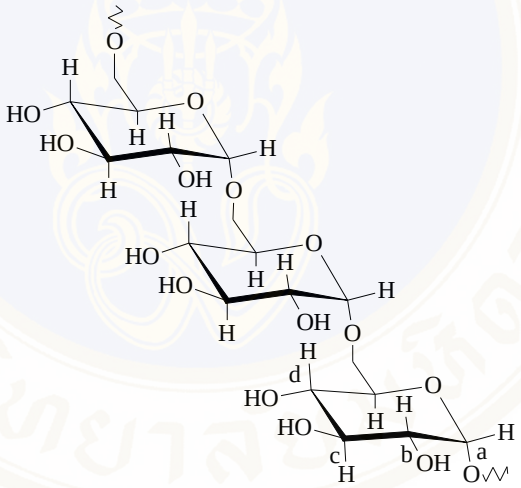


Figure 2.1: Chemical structure of dextran; the position of anomeric hydrogen (a) and secondary hydroxyl groups, OH-2 (b), OH-3 (c) and OH-4 (d) on glucopyranosyl residue of dextran.

Commercial dextran is produced by *Leuconostoc mesenteroides.* Dextran contains only one type of reactive groups, hydroxyl, with three groups in each repeat unit (Figure 2.1). A small percentage of the hydroxyl groups in dextran are primary since their glucopyranoside residues are linked to each other by α (1→3) glycosidic linkages. Therefore, the reactivity of dextran involves a study of relative reactivity of the secondary, equatorially orientated hydroxyl groups, OH-2, OH-3 and OH-4. It was reported that the reactivity at OH-2 towards methylation is higher than at OH-3 or OH-

4 [3]. This is rationalized in terms of the higher acidity of the OH-2 due to its proximity to the anomeric center. The position of anomeric proton and secondary alcohol was presented in Figure 2.1.

The molecular weight of native dextran is available in multiple ranges in commercial. Since higher $\overline{Mw}$ is ascribed to the increase in viscosity and branching and made them not suitable for commercial application [4], dextrans with molecular weight in the range of 10,000 to 70,000 Da (Dextran 10-70) is preferable as a starting material to synthesize dextran derivatives. Water and some polar solvents as DMF and DMSO are good solvents for dextran which allow expansion of dextran coils. On the other hand, they are rather compact in poor solvent and become crystalline in monohydric alcohol and most ketones [5]. Its solubility in organic solvents enables the feasibility to synthesize dextran derivatives.

2.1.2 Biomedical applications of native dextran

Dextrans offer many attractive features for synthesis of clinical and technical products due to their solubility in water and some polar solvents, excellent biocompatibility and clinical safety records, biodegradability and good availability. Dextrans have been used in medical field for several decades. The early idea of utility of dextran was as plasma substituent. Dextran 40 and 70 is worldwide used as intravenous solutions, dextran solution in normal saline, with dextran function both as plasma volume expanders and means of parenteral nutrition. Dextran solution provides an osmotically neutral fluid that once in the body is ingested into glucose and free water. It also increases blood sugar levels. Dextran solution is used to replace lost blood in emergency situations recommended for treatment of impending shocks due to hemorrhage, burns, surgery or trauma [6]. Dextran 40 and 70 are used medicinally as an antithrombotic (anti-platelet), to reduce blood viscosity, thus improve blood flow. Binding of dextrans with erythrocytes, platelets, and vascular endothelium increases their electronegativity and thus reduces erythrocyte aggregation and platelet adhesiveness. Moreover, dextran 70 is commonly used in some artificial tear or eye drops as a lubricant for the relief of various ocular irritation syndromes. Soluble low molecular weight Dextran (Dextran 20) is used for reducing plaque by inhibiting the

adherence of bacteria to the dental enamel and by inhibiting the synthesis of insoluble glucan by glucosyltransferases in the oral cavity [7].

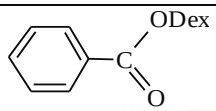
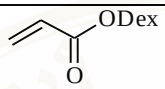
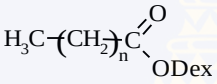
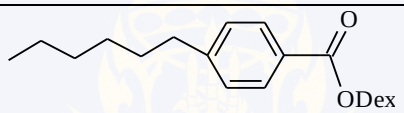
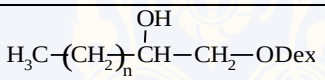
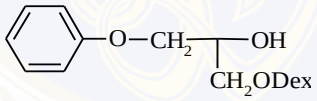
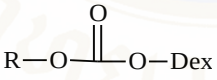
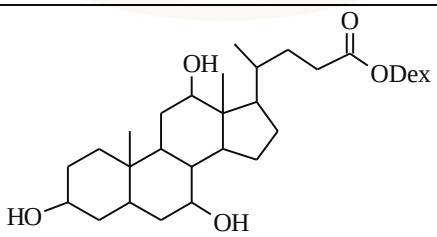
2.2 Modification of dextran

Since dextrans contain a large amount of hydroxyl groups, they can be easily modified with several functional hydrophilic or hydrophobic groups. Modification of dextrans with hydrophobic groups gives amphiphilic dextrans which have been suggested for several applications including polymeric surfactants [8], hydrogels formation [9] and nanoparticles formation [10]. Amphiphilic dextran can be derived from grafted of synthetic polymers as poly(ε-caprolactone), polylactide, poly(methyl methacrylate), poly(isobutylcayanoacrylate) and poly(ethylene glycol) [11-13] or hydrophobic functional groups as ester and ether. A number of reactants used as hydrocarbon tails were attached to dextran mainly via ester and ether functions (Table 2.1).

Because of differences in chemical environment and steric hindrance, the three hydroxyl groups of each glucopyranose unit, exhibit distinct reactivities [14]. Consequently, the distribution of hydrocarbon substituents within sugar rings is controlled by the order of reactivity, the followed synthesis strategy and the DS. The resulting substitution pattern has been reported in some cases but no general study is available. Examples of reactants for modification of dextran and their regioselectivity are shown in Table 2.2.

The control of the position of the chemical modification of dextran can be achieved by the use of reactants involving a specific mechanism like in the case of ketodextran synthesis by oxidation of dextran [15, 16]. Hydrogen bond formation in reaction intermediates can also induce regioselectivity like in acylation reactions with acetic anhydride as compared to that involving acetyl chloride [17].

Table 2.1: Chemical structure of hydrophobic groups and chemical bonds for modification of dextrans.

Hydrophobic groups		Chemical bonds	References
Benzoate		ester	[24]
Acrylate		ester	[18]
Adipate		ester	[20, 25]
Carboxyl groups	 n ≤16	ester	[26-30]
4-hexylbenzoate		ester	[31]
Epoxides	 n = 5, 7	ether	[8]
Phenoxy group		ether	[32]
Carbonate groups	 R = Cyclic or alkyl group	carbonate	[33-35]
Cholate		ester	[36]

For esterification or transesterification reactions, the hydroxyls in positions 2 and 3 are preferably substituted. Ferreira *et al* [18] showed that the use of enzymatic catalysis could influence strongly the substitution pattern according to the chosen enzyme (Table 2.2). The resulting substituent distribution can be either very similar to that obtained with chemical catalysis or significantly different. The choice of enzyme

is thus an additional parameter for the control of substitution pattern in dextran modification. To our knowledge, no detailed study of the effect of the substituent position onto physico-chemical properties has been reported for dextran derivatives. This might be a consequence of the difficulty to obtain regioselective modification of sugar rings with functional substituents. Nevertheless, we must mention here that results were reported about the effect of the position of the oxidized groups within sugar rings on the rate of enzymatic hydrolysis of ketodextrans [19].

Table 2.2: Regioselectivity of dextran modified with different reactants as reported in various studies

Modification	Reactant	Reactivity order	Ref.
Esterification	Acetyl chloride	C-2 > C-4 > C-3	[17]
	Acetic anhydride	C-2 ≈ C-3 > C-4	
Esterification	Not given	C-2 > C-3 > C-4	
Esterification	Acetic anhydride in aqueous NaOH (pH 8)	C-2 ≈ C-3 ≈ C-4	[37]
	Acetic anhydride in formamide-pyridine	C-2 > C-3 ≈ C-4	
Esterification	1-naphthylacetic acid, 2-(6-methoxy-2-naphthyl)propionic acid, nicotinic acid	C-2 > C-4 > C-3	[38]
Esterification	Chloroacetyl chloride	C-2 > C-3 > C-4	[34]
Transesterification	Glycidyl methacrylate	C-2 ≈ C-3 > C-4	[39]
Transesterification	Vinyl acrylate (Proleather catalysis)	C-2 ≈ C-3 >> C-4	[18]
	Vinyl acrylate (*C. rugosa* lipase catalysis)	C-3 > C-2>>C-4	
	Vinyl acrylate (4-DMAP chemical catalysis)	C-2 ≈ C-3 >>C-4	
Ether formation	Ethylene oxide	C-2 > C-4 > C-3	[40]
Nucleophilic substitution	Ethyl chloroformate	C-2 > C-4 > C-3	[41]
Sulphation	SO_3 – pyridine	C-3 > C-2 > C-4	[42]
Cyanoethylation	Acrylonitrile	C-2 > C-4 > C-3	[43]
Silylation	1,1,1,3,3,3-Hexamethyldisilazane	C-2 > C-4 > C-3	[44]
Carboxymethylation	Not given	C-3 ≥ C-2 > C-4	[45]
Carboxymethylation	Chloracetic acid	C-2 > C-4 > C-3	[46]
Oxidation	Bromine	C-4 ≥ C-2 > C-3	[15]
	Acetic anhydride, DMSO	C-3 > C-2, C-4	[16]
Ether formation	Methyl iodide	C-2 > C-4 ≥ C-3	[47]

Recently, a few studies appeared about the use of enzymatic catalysis in the chemical modification of modified dextran. The use of soft conditions with the ability to obtain homologue series with regularly varying structural parameters is a strong advantage for optimizing end-use properties. Enzymatic catalysis allows the preparation of dextran derivatives (esters) which would otherwise require chemical catalysis [9, 18, 20]. In addition, it is known that enzymatic catalysis can provide regioselective modifications of polymers that could be very difficult to obtain by chemical strategies [21-23].

2.3 Biomedical application of dextran derivatives

As mentioned in section 2.1, several properties of dextran highlight their benefits as material for biomedical applications. The advantage of dextran over other polysaccharides is solubility of dextran in water. With $\alpha(1\rightarrow6)$ glucosidic linkages, dextran is classified as very flexible and extended polymer. Dextran solution with different $\overline{Mw}$ and concentrations could provide different colloidal ostomic pressure which is an important parameter for biomedical applications [6]. However, the price of dextrans is a factor that restricts the widespread commercial uses compared with starches and celluloses. Consequently, applications of dextrans are focused mainly in the high quality or high technology products with proven technical or biological effects. Several dextran derivatives are well known for biomedical applications. Fore instance, dextran sulfate is known as a possible substitute for heparin in anticoagulant therapy [6]. Iron-dextran complex solution is used for treatment of human and veterinary anemic iron deficiency. Diethylaminoethyl dextrans prepared from Dextran 500 have been reported to reduce serum cholesterol and triglycerides.

For several years, the dextran derivatives are shown to extend possibility for drug delivery as following attempts: increasing the longevity of therapeutic agent in the circulation, drug targeting to specific sites and increasing *in vivo* stability and decreasing the *in vivo* immunogenicity of proteins or enzymes. Several strategies have been developed for utility of dextran for drug delivery.

2.3.1 Dextran conjugates

Dextran conjugated to bioactive substances has gained an interest for drug carrier system as prodrugs. Dextran conjugates may offer a solution to drug design problem. Several techniques have been introduced for conjugation with dextran including periodate oxidation leading to the introduction of dialdehyde groups that can react with amino-groups in proteins [48, 49], coupling procedures via cyanogens bromide activation [50], introduction of oxirane groups by reaction with epichlorohydrin [51], conjugation with amino or carboxyl [52] groups and introduction of non-steroidal esters as anti-inflammatory drug [53]. It has been reported that dextran conjugates showed the prolongation of the effect and reduction in the immunogenicity of drugs or proteins. However, stability of dextran conjugates is low after oral administration. Therefore, most application of dextran conjugates is via the injection [54].

2.3.2 Dextran-based hydrogels

Hydrogels are three-dimensional, cross-linked networks of hydrophilic polymers which capable to absorb large amount of water. Hydrogels are commonly used in clinical practice and experimental medicine for a wide range of applications, including tissue engineering and regenerative medicine [55], immobilization of enzyme or cells [56], separation of biomolecules or cells [57], and delivery systems for gene therapy and protein controlled-released systems [58]. The unique physical properties of hydrogels have gained considerable interest in their use in drug delivery applications. The porosity of hydrogels can be controlled thereby the density of cross-links in the gel matrix and the affinity of the hydrogels for swelling conditions. Their porosity allows loading of drugs into the gel matrix. Subsequently, the release of drugs depends on the diffusion coefficient of the loaded molecules through the gel network. The advantages of hydrogels for drug delivery are prolongation of drugs life time and slow release to the surrounding tissues over an extended period [59].

Dextran-based hydrogels have been reported for their uses in drug delivery system. Dextran-acrylate hydrogels have been evaluated for their biocompatibility by *in vivo* subcutaneous and intramuscular implantation in rats. These hydrogels have been suggested for suitable applications as implantable longterm peptide/protein

delivery systems or scaffolds for tissue engineering [9]. Hydrogels based on dextran cross-linked with diisocyanate have been proposed for colon-specific drug delivery. These have been found to be fully degradable by dextranase *in vitro* and *in vivo* in the rat cecum [60]. Dextran hydrogels can be used as possible drug carriers for specific target sites. However, some limitations have to be considered for the use as drug carriers including limitation in quantity and homogeneity of drug loading for hydrophobic drugs. Moreover, tensile strength, pore size and water content of hydrogels are important parameters for localization at targeted site and controlling drug release [59]. To design hydrogels with a desired release profile for a specific drug, it is necessary to examine the chemical properties of hydrogels including degree of swelling, mechanical strength, degradability and release properties.

2.3.3 Derivatives of dextran as polymeric surfactants and stabilizers

Dextrans grafted with hydrophobic groups or polymers have been reported for their uses as polymeric surfactant applying as stabilizer in drug delivery system. The advantages of polymeric surfactants over molecular surfactants are low side effects, particularly during or after parenteral application and high emulsifying activities due to quasi-irreversible adhesion on the interface. Polymeric surfactants exhibit valuable properties for aqueous formulations. Therefore, polymeric surfactants have been applied as stabilizers in variety of applications including adhesive technology and coating, drug delivery systems, medical diagnostic tests, separation media, etc [61]. Therefore, polymeric surfactant derived from dextran is used to covers nanoparticles and found a potential application as drug carriers. Poly(ethylene glycol)-dextran have been reported as a combined stabilizer and surface modifier to produce resorbable poly(D,L-lactide-co-glycolide) (PLG) microparticles proposed for using as drug carriers [62]. Water-soluble dextran fatty acid esters have proved to be good stabilizers for pharmaceutical or cosmetic preparation with low hemolytic activity [27, 30]. Hydrophobically modified dextrans derived from dextran grafted with phenoxy groups have been used as a surface coverage of synthetic polymer nanoparticles [63, 64].

2.3.4 Dextran-based nanoparticles

Nanotechnology is attractive for the use in biomedical application especially in drug delivery system. Nanoparticles for drug delivery applications are defined as submicron (< 1 μm) colloidal particles. The drug incorporation is concerning adsorption or dispersion throughout the matrix or covalent attachment to the surface or into the matrix of nanoparticles. The drug is released from throughout diffusion, swelling, erosion, or degradation. The advantages of nanoparticles as drug carriers are high stability, high carrier capacity, feasibility of incorporation of both hydrophilic and hydrophobic substances, and feasibility of variable routes of administration. The aims of using nanoparticles as a drug carrier are enhanced delivery to, or uptake by, target cells and reduction in toxicity of drug to non target organs. Nanoparticles for drug delivery are constructed from such biocompatible and biodegradable materials as biopolymers including natural or synthetic or solid lipids.

Recently, nanoparticles prepared from self-assembling of amphiphilic polymers have been efficiently developed. Amphiphilic polymers can be synthesized by hydrophobic modification of hydrophilic polymers as polysaccharides. Grafting of hydrophobic groups on dextran backbone dextrans becomes considerable strategy to achieve amphiphilic structure. Numerous studies reported for nanoparticle preparation from amphiphilic dextrans.

Fabrication of nanoparticles form dextran modified with phenoxy or epoxide groups via nanoprecipitation or emulsion/solvent evaporation was addressed by Aumelas *et al* [10]. In that study, highly modified dextrans were suggested as core of nanoparticles whereas water-soluble modified dextrans were used as surface coverage for improving colloidal stability of nanoparticles. Degradation of modified dextran by dextranase was also evaluated.

Polylactide-grafted dextran copolymers were synthesized progressively by three-step strategy, silylation, ring opening polymerization and deprotection. Water-soluble dextrans modified with phenoxy or epoxide groups, or grafted with polylactide were used as stabilizers. Nanoparticles were prepared by emulsion/solvent evaporation procedure as reported by Nouvel *et al* [12]. Nanoprecipitation method was used to prepare nanoparticles from polylactide-grafted dextran copolymers as reported by Gavory *et al* [65].

Nanoparticles fabricated from dextran functionalized with small carboxylic, unsaturated carboxylic and pyroglutamic acid were reported by Hornig *et al* [66]. The esterification of dextran was undertaken via *in situ* activation of the carboxylic groups with *N*, *N*′-carbonyldiimidazole. They suggested that, in that case, a DS at least 2.0 is necessary for nanoparticles formation. Self-assembling of modified dextrans into nanoparticles was undertaken during dialysis process. Afterward, the study of the preparation of nanoparticles from dextran functionalized with poorly water-soluble drugs as ibuprofen or naproxen was examined by Hornig *et al* [67]. Hydrophobic derivatives self-assemble into nanoparticles with high loading efficiency during nanoprecipitation.

Recently, nanoparticles obtained from dextran modified with long chain ester as decanoate was reported by Ge *et al* [68]. The synthesis of dextran ester was undertaken via transesterification catalyzed by lipase. Nanoparticles of modified dextran with 23% DS were constructed by dialysis method.

Regarding to the application of amphiphilic dextran as stabilizer or core of nanoparticles, one challenge is to create nanoparticles that resist protein adsorption whereas maintaining suspension stability. This can be accomplished by immobilizing hydrophilic and neutral polymers on the particle surface, either by physical adsorption or by covalent coupling.

2.4 Enzymatic synthesis of dextran esters

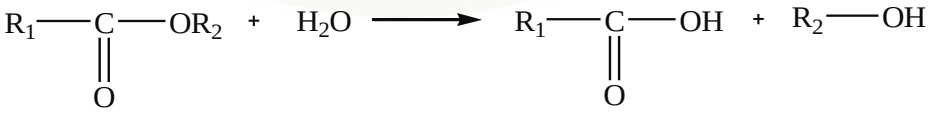
Dextran esters are synthesized by introducing esters to dextran backbone via esterification or transesterification. The chemical synthesis of dextran ester have been performed in treating polysaccharide with acid chloride in absolute pyridine or using *NN'*dicyclohexylcarbodiimide and nucleophilic catalyst as 4-dimethylaminopyridine which is highly toxic and also corrosive. The use of organic compound as carbonyldiimidazole is also recognized for esterification. However, the reaction requires heat or the presence of potent nucleophiles as sodium ethoxide and other strong bases. Instead of using highly toxic and highly corrosive catalysts, enzymatic catalysis allows the mild condition for preparation of dextran esters which

would otherwise require chemical catalysis [9, 18, 20]. In addition, it is known that enzymatic catalysis can provide regioselective modifications of polymers that could be very difficult to obtain by chemical strategies [21-23].

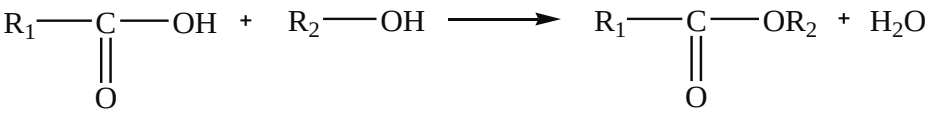
2.4.1 Enzyme

As mention above, the synthesis of dextran ester can be undertaken via esterification and transesterification. Lipase (EC 3.1.1.3) and protease (EC 3.4.21.62) are responsible for those reactions. In aqueous medium, lipases catalyze hydrolysis of ester bond whereas proteases hydrolyze the peptide bond. In non-aqueous solvent, the thermodynamic equilibrium of the reaction is shifted towards synthesis reaction instead of hydrolysis. Both hydrolysis and synthesis reaction depend on the substrates as show in Figure 2.2. For esterification, ester bonds of dextran ester are formed by reacting of carboxylic acids and alcohols which are hydroxyl groups in dextran backbone. For transesterification of dextran ester, exchanging of organic groups in ester with organic groups of alcohol which are glucose units in dextran backbone takes place in non-aqueous solvent. The interest in the use of proteases and lipases as synthetic catalysts has risen rapidly in recent decades [18, 20, 69-71]. Lipase and protease play important roles in dextran ester synthesis due to their stability in organic solvents as DMSO and DMF in which lipophilic substrates are soluble, no cofactor requirement, broad substrate specificity and high regioselectivity [72, 73].

a) Hydrolysis



b) Esterification



c) Transesterification

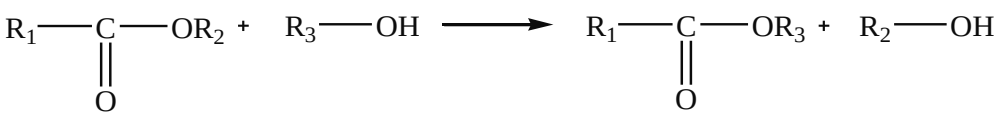


Figure 2.2: Schematic representation of a) hydrolysis, b) esterification and c) transesterification catalyzed by lipase and protease.

From the literature, protease from *Bacillus subtilis*, subtilisin, and lipase from *Candida rugosa* have been found to be the effective enzymes to catalyze the transesterification between sugar and ester in organic solvents [35, 48]. Protease from *Bacillus subtilis* (Proleather FG-F) exhibited the highest efficiency in catalyzing the transesterification of sucrose with divinyladipate in dimethylformamide. This enzyme was found to have high regioselectivity at secondary alcohol groups by giving sucrose 2-*O* vinyladipate as the major product of the reaction with divinyladipate [20]. Our previous study reported the screening of enzyme for transesterification between dextran and vinyl decanoate. It was found that the synthesis of dextran decanoate by lipase AY from *Candida rugosa* gave higher %DS than that of Proleather FG-F [74].

The enzyme-catalyzed esterifications have been reported for the synthesis of sugar ester [75-77]. However, direct esterification of a carboxylic acid with a hydroxyl group leads to the production of water which is thermodynamically unfavourable for ester synthesis. Water content in the reaction medium was found to be important parameter for esterification of sugar ester. Water must be removed from the medium to assure a high synthetic activity [78].

2.4.2 Enzyme in organic solvent

For several decades, the use of enzyme in organic solvents has been well recognized. The advantages of using enzyme in organic solvents are higher stability of enzyme and chemo-, regio-, and stereoselectivity. These are of interest for synthesis of pharmaceutical, fine chemicals and functional polymers with well-defined molecular structures that correspond to their required properties. Moreover, there are some reasons described the advantages of enzymatic conversions in organic solvents including feasibility to recover or reuse insoluble enzyme in organic solvents, less undesirable side reactions and worse in thermodynamically favorable in organic solvents [70].

The properties of solvent affect the catalytic activity of lipase in organic solvents. The effect of solvent hydrophobicity on the regioselectivity of acylation of phenyl 6-*o*-trityl-*β*-D-glycopyranosides using *Pseudomonas cepacia* lipase was investigated. The ratio of the two products formed, namely the 2-acetate and the 3-acetate, was found to be markedly affected by the nature of the reaction medium. In

solvents of low log P, values up to 85% of the 3-acetate was formed compared to less than 40% in solvents of high log P values [79].

It is worth noting that, as in case of catalytic activity, enzyme stability is enhanced in pure solvent. Nevertheless, several attempts of using an enzyme powder in an organic solvent are needed to be considered. The polarity of the solvents plays important role for enzymatic conversion. Hydrophobic solvents are preferable to hydrophilic ones since the latter have a greater characteristic to strip tightly bound water from the enzyme molecules, although some enzymes remain catalytically active even in the most hydrophilic organic solvents. Enzyme preparation in prior optimal pH aqueous solutions is required. The use of enzyme in organic solvent is not a conventional wisdom. Microemulsion of enzymes in water and water immiscible solvents or direct suspension of powdered enzymes are two attempts to use enzyme in organic solvents. For direct suspension in organic solvents, the small enzyme particles should be obtained to increase the accessibility of substrates [71]. Water content of organic solvent influenced enzymatic synthesis in organic solvents, thus controlling of water content is important in some case [80].

Although numerous enzymes are catalytically active in a vast variety of organic solvents, in some case, a crucial problem is the much lower activity of enzymes in anhydrous media than in water. The important reason for diminished enzymatic activities in organic solvents results from reduced structural flexibility of enzyme. In aqueous environments, enzymes possess the conformational mobility necessary for optimal catalysis [81]. On the contrary, the conformation of enzyme is rigid in organic solvent. Fortunately, this property results the increase of stability of enzyme that is advantageous when conducting reaction at high temperature. At high temperature in aqueous solution, the unfolding of enzyme molecule occurs easily due to the flexible conformation of enzyme [82]. Depending on the type of solvent, the activity and stability of enzyme can be increased.

The disadvantages of using polar organic solvent as reaction medium for enzymatic reaction have been mentioned in the above paragraph. As the matter of fact, very few enzymatic catalyses have been realized in strong polar solvent, as DMF and DMSO known as universal solvents and particularly useful for dissolving high molecular weight substrates. DMSO and DMF are denaturing solvent for enzyme due

to their dissociate capacity to tertiary structure of enzymes leading to enzyme unfolding and deactivation [83, 84]. Moreover, the stripping of water from the surface of enzyme results in dehydration and consequently inactivation [85].

The difficulty encountered in enzymatic transesterification of dextran is the need to conduct the reaction in DMF or DMSO since dextran is soluble only in such solvents. Therefore, the enhancement of lipase stability and activity in those stringent conditions is the crucial importance. Several strategies have been proposed for the improvement of catalytic activity and stability of enzyme in organic solvents.

2.4.3 Improvement of enzyme activity and stability in organic solvents

It is noteworthy that the preparation of enzyme prior to use in organic solvent is of relevant importance. It has been documented that the enzymatic activity in purely aqueous solution is independent of the enzyme's history since in this solution enzyme conformation remains flexible. For this reason, the enzyme assays in such system are inherently reproducible. This is not the case, however, in non-aqueous media. The lack of the water acting as the molecular lubricant, leads to the rigidity of enzymes molecules in this unconventional media. Consequently, the enzymatic activity in a given system may be profoundly affected by how the enzyme is prepared, for example by its history [86]. Therefore, the suitable method of enzyme preparations for their use in peculiar solvent must be known. Many strategies have been reported to improve stability of enzyme in organic solvent.

2.4.3.1 Lyophilization

Lyophilization is the traditional method for improvement of activity and stability of enzyme in organic solvents. This method provides the change of secondary structure of many different proteins. In most of proteins a marked reversible change in proteins secondary structure has been observed. Griebenow *et al* [87] reported that lyophilization promotes the decrease in α-helices and a concomitant increase in β-sheet. Consequently, lyophilization made the proteins structure more ordered and more rigid. On the other words, one can say that all proteins undergo a significant reversible denaturation on lyophilization, leading to a plunge in enzymatic

activity. Lyophilized enzymes suspended in organic solvent have proven to be useful synthetic catalysts [87]. Secondary structures of lyophilized α-chymotrypsin and subtilisin Carlsberg in polar and nonpolar solvents were studied by Dong *et al* [88]. They reported that secondary structure of lyophilized α-chymotrypsin after suspension in ethanol and hexane did not alter the unfold structure observed in dry powder. For subtilisin Carlsberg**,** the perturbation structure was observed for dried enzyme suspended in ethanol whereas the native-like structure was observed in hexane and pyridine.

2.4.3.2 pH adjustment

As mentioned previously that enzyme‘s history influences enzyme activity in organic solvents. It has been reported that enzymes have a pH memory of the last aqueous solvent. pH is an crucial parameter affecting enzymatic activity in aqueous solution, but it is not in organic solvent. Nevertheless, lyophilization of enzyme in buffer at optimal pH enhances the activity of enzyme in organic solvent regarding to their memory of the last aqueous solution to which they were exposed. This thought to be due to ionogenic groups of protein that retain their last ionization state on either dehydration or subsequent placement in organic solvent. Lyophilization of enzyme in aqueous solution of pH optimal for catalysis can improve enzymatic activity in organic solvent [82]. The pH of buffer solutions plays an important role on the charge density of the enzyme surface. The conformation and surface charge are then fixed in the anhydrous organic solvent [89]. It has been reported that pH-adjusted lipase AY (lipase from *C. rugosa*) at phosphate buffer pH 7.5 before lyophilization significantly enhanced transesterification activity of dextran with vinyl decanoate in DMSO [74].

2.4.3.3 Use of enzyme conformational preservative

Although lyophilization have been proven to enhance enzyme activity in organic solvent, in some case, the changing of secondary structure of enzyme by decreasing in α-helices and increasing in *β*-sheet upon lyophilization can cause reversible denaturation leading to the loss of enzyme activity. One way to prevent reversible denaturation during lyophilization process is to lyophilize enzyme

in the presence of their substrate. Binding of substrate to the active site could protect the enzyme from dehydration-induced inactivation during lyophilization. Another way to protect enzyme is to co-lyophilize with lyoprotectants such as sorbitol, sugars poly(ethylene glycol) [90] and certain inorganic salts [91]. Macrocyclic compounds such as crown ethers have been found to be effective molecules to activate various enzymes [92, 93]. In addition, cyclodextrins are another class of macrocyclic compounds used to increase the enzyme activity in organic solvents [94]. It has been reported that lyophilization of enzyme in the presence of these substrates preserved enzyme conformation in organic solvent by fixing the enzyme into a conformation that is favorable for catalysis during lyophilization. This process can protect the enzyme from the loss in the activity [95].

2.4.3.4 The mixture of two solvents as reaction media

The use of strong polar organic solvent as DMF and DMSO as reaction medium for enzymatic synthesis of sugar ester has major drawback in the loss of enzyme activity and stability. Since the polar solvent is needed for dissolving sugar substrates and retaining of enzyme activity. Thus, the interest of using solvent mixture to compromise these two attempts has been raised. In the literature, the two-solvent mixtures were applied as the media for transesterification of sugar with fatty acid ester. In the enzymatic synthesis of fatty acid esters of sucrose, maltose, leucrose and maltotriose using mixtures of miscible solvents as DMSO and 2-methyl-2-butanol as reaction medium, the molar ratio sucrose diesters versus sucrose monoesters was found to be enhanced by decreasing the hydrophilicity of the medium (lowering the percentage of DMSO [96]. The synthesis of sugar esters by transesterification of sugars with fatty acid vinyl esters catalyzed by lipases from *Thermomyces lanuginosus* (immobilized by granulation with silica) and C*andida antarctica* B in 2-methyl-2-butanol:DMSO mixtures was studied. Both lipases were similarly effective for the regioselective synthesis of 6′-*O*-palmitoylmaltose and 6-*O*-lauroylglucose. It was suggested that the mixture of these two solvent increased enzyme stability when compared with pure DMSO. The mixture of solvent compromises stability of enzyme and solubility of sugar [97, 98].

2.4.3.5 Enzyme Immobilization

Immobilization is often the key to optimizing the operational performance of an enzyme in inorganic solvents. A benefit is often to enhance the stability under operational conditions, e.g., towards denaturation by heat or organic solvents or by autolysis. Different methods for the immobilization of enzymes have been proposed including the binding to a prefabricated support (carrier), entrapment in organic or inorganic polymer matrices, and cross-linking of enzyme molecules. These techniques can prevent the structural changing of enzyme, providing the active conformation of enzyme in organic solvents. Recently, the synthesis of modified dextran in DMSO using the encapsulated lipase in nanogel was reported by Ge *et al* [99]. They suggested that polyacylamide nanogel prevented the extraction of essential water and preserved the native configuration of encapsulated lipase in anhydrous DMSO leading to the enhancement of lipase stability.

2.4.4 Lipase catalyzed-transesterification of dextran

Dextran consists of large amount of OH groups which can be modified with ester via transesterification. Since dextran is soluble in polar solvents such as DMSO and DMF known as enzyme denaturing solvents, pretreatment of enzyme is necessary for improving enzyme activity and stability in those solvents. The stability of lipases in solvents as DMSO and DMF proposed the usefulness for enzymatic catalyst. Moreover, the advantages of the enzyme are selectivity and specificity in catalysis reaction. The enzyme catalyzed chemical reaction occurs on three levels, chemo-, regio- and stereoselectivity. Chemoselectivity is the activity of the enzyme to direct the catalytic action to specific functional group in the molecule such as to distinguish between OH or NH. Regioselectivity is the capability of enzyme to catalyze the reaction of one particular OH-group situated in specific region of molecule amongst various OH groups. Stereoselectivity is the capability of enzyme to alter stereoisomers from a single reactant.

Recently, the pH adjustment of lipases at optimal pH prior to lyophilization has been reported in the work of Ferreira *et al* [18] for catalysis by lipase from *C. rugosa* (lipase AY) in DMSO. The enzymatic transesterification of dextran with vinyl acrylate has been demonstrated. Enzymatic modification of dextran

and the study of its polymerization in order to prepare hydrogels have been reported. Interestingly, after 12 hour of reaction both enzymes seem to loss their stability. After 72 hours of the reaction, it was found that degree of substitution was 25% in the presence of active lipase AY. Regioselectivity of lipase AY was evaluated. It was found that two positional isomers of dextran modified at position 2 and 3 of glucopyranosyl residue were obtained. In that study, lipase could not distinguish perfectly two secondary hydroxyl groups at 2 and 3 positions. However, the regioselectivity of enzymatic transformation over chemical transesterification was observed.

Since the repeated glucopyranoside residues of dextran are linked to each other at position 1 and position 6, the only small amount of primary hydroxyl group is available. For this reason, the reasonable comparison of the selectivity between hydrolytic enzyme-catalyzed acylation of dextran and protected glucose molecules, glucose that OH group at position 6 (O-6) is occupied with akyl groups, were analyzed as shown in Table 2.3.

Table 2.3: Enzyme-catalyzed transesterification reaction involving protected glucose molecules

Protected glucose	Acyl donor	Catalysts	C2:C3 ratio
6-O-butyryl glucose [100]	2,2,2-trichloroethyl butyrate	Lipase form *Candida cylindracea*	60:40
6-O-triaryl-D- glucose [101]	vinyl acetate	Lipase from *Pseudomonas cepacia*	86:14
Dextran [18]	vinyl acrylate	4-dimethylamino pyridine (4-DMAP)	85:15
Dextran [18]	vinyl acrylate	Lipase from *C. rugosa*	28:72
Dextran [18]	vinyl acrylate	Proleather FG-F	43:57
Dextran [74]	vinyl decanoate	Lipase AY form *C. rugosa*	48:52
Dextran [99]	vinyl decanoate	L-1754 from *C. rugosa*	70:30

The difference in regiospecificity observed in the experiment shown in Table 2.3 might be due to the nature of acylating agents used [102], the nature of DMSO as opposed to more non polar solvents, or to the specific 3D architecture of

dextran in solution, which may favor the acylation at position 3, or to a combination of each factor.

Three different types of acyl donors are widely used in the synthesis of sugar ester including vinyl ester, alkyl ester and carboxylic acid. For vinyl ester and alkyl ester, the sugar esters are synthesized via transesterification while sugar esters are synthesized via esterification by using carboxylic acid as an acyl donor. These reactions are mostly performed in organic solvent. Amongst those acyl donors, vinyl ester is the most popular largely due to its high reactivity. The unique reactivity of vinyl ester is due to the production of enol which subsequently tautomerizes into acetaldehyde (Figure 2.3).

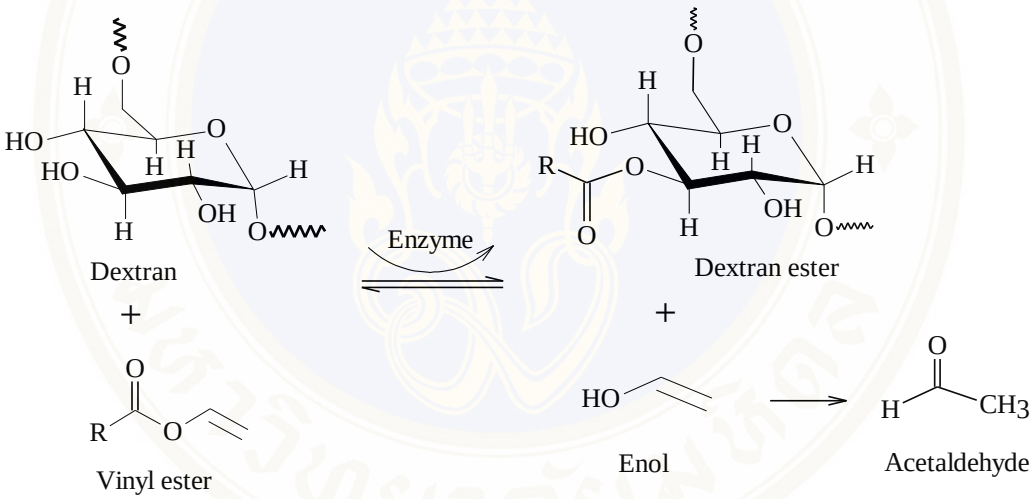


Figure 2.3: Schematic representation of enzymatic transesterification of dextran with vinyl ester.

This makes the reaction irreversible providing products in high chemical and optical purity. However, the accumulation of acetaldehyde by product can cause the inactivation of enzymes which limits the use of vinyl ester [103]. Using carboxylic acid to synthesize sugar ester via esterification shows the minor drawback in the promoting of water during the reaction, the hydrolysis reaction may occur. Our previous study demonstrated the lipase catalyzed the synthesis of dextran decanoate by using different acyl donors including vinyl decanoate, methyl decanoate and decanoic acid. Similar to the literature, highest DS of dextran decanoate was obtained from

vinyl decanoate in the presence of lipase AY whereas methyl decanoate and decanoic acid gave very low DS [104].

Another property of acyl donor that can affect its reactivity is chemical structures including number of carbon in carboxylic acid chain, chemical bond (saturated or unsaturated bond) and type of hydrocarbon chain (aromatic hydrocarbon, linear or branched alkane or cycloalkane). Generally, the smaller size is more reactive acyl donor. Changing acyl group of acylating agents was found to be significant for enantioselectivity in the enzyme catalyzed transesterification. The enantioselectivity was enhanced markedly by changing the acylating agent from small vinyl ester to medium vinyl ester, and dropped substantially with longer acyl donors [105].

For the synthesis of dextran ester in organic solvent, several parameters are needed to be optimized including temperature, reaction time, concentration of enzyme and the mole ratio of the two substrates. Our previous study reported the systematic optimization of transesterification of dextran with vinyl decanoate catalyzed by pH-imprinted lipase AY. The significant increase of transesterification in DMSO in the presence of pH-imprinted lipase AY was observed when compared with native lipase AY and reaction with enzyme. At optimal condition, the DS of dextran decanoate reached 62% after 103 h with the presence of pH-imprinted lipase AY [74].

More recently, the study of transesterification of dextran with vinyl decanaote in DMSO was catalyzed by lipase encapsulated in nanogel [99]. Lipase from *C. rugosa* was encapsulated in polyacrylamide gel. The DS was 23% at 60°C after 240 h of the incubation. The substitution was preferential at 2-position. Degradation of obtained dextran decanoate by lipase was also reported. Nanoparticle formation of dextran decanoate was undertaken from 25%DS polymer by dialysis method. The possibility of nanoparticles to encapsulate protein was examined for approaching the use as drug carrier [68].

To improve the enzymatic reaction in DMSO, several strategies as described in section 2.4.3 are needed for increasing of DS of dextran ester. Highly modified dextran enables the possibility for the use in drug delivery system.

2.5 Degradation of dextran

Dextrans are biopolymers that have been used in biomedical field for several decades due to their degradability in human body by dextranase. This property enables the development of dextran derivatives for multiplying their application in biomedical field.

2.5.1 Dextranase degradation

Dextranase (EC 3.2.1.11; α-1,6-glucan 6-glucanohydrolase) hydrolyzes the α-1,6-glycosidic linkage in dextran polymers. The dextranase cleaves the linkages within the dextran molecule and the products are essentially low molecular weight sugars with reducing end as glucose and isomaltose (Figure 2.4). Many bacteria produce extracellular dextranases that split dextran into low molecular weight sugars. Examples of these are *Lactobacillus, Cellvibrio, Cytophaga, Penicillium, Verticillium* and soil *Bacillus spp.* Dextran products are readily absorbed into the natural environment. In human body, dextranase is found in different tissues such as colon, spleen, lung and kidney but not present in blood. The presence of dextranase in those tissues enables the possibility to use dextran as drug carrier targeted to specific sites.

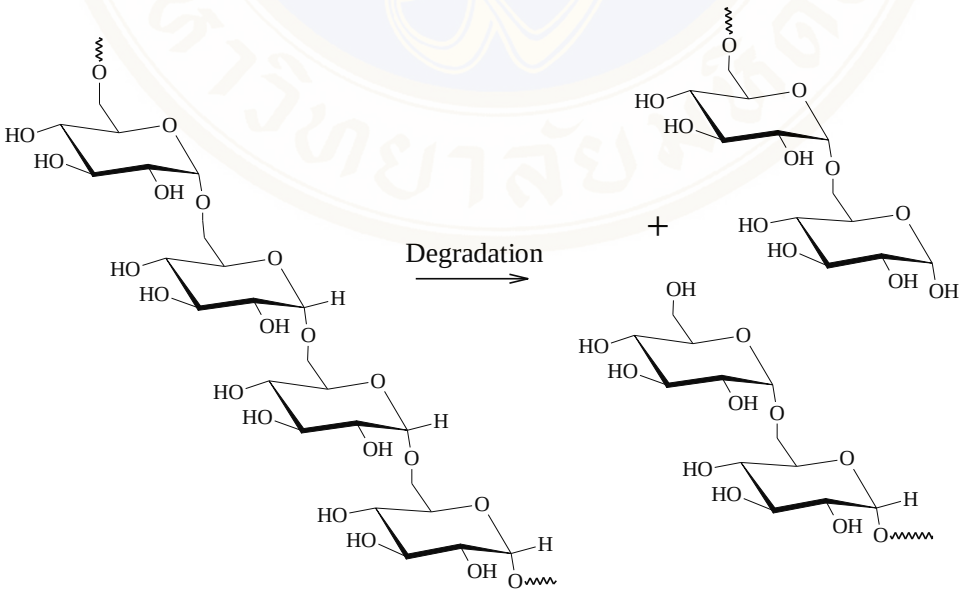


Figure 2.4: Degradation of dextran by dextranase

A large number of dextran conjugates have been reported for their release of active substance by dextranase. Several dextran conjugated with drug known as

prodrug, for examples, dextran–nalidixic acid ester [106], dextran-5-aminosalicylic acid ester [107], dextran–budesonide [108], etc., have been widely used for targeting specific sites that present in dextranase. The dextranase degradation of different amphiphilic dextran derivatives including dextran modified with small ester, phenoxy or epoxide groups, and dextran grafted with polylactide have been reported [10, 12, 28, 109]. Analogous results were observed from those studies in which the increase of DS reduced the degradation of modified dextran. The biodegradability of amphiphilic dextrans permits their use as material for drug delivery system.

2.5.2 Acid hydrolysis

Dextran is generally stable in pH 4-10 at room temperature. However, it can be partially hydrolyzed or depolymerized in the acid condition depending on acid concentration, molecular weight and branching of dextran. Chemical degradation of dextran by pH 0.5 perchloric acid at 90°C was studied at various time of incubation. After 3 h, glucose and dextran oligomer with degree of polymerization from 2 to 5 were observed [110]. Acid hydrolysis is one concern for the use of dextran derivative as drug carriers for oral administration due to the pH in stomach is around 2. However, many studies reported the stability of modified dextran at acidic conditions. Jung *et al.* reported chemical stability of dextran-5-aminosalicylic acid ester, a colon-specific prodrug, in pH 1.2 or 6.8 at 37°C for 6 h. Drug was not released in such condition [107]. Dextran–budesonide conjugates used as colon specific prodrugs for treatment of ulcerative colitis were reported for their stability in 0.1 M HCl, and phosphate buffer solutions pH 6.8 and 7.4 at 37 °C within 6 h [108].

2.5.3 Assay of dextran degradation

The degradation of dextran can be determined by measuring the number of reducing end. The most common reagent for determination of reducing end is 3,5-dinitrosalicylic acid (DNS). This method determines the content of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in glucose. Simultaneously, DNS is reduced to 3-amino-5-nitrosalicylic acid and the color is developed from yellow to orange-red under alkaline conditions as shown in Figure 2.5.

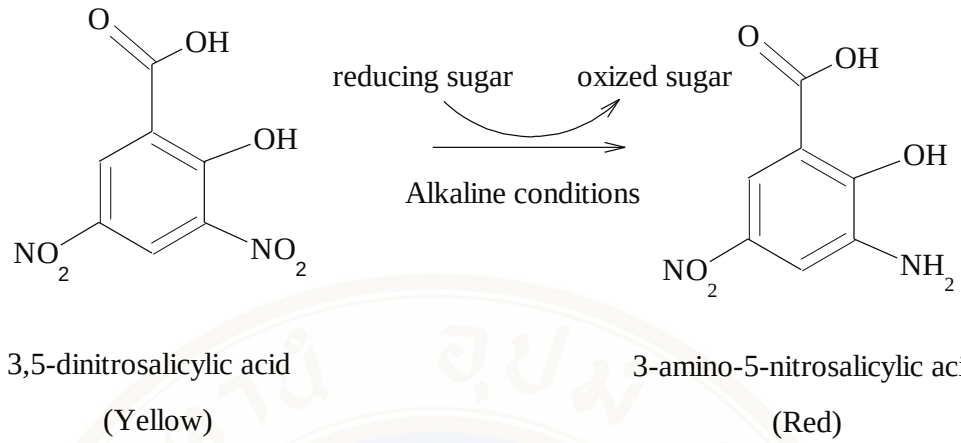


Figure 2.5: Schematic of reduction of 3,5-dinitrosalicylic acid with reducing sugar

The above reaction scheme shows that one mole of sugar will react with one mole of 3,5-dinitrosalicylic acid. Different reducing sugars generally yield different color intensities. For dextran degradation, glucose can be used as a standard sugar. Absorbance is measured at λ_{max} of 620 nm by spectrophotometer.

2.6 Nanoparticles formation

Amphiphilic polymer can effectively form nanoparticles by self-organization in aqueous solution. Hydrophobic part is essentially in the core of nanoparticles whereas polysaccharide backbone is located in the outer part as depicted in Figure 2.6.

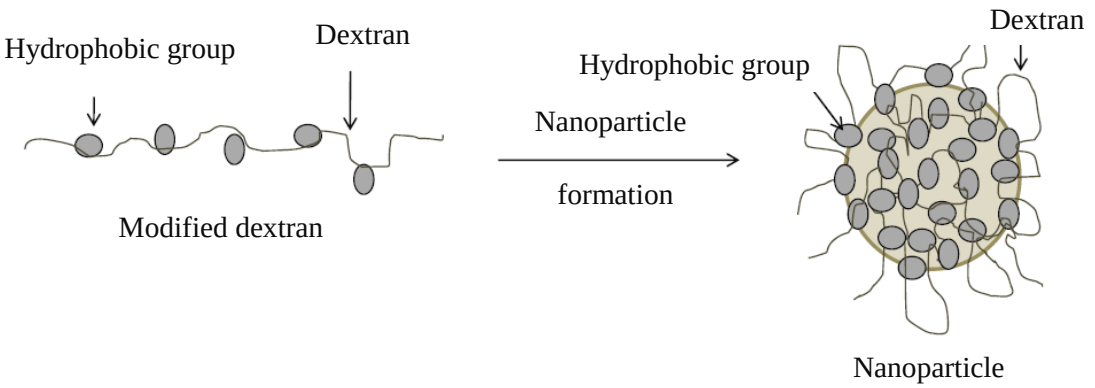


Figure 2.6: Nanoparticle formation of amphiphilic dextran by self-assembling (adapted from the work of Aumelas *et al* [10])

It has been reported that some amphiphilic molecules can be prepared by simple dispersion in aqueous solution. Liu *et al*. reported the self-aggregation of nanoparticles of chitosan grafted with polycaprolactone and poly(ethylene glycol) by simple dispersion in 1% acetic acid solution [111]. Another study of Huang *et al*. demonstrated the preparation of nanoparticles by self-assembling in aqueous solution of N-phthaloylchitosan-g-polycaprolactone [112].

From the literature, several nanoparticles preparation techniques have been reported for formation of amphiphilic polymers including simple dispersion, dialysis, emulsion/evaporation and nanoprecipitation.

2.6.1 Dialysis method

This method works on the principles of the molecular organization of amphiphilic polymer throughout the diffusion of the water and water-miscible solvents across the membrane. The method is suitable for the amphiphilic polymer that is soluble in water-miscible solvent. As presented in Figure 2.7, amphiphilic polymer dissolved in solvent is inside the membrane while the water is outside. The movement of solvent from areas of higher to lower concentration through the membrane causes changing of solution polarity in membrane initiating self-organization of nanoparticles until equilibrium is reached.

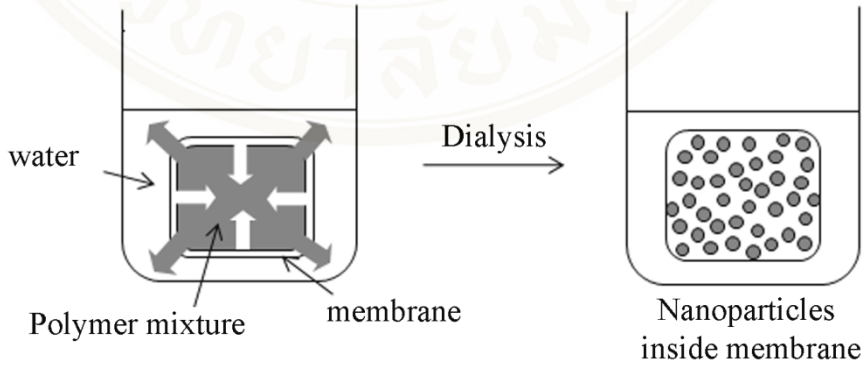


Figure 2.7: Steps of nanoparticle formation by dialysis method

Several studies demonstrated nanoparticles formation by dialysis method. Yu *et al*. demonstrated the nanoparticle preparation from chitosan grafted with polycaprolactone by dialysis method using tetrahydrofuran (THF)/water mixture as a

solvent [113]. The formation of nanoparticle from dextran ester by dialysis method was reported by Hornig *et al*. Dextran ester was dissolved in DMSO before dialysis [66]. Recently, Ge *et al*. reported nanoparticle of dextran decanoate obtained from purification after the synthesis reaction in DMSO by progressive dialysis in ethanol/water mixture (for removing non-grafted decanoate) followed by dialysis in pure water [68].

2.6.2 Emulsion/solvent evaporation

The emulsion/solvent evaporation process allows nanoparticles formation in aqueous solution. In that process, the amphiphilic polymer is dissolved in highly volatile organic solvent which is immiscible in water. This polymer solution is emulsified by applying energy throughout ultrasonication or homogenization in an aqueous solution. Then solvent evaporation is carried out under reduced pressure to prevent Ostwald ripening. A suspension of nanoparticle is obtained after complete evaporation of solvents. The steps of nanoparticle formation are depicted in Figure 2.8. This method is widely used for nanoparticle formation prepared from amphiphilic polymer.

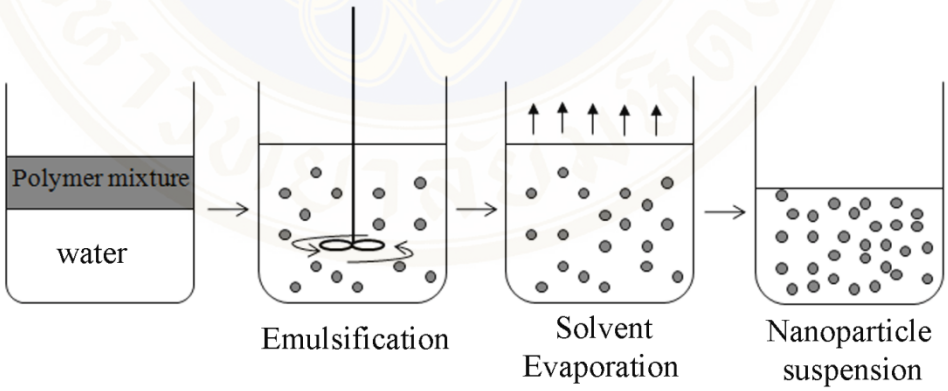


Figure 2.8: Steps of nanoparticle formation by emulsion/solvent evaporation

Preparation of nanoparticles from dextran grafted with poly(caprolactone) using dichloromethane (CH_2Cl_2) as a solvent for emulsion/solvent evaporation was reported by several groups of researchers [11, 114, 115]. Nanoparticles of dextran modified with phenoxy or epoxy groups prepared by emulsion/solvent evaporation using CH_2Cl_2 or CH_2Cl_2/DMSO mixture as solvents was demonstrated by Aumelas *et al* [10]. Recently, dextran grafted with polylactide was used to constructed

nanoparticles by the same procedure was reported by Nouvel *et al* [12]. This process is convenient for encapsulation of oil-soluble molecules which exhibit a partition coefficient highly favorable to the organic solvent.

2.6.3 Nanoprecipitation

Nanoprecipitation is performed by a classic technique which avoids the use of high energy input for emulsification. Volatile solvents which are water-miscible as THF or acetone are used for dissolving polymer. The mixture of polymer is added drop-wise into aqueous solution with agitation. Solvent is then evaporated and subsequently nanoparticle suspension is obtained as depicted in Figure 2.9. Recently, nanoprecipitation was performed to fabricate nanoparticle form dextran modified with phenoxy or epoxpy groups in THF [10]. Moreover, nanoparticles prepared from dextran grafted polylactide using THF or acetone as solvents were compared to study effect of solvent on nanoparticle formation by nanoprecipitation [65]. The method is benefitial for encapsulation of heat sensitive drug as proteins. However, the encapsulation efficiency might be low since there is no partition between two liquid phases during nanoparticle formation.

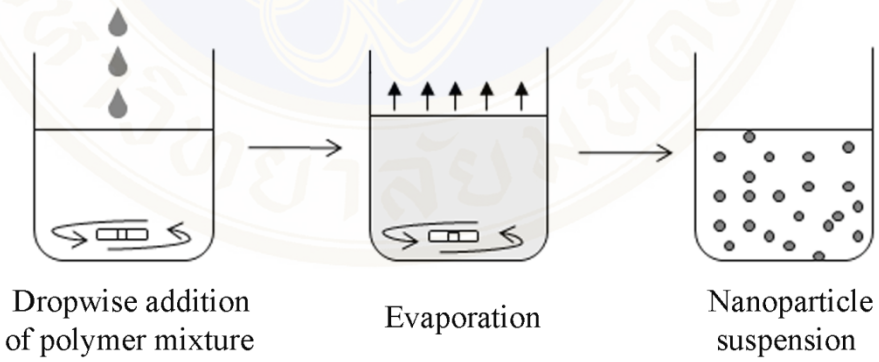


Figure 2.9: Steps of nanoparticle formation by nanoprecipitation

2.7 Analysis and characterization

2.7.1 Structure analysis

The common methods for structure analysis of modified dextrans are ^{1}H NMR and ^{13}C NMR spectroscopy. These methods are used to confirm or elucidate the

structure of starting material or product after the synthesis. Quantitative estimation of the degree of substitution of modified dextran by these methods has been reported in several researches.

2.7.1.1 ^{1}H NMR Spectroscopy

Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy is a powerful method used for the determination of the structure of unknown organic compounds. ^{1}H NMR has been routinely used to analyze the structure of synthesized polymers. The chemical shifts, splitting signals or spin systems are dependent upon the instrument's magnetic field strength. For unknown compound, the relationship of protons in different environments in the structure can be determined by H-H correlation spectroscopy (COSY) spectral information produced by 2D-NMR spectroscopy.

The ^{1}H NMR spectroscopy has been the convenient method to characterize the structure of dextran. Figure 2.10 represents the ^{1}H NMR signals (δ, ppm) of native dextran in DMSO-d_6; 4.91 (O-H4) 4.86 (O-H3), 4.66 (H1), 4.51 (O-H2), 3.73 (H6), 3.61 (H5), 3.49 (H6), 3.41 (H3), 3.2 (H2) 3.15 (H4). Most studies used this technique to determine the DS of modified dextran. Calculation of the DS of modified dextran has been reported by using ^{1}H NMR spectra. It has been reported that dextrans modified with vinyl acrylate catalyzed by Proleather FG-F and lipase AY were determined by ^{1} H NMR (300 MHz) recorded in D_2O. Moreover, ^{1}H-^{1}H COSY spectra, a bidimensional spectra were recorded by (500MHz) and ^{1}H-^{13}C HMQC spectra were recorded in D_2O [18].

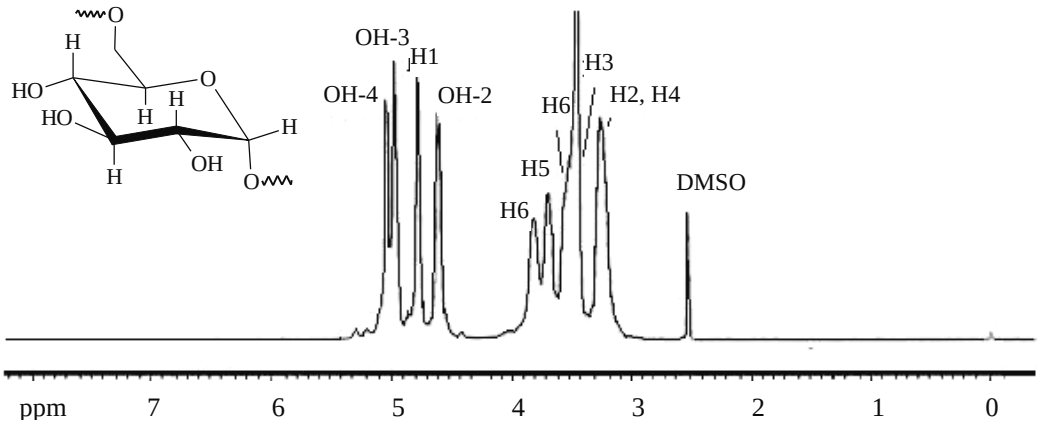


Figure 2.10: ^{1}H NMR spectrum of native dextran in DMSO-d_6.

2.7.1.2 ^{13}C NMR Spectroscopy

Carbon Nuclear Magnetic Resonance (^{13}C-NMR) Spectroscopy is the analysis used to determine the carbon in the unknown molecule. The^{13}C-NMR spectrum of an organic compound provides the different types and different environments of carbon atoms present in the molecule and also the number of neighbor carbons. The HMQC is 2D-NMR spectroscopy represented the H/C correlation techniques that allow for the determination of carbon to hydrogen connectivity. Similar to ^{1}H NMR, the expected peaks is promoted comparing with the native spectra. Quantitative ^{13}C-NMR spectroscopy has been suggested for calculation of DS of modified polysaccharide [116]. This method has been reported for the determination of selectivity of modified polysaccharide [41]. ^{13}C-NMR spectrum of native dextran in DMSO-d_6 is presented in Figure 2.11. ^{13}C NMR (δ, ppm) signals have been assigned as follows: 98.25 (C1), 73.35 (C3), 71.89 (C2), 70.39 (C5), 70.17 (C4), 66.17 (C6), 60.89 (C6).

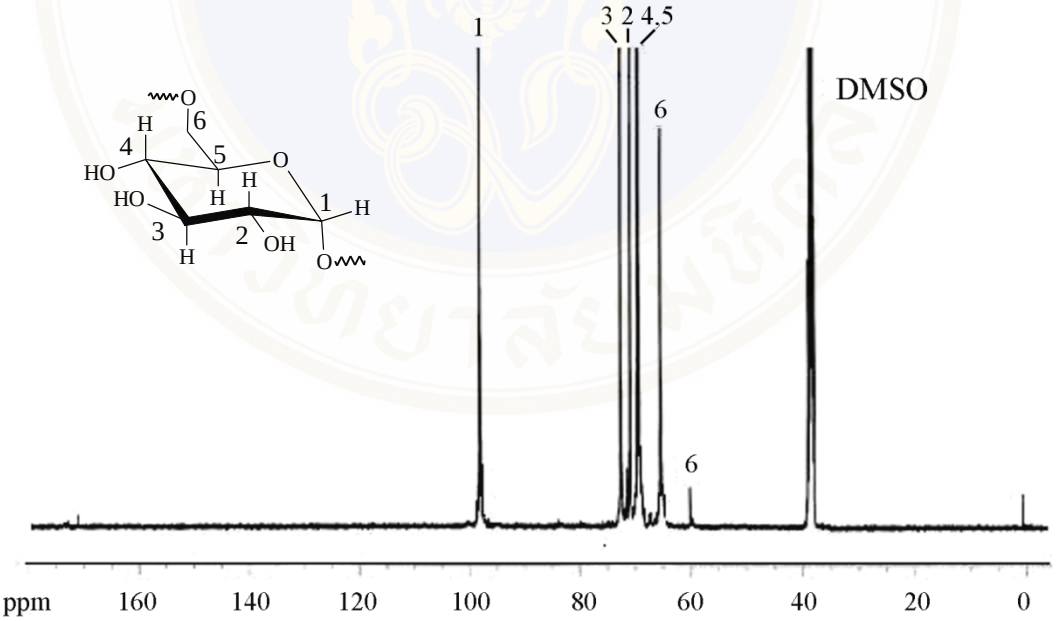


Figure 2.11: ^{13}C NMR of native dextran in DMSO-d_6**.**

It has been reported that ^{13}C NMR spectra were commonly used to determine the position of transesterification of dextran with vinyl acrylate in glucopyranosyl residues. The two positional isomers at position 2 and 3 of modified

dextran were determined. The ratios of these two isomers were calculated from the integration of the chemical shift at 95.5 and 75.2 ppm [18].

2.7.2 Physico-chemical analysis

2.7.2.1 Surface pressure analysis by Langmuir–Blodgett method

This technique uses for measurement of surface phenomena due to the compression. A Langmuir–Blodgett film is monolayer of an organic material deposited from the surface of a liquid onto a solid by immersing the solid substrate into the liquid. A volatile organic solvent is used to dissolve the material and then the solution is deposited onto the surface of an aqueous subphase. After the evaporation of the solvent, the molecules spread over the water surface. The monolayers, so-called Langmuir monolayer, are assembled vertically and are usually composed of amphiphilic molecules with a hydrophilic head and a hydrophobic tail. Figure 2.12 represents the fabrication of Langmuir-Blodgett films by immersing a substrate solution into the monolayer-covered aqueous subphase.

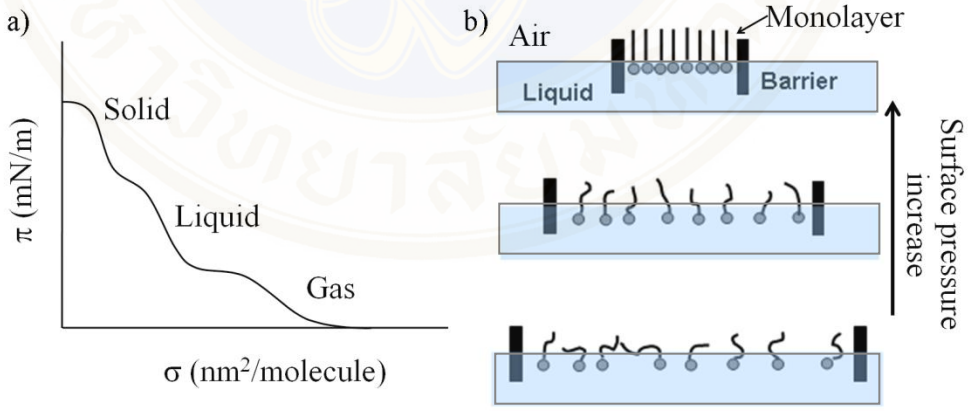


Figure 2.12: Surface pressure - area (π-σ) isotherms (a) and molecular configuration (b) of amphiphilic molecules over the compression Langmuir–Blodgett method (adapted from the report of Osvaldo and Oliveira [117]).

The compression of monolayer is undertaken by Langmuir-Blodgett trough, an instrument for determination this phenomena. The decrease of surface area by reducing the distance between the barriers aligns the molecules in a regular

arrangement (Figure 2.12 (b)). The behavior of Langmuir monolayer is greatly important for understanding the structure, stability and deposition transfer ratio of monolayer. By measuring and analyzing the surface pressure-area (Π-A) isotherm and elasticity of monolayer, one would be able to understand the state of monolayer and its changes, for example, molecular arrangement, phase structure, and phase transition etc. The arrangement of amphiphilic molecules is progressively aligned at gaseous, liquid and solid phase over the compression (Figure 2.12 (a)).

2.7.2.2 Surface tension analysis

The surface tension is a direct demonstration of intermolecular forces at any surface which can be defined as a property of surface that can resist an external force. The cohesive forces of surface molecules of a liquid or solid material leading to additional energy of surface are responsible for surface tension phenomena. The intermolecular forces among the surface molecules increase when those molecules are in contact with surrounded air causing the surface tension increases. Interfacial tension is known as the additional energy at the interface of the contact of a material surface with other material different than air. The magnitude of the interfacial tension depends on the surface tension of each contact surface. Surface tension is a measurement of the cohesive energy present at an interface by using force tensiometers or optical tensiomters (also known as contact angle meter or goniometer). The measurement of surface tension permits the understanding of the property of surfactant. Surface tension plays a role to the emulsions formation.

Wilhelmy technique is the method utilizing the interaction of a platinum ring with the surface being tested (Figure 2.13). This technique is based on the measurement of this maximum force to emerge the ring from the liquid surface. Surface tension has the dimension of force per unit length or of energy per unit area**.**

Water-soluble amphiphilic dextrans have been reported as polymeric surfactant which lowers surface tension of water [118]. It has been reported that the presence of amphiphilic dextran surfactant can provide a decrease in surface tension, which permits stability of oil droplets in emulsion solution [64].

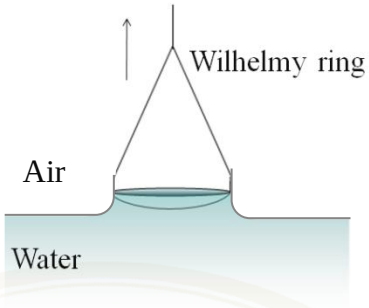


Figure 2.13: Illustration of Wilhelmy ring method for surface tension determination.

2.7.2.3 Capillary viscometry

This technique is used for measurement of viscosity of a fluid moving though the stationary object which commonly uses glass capillary viscometers or Ostwald viscometers. A liquid is introduced into the reservoir then sucked through the capillary and measuring bulb. The time taken for the travelling of liquid back through the measuring bulb passing through two calibrated marks is a measure as viscosity. Usually the viscosity of a polymer solution is compared to a pure solvent with a polymer dissolved in it. It turns out that the flow time for either polymer solution or pure solvent is proportional to the viscosity, and inversely proportional to the density. The relative viscosity (η_r) is expressed in the equation 1.

$$\eta_r = \frac{\eta}{\eta_0} = \frac{t}{t_0} \quad (1)$$

where t_0 and ρ_0 are the flow time and density of the pure solvent. When the solution is very diluted the so-called specific viscosity (η_{sp}) becomes:

$$\eta_{sp} = \eta_r - 1 = \frac{t - t_0}{t_0} \quad (2)$$

This specific viscosity is related to the concentration of the polymer solution through the intrinsic viscosity [η] as shown in equation 3:

$$\frac{\eta_{sp}}{C} = [\eta] + k[\eta]^2 C + \ldots \quad (3)$$

where $\frac{\eta_{sp}}{C}$ is called reduced viscosity $\eta_{red.}$

In fact, the plotting of η_{sp} versus C permits the determination of the intrinsic viscosity which can be determined experimentally by measuring the viscosity number as function of concentration as the Y-axis intercept.

Several mathematical equations are available in the literature for determining the intrinsic viscosity $[\eta]$ of a polymer solution, by graphical extrapolation. One of most commonly employed equations is Huggins equation (equation 4). In order to apply these relations, practical determinations using different concentrations of polymer solutions are carried out by counting the efflux time of these solutions through a capillary.

$$\eta_{red} = [\eta] + k_H[\eta]^2 C \qquad (4)$$

Some relations have been proposed for determining the intrinsic viscosity from a single point viscosity measurement of a dilute solution. This method has the advantage of being more rapid and adequate when a large number of samples have to be analyzed in short periods of time. Moreover, it has the advantage of being independent of a constant value, in contrast to graphical extrapolation determinations.

Solomon and Ciuta derived their intrinsic viscosity relationship (equation 5) by combining equations 2 and 3.

$$[\eta] = \frac{\sqrt{2(\eta_{red} C - \ln \eta_{red})}}{C} \qquad (5)$$

Viscosity of a polymer solution depends on concentration, size and behavior of dissolved polymer. Measuring the solution viscosity permits the possibility to get an idea of conformational behavior of polymer in solvent.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Dextran T40 (DexT40), (from *Leuconostoc mesenteriodes*) with weight average of molecular weight ($\overline{Mn}$) 40,000 g/mol, respectively, according to size exclusion chromatography measurements was purchased from Pharmacosmos (Holbaek, Denmark). Vinyl acetate, vinyl propionate, vinyl acrylate, vinyl methacrylate, vinyl decanoate, vinyl crotonate, vinyl pivalate, dimethyl sulfoxide (DMSO, CROMASOV®), 1,4,7,10,13,16-Hexaoxacyclooctadecane (18-crown-6), 2-methyl-2-butanol (*tert*-Amyl alcohol), dodecane, Dextranase from *Penicillium* sp. and lipase from porcine pancreas Type II were purchased from Sigma-Aldrich. Vinyl laurate and glycerol tributyrate (tributyrin) were purchased from Fluka. The dialysis membrane with MWCO 6,000-8,000 was purchased from Spectrum Laboratories Inc. (CA, USA). Mygliol 810® was purchased from Sasol Germany GmbH. All other chemicals and solvents used in this work were analytical grade. Lipase AY, a commercial lipase (EC 3.1.1.3) from *Candida rugosa*, Lot: LAYE0151016, was purchased from Amano Enzyme Co.(Nagoya, Japan). According to specification sheet (Appendix A), it is a crude lyophilized powder with the activity 32,800 U/g.

3.2 Enzymatic synthesis of dextran fatty acid ester

3.2.1 Preparation of enzymes co-lyophilized with 18-crown-6

One gram of lipase AY was dissolved in 20 mL of 20 mM phosphate buffer pH 7.5 in the presence of 18-crown-6 at 11, 23, 46 or 92 μmol. The enzymatic mixture was stirred at room temperature for 1 h. Then, the solution was flash-frozen in liquid nitrogen followed by lyophilization with freeze dryer (Labcongo, USA) for 48 h. The water content of lyophilized powder was determined by moisture analyzer (Sartorius MA 30, Medtel, Australia). The lyophilized pH-imprinted enzyme

contained approximately 15% of water as shown in Table 3.1. The obtained lyophilized pH-imprinted enzyme contained 25, 50, 100 and 200 mole equivalent of 18-crown-6 respectively.

The hydrolytic activity of pH-adjusted lipase was determined using the titration method. Mixture of 20 mM of tributyrin in 20 mL phosphate buffer at pH 7.5 was emulsified in the presence of 2 % of Gum Arabic by using homogenizer (IKA Ultra Turrax T25, IKA Labortechnik, USA) for 20 s at 10,000 rpm. The operation was repeated 3 times. Then the temperature and pH of the solution were adjusted to 37°C and 7.5, respectively. The enzyme activity was measured by titrating released fatty acid with 0.05 N sodium hydroxide solution using a pH-stat titrator (Mettler toledo DL50, Schwerzenbach, Switzerland). In addition, the stability of pH-adjusted enzyme co-lyophilized with 50 mole equivalent of 18-crown-6 in DMSO was also investigated. One enzyme unit hydrolyzes 1.0 micro equivalent of fatty acid from a triglyceride in 1 h at pH 7.5 and at 37°C.

Table 3.1: Moisture content of the dry sample

Sample	%Moisture content
Normal lipase AY	14.61 ± 1.82^{a}
pH-adjusted lipase AY	14.00 ± 1.05^{a}
lipase AY co-lyophilized with 18-crown-6 (50molar equiv.)	13.27 ± 1.97^{a}
lipase AY co-lyophilized with 18-crown-6 (200molar equiv.)	13.54 ± 1.20^{a}
Dextran	9.18 ± 1.72^{b}

- Each value is the mean±SD of triplicate measurements. Values with a column with different letter (a, b) differ significantly ($p<0.05$) by Tukey test.

3.2.2 Stability of lipase in DMSO and *tert*-amyl alcohol mixture

One milligram of lipase AY pH-adjusted was incubated in 50 µl of DMSO and *tert*-amyl alcohol mixture (30 and 100% *tert*-amyl alcohol) at 37°C or at 50°C for 1, 2, 3, 4 and 5 h. Then, their units of hydrolytic activity were measured using a modified assay as described in section 3.2.1. The experiments were done in triplicate for each incubation test. The percentage of residual activity was calculated by using equation 6.

$$\%\text{residual activity} = \left(\frac{U_t}{U_0}\right) \times 100 \quad (6)$$

Where; U_0 was unit of activity of incubated enzyme solution at time zero. U_t was unit of activity of incubated enzyme solution at various times.

3.2.3 Lipase-catalyzed transesterification of dextran with vinyl esters

3.2.3.1 Effect of reaction medium on DS

The effect of reaction on the DS was studied by using the co-solvent of DMSO/*tert*-amyl alcohol. The optimal condition for pH-adjusted lipase AY-catalyzed transesterification has been determined and was used throughout this study [74]. A mixture reaction used for transesterification contained 0.1 M of DexT40 (expressed in moles of hydroxyl groups of the polymer, DexT40, 1 hydroxyl groups per repeat unit) and 0.4 M of vinyl decanoate ($[OH]_0$ / $[ester]_0$ = 1/4) in 5 mL of DMSO/*tert*-amyl alcohol mixture at the ratio of 95/5, 90/10, 80/20 and 70/30, with the DexT40 being dissolved in 5 mL co-solvent prior to its mixing with the vinyl decanoate. The reaction mixture temperature was raised to 50^{o}C and kept constant for 5 min before addition of 26 mg of pH-imprinted enzyme. The preparation of pH-imprinted lipase AY has been described previously [119]. The enzymatic solution was left under stirring at 50^{o}C for 32 h. The reaction was stopped and the DMSO solution was dialyzed against ethanol water mixture (85:15 %, v/v) for 2 days followed by mixture of 60:40 (% v/v) for 2 days and then with distilled water for 2 days. Finally, the aqueous solution was lyophilized for 48 h. Control experiments using pure DMSO as a reaction were carried out with the same procedure. The DS of polymer samples (%) was determined by ^{1}H NMR (300 MHz) in DMSO-d_6 described in section 3.2.4.1.

To study the behaviour of DexT40 dissolved in different co-solvents, viscometric measurements of polymer solutions were carried-out using an Ostwald-type capillary viscometer (0.46 mm diameter). The temperature was regulated by a circulating bath. Prior to measurements, the polymer solutions were filtered through 0.2 μm filters. No kinetic corrections were required since we verified that the flow time was proportional to the kinematic viscosity. The densities of the polymer solutions were assumed to be identical to that of pure solvent within the concentration range explored (up to 60 g/L). With that assumption, the ratio of flow times of two

solutions is considered equal to the ratio of their viscosities. The reduced viscosity (η_{red}) of a polymer solution is then defined according to equation 7.

$$\eta_{red} = \frac{t - t_0}{t_0 C} \quad (7)$$

In equation 7, t and t_0 are the flow time of the polymer solution and of the pure solvent, respectively, η_{red} is the reduced viscosity of the solution (L/g), C is the polymer concentration (g/L). For dilute polymer solutions, the reduced viscosity varied linearly with polymer concentration which is consistent with Huggins equation (equation 4 in literature).

3.2.3.2 Effect of 18-crown-6 on DS

The effect of enzyme pretreated with 18-crown-6 at various mole equivalents on DS was studied. The transesterification catalyzed by pH-adjusted lipase AY co-lyophilized with 25, 50, 100 and 500 mole equivalent of 18-crown-6 was carried-out in DMSO the same procedure as described in section 3.2.3.1. The water content of the reaction systems was determined by Karl Fischer titration (C30 Compact Karl Fischer Coulometer, Mettler Toledo, Germany). The control reactions were performed by adding 18-crown-6 in the reaction solvent with the same amount corresponding to 25, 50, 100 and 500 mole equivalent of 18-crown-6 co-lyophilized with lipase.

3.2.3.3 Stepwise transesterification of DexT40 with VD

The rate of stepwise transesterification was studied by following the modification at different time of reaction. Fourteen tubes with screw cup were prepared. In each tube, DexT40 (0.1 M), vinyl decanoate (0.4 M), pH-imprinted lipase AY (26 mg) and 5 mL of DMSO were mixed and stirred at 50^{o}C. The transesterification of DexT40 with vinyl decanoate was performed in the previously described optimal conditions described in section 3.2.3.2. Then, the tube was periodically sampled according to the specific time of reaction. After 52 h of the reaction, the second step of transesterification was performed by freshly adding the same amount of vinyl decanoate and pH-imprinted lipase AY in all the rest of the tube. The same procedure was repeated again after 96 h of the reaction. The reaction was

performed for 144 h (the third step of transesterification). The control experiment was undertaken by using total amount of VD and enzyme as stepwise reaction and incubated for 48, 96 and 144 h (Table 3.2). The modified dextrans were purified and determined in different times of the reaction by ^{1}H NMR (300 MHz) in DMSO-d_6 described in section 3.2.4.1.

Table 3.2: The addition of enzyme and VD at various periods of incubation in stepwise and batch reaction.

Time of incubation (h)	Reaction ingredients		
	DexT40 (mg)	VD (mg)	Enzyme (mg)
Stepwise reaction			
0	84	396	26
52	-	396	26
96	-	396	26
Batch reaction			
0	84	1188	78

3.2.3.4 Effect of acyl donor structure

The effect of chemical structure of acyl donor was investigated by using different acyl donors in transesterification at the same condition as performed with vinyl decanoate. Saturated acyl donors (vinyl acetate, vinyl propionate or vinyl laurate), unsaturated acyl donors (vinyl acrylate, vinyl methacrylate and vinyl crotonate) and steric hindered acyl donor (vinyl pivalate) were used for transesterification. The DS and positional substitution was characterized as described in section 3.2.4.

3.2.3.5 Transesterification of DexT40 with VD at various conditions

To obtain the wide range of modified dextran with different %DS, transesterifications of DexT40 and VD were conducted by chemical and enzymatic reactions. The conditions for transesterification were presented in Table 3.3.

Table 3.3: Conditions used for the synthesis of dextran derivatives.

Sample No.	pH-adjusted lipase[b] (mg)	Dex:VD[c]	Time (h)
1	-[a]	1:2	218
2	-[a]	1:4	240
3	25	1:4	0.5
4	25	1:4	2
5	25	1:4	24
6	25	1:4	48
7	25	1:2	4
8	133	1:4	32
9	25 (×2)[d]	1:4	48

[a] Control experiment without enzyme.

[b] pH-adjusted lipase AY co-lyophilized with 50 molar equivalent of crown ether.

[c] Molar ratio of dextran repeating units to vinyl decanoate.

[d] Two steps addition of pH-adjusted lipase AY co-lyophilized with 50 molar equivalent of crown ether.

3.2.4 Determination of DS and positional substitution

3.2.4.1 Determination of DS

^{1}H NMR spectrum was recorded on a BRUKER 300 MHz spectrometer in DMSO-d_6 solution for DexT40-VD. ^{1}H NMR Spectra of native dextran, vinyl decanoate and dextran decanoate were presented in Figure 3.1. ^{1}H NMR (δ, ppm) peaks of VD in $CDCl_3$ were 7.64-4.52 (CH=CH_2), 2.29 (-C$\underline{H}_2$-C=O), 1.25 (-C$\underline{H}_2$-CH_2-C=O), 1.23 (-CH_2-, 8H), 0.84 (-CH_3). ^{1}H NMR (δ, ppm) peaks of native dextran in DMSO-d_6 were 4.91 (O-H4), 4.86 (O-H3), 4.66 (H1), 4.51 (O-H2), 3.73 (H6), 3.61 (H5), 3.49 (H6), 3.41 (H3), 3.2 (H2) 3.15 (H4). ^{1}H NMR (δ, ppm) peaks of DexT40-VD in DMSO-d_6 were 4.91 (O-$\underline{H}$4) 4.86 (O-$\underline{H}$3), 4.66 (H1), 4.51 (O-$\underline{H}$2), 3.73 (H6), 3.61 (H5), 3.49 (H6), 3.41 (H3), 3.2 (H2) 3.15 (H4), 2.29 (-C$\underline{H}_2$-C=O), 1.25 (-C$\underline{H}_2$-CH_2-C=O), 1.23 (-CH_2-, 8H), 0.84 (-CH_3).

The signal of methylic proton of DMSO, used as reference signal, was set at δ 2.47 ppm. For the DS (%) calculation, signal one (I_1) at 0.84 ppm corresponds to 3 methylic protons of the hydrocarbon chain of decanoate (I_1 = 3H) and signal (I_2) of 1

proton of anomeric carbon and 3 hydroxyl proton of glucopyranoside unit of DexT40, respectively (Figure 3.1). The DS (%) is given below (equation 8).

$$\%DS = \left[\frac{\frac{I_1}{3}}{\frac{I_1}{3} + \left(\frac{I_2 - I_1}{4} \right)} \right] \times 100 \qquad (8)$$

This value represents the molar ratio of attached ester groups to glucopyranoside units of DexT40 modified by vinyl decanoate. To calculate % DS of dextran modified with other acyl donors, the same equation was used with different proton signals in ester group as presented in Table 3.4.

Table 3.4: Chemical shift of I_1 protons of different acyl donor in DMSO-d_6 used for the calculation of DS of modified dextran.

Acyl donors	Group of proton signals	I_1 (δ, ppm)
Vinyl acetate (VA)	$-CH_3$	2.05
Vinyl propionate (VPP)	$-CH_3$	0.97
Vinyl laurate (VL)	$-CH_3$	0.84
Vinyl acrylate (VAC)	$-CH=CH_2$	5.8-6.4
Vinyl methacrylate (VM)	$-CH_3$	1.89
Vinyl crotonate (VC)	$-CH_3$	1.89
Vinyl pivalate (VPV)	$-3CH_3$	1.10*

*I_1 was the signal of 9 protons from 3 methyl groups in pivalate. For the calculation of %DS, I_1 of pivalate was divided by 3 before applying in the equation 8.

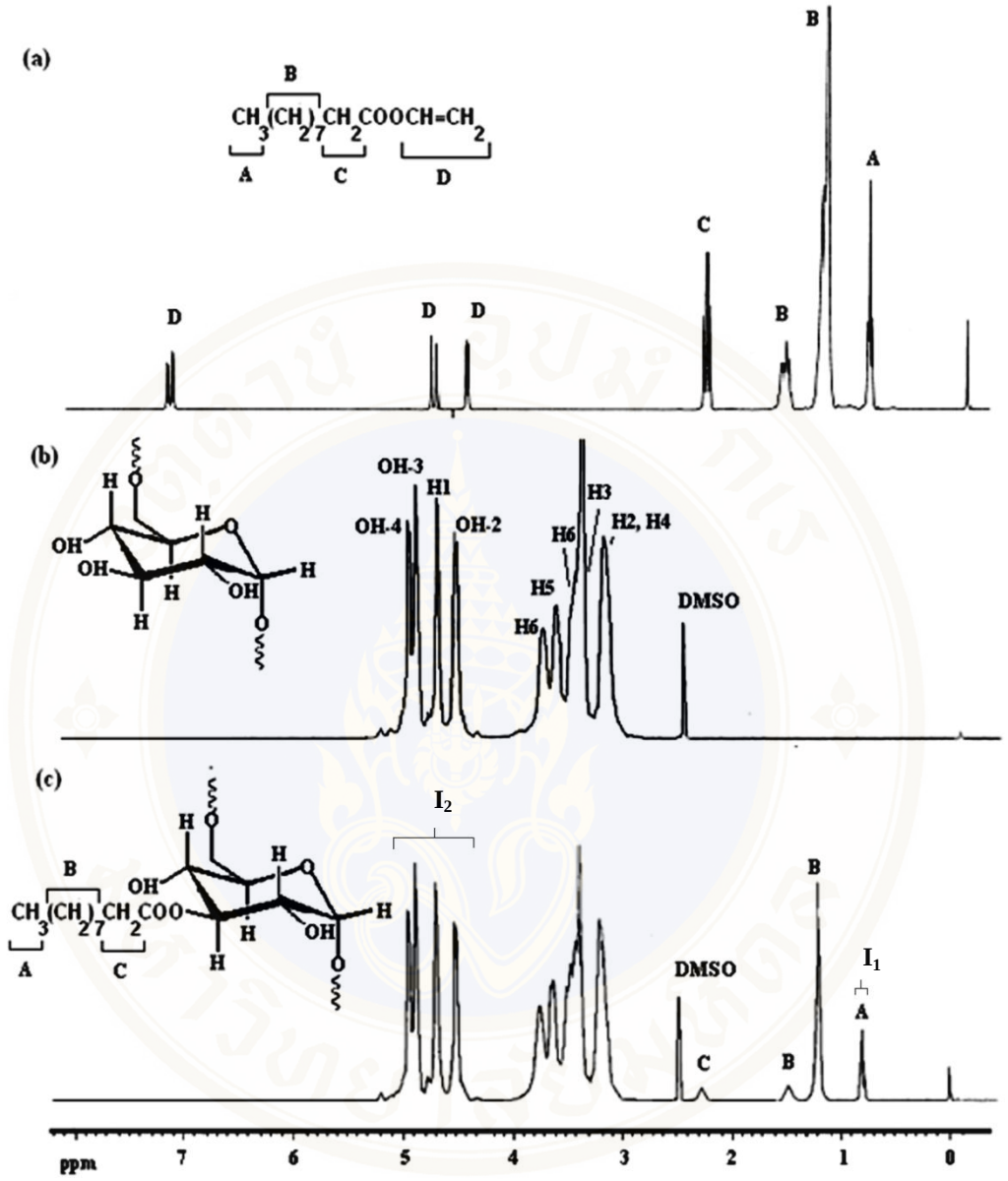


Figure 3.1: ^{1}H NMR (300 MHz) Spectra of VD (a), native DexT40 (b) and DexT40-VD (c).

3.2.4.2 Determination of proportional substitution

The ^{13}C NMR pattern was recorded on a BRUKER 500 MHz spectrometer in DMSO-d_6 solution for DexT40-VD. The denotation Cx-Sy means that the carbon x is adjacent to a substituted carbon y. ^{13}C NMR (δ, DMSO-d_6, ppm) signals were assigned as follows: 172.7 (C=O), 98.25 (C1), 95.49 (C1-S2), 74.99 (C3-S3), 73.35 (C3), 71.89 (C2), 70.39 (C5), 70.17 (C4), 66.17 (C6), 60.89 (C6), 34 (-$\underline{C}H_2$-C=O), 31 (-$\underline{C}H_2$-CH_2-CH_3), 28.72 (-$\underline{C}H_2$-, 4 C), 23 (-$\underline{C}H_2$-CH_2-C=O), 22.09 (-

$\underline{C}H_2$-CH_3), 13.99 (-$\underline{C}H_3$). The chemical shifts of C3-S2, C2-S2, C2-S3 and C4-S3 are not presented as interference occurred from the carbon peak of native DexT40.

For quantitative ^{13}C NMR, the decoupler was gated on during acquisition and off during delay, to suppress the Overhauser effect. The calculation of decanoate attachment ratio on the 2 position to 3 position of glucopyranosyl residues was done by dividing the intensity of two well separated signal corresponding to C1-S2 at 95.49 ppm by the one of C3-S3 at 74.99 ppm as shown in Figure 3.2.

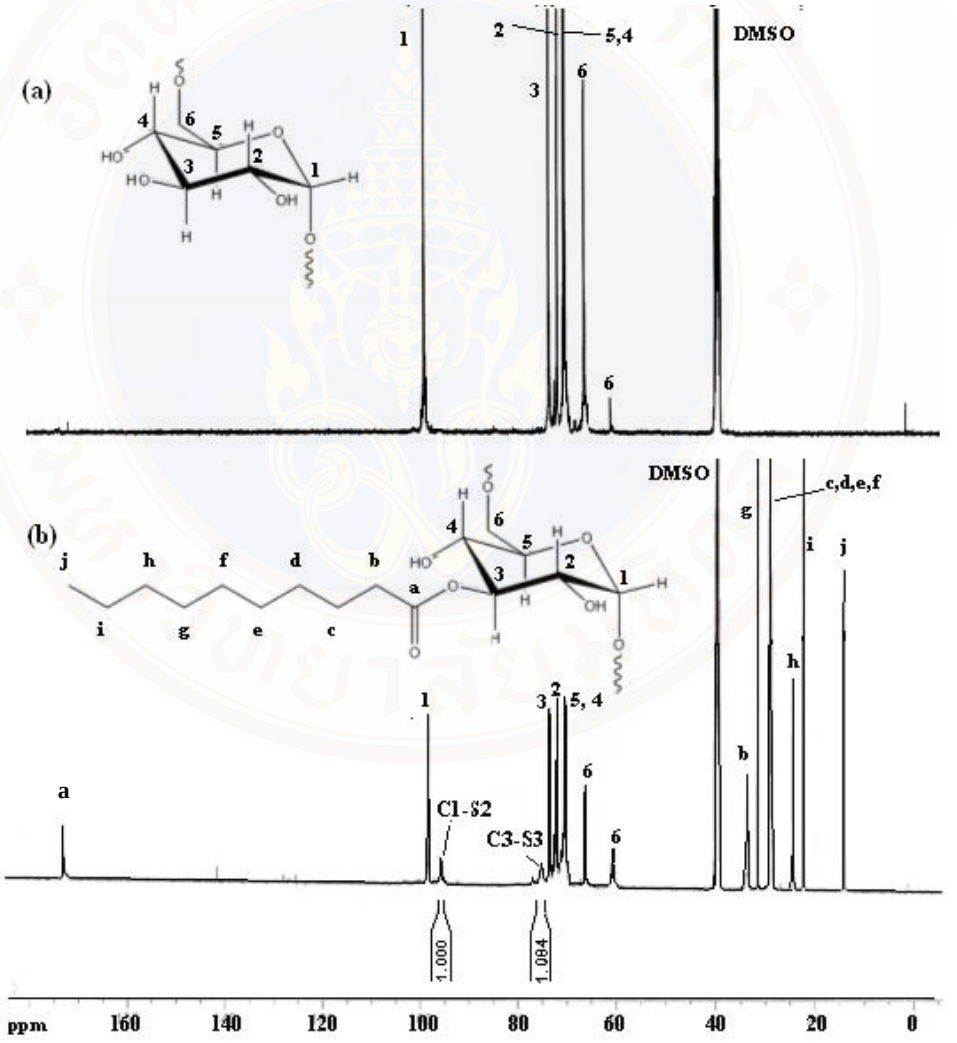


Figure 3.2: ^{13}C NMR spectra of native DexT-40 compared with DexT-40VD of 48.32% modification.

3.3 Physical characterization of dextran fatty acid ester

3.3.1 Fractionation of modified dextran

Modified dextrans with various modifications were progressively fractioned by ethyl acetate, methanol and water. In the first step, 1 g of modified dextran was suspended in 200 mL of ethyl acetate with vigorous stirring for 1 h. Then, the suspension was filtrated through borosilicate filter No. 4. Supernatant was evaporated under vacuum by rotary evaporator. Precipitate and solid residue from supernatant was dried under vacuum for 24 h. Dry weights of soluble and non-soluble polymer in ethyl acetate were measured. DS of both parts were determined by ^{1}H NMR analysis. The non-soluble polymers were further fractioned in methanol with the same procedure. Finally, the fractionation in water of the methanol insoluble part was done with the same proportion of polymer and solvent as described above for 1 h. The suspension was centrifuged at 3000 rpm for 15 min. Supernatant was separated and precipitate was washed 3 times with milliQ water. Supernatant and precipitate were flash-frozen in liquid nitrogen and dried by using freeze dryer for 48 h. Dry weights and % DS were determined as described above for the fractionation with ethyl acetate.

3.3.2 Solubility of modified dextran obtained from fractionation

One g/L of water-insoluble modified dextrans obtained from solvent fractionation were tested for their solubility in THF and 90:10 (v:v) of THF:water mixtures at 1 g/L. The polymer solution was filtrated through 0.4 μm filter. The supernatant was then evaporated and the dried weight of soluble polymer was measured.

3.3.3 Interfacial tension measurements

Interfacial (oil/water) tension measurements were carried out at 25°C using a K8 surface tensiometer (Krüss, Germany). For water-soluble polymers, solutions were prepared with water MilliQ water purification system, Millipore) and then allowed to stand overnight at 25°C before measurement. Interfacial tension was measured with a platinum ring at air/water interface using the Wilhelmy technique. All samples were equilibrated for a sufficient time to reach constant readings. Afterward, interfaces were prepared by adding slowly oils on water solution and were further

equilibrated for 20 min to reach constant readings before measurements. Interfacial tension was determined with a platinum ring at water/oil interface (Lecomte de Noüy method). In each case, the experimental errors were in the 0.2 mN/m range.

3.3.4 Capillary viscometer

Modified dextrans with different DS were dissolved in DMSO at various concentrations. The mixtures were prepared 24 h before the measurement. Reduced viscosity (η_{red}) measurements were made using a S5 Lauda injection pump, Lauda E200 Water Bath (set to 30 °C), Lauda Processor Viscosity System and a Schott 53110 Capillary Viscometer Tube, 0.53 mm diameter. Reduced viscosity (η_{red}) was calculated by equation 7. The intrinsic viscosity [η] of polymer solution was determined using following Solomon-Ciuta equation 5 in literature section.

3.3.5 Langmuir–Blodgett films experiment

Monolayers of water-insoluble polymer were formed by spreading of polymer with a microsyringe on the pure water as subphase. A polymer concentration of 1 g/L in organic solvent was prepared 24 h before measurement. The films were prepared in a trough having a moving barrier. Compression was initiated 10 min after spreading the amphiphilic molecules, which allowed a polymer layer to be formed on pure water. The π–σ isotherms were measured at various barrier speeds (1, 2, 5 and 10 mm/min) by a computer-controlled KSV-1100 film balance system (KSV Instruments, Helsinki, Finland) and the surface pressure by a platinum Wilhelmy plate. The maximum surface area was 242 cm^2 and the subphase was maintained at a temperature of 25°C.

3.4 Nanoparticles preparation and characterization

3.4.1 Preparation of nanoparticles

According to the solubility of modified dextran in water-miscible solvent as THF, nanoprecipitation was the method used for nanoparticle preparation. Modified dextrans were dissolved in 5 or 10 mL of THF or in THF/water mixture (90/10, v/v) at various concentrations (5, 10 and 20 g/L). Then, the mixture was added drop-wise into

10 mL of an aqueous solution with and without 1 g/L of low modified dextran under vigorous magnetic stirring. Nanoparticles suspension was obtained after evaporation at 37°C for 4 h**.**

3.4.2 Nanoparticles characterization

The mean diameter (d_z) of nanoparticles was measured in 10 mM NaCl at 25°C by dynamic light scattering (DSL), using a High Performance Particle Sizer (Malvern). Scanning electron microscopy was analyzed by using a Hitachi S-2500 scanning electron microscope. Stability of nanoparticles was investigated by measuring the dry weight of both precipitate and suspension after centrifugation at 1500 ×g for 15 min at 25°C. The stability of colloidal dispersions toward added electrolyte was assessed in NaCl by turbidimetry, as described by Long *et al* [120]. Experimentally, 100 µl of nanoparticles suspension were added to 3 mL of NaCl at various concentrations (10^{-3} – 4 M). The solution turbidity was measured in the range of λ varied from 400 to 700 nm. The plot of log absorbance (*A*) versus log wavelength (λ) gives the slope *n* indicating the size of particle. The critical NaCl flocculation concentration was determined at the slope breaking of *n* as a function of NaCl concentration.

3.4.3 Dextranase degradation

About 10 mL of a dextran water solution (10 mg dextran/mL) were incubated with dextranase (20 µg/mL, 41 U/mg) at 37°C, pH 5.6. Samples were taken at different times, and heated at 95°C for 10 min to inactivate the enzyme. The concentration of reducing oligosaccharides was determined as described by Franssen *et al* [121]. Typically, 4 mL of dextran solution were incubated with 6 mL of Sumner reagent for 15 min at 95 ◦C. After cooling, the absorbance was measured at λ_{max} of 620 nm. Glucose solutions were used as references for calculating number of glucose unit in fragment.

3.4.4 Lipase degradation

Twenty mL of modified dextran in 20 mM phosphate buffer solutions (10 mg/mL) was incubated with porcine pancreatic lipase (200 U/mL) at 37°C, pH 7.7 for

4 h. Free fatty acid liberated from hydrolysis of ester bond of DexT40-VD was determined by titration with 0.05 M NaOH using Mettler Toledo DL50 titrator.

3.4.5 Encapsulation of lidocaine

Various concentrations of lidocaine (0.5, 1.0, 2.5 and 5 g/L) were dissolved in the mixture of modified dextran/THF (5 g/L). The mixtures were added drop-wise into water solution with and without low modified dextran to form nanoparticles as mentioned previously. Size and stability of nanoparticles loaded with lidocaine were characterized. Amount of encapsulated lidocaine was calculated from the lidocaine remaining in supernatant after centrifugation of nanoparticles at 24,000 $\times g$ at 4°C for 30 min, using Beckman Coulter (Optima L-100XP Ultracentrifuge). The concentration of lidocaine was measured by spectrophotometer at λ_{max} 262 nm. In parallel, the amount of encapsulated lidocaine could be determined directly after degradation of the particles in acid conditions. 20 µL of 37% HCl was added into 10 mg/mL of nanoparticles suspension obtained after centrifugation. The mixture was heated at 95°C for 15 min then neutralized by adding 50 µl of 5 N KOH before analysis.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Optimization of lipase AY activity

The transesterification of DexT40 with vinyl decanoate was catalyzed by lipase in DMSO as presented in Figure 4.1. It is well known nowadays that the activity of most enzymes in organic solvents is decreased due to a reduction of conformation mobility, unfavorable substrate desolvation energetics, transition state destabilization and conformational change [122]. The situation is even more dramatic in lipase-catalyzed transesterification of dextran with vinyl ester since the DMSO used as solvent and the by-product as acetaldehyde denatured the enzyme. The pre-treatment enzyme by pH adjustment in aqueous solution appeared to retain lipase AY stability at 50°C in DMSO for about 11 h and about 40 % of dextran modification was achieved [119]. This DS might be too low for some of the targeted applications of modified dextrans particularly as the core of nanoparticles. Therefore, another lipase AY pre-treatment was considered in order to reach higher DS.

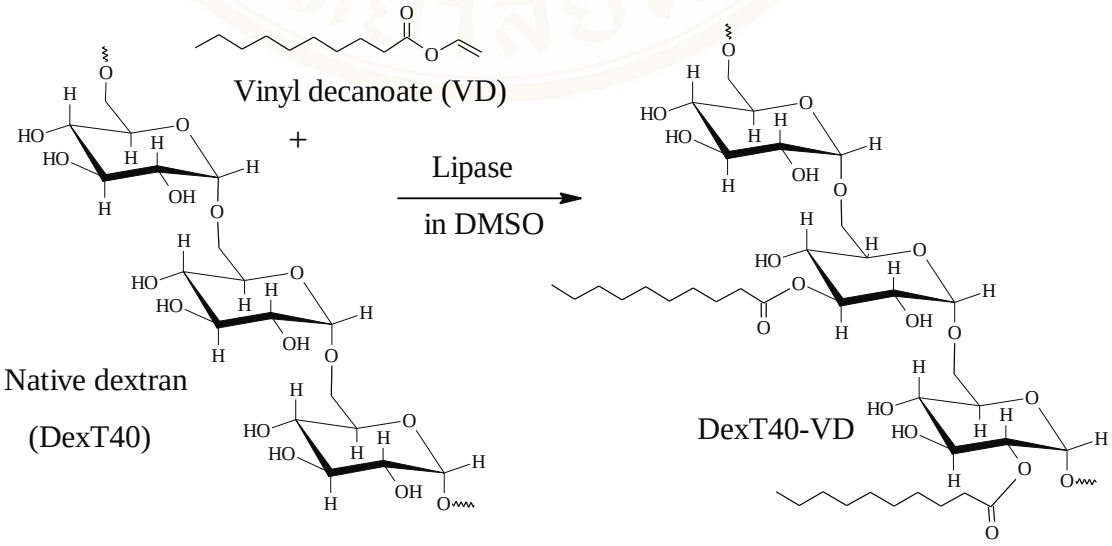


Figure 4.1: Schematic presentation of transesterification of DexT40 and VD

4.1.1 Using the mixture of DMSO and *tert*-amyl alcohol

A first strategy to increase the degree of substitution of the modified dextrans was to change the solvent used for the transesterification reaction. As mentioned previously, although DMSO is needed as reaction media to dissolve substrates of reaction, both DexT40 and VD, the drawback is the loss of activity of enzyme in such solvent. The solvent mixture has been reported to compromise the solubility of substrates and enzyme stability. Generally, a medium constituted of two solvents suitable both for solubilization of substrates and for retaining the stability of lipases was used in the enzyme catalyzed acylation of sucrose [96, 123]. More specifically, approximately 20% of DMSO was added in *tert*-amyl alcohol to increase the solubility of sucrose while *tert*-amyl alcohol in which most lipases exhibit high stability was used to maintain the enzyme activity [124].

It was found that DexT40 and VD can be dissolved in co-solvent constituted of DMSO and *tert*-amyl alcohol at the ratio of 7:3 (v/v). The stability of pH-adjusted lipase AY in this solvent mixture was determined compared with in pure DMSO and pure *tert*-amyl alcohol as shown in Figure 4.2.

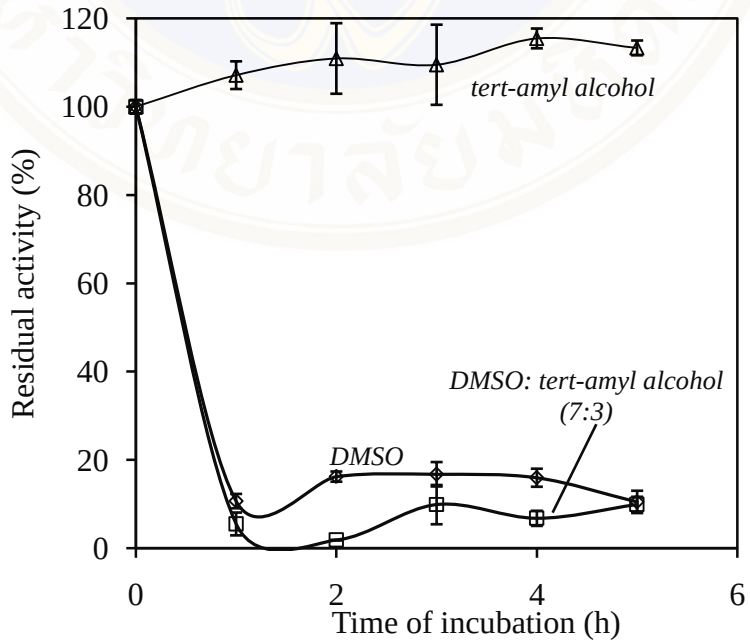


Figure 4.2: Residual activity (%) of pH-imprinted lipase AY after 5 h of incubation in *tert*-amyl alcohol, DMSO and the mixture of DMSO to *tert*-amyl alcohol (7:3 v/v) at 50 °C. Each value is the mean ± S.D. of triplicate measurement.

The residual hydrolysis activity of pH adjustment lipase AY remained constant for at least several hours in *tert*-amyl alcohol, which is much better than what is observed in DMSO or in mixtures of both solvents containing 70% of DMSO. This result implies that the deactivation effect of DMSO was not decreased at 30% of *tert*-amyl alcohol. These results were not in line with the activation effect of DMSO/*tert*-amyl mixture reported in the literature.

From the literature, the use of co-solvent constituted of DMSO and *tert*-amyl alcohol gave rise to a remarkable acceleration of the lipase catalyzed transesterification of sucrose with vinyl laurate thought to be due to the better solubilization of sucrose in DMSO [96]. The contrasting results obtained in our study might be explained by the insolubility of dextran in *tert*-amyl alcohol. Although DMSO is a very good solvent for dextran, it is not the case of *tert*-amyl alcohol in which the polysaccharide is almost insoluble. However, the synthesis activity of pH-adjusted lipase AY should remain constant whatever the ratio of *tert*-amyl alcohol added to DMSO. To confirm the hypothesis, the synthesis activities of lipase AY in the mixture of DMSO/*tert*-amyl alcohol at various ratios were studied. The results were shown in Figure 4.3.

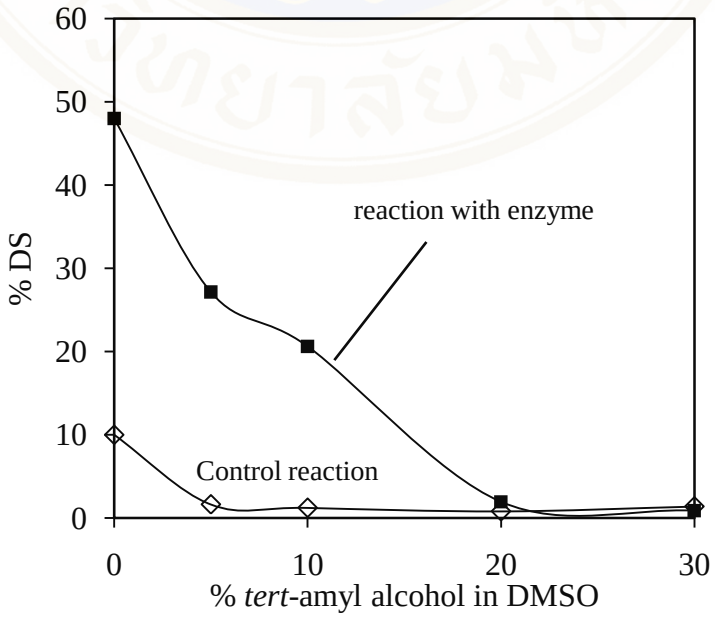


Figure 4.3: Effect of ratio of *tert*-amyl alcohol (5, 10, 20 and 30%) to DMSO on %DS of DexT-40 by vinyl decanoate, reaction time 32 h at 50 ˚C in DMSO.

Surprisingly, in a series of reactions carried-out in DMSO/*tert*-amyl alcohol mixtures with DMSO contents ranging from 70 to 100%, as can be seen, a continuous decrease of the obtained DS is observed when the content of *tert*-amyl alcohol in the reaction solvent is increased.

From the result, it was found that conversion of reaction without enzyme was also decreased when *tert*-amyl alcohol content increased. The loss of activity of pH-adjusted lipase AY in pure DMSO and in the mixture of DMSO/*tert*-amyl alcohol was similar (Figure 4.2). The loss of enzyme activity cannot account for this phenomenon. The most likely explanation was, therefore, due to the decrease of dextran solubility in the mixed solvent. This phenomenon is directly attributed to the polymeric nature of dextran since it is not observed with sucrose. To get further insight on dextran conformation in mixed solvent, viscometric experiments were carried out with dextran. The viscometric parameters related to the conformation fin solution (intrinsic viscosity and Huggins coefficient, see experimental) were determined for various solvent compositions (Table 4.1). For solutions of dextran in DMSO at temperatures ranging from 25 to 45^{o}C, Catiker *et al.* proposed Mark-Houwink relationships [125]. Using their results, the intrinsic viscosity of the studied dextran sample can be estimated to 23.7 mL/g which is in satisfactory agreement with the experimental value of 24.2 mL/g.

Table 4.1: Viscometric results obtained with DexT40 in binary mixtures of DMSO and *tert*-amyl alcohol at 25^{o}C.

DMSO:*tert*-amyl alcohol (v/v)	Intrinsic viscosity [η] (mL/g)	Huggins coefficient (k_H)
100:0	24.2	0.4
90:10	20.0	0.6
80:20	16.1	0.8
70:30	≈ 4.0	Not determined[a]

[a] Because of the limited solubility of dextran in that binary mixture, the number of experimental points did not allow a reliable determination of k_H.

As expected, with increasing amounts of *tert*-amyl alcohol, the intrinsic viscosity decreased and the corresponding Huggins coefficient increased. These variations both indicated that the solvent quality was decreased (k_H rose from 0.4 to more than 0.8) when *tert*-amyl alcohol was added and that the polymer coils collapsed into more compact conformations ($[\eta]$ was divided by more than 3). A continuous contraction of dextran coils with increasing amounts of *tert*-amyl alcohol seemed consistent with a decrease in the extent of reaction presented in Figure 4.3.

We may conclude that the use of solvent mixtures for maintaining enzyme activity is not a convenient strategy because of the polarity of dextran which involves that only polar solvents can dissolve it. Even when limited amounts of *tert*-amyl alcohol are added into DMSO, the reduction of hydrodynamic dimensions of macromolecules leads to a significant decrease of the extent of reaction. Consequently, the use of DMSO as the solvent of reaction was retained for the rest of the work.

4.1.2 Lyophilization of pH-adjusted Lipase AY together with 18-crown-6

For the transesterification of DexT40 and vinyl decanoate, the challenge was to design reaction conditions so as to enhance enzyme activity within a polar solvent like DMSO. Most enzymes are generally less active in organic solvents than in water because of conformation and mobility modification induced either by the solvent environment or by additional steps like lyophilization that are required when using enzymes in those conditions [92]. For instance, intramolecular salt bridges may be formed between ε-ammonium groups of lysine residues and anionic amino acid residues like aspartic and glutamic acid in enzyme [126]. This activity reduction might be overcome by using crown ether involving in the complexation of buffer cations present in the enzyme preparation, complexation of charge residues and water at the surface and/or in the active site of the enzyme and contributing to the enzyme activation [92]. It has been reported that the co-lyophilization of proteases with crown ether significantly enhances the enzymatic activity in polar and nonpolar organic solvents. Other successful results have also been reported for the improvement of activity of Substilisin Carlsberg in THF and acetonitrile [127] and α-chymotrypsin in hexane, octane, dibutyl ether and toluene [126, 128]. However, the mechanism of

crown ether induced “specific activation” of these enzymes in non-aqueous media has not yet been understood in detail. Several hypotheses have been suggested. One possible explanation is that crown ether can protect the enzyme during lyophilization against their conformational changes by binding with the active site during freezing and dehydration, thus preserving enzymes in their active conformation. The best crown ether for such purpose reported in the literature is 18-crown-6 (Figure 4.4) [129]. The activation effect of this crown ether was investigated in this study.

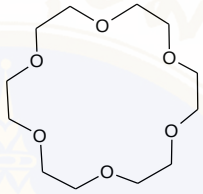


Figure 4.4: Chemical structure of 18-crown-6

18-crown-6 at various molar equivalents was added to the phosphate buffer used for the pH adjustment of lipase AY prior to lyophilization. This lipase AY was named “pH-imprinted lipase AY co-lyophilized with 18-crown-6”. After that, stability of those pretreated enzymes in DMSO was determined using standard lipase assay as described in section 3.2.2 in chapter III. The results were shown in Figure 4.5.

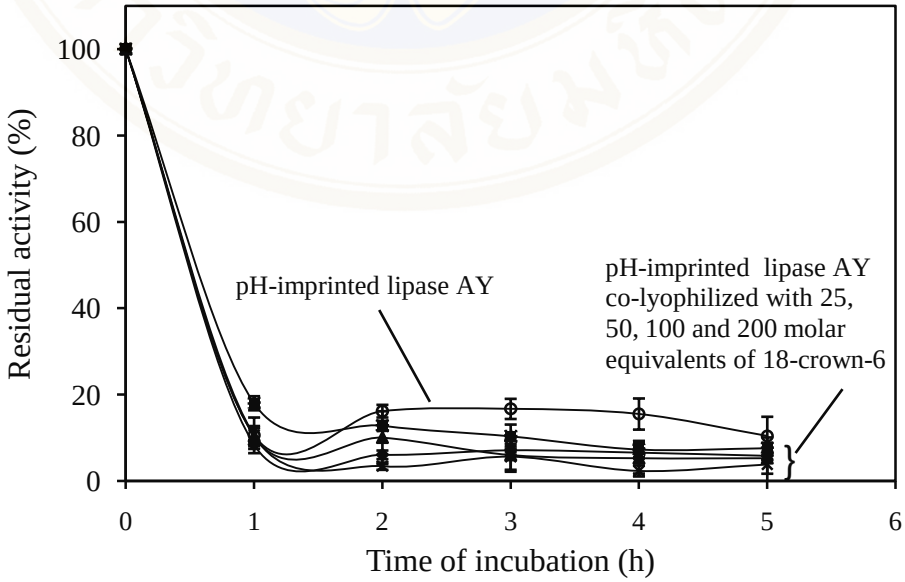


Figure 4.5: Residual activity (%) of pH-imprinted lipase AY (○)and pH-imprinted lipase AY co-lyophilized with 25 (●), 50 (■), 100 (▲) and 200 (×) molar equivalent of 18-crown-6 after 5 h of incubation in DMSO at 50°C.

In all experiments, residual activity in DMSO was decreased rapidly after 1 h of incubation. It was found that the stability in DMSO of pH-imprinted lipase AY co-lyophilized with various concentrations of 18-crown-6 was similar to that of untreated enzyme. This inferred that 18-crown-6 did not help to retain hydrolysis activity after incubation in DMSO. Santos *et al* has reported that the activity of Substilisin Carlsberg co-lyophilized with 18-crown-6 was in a linear fashion with time when it was stored at 5^{o}C. In addition, at 25^{o}C, the complete inactivation of this enzyme was observed in 2 days [127]. More recently, Secundo *et al*. has demonstrated that the activity of lipase in water was decreased in the presence of 18-crown-6 when compared with native enzyme. They suggested that crown ether causes conformation change in water leading to enzyme inactivation. The contrast situation was observed in organic solvent since lipase co-lyophilized with 18-crown-6 at low concentration can increase transesterification activity of enzyme. When 18-crown-6 was added to enzyme solution before lyophilization, transesterification activity of sulcatol with vinyl acetate in toluene catalyzed by *Burkholderia cepacia* lipase and *Candida antarctica* lipase B increased up to 2.5- and 1.4-fold, respectively [80]. Moreover, addition of crown ethers to α-chymotrypsin, subtilisin and other proteases considerably enhanced the activity of these enzymes in transesterification reactions of *N*-acetyl-alanine and *N*–phenylalanine esters in organic solvents. In that specific case, up to 640-fold of activity enhancement were observed by prior lyophilization of the enzymes in the presence of 18-crown-6 ether [129].

In our study, transesterification of DexT40 with VD catalyzed by lipase co-lyophilized with 25, 50, 100 and 200 molar equivalents of 18-crown-6 was demonstrated. The effect of 18-crown-6 on lipase-catalyzed transesterification of DexT40 in DMSO was compared with addition of 18-crown-6 to reaction media at the same quantity as presented in Figure 4.6.

It was found that the DS increased with increasing molar equivalent and reached the highest value (64% DS) at 50 molar equivalent of 18-crown-6 (enhanced DS up to 1.4-fold when compared with pH-adjusted enzyme alone). It has been reported previously that crown ether could play role as activator or inhibitor depending on the polarity of solvent. Indeed, the addition of crown ether to enzyme solution before lyophilization does strongly enhance the transesterification activity of α-

chymotrypsin and lipases in non-polar organic solvent and the activation level decreased with the increase of polarity of organic solvent [80, 130]. In accordance with the earlier published results, the activation by 18-crown-6 added before lyophilization was observed in strong polar solvent as DMSO when molar ratios of 18-crown-6 to lipase were equal to 50. The increase of 18-crown-6 amount above 50 molar equivalents, however, resulted in a decrease of the degree of substitution. In contrast, the addition of 18-crown-6 into reaction media did not affect the transesterification activity of enzyme when compared with the reaction without 18-crown-6.

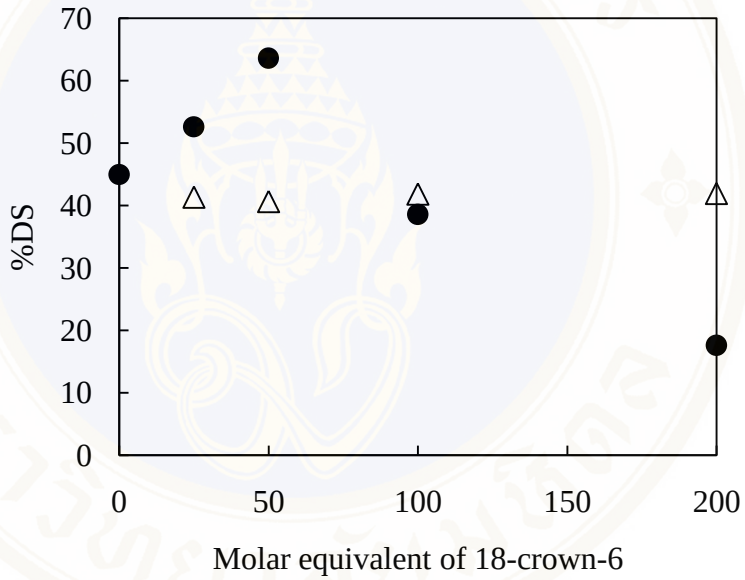


Figure 4.6: Effect of molar equivalent of 18-crown-6 co-lyophilized lipase (●) and added to reaction media (△) on %DS of DexT40 by VD, reaction time 32 h at 50°C in DMSO.

These results agreed with the previous study of transesterification of sulcatol with vinyl acetate catalyzed by lipases in toluene reported by Secundo *et al*. It has been suggested that the decrease of activity in organic solvent at the higher molar ratios of 18-crown-6 to lipase relates to the inactivation of lipase in water when concentration of 18-crown-6 increased. The enzyme activity becomes deleterious when enzyme is lyophilized [80]. Moreover, reequilibration of water may probably be

the explanation of this behavior. When crown ether is in excess, it will be slowly released from the enzyme molecules into the organic solvent. Due to an increased solvent polarity, the solvent becomes more "water-demanding". Therefore water will redistribute between the crown ether containing organic solvent, and the enzyme. As a result, the enzyme becomes dehydrated and will consequently become inactive [122]. Due to this phenomenon, the optimum is shifted towards 50 molar equivalents of 18-crown-6.

The activation of lipase by 18-crown-6 seems to be originated from the increase of activity, not from the increase of stability. The kinetic study of pH-adjusted lipase AY co-lyophilized with 50 molar equivalents of 18-crown-6 comparing with the one without crown ether should provide the evidence on this phenomenon. The results of kinetic studies were shown in Figure 4.7.

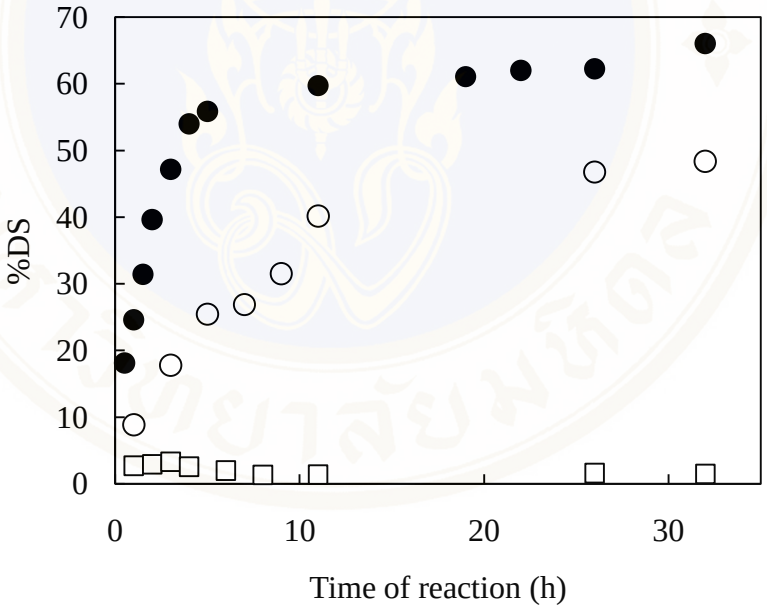


Figure 4.7: The kinetic of transesterification of lipase co-lyophilized with 18-crown-6 (●) on % modification of DexT40 by vinyl decanoate compared with pH-adjusted enzyme (○) and native enzyme (□), reaction time 32 h at 50°C in DMSO.

The rate of transesterification catalyzed by lipase co-lyophilized with 18-crown-6 on %DS of DexT40 by vinyl decanoate was compared with those catalyzed by pH-adjusted enzyme and by native enzyme. The difference in rate in transesterification was demonstrated clearly during 7 h. As presented in Figure 4.7, %

modification increased rapidly in the presence of lipase co-lyophilized with 18-crown-6 at 50 molar equivalent when compared with pH-adjusted lipase AY and native lipase AY.

It is important to be aware of the water content in the reaction mixture that has been reported to affect the degree of substitution [131]. To that the water content of reaction mixtures containing different enzyme preparations was determined by Karl Fischer titration. It appears that the water content of reaction mixtures containing different enzyme preparations were not significantly different in all cases (Table 4.2). Thus water activity cannot be responsible for the observed differences.

Table 4.2: Water content of reaction mixtures and pure DMSO determined by the Karl Fischer titration.

Sample	% water content
Reaction mixture with normal lipase AY	0.39±0.01[a]
Reaction mixture with pH-adjusted lipase AY	0.33±0.01[a]
Reaction mixture with lipase AY co-lyophilized with 18-crown-6 (50 molar equiv.)	0.34±0.02[a]
Reaction mixture with lipase AY co-lyophilized with 18-crown-6 (200 molar equiv.)	0.35±0.02[a]
DMSO+VD	0.08±0.01[b]
DMSO	0.05±0.01[b]

- Each value is the mean±SD of triplicate measurements. Values with a column with different letter (a, b) differ significantly (p<0.05) by Tukey test.

Several complexation processes resulting from crown ether may contribute to the enzyme activation such as buffer cations present in the enzyme preparation, charged residues on the enzyme surface, and water at the surface and/or in the active site of the enzyme [129]. Crown ether was reported to induce activation of enzymes in non-aqueous media, and the increased V_{max} values of crown ether-treated enzyme most probably originated from conformational changes, which alter k_{cat} as well as the amount of catalytically active enzyme [122].

From the result, we can conclude that co-lyophilization of enzyme with 18-crown-6 potentially enhances transesterification activity in DMSO. However, the kinetic of transesterification by this enzyme reached the plateau within 5 h. At this state, we can conclude that these was not the method to maintain the stability of lipase AY in DMSO. Whatever the method applied, pH-adjustment, mixture of DMSO/*tert*-amyl alcohol and co-lyophilization with 18-crown-6, the stability of lipase was maintained only for 5 h. After that, the lipase AY seems to lose its stability rapidly in DMSO. Therefore, the increase of lipase AY stability in DMSO was not an appropriated strategy to increase %DS. To this end, the addition of active enzyme into reaction was investigated in following section.

4.1.3 Tuning the degree of substitution by stepwise addition of enzyme and vinyl ester

Stepwise reaction was studied to increase the DS of dextran. The idea consists in the addition of new lot of active enzyme once the other one lost its activity in DMSO. This might open way to boost up the modification extent of dextran without the need to improve the lipase AY stability. Following this idea, the rate of lipase-catalyzed transesterification of DexT-40 with VD was carried out in three consecutive steps as presented in Figure 4.8.

It was found that the increase of DS of dextran was observed when new lot of active lipase AY was added in the second and the third step. The conversion of glucopyranosyl units in the first step reached approximately 60 % after 24 h. This conversion was followed until 52 h to confirm that the plateau was attained. This plateau could not be due to the reaction equilibrium since the vinyl ester was used to ensure a good irreversibility. The lost of enzymatic activity was, therefore, strongly ascertained. This was confirmed by the immediate increase of conversion percentage when the enzyme was freshly added at 52 h (the second step) similar to that of in the third step (96 h). Finally, 96 % of conversion was obtained after 112 h of reaction. The result obtained from stepwise reaction was compared with batch reaction which total amount of enzyme and VD used in 3 steps reaction was initial substrate and enzyme concentration of this reaction. As shown in Figure 4.8, although the high amount of enzyme and VD was used in batch reaction, the DS was lower when compared with

stepwise reaction. This result confirms that enzyme loses their stability over long time of reaction. It is worth noting that the slight variation of modification extent of dextran, especially in the third step, was observed. This might be due to the difference in branching percentage of DexT40 used as acyl acceptor. In fact, a small percentage (5%) of glucopyranose units of dextran was linked by α(1→3) glucosidic linkages [132]. It was confirmed by ^{1}H-^{1}H COSY spectrum that all the glucopyranosyl residues of dextran modified by vinyl ester are mono-substituted [18, 99]. The units of dextran linked by α(1→3) glucosidic linkages, therefore, might not be available for substitution reaction catalyzed by lipase.

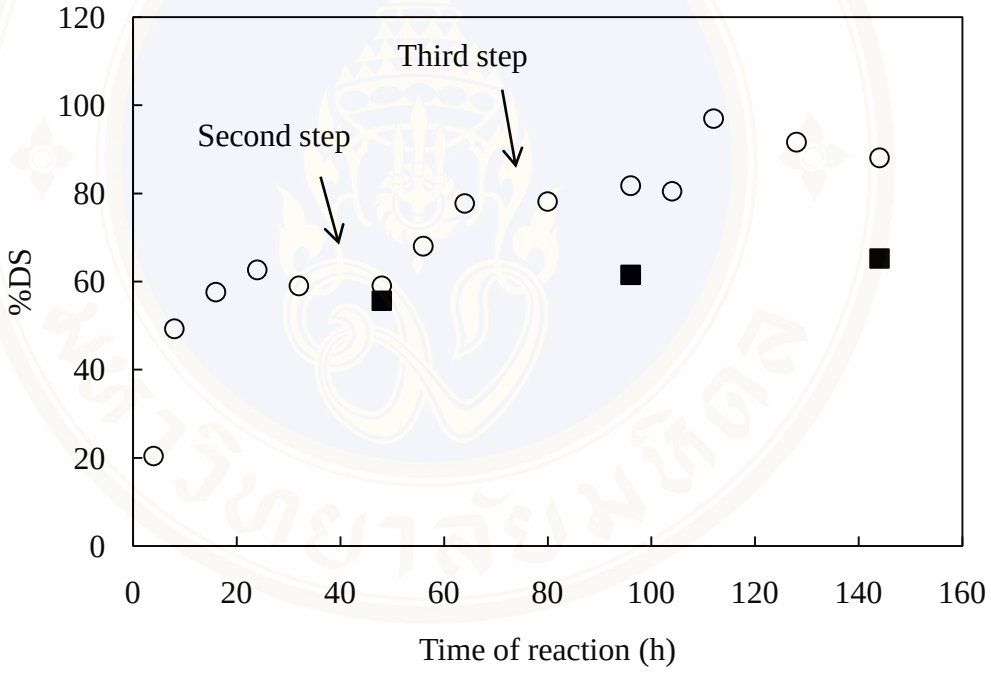


Figure 4.8: Time course for the conversion percentage of transesterification of DexT40 with VD batch (■) and 3 successive stepwise (○) reaction. For each step, 26.6 g of pH-adjusted lipase AY was added to catalyze the transesterification of 1 mole of dextran with 4 mole of VD in 5 mL of DMSO at 50°C. The duration of first, second and third step was 0-52 h, 52-96 h and 96-144 h, respectively.

A kinetic study would be useful to design the experimental condition required to obtain modified dextran of desirable modification extent. Our attempts to

obtain the highest degree of modification of dextran were successful by using the stepwise addition of the new active pretreated-lipase AY. This simple method can increase the yield of enzyme-catalyzed reaction in organic solvent.

4.1.4 Influence of vinyl fatty acid esters on the DS of modified dextrans

The effect of various types of fatty acid vinyl ester employed as acyl donors were demonstrated in our study. Using regioselectivity information provided in this study, one can design the well-defined structure of hydrophobically modified dextran with tunable degree of modification, which will lead to controllable physico-chemical properties of the target polymeric surfactants.

The nature of acyl donor may have influence on the regioselectivity of lipase in catalyzing the transesterification of DexT40 with vinyl ester. Numerous studies on regioselectivity of lipase catalyzed-transesterification of oligosaccharides have been published. Most of lipases are able to acylate primary hydroxyl groups at the 6-position of oligosaccharides due to its distinct reactivity comparing with the secondary hydroxyl groups at 2-, 3-, 4- positions of the glucopyranosyl ring. Ferrer *et al*. synthesized fatty acid esters of maltose using lipase from *Thermomyces lanuginosus* in a reaction medium containing 2-methyl-2-butanol/DMSO. They found that hydroxyl at position 6’ of the non-reducing end of maltose was acylated in high yields of 72% in 24 h using 5% DMSO mixture [133]. The regioselectivity of enzymatic acylation was demonstrated, therefore, only on the primary hydroxyl groups of oligosaccharides and depends on the source of enzyme, type of sugar, acyl donors, reaction medium and the support used for enzyme immobilization [96, 134].

In case of dextran, its repeated glucopyranosyl rings are linked to each other at 1- and 6-position and therefore lack of primary hydroxyl group. The acylation of its secondary hydroxyl groups is difficult due to their low reactivity and only some rare lipases such as the one from *C. rugosa* are able to catalyze this acylation reaction [20, 74]. Our previous results showed that activity of *C. rugosa* lipase was toward the more sterically hindered and less reactive 2- and 3-OH of dextran as demonstrated in this study [74]. In contrast, Ge *et al*. reported the chemical structure of DexT40-VD with a DS of 23% when the reaction was catalyzed by nanogel encapsulated lipase L-

1754, the regioisomer was favorable at 2-OH (ratio of 2- to 3-position was 7 : 3). Lipase AY and lipase L-1754 showed, thus, the different activity in catalyzing the transesterification between DexT40 and VD. The difference was probably due to the different isoforms of *C. rugosa* lipase produced by different fermentation conditions [135]. Consequently, the activity and regioselectivity of lipase L-1754 and lipase AY toward glucose unit of dextran might be different.

The specificity of lipase-catalyzed transesterification of dextran and acyl donors, however, few studies has been reported on the relationship between the acyl donor type and lipase regioselectivity. In order to understand this topic, pH-adjusted lipase AY catalyzed-transesterification of DexT40 was carried out by using acyl donors of different structure. The studied vinyl esters correspond to fatty acids of different chain length and saturation: saturated fatty acids; vinyl acetate, vinyl propionate, vinyl pivalate, vinyl decanoate and vinyl laurate, unsaturated fatty acids; vinyl acrylate, vinyl methacrylate and vinyl crotonate. The structure of vinyl esters was presented in Figure 4.9.

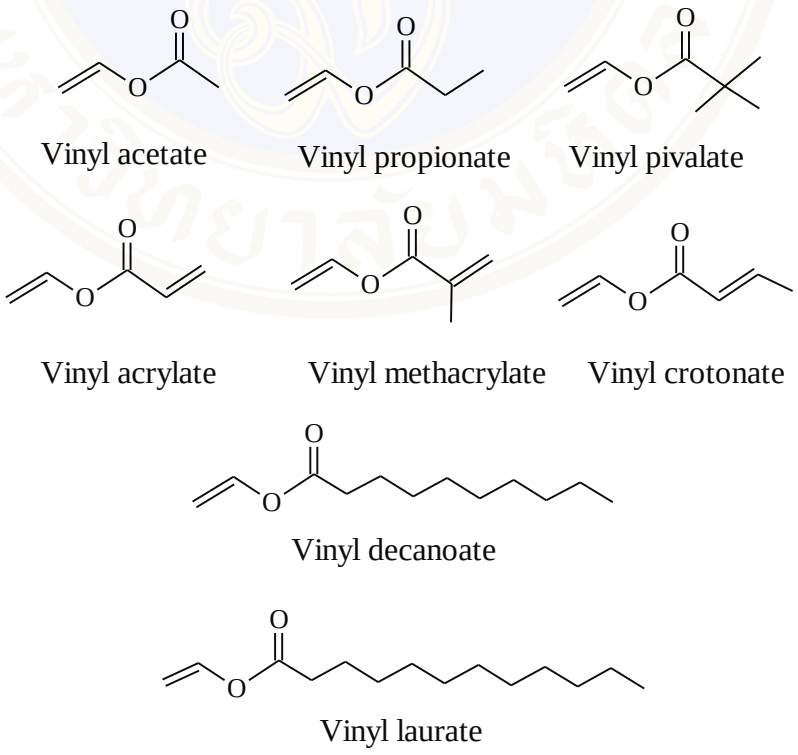


Figure 4.9: Structure of vinyl esters used as acyl donor.

The enzymatic transesterifications between DexT40 with acyl donor of different structure were conducted under the same conditions as for VD. ^{1}H NMR spectra of DexT40 modified with different vinyl ester were presented in Figure 4.10.

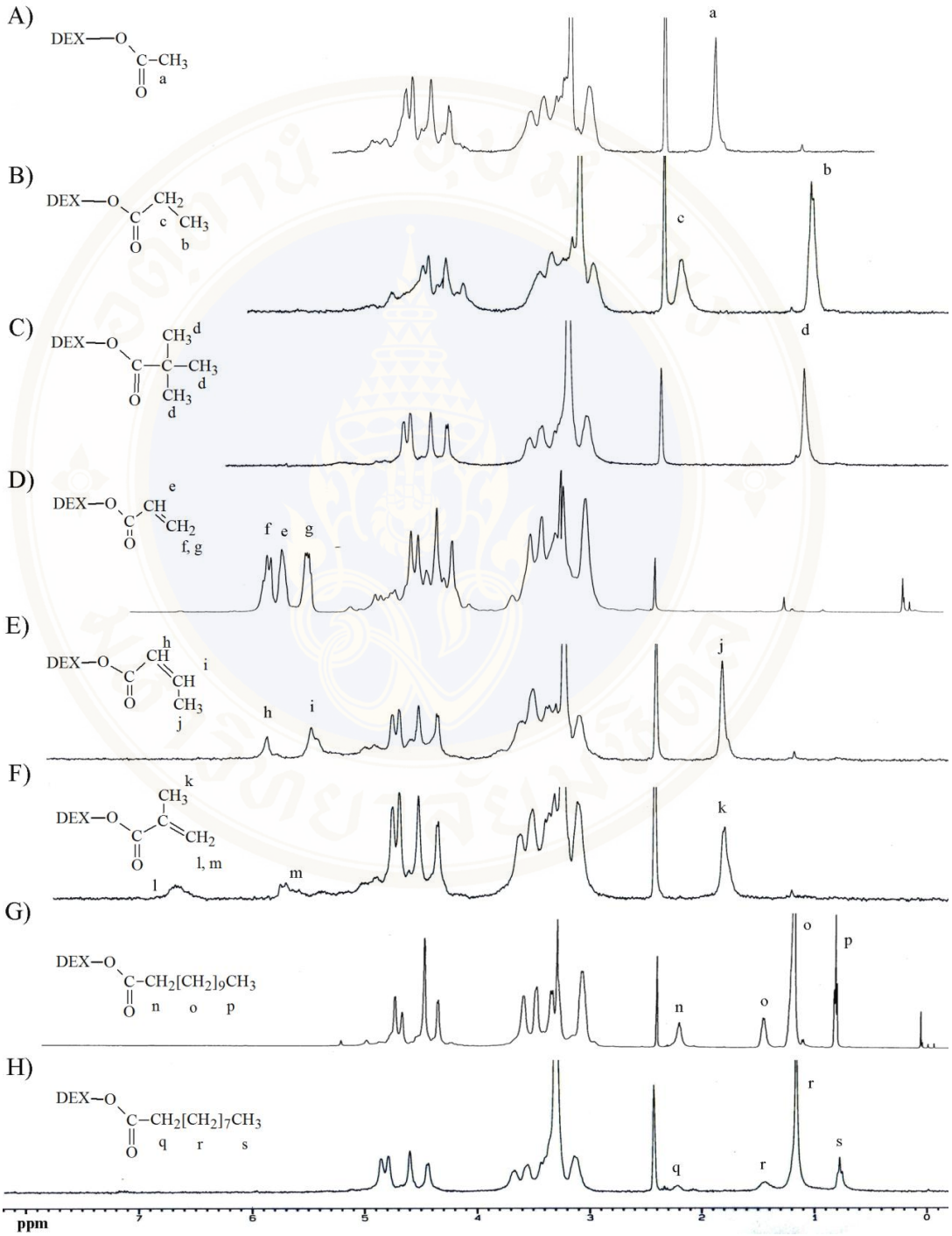


Figure 4.10: ^{1}H NMR spectra of DexT40 modified with vinyl acetate (A), vinyl propionate (B), vinyl pivalate (C), vinyl acrylate (D), vinyl methacrylate (E), vinyl crotonate (F), vinyl decanoate (G) and vinyl laurate (H).

The performance of lipase AY toward C-2 and C-3 in the presence of different vinyl ester was investigated using ^{13}C NMR as described in section 3.2.4.2.

The effect of acyl donors on %DS and regioselectivity of DexT40 catalyzed by pH-adjusted lipase AY was demonstrated. The results are shown in Table 4.3.

Table 4.3: Effect of acyl donors on the degree of substitution and positional substitution of DexT-40. The transesterification was catalyzed by pH-adjusted lipase AY with 1:4 mole ratio of dextran to vinyl ester at 50°C for 32 h in DMSO.

Type of acyl donors	Control		Enzyme	
	DS (%)	C2:C3 (%)	DS (%)	C2:C3 (%)
Saturated, Short chain				
Vinyl acetate	31.8	34 : 66	60.5	49 : 51
Vinyl propionate	8.2	36 : 64	58.6	49 : 51
Saturated, Long chain				
Vinyl decanoate	2.8	47 : 53	48.4	51 : 49
Vinyl laurate	0.7	*N/D*	43.2	46 : 54
Unsaturated				
Vinyl acrylate	25.5	55 : 45	74.3	80 : 20
Vinyl methacrylate	1.7	*N/D*	51.2	85 :15
Vinyl crotonate	0	*N/D*	25.0	77 : 23
Sterically-hindered				
Vinyl pivalate	1.5	*N/D*	13.4	29 : 71

When vinyl acetate was used as acyl donor, %DS of DexT40 increased from 31.8% to 60.5% in the presence of enzyme. While the enzyme accelerated the reaction with vinyl propionate up to 58.6% compared to 8.2% without enzyme. When saturated long hydrocarbon chain such as vinyl decanoate (C10) and vinyl laurate (C12) was employed, enzymatic modification of DexT40 caused notably high extent

of modification between 40-50%, as compared to less than 3% without enzyme. Although the DS in the control reactions is almost inversely proportional to the length of the fatty chain of acyl donor, this decrease is much less pronounced in the case of enzyme-catalyzed reactions. The transesterification of unsaturated fatty acid esters such as vinyl acrylate, vinyl methacrylate, and vinyl crotonate with DexT40 in DMSO demonstrated high extent of modification of 74.3%, 51.2%, and 25.0% with addition of enzyme, compared to 25.5%, 1.7% and 0% without biocatalyst. It should be noted that transesterification of DexT40 using vinyl crotonate as an acyl donor was not possible within 32 h reaction without pH adjusted-lipase AY. In case of vinyl pivalate which processes *tert*-butyl group, the highly sterically hindered structure resulted in the low percent modification at 13.4 %, the lowest value observed in this study. The low modification percentage obtained, with highly sterically hindered acyl donors might be correlated with the size of tunnel of active site of *C. rugosa* lipase. It was reported that the binding pocket of *C. rugosa* lipase is in the form of tunnel and is only large enough to lodge linear fatty acid chain. The steric fatty acid chain of vinyl pivalate might have a difficulty to protrude through such tunnel [136].

The effects of acyl donor chemical structure on regioselectivity can be evidenced from that series of results. In the absence of enzyme, regioselectivity of transesterification of DexT40 with saturated acyl donors of both short and long alkyl chains was comparatively higher at 3-position, while the substitution was distributed equally between 2-OH and 3-OH in enzymatic reaction. The effect of enzyme on the position of the substituent was not significant either in short and long hydrocarbon chain of fatty acids of vinyl esters. With unsaturated and reactive acyl donors, the substitution on hydroxyl group located on 2-OH is favored as compared to the control reaction. Substituting one of the hydrogen atoms of the double bond by a methyl group does not induce any significant change in the substitution pattern. Finally, with sterically hindered acyl donors, the substitution on the hydroxyl group of 3-OH is favored.

Both the order of reactivity of hydroxyl groups of dextran and the active site's conformation of *C. rugosa* lipase might intervene in the control of lipase-catalyzed transesterification with vinyl ester of different structure. The major substitution on the hydroxyl group located at 2-OH, with unsaturated acyl donor,

seems to suggest that the reaction might be control chiefly by the order of reactivity of hydroxyl group of dextran. In fact, the hydroxyl group located on 2-OH is the most reactive due to its proximity to anomeric center. The similar regioselectivity observed in the case of saturated acyl donor of different linear hydrocarbon chain length might be explained by the presence of an active site in the form of hydrophobic tunnel as mention previously [137]. The structures of *C. rugosa* lipase-Inhibitor complex demonstrate that the tunnel can accommodate a fatty acid chain length of 2 to 18 carbons in an identical manner [136]. Only small side chain movements of residues lining the tunnel are required to accommodate the scissile fatty acid chain of different chain length. According to the X-ray structure of the acyl moiety binds in a narrow tunnel, which starts at the active site serine at the surface and protrudes through the entire lipase molecule. This result is also consistent with the variation of the degree of substitution with fatty acid chain length as compared to the control reaction. The switch of regioselectivity and low modification percentage obtained, with highly sterically hindered acyl donors might be correlated with the size of tunnel. The narrow size of binding pocket of *C. rugosa* lipase is only enough to lodge linear fatty acid chain. The bulky fatty acid chain as vinyl pivalate might have a difficulty to protrude through the tunnel and might expose its hydrophilic head in the suitable position for the nucleophilic attacked by less hindered hydroxyl group on the 3-OH of glucopyranosyl ring.

4.1.5 Transesterification of DexT40 with VD at various conditions

Since the properties of modified dextrans depend on their DS, the wide range of DS is needed for studying the properties of DexT40-VD. Various conditions of transesterification of DexT40 with VD described in section 3.2.3.5 were conducted. The wide range of %DS (7-77%) was obtained as shown in Table 4.4. The effects of substrate ratio, concentration of enzyme, step of enzyme addition and time of reaction on DS of modified dextran were discussed in this section.

Table 4.4: DexT40-VD with various DS obtained from different transesterification conditions.

Sample No.	DS (%)
1	11
2	12
3	7
4	18
5	35
6	31
7	27
8	63
9	77

It was found that the DS was influenced by substrate ratio, concentration of enzyme and step of enzyme addition. For reaction without enzyme, DS did not exceed 12% after 240 h reaction although the highest molar ratio was used (sample 1 and 2). In the presence of lipase co-lyophilized with 18-crown-6, transesterification occurred rapidly, reaching 18% within 2 h at low concentration of enzyme (sample 4). It was possible to reach 77% substitution after 48 h by the stepwise reaction (sample 9). The effect of substrate ratio was reported in previous studies. The optimal ratio was 1:4 for the lipase-catalyzed transesterification of dextran with vinyl ester [74]. Co-lyophilization of enzyme with 18-crown-6 and stepwise reaction were found to increase transesterification of dextran with vinyl ester in DMSO as reported previously [138]. In this study, addition of enzyme in two steps increased DS (sample 9) in a more important way than batch reaction with higher amount of enzyme (sample 8). This result shows that addition of enzyme in two steps allowed a significant reduction of the required amount of catalyst while keeping high DS values and similar reaction times.

4.2 Characterization of modified dextran

4.2.1 Fractionation of reaction products

For modified dextran of the same structure, its property depends on the DS which is determined as average value by ^{1}H NMR. It is crucial to know the distribution of modified dextran chains of different DS as well as their proportion. Nevertheless, there was no analytical technique to determine such chain distribution. As the solubility of modified dextrans in the organic solvents was different depending on the DS, the fractionation of modified dextrans by solvents of different polarity is possible. Modified dextrans of wide range of %DS obtained from section 4.1.5 were used as starting polymers for fractionation in order to obtain modified dextran of specific DS.

To design the solvents used for fractionation, the solubility tests of modified dextran presented in Table 4.4 were performed. It was found that these polymers were completely soluble in DMSO but they were only partly soluble in most solvents. Three solvents of increasing polarity; ethyl acetate, methanol and water were chosen for fractionation study as shown in Table 4.5.

Table 4.5: Polarity index of solvents

Solvents	Polarity index
Ethyl acetate	4.4
Methanol	5.1
DMSO	7.2
water	10.2

For %DS of each recovered fractions, %DS was determined by ^{1}H NMR. The consistency of DS values with the weights of recovered fractions was checked by comparing the experimental DS value of initial sample to the one calculated on the basis of fraction results by equation 9.

$$DS_1 = \frac{DS_2 + DS_3\left[\left(\frac{m_3}{Mav_3}\right)\Big/\left(\frac{m_2}{Mav_2}\right)\right]}{1+\left[\left(\frac{m_3}{Mav_3}\right)\Big/\frac{m_2}{Mav_2}\right]} \qquad (9)$$

where DS_1 was calculated %DS, DS_2 and DS_3 were %DS of soluble and insoluble fraction, respectively, m_2 and m_3 were dried mass (mg) of soluble and insoluble fraction, respectively and Mav_2 and Mav_3 were average molar mass (g/mol) of repeated unit in modified dextran of soluble and insoluble fraction, respectively, which were obtained from equation 10.

$$Mav = 162 + \left[N_{sub} \times \left(M_{sub} - 1\right)\right] \quad (10)$$

where N_{sub} was average number of substituted groups per one glucose unit (N_{sub} = DS/100) and M_{sub} was molar mass of substituted groups. In the case of decanoate, the M_{sub} was equal to 155.

Four fractions obtained from the fractionation were shown in Table 4.6. It was found that, for all experiments, the experimental DS was consistent to the calculated one based on fractionation. Fraction 1, 2, 3 and 4 were soluble part in ethyl acetate, soluble part in methanol, insoluble part in water and soluble part in water respectively. For chemical reaction without enzyme (sample 1 and 2), only one or two fractions (fraction 3 and 4) were obtained with approximately 88% of weight fraction soluble in water. For enzyme reaction at 30 min of incubation (sample 3), the result was similar to chemical reaction with the same weight % of water soluble fraction. When the DS of starting modified dextran increased, % weight fraction of fraction 4 decreased whereas % weight fractions of fractions 1, 2 and 3 increased. When the DS of starting modified dextrans was approximately 27-77% (sample 5 to 9), DS of dextran esters obtained after fractionation were in a range of 8 to 150 %.

These results demonstrate that, DS of products after fractionation contain macromolecules with DS up to 150 % despite the lower the lower DS obtained after transesterification reaction. Such high DS values are difficult to obtain by usual chemical modification techniques. For all polymers obtained after at least 24 h reaction in the presence of enzyme, DS values of given fractions fall in the same range: 113-156 % for fraction 1 of soluble in ethyl acetate, 81-89 % (except for sample 6) for fraction 2 of soluble in methanol, 22-29 % for fraction 3 of insoluble in water and 10-19 % for fraction 4 of soluble fraction in water.

Table 4.6: Fractionation of modified dextrans with various DS obtained from enzyme-catalyzed transesterification of DexT40 with VD. Fraction 1: soluble in ethyl acetate; fraction 2: soluble in methanol; fraction 3: insoluble in water; fraction 4: souble in water. For sample numbers see Table 3.3.

Sample Number	Fraction 1		Fraction 2		Fraction 3		Fraction 4		DS_{exp} (%)	DS_{calc} (%)
	Weight fraction (%)	DS (%)	Weight fraction (%)	DS (%)	Weight fraction (%)	DS (%)	Weight fraction (%)	DS (%)		
1	0.6	n.d.	0.6	n.d.	0.0	---	88.1	10	11	10
2	0.3	n.d.	0.2	n.d.	0.7	12	86.8	11	12	11
3	0.2	n.d.	7.4	37	0.0	---	87.8	3	7	5
4	4.8	76	21.9	70	1.2	28	49.6	6	18	23
5	20.1	154	2.5	83	62.0	22	6.3	16	35	39
6	18.2	150	2.6	49	70.9	23	2.4	19	31	37
7	7.0	113	19.0	80	6.8	46	51.4	8	27	28
8	43.3	150	5.2	81	26.2	25	10.4	17	63	71
9	42.3	156	9.3	89	24.9	29	11.7	11	77	73

For the reaction without enzyme, fractionation did not allow extracting significant amounts of highly modified chains. When comparing to samples obtained after 30 min or 2 h in the presence of enzyme (these sample have DS values below and above 12 %), it appears that without enzyme highly modified chains cannot be formed, even after very long reaction times. Indeed, even by extending reaction time up to 240 h, it was not possible to obtain DS values higher than 12 %. One possible explanation for the higher and wider range of %DS obtained from enzymatic reaction was the higher affinity of enzyme to catalyze the already modified dextran than the native one. Thus, the transesterification of native dextran was compared with the one of 25% modified dextran. The results were shown in Figure 4.11.

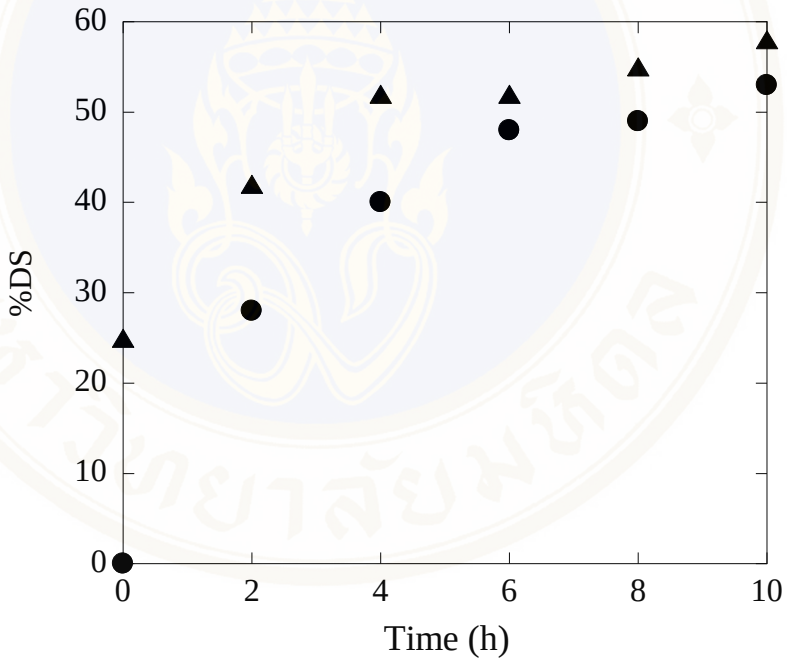


Figure 4.11: Variation of degree of substitution as a function of reaction time for lipase-catalyzed transesterification of native dextran (●) and already modified dextran with DS = 25 % (▲).

The slope of the linear relating to %DS with the time of reaction represented the rate of reaction catalyzed by enzyme. As shown is Figure 4.11, the increase of %DS as a function of time of reaction was slightly faster with the native dextran used as a substrate. This inferred that enzymatic reaction was a bit faster.

Nevertheless, the %DS obtained from 25% modified dextran and native dextran were not different after 6 h. Thus, starting from a modified dextran with certain DS did not allow the faster reaction and higher DS. Enzyme-catalyzed transesterification seems slower with modified dextrans than with the native polysaccharide. This point will be discussed in following section considering in more detail of the distribution of DS within the product of reaction.

4.2.2 Significance of solvents and solubility of modified dextran

Fractionation results show that polymers obtained after enzyme-catalyzed transesterification contain modified dextran molecules which cover a wide range of DS that may be associated to a range of polarity. A semi-quantitative interpretation of these results can be proposed on the basis of Hansen solubility parameters. This parameter is useful to predict solubility of polymer. It was assumed that dispersion, polar and hydrogen bonding parameter were valid simultaneously. Hansen defined solubility parameters by the equation 11.

$$\delta^2 = \delta_D^2 + \delta_P^2 + \delta_H^2 \qquad (11)$$

where δ is Hansen solubility parameter of dextran, δ_D is the dispersive term, δ_P is the polar term and δ_H is hydrogen bonding term. Only a few papers dealt with the calculation of Hansen solubility parameter of dextran and its various contributions (dispersion, polar and hydrogen bonding, Table 4.7) [139-144].

Table 4.7: Hansen solubility parameter of dextran (δ) and its dispersion (δ_D), polar (δ_P) and hydrogen bonding (δ_H) contributions.

δ ($J^{1/2}/cm^{3/2}$)	δ_D ($J^{1/2}/cm^{3/2}$)	δ_P ($J^{1/2}/cm^{3/2}$)	δ_H ($J^{1/2}/cm^{3/2}$)	Calculation method	Ref.
40.4	---	---	---	Van Krevelen and Hoftyzer	[140]
46.3	---	---	---	Hoy	[140]
38.6	24.3	19.9	22.5	Not detailed	[141-143]
31.4	13.2	15.0	24.2	Van Krevelen and Hoftyzer	[139]
30.3	13.3	18.0	20.4	Hoy	[139]

To the best of our knowledge, no application of Hansen solubility parameter of dextran esters has been reported yet. Although all published values are reported to result from group contribution method, it is evident from Table 4.7 that there are significant differences in the obtained values. The exact reason for the discrepancy of calculated data is unclear. In this study, the use of group contribution method from Van Krevelen and Hoftyzer to calculate the Hansen solubility parameters of dextran fatty esters of theoretical DS varying from 100, 200 to 300% were determined, considering that all repeats units were uniformly substituted by 1, 2 or 3 fatty acid chains. The calculated Hansen solubility parameters of dextran and dextran fatty esters were presented in Table 4.8.

Table 4.8: Hansen solubility parameter of dextran and dextran fatty esters calculated by group contribution method from Van Krevelen and Hoftyzer.

Dextran fatty ester	δ ($J^{1/2}/cm^{3/2}$)	δ_D ($J^{1/2}/cm^{3/2}$)	δ_P ($J^{1/2}/cm^{3/2}$)	δ_H ($J^{1/2}/cm^{3/2}$)
DS = 0 %	46.3	26.0	17.9	33.8
DS = 100 %	24.8	18.9	4.6	15.3
DS = 200 %	20.9	18.0	2.6	10.1
DS = 300 %	18.9	17.5	1.8	6.9

The diagram proposed by Bagley proposed the relationship between δ_H in ordinate and $\delta_V = (\delta_D^2 + \delta_P^2)^{1/2}$ in abscissa as shown in Figure 4.12. This diagram is used for quantitative comparison of polymer and solvent characteristics. The diagram shows that the main effect explaining the variation of solubility comes from the reduction of hydrogen bonding contribution due to the substitution of hydroxyl groups with fatty esters. Consequently, the more substitution of dextran, the modified dextran was less soluble in water or polar organic solvent. δ_V-δ_H diagram defined solvents and polymer as points in diagram. If a solvent point is close to a polymer point, then the polymer can be dissolved by the solvent. We focused on 5 solvents: water, DMSO, methanol, THF and ethyl acetate. In this diagram, the representative point of modified polysaccharide is displaced from the vicinity of water (for DS = 0%) to that of ethyl acetate (for DS = 200 and 300%) upon hydrophobic substitution. The point

representing methanol is located between that of native dextran and that of DS = 100%. Finally, the point of DMSO is close to those of dextran esters. These theoretical results are qualitatively consistent with the experimental DS of the four fractions contained in most of modified dextrans (Table 4.6).

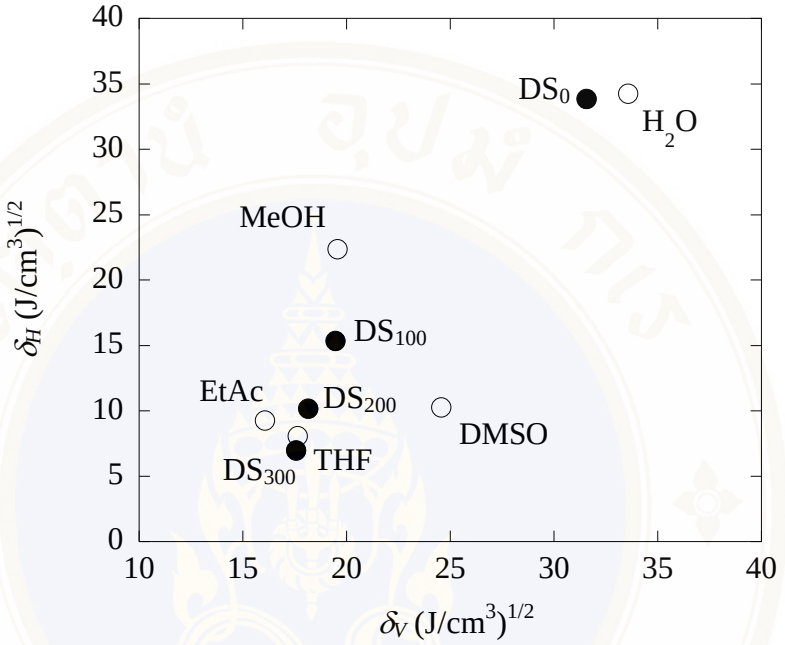


Figure 4.12: Representative points of modified dextrans DS= 0, 100, 200 and 300 (●) and various solvents (○) in the δ_V-δ_H diagram.

Another experimental illustration of that result was obtained when comparing the solubility of dextran derivatives of various DS in THF and in THF:Water (90:10 v:v) mixture (Table 4.9). It was found that the polymers with %DS ranging from 80-150 were completely soluble in pure THF and partially soluble in 90% THF/water mixture, whereas polymer with 22% DS was completely soluble in 90% THF/water mixture. When the DS decreased further, the polymer trended to be insoluble in THF/water mixture but soluble in pure water. The addition of 10% of water strongly modified the solubility characteristics of dextran derivatives favoring dissolution of low-modified polymers while restraining the solubility of highly modified derivatives.

Table 4.9: Solubility of dextran derivatives in THF, THF:Water (90:10 v:v) mixture and water. The experiments were carried out with feed compositions equivalent to 1 g/L polymer solutions.

DS (%)	THF	THF:Water (90:10 v:v)	Water
150	*s*	*p s* (87 %)	*i*
113	*s*	*p s* (80 %)	*i*
80	*s*	*p s* (77 %)	*i*
22	*i*	*s*	*i*
17	*i*	*i*	*s*
8	*i*	*i*	*s*

i, insoluble, *p s*, partly soluble (with weight percent of solubilized material in parentheses), *s*, fully soluble.

4.2.3 Mechanism of enzyme-catalyzed transesterification of dextran

To understand the mechanism of enzyme catalyzed transesterification of dextran with vinyl ester, the plot of %DS or %weight fraction versus time of reaction was analyzed (Figure 4.13 and 4.14). The enzyme-catalyzed transesterification of dextran was monitored by characterizing the relative amounts of fractions 1 to 4 as well as their DS values over time of incubation for 48 h. The modified dextran sampling at specific time of reaction was fractionated in series of three solvents following the same procedure as section 4.2.1. For each recovered fraction, its %DS and %weight was determined. As seen in Figure 4.13, the % DS of fraction 4 was continuously increased from 3 to 19 % with the time of reaction. For the fraction 1 which is soluble in ethyl acetate, the DS reached 150 % within 24 h and did not increase further after 48 h. On the contrary, in water-insoluble fraction (fraction 3), the DS did not significantly change over the time of incubation and the DS remained in a narrow range (22-28%). Finally, the DS of the methanol-soluble fraction (fraction 2) reached a maximum (83%) after 24 h and decreased after 48 h of the reaction. It was found that the maximum DS was 150% even after 48 h of reaction. Thus, it seems that enzyme-catalyzed transesterification provides 150%DS as a maximum modification with two hydrocarbon chains grafted per glucose unit within dextran chains. In

addition, low modified chains were progressively converted into more modified ones, as illustrated by the continuous increase of DS in the water-soluble fraction, up to the limit of 20% (Figure 4.13). Based on such hypothesis, one can imagine to increase the %DS by conducting the reaction at long reaction in excess of fatty acid ester comparing to the hydroxyl group concentration of dextran. Nevertheless, the stability of lipase in such conditions was still the problem to overcome.

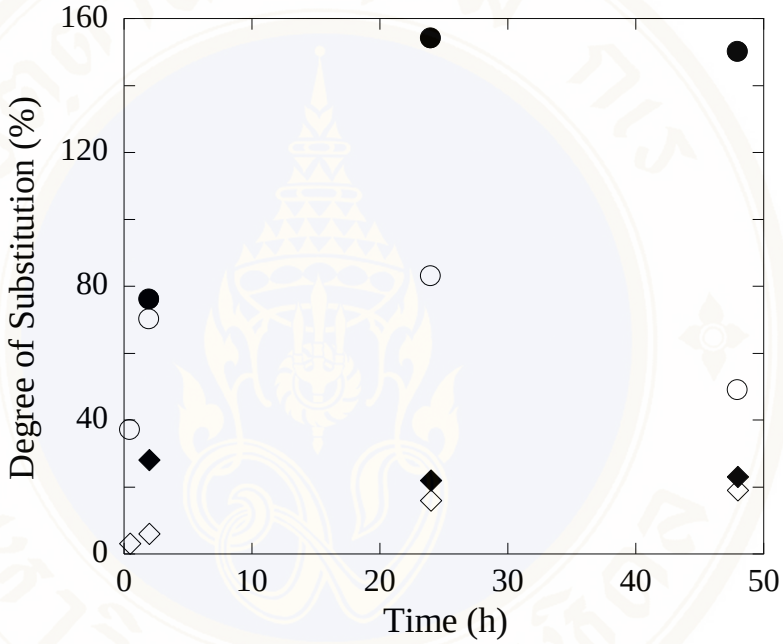


Figure 4.13: Variation of degree of substitution of various fractions extracted from transesterification products as a function of reaction time: fraction 1 (●), fraction 2 (○), fraction 3 (◆), fraction 4 (◇).

The plot between the weights of fractions against time of reaction showed a consistent trend with the plot of %DS against time of reaction of fraction 1 to 4 (highly modified to the low modified dextran). It was found that the %weight of water soluble polymers fraction (fraction 4) decreased monotonically over the reaction time from 100% to 2%wt. In contrast, for water insoluble fraction (fraction 3), %weight fraction continuously increased from 0 to 71%wt after 48 h of reaction. Highly modified polymers as fraction 1 increased from 0 to 18%wt with time. Thus the final product of enzyme-catalyzed transesterification after 48 h of average DS contained

essentially a low-modified fraction (71%wt) with a DS equal to 23% and a highly modified fraction (18%wt) with a DS equal to 150%.

It was found that decreasing the amount of vinyl ester in the feed did not induce any significant variation as compared to previous results. On the contrary, amount of enzyme used either for the batch or stepwise reaction significantly influenced the increase of weight fraction of highly modified polymers (up to 43%wt as compared to 18%wt) while the weight fraction of low modified polymers was decreased down to 37%wt. Nevertheless, even with higher amounts of enzyme in the reaction medium (either initially or by the addition of fresh enzyme during the reaction) the maximum value of DS was still 150% in the highly modified fraction. These results were consistent with all cases of enzyme-catalyzed reactions. The limit of DS at 150% might be due to the change of solubility behavior of highly modified dextran in the reaction media as DMSO. To understand such behavior, the viscometric study of various DS of modified dextran dissolved in DMSO was undertaken as described in section 4.2.4.

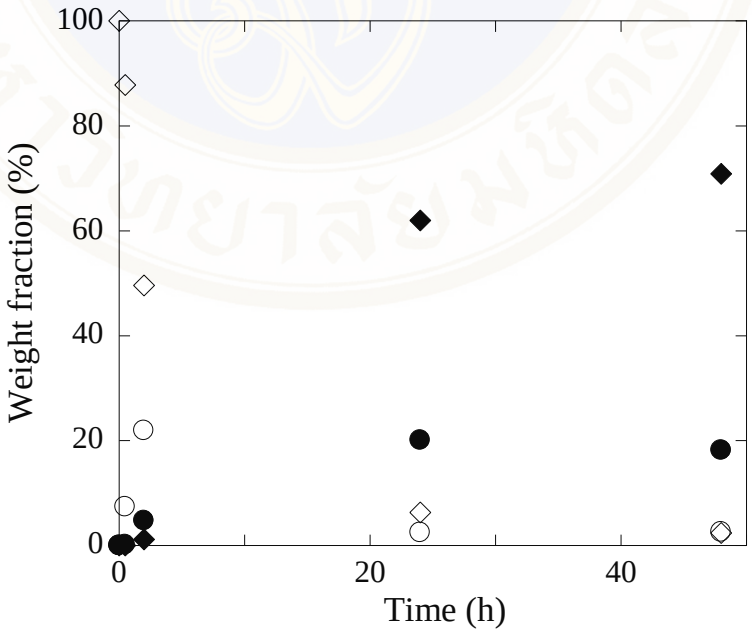


Figure 4.14: Variation of weight percent of various fractions fractionated from transesterification products as a function of reaction time: fraction 1 (●), fraction 2 (○), fraction 3 (◆) and fraction 4 (◇).

4.2.4 Viscometric study

Since DMSO is a powerful solvent that can dissolve all modified dextran obtained from fractionation, viscosity of modified dextrans in DMSO with different DS was evaluated and compared to native dextran as presented in Table 4.10. Reduced viscosity of dilute solutions of modified dextrans in DMSO at 25 °C were measured and the intrinsic viscosity [η] of the polymers was estimated using the one point methods proposed by Solomon [145] and Deb and Chattergee [146] (both giving very similar values). According to the supplier, the number-average molar mass of native dextran ($\overline{Mn}$) should be around 20,000 g/mol. Using the Mark-Houwink relation established by Catiker *et al*. [147], we can calculate a value of 20.6 mL/g for native dextran. This estimated value seems reasonably close to the experimental value of 25.1 mL/g.

Table 4.10: Intrinsic viscosity of native and modified dextrans in DMSO at 25°C

Polymer	[η] (mL/g)[a]
Native dextran	25.1
15 %	22.6
21 %	24.7
89 %	7.4
150 %	4.7

[a] Estimated by one-point methods (see text) from one measurement at 5 g/L.

From the results, lower [η] was obtained when the DS was increased indicating less solubility of modified dextran with higher DS in DMSO. Even if fractionation of polymer samples may have induced variations of molar mass between the four separated fractions, this would not account for the 4-fold decrease observed when DS changes from 15% to 150%. The attachment of hydrocarbon chains onto polysaccharide backbone might decrease solvent quality especially in the case of a polar solvent like DMSO. These trends are consistent with the previous discussion about fractionation results and the role of hydrogen bonds. From these results, coil

contraction upon attachment of hydrocarbon tails may be one reason for the limitation of modification extent of dextran molecules in DMSO.

4.2.5 Adsorption of modified dextrans at air/water and oil/water interfaces

Dextrans modified with hydrophobic groups as phenoxy, epoxy or ester group have been reported for their surface-active properties and their potential use as polymeric surfactant [8, 30, 64, 148]. Water soluble dextran decanoate obtained from fractionation was evaluated for its surfactant potential. Interfacial tension was measured between aqueous polymer solutions and various oils, using a dextran derivative with DS = 12 % (Figure 4.15). Polymer concentration was varied between 5×10^{-4} and 10 g/L. Three oils differing by their polarity were used: dodecane, Mygliol 810® and silicone oil.

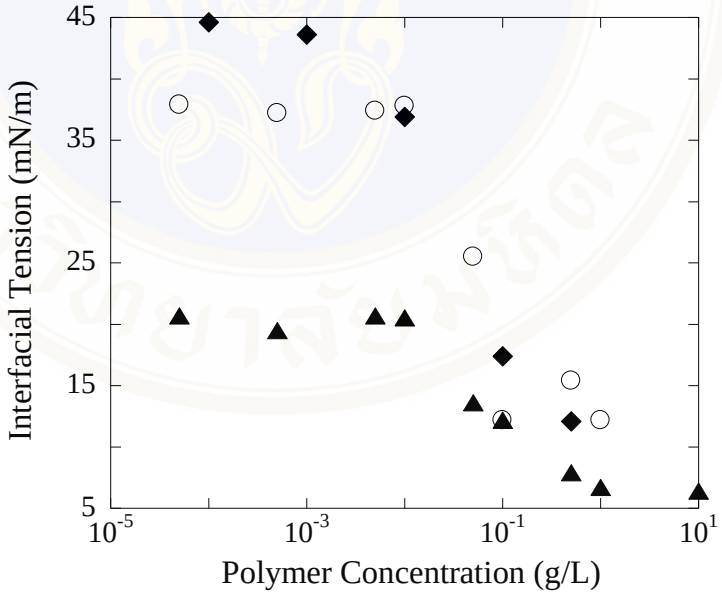


Figure 4.15: Interfacial tension at 25 °C between various oil and water as a function of modified dextran (DS = 12%) concentration in water. Oils: Mygliol 810® (▲), Silicone oil (○) and Dodecane (◆).

In all cases, a significant lowering of interfacial tension was observed within a similar concentration range (10^{-2} – 1 g/L) of modified dextran. Therefore, the water-soluble modified dextran (DS lower than 20%) may be used as stabilizers for

dispersions of hydrophobic particles in aqueous medium. The capacity to decrease interfacial tension was thought to be due to their adsorption at oil/water interface giving rise to the formation of a dense layer of hydrophilic loops providing steric repulsions between particle surfaces [10]. It have been reported that dextrans with an average molecular weight of 5,000 to 100,000 modified with decanoate and laurate at DS between 0.2 to 5% could be potentially used as stabilizer for pharmaceutical and cosmetic active substances [27].

In the case of water insoluble dextran derivatives, adsorption at air/water interface was characterized by compression isotherms. Pressure per area of monolayer film of modified dextrans on pure water was determined. It has been reported that the equilibrium isotherm strongly depends on the speed of barrier [149]. Speed of barriers was thus fixed at 1, 2, 5 and 10 mm/min. The surface isotherms of the copolymers at the air–water interface are plots of pressure (π) versus area per molecule. The 150% DS modified dextran soluble in ethyl acetate was used in that study.

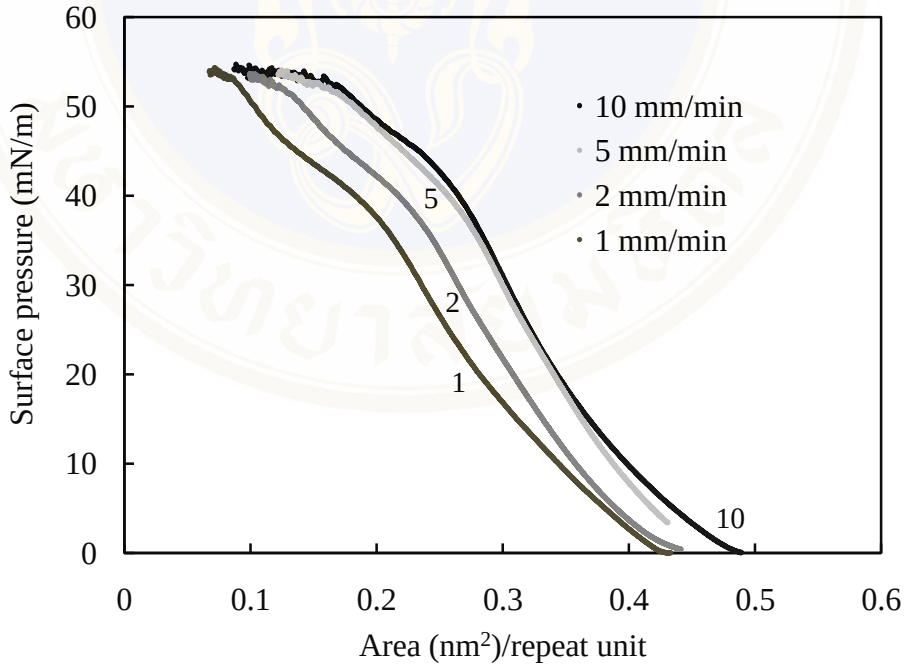


Figure 4.16: Surface pressure-Area isotherms of 150% modified dextran at 25°C with different speeds of barriers.

As presented in Figure 4.16, smaller area per molecule in gas-liquid phase was observed for lower speed of barriers. However, a similar pattern of the folded

conformation at surface was observed for all speeds. The speed at 2 mm/min was chosen for further experiment

Compression isotherms of monolayers obtained with 4 dextran derivatives (DS = 20% deposited on THF/water mixture and DS = 80, 113 and 150% deposited from THF) were characterized as presented in Figure 4.17.

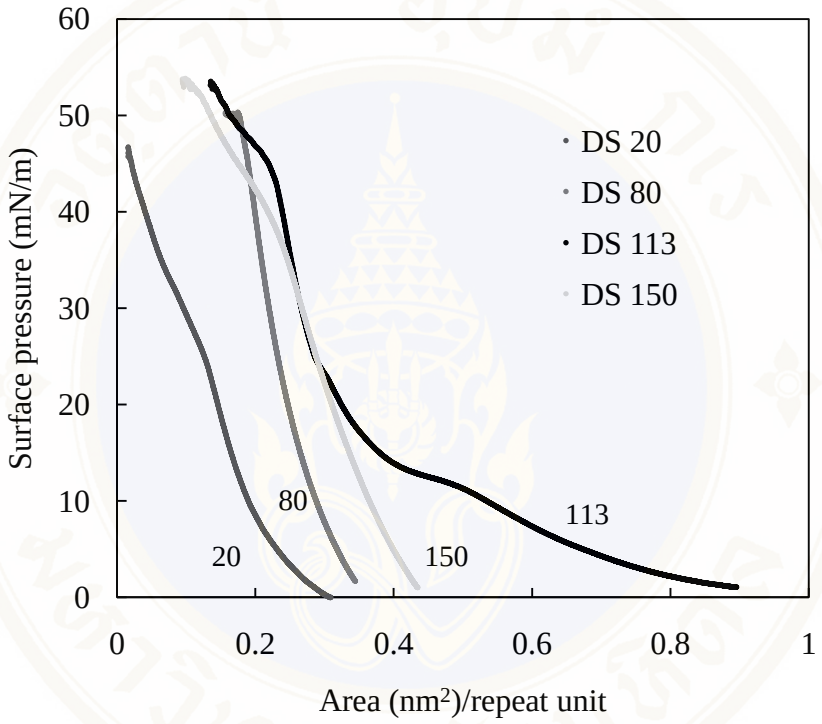


Figure 4.17: Surface pressure of modified dextrans with different DS in THF (DS=80, 113 and 150) or THF/water mixture (DS=20) at 25°C.

For 20, 80 and 150%DS, the surface isotherms show raising curves with narrow or no plateau regions despite the use of different solvent for 20%DS (Figure 4.17). This indicated that the modified dextran can form stable monolayer, in which a steep rise surface pressure and a high collapse pressure could be observed. Because of high density of hydrocarbon tails, interactions with aqueous subphase are reduced and macromolecules are present in the form of folded conformations at interface. Surprisingly, for 113%DS, the plateau region was observed indicating much more expanded macromolecules when compared with other polymers.

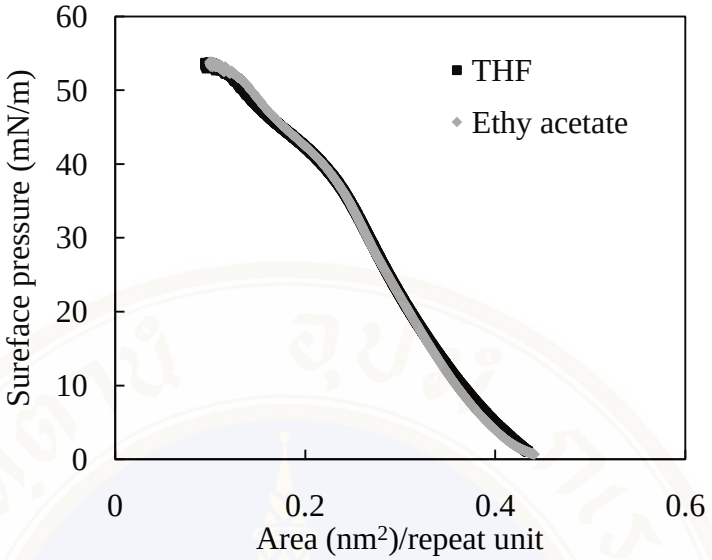


Figure 4.18: Surface pressure of modified dextran with 150%DS in ethyl acetate and THF.

The effect of solvent on compression isotherm was studied. In the case of a highly modified dextran with DS = 150%, no significant different compression isotherm was observed when THF or ethyl acetate were used to form the layer (Figure 4.18). This was consistent with the high density of hydrocarbon tails grafted along the polysaccharide backbone which strongly limited interactions with the aqueous subphase, whatever the solvent polarity (either miscible with water or not).

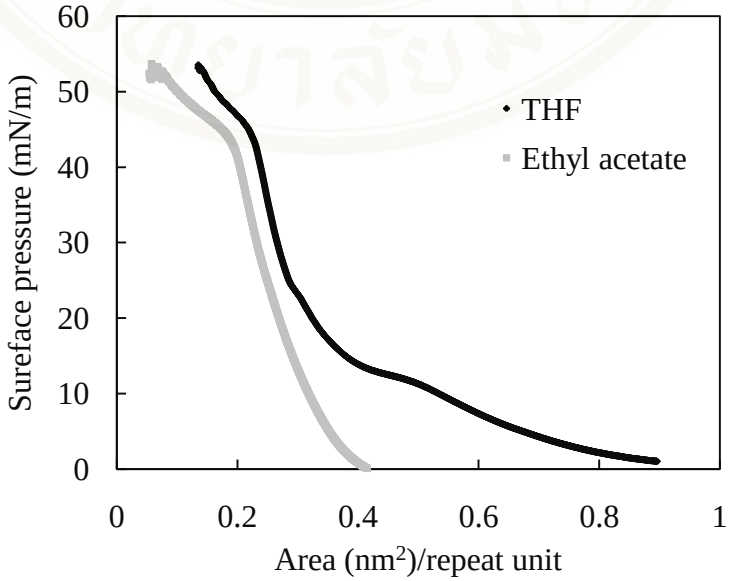


Figure 4.19: Surface pressure of modified dextran with 113%DS in ethyl acetate and THF.

A very different result was observed with a less modified dextran (DS = 113%, Figure 4.19). When ethyl acetate was used, the shape of compression isotherm was similar to that of DS = 150%. On the contrary, when THF was used, compression isotherm exhibited a plateau region indicating much more expanded macromolecules. It seems that this polymer covers a range of DS values for which THF is particularly favorable for chain extension that strongly modified chains conformation at air/water interface, promoting interactions with aqueous subphase. A more detailed study would be required to get deeper insight into the link between spreading solvent, DS and compression isotherm.

4.3 Nanoparticle formation and characterization

The nanoprecipitation method was used to prepare the nanoparticles from modified dextran. In the present work, nanoparticles were prepared from DexT40-VD derivatives with different DS. DexT40-VD was synthesized by enzyme-catalyzed transesterification in DMSO between DexT40 and VD as described in section 3.2.3.5.

To the best of our knowledge, potentialities in the elaboration of drug delivery systems of dextran esters carrying long hydrocarbon chains and having DS varying over a wide interval have never been reported. Thanks to enzyme-catalyzed modification of dextran, it was thus possible to design nanoparticles having an inner core with variable polarity according to the density of hydrocarbon tails grafting. Schematic representation of dextran decanoate based nanoparticle is presented in Figure 4.20. Colloidal stability of the obtained nanoparticle suspensions depends on the hydrophilicity of the nanoparticle surface. Two possibilities were envisaged in that work. First, self-organization of dextran ester macromolecules during nanoprecipitation may provide hydrophilic layer of unmodified hydroxyl groups on the surface of nanoparticles. Obviously, sufficient amount of unmodified hydroxyl groups in macromolecules is needed to obtain an efficient hydrophilic barrier against particle aggregation. A second strategy was attempted, which was to prepare nanoparticles which would combine low and high DS dextran derivatives. The high DS polymer would form the inner core while the low DS polymer would generate a hydrophilic corona at the surface of nanoparticles.

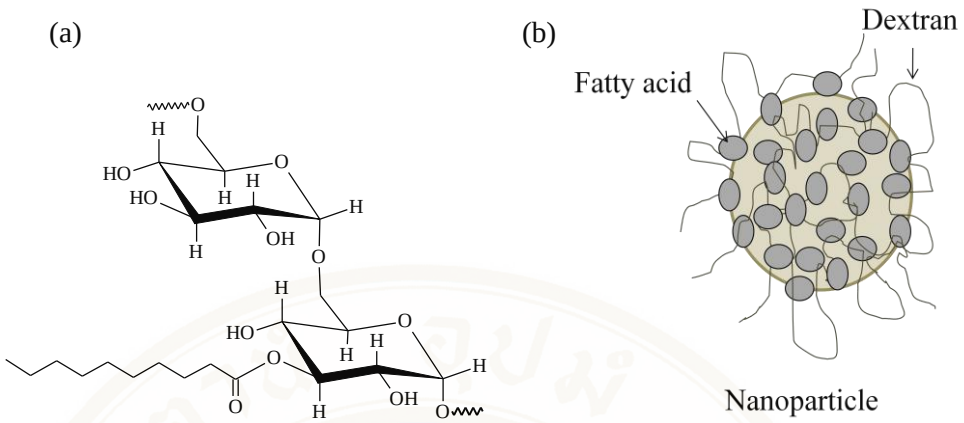


Figure 4.20: Formula of dextran modified with decanoate (C10) (a) and schematic representation of dextran decanoate nanoparticle (b) (adapted from the literature [10]).

In this study, DexT40-VD with various DS were obtained after progressive fractionation by ethyl acetate, methanol and water, as reported in section 4.2.1.

4.3.1 Conditions for nanoparticle preparation

The idea of solubility of modified dextran in organic solvent is necessary for preparation of nanoparticles by nanoprecipitation. The solubility of modified dextran was studied in Table 4.9 section 4.2.2.

Highly modified dextrans (DS > 80%) were soluble in THF. Modified dextrans with 20-25%DS were soluble in a THF/water mixture (90/10 v/v). Modified dextrans with DS lower than 17% were water-soluble polymers. The solubility of modified dextran in water-miscible solvent as THF makes it possible to prepare nanoparticles by nanoprecipitation method. This method has already been applied to prepare nanoparticles from modified dextran [10, 65, 151-155].

4.3.2 Effect of polymer concentration on average size and colloidal stability of nanoparticle suspensions

Based on the solubility in water-miscible solvent, three modified dextran with increasing DS were used to prepare nanoparticles by nanoprecipitation method. In order to obtain the nanoparticles with designed size and solubility, the concentrations of modified dextran were varied. The different conditions are gathered in Table 4.11. In all the cases, nanoparticle suspensions were successfully obtained. As

shown in Table 4.11, dextran nanoparticles with the size ranging from 86 to 256 nm were obtained without surfactant in the aqueous phase. The smallest sizes were obtained when the polymer concentration in the organic phase was 5g/L (sample 1, 4 and 8). For all the dextran derivatives, particle size of nanoparticles increased with polymer concentration in organic solvent (sample 2, 5, 6, 9 and 10). It is worth noting that, the modified dextran with 25%DS was not completely soluble in the 90/10 THF/water mixture at a concentration of 20 g/L. Therefore, the nanoparticles formation at this concentration was not conducted.

The colloidal stability of the suspensions was evaluated by considering the dry extract of remaining suspensions after centrifugation at 1500×g for 15 min at 25°C, either directly after nanoprecipitation or after 1 week storage at room temperature.

For all polymers, formation of aggregates during nanoprecipitation was favored by increasing polymer concentration in organic solvent as indicated by both the strong decrease of dry extract of suspensions after centrifugation and the higher particle diameters before centrifugation. The amount of submicronic particles in the suspensions was found to decrease with time and the aggregation process was accelerated upon increasing polymer concentration in the organic phase, i.e. the solid content of the suspensions. Indeed, after 1 week storage, the solid content of nanoparticles strongly decreased especially when high polymer concentrations were used except for nanoparticles prepared from 80% modified dextran at 5 g/L. This better stability may be due to the smaller particle size obtained in this case as well as to the DS value of the polymer (Table 4.11).

The effect of solvent to water volume ratio was examined by increasing volume of solvent from 5 to 10 mL (sample 3, 7 and 11). Samples with the same solid content but different volume ratio were compared (sample 6 and 7, sample10 and 11). For 80% modified dextran, the volume ratio of THF to water did not affect the particle size. On the contrary, higher volume of solvent led to smaller particle size for 150% modified dextran. However nanoparticles were less stable. The low solubility of modified dextran in lower volume of THF may be responsible for the larger size of nanoparticle. In addition, the slower evaporation of higher amount of solvent may influence the formation of the nanoparticles.

Table 4.11: Particle size and the content of dried extract (%DW) after centrifugation of nanoparticle suspensions obtained with different nanoprecipitation conditions.

%DS	Sample Number	Conc.(g/L)[a]	Vol.(mL)[b]	D_z (nm)[c]	Centrifuge after preparation		Centrifuge after 1 week	
					% DW[d]	D_z (nm)[c]	% DW[d]	D_z (nm)[c]
25	1	5	5	206	52	169	13	233
	2	10	5	256	2	211	0.1	183
	3	10	10	237	3	209	0.1	196
80	4	5	5	86	96	95	96	107
	5	10	5	120	89	120	41	136
	6	20	5	155	62	139	10	151
	7	10	10	148	53	127	16	147
150	8	5	5	126	85	127	33	130
	9	10	5	203	29	179	7	181
	10	20	5	215	2	242	0.1	201
	11	10	10	155	4	268	2	265

[a] Concentration of polymer in THF or THF/water mixture.

[b] Volume of polymer/THF mixture used for 10 mL of water in nanoprecipitation.

[c] Mean diameter of nanoparticles.

[d] Dry weight of nanoparticles remaining in suspension after centrifugation.

From these results, it can be concluded that polymer concentration plays an important role in nanoparticles formation upon solvent diffusion. The agglomeration of nanoparticles is enhanced when solid content of polymer in suspension increases. Analogous phenomenon has been reported by Gavory *et al*. for nanoparticles without surfactant [65]. The concentration of 5 g/L was thus chosen for further experiments in order to optimize both solid content and particle size.

4.3.3 Effect of DS on size and colloidal stability of nanoparticles

The effect of the degree of modification of the modified dextran used for forming the core of the nanoparticles on the size was investigated. Generally speaking, increasing DS should induce an increase in particle size because more hydrophobic units are available to form hydrophobic cores while non-modified units are expected to gather at the interface with the aqueous medium. As shown in Table 4.11, nanoparticles size increased from 86 to 126 nm when DS was extended from 80 to 150%. Similar result has been reported previously for nanoparticles prepared from dextran grafted with phenoxy at different DS [10]. Unexpectedly, larger particle size was obtained for the 25% modified dextran. This size increase may be due to the less numerous interactions between hydrophobic groups of low modified dextrans leading to a loose hydrophobic core whereas numerous interactions of highly modified ones led to dense hydrophobic core. Indeed, in the case of dextran derivative with DS = 25%, the weight fraction of hydrophobically modified units is 40%, which is much lower than for dextran derives with DS = 80 or 150% for which the weight fraction of hydrophobic units is 89 and 100% respectively. Moreover, the difference of solvent used in nanoparticles preparation could affect the size of nanoparticles. The differences between the particles obtained with the high and low modified dextran evidenced by DLS were confirmed by SEM images (Figure 4.21).

It was found that the regular spheres with smooth surfaces of nanoparticles were found when DS were 80 and 150% whereas the irregular particles were formed by using 25% modifeid dextran. However, the spherical nanoparticle with the approximately 270 nm diameter obtained from dextran decanoate with 23%DS by dialysis method (1.25 mg/mL of polymer concentration in aqueous solution) was

reported by Ge *et al* [99]. The different method of preparation might affect the size and shape of nanoparticles.

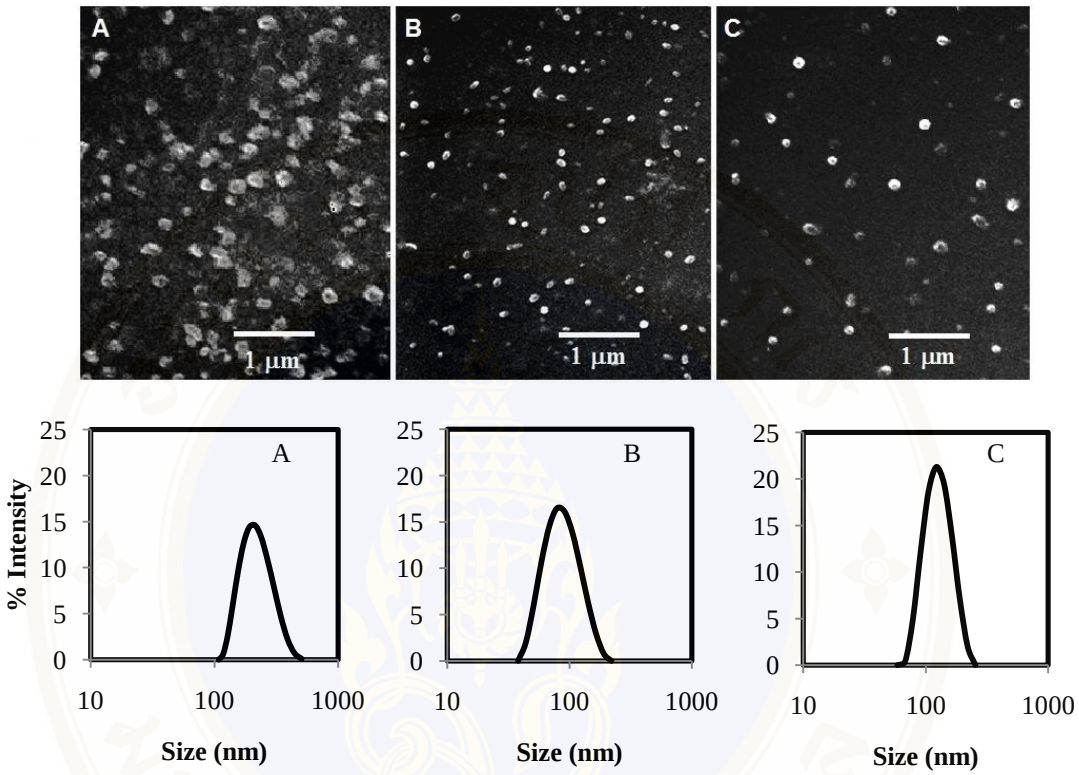


Figure 4.21: SEM images and DLS results of nanoparticles obtained from dextran derivatives with various DS: (A) DS = 25%, (B) DS = 80% and (C) DS = 150%. [Dextran ester] = 5 g/L; Volume of organic phase = 5 mL.

4.3.4 Effect of polymeric surfactant on size and colloidal stability of nanoparticles

Further investigations were carried out to improve stability of nanoparticles by using polymeric surfactant in aqueous solution. Water-soluble modified dextran has been demonstrated to be efficient polymeric stabilizer to improve stability of nanoparticles prepared from polylactic acid and modified dextran [10, 63, 156]. In this study, water-soluble modified dextran (DS=15%) was used as polymeric surfactant during nanoprecipitation in order to from a hydrophilic shell on the nanoparticles. As presented in Table 4.12, the difference in size of nanoparticles obtained with and without surfactant was not significant. Smaller polydispersity index,

indicating narrower size distribution, was however obtained in the presence of 15% modified dextran. This confirms that during nanoprecipitation the size of obtained particles is mainly controlled by thermodynamic parameters like nature of solvent or polymer concentration but not by the use of a stabilizer.

Table 4.12: Mean diameter of nanoparticles prepared by nanoprecipitation with and without 1 g/L of 15% modified dextran in the aqueous phase using 5 g/L of modified dextran in 5 mL of THF or THF/water.

DS of modified dextran for nanoparticles core	Without surfactant[a]		1 g/L of Surfactant[a]	
	D_z	PDI[b]	D_z	PDI[b]
25	206	0.11	208	0.09
80	86	0.11	84	0.06
150	126	0.09	114	0.03

[a] Nanoprecipitation condition described in section 3.4.1.

[b] Polydispersity index.

The colloidal stability of those nanoparticles was examined at various concentration of NaCl. The critical flocculation concentration was determined at the slope breaking of *n* as a function of NaCl concentration as presented in Figure 4.22. The slope *n* was calculated from equation 12.

$$\text{Slope } n = \frac{d(\log A)}{d(\log \lambda)} \qquad (12)$$

It was found that nanoparticles without surfactant were no longer stable when NaCl concentration was above 10^{-3} M. The flocculation was caused by the screening of residual surface charges by electrolyte. Van der Waals attractions between particles occurred upon increasing ionic strength. In the presence of surfactant, nanoparticles prepared from 80 and 150% modified dextran were stable up to 1 M NaCl as presented in Figure 4.22.

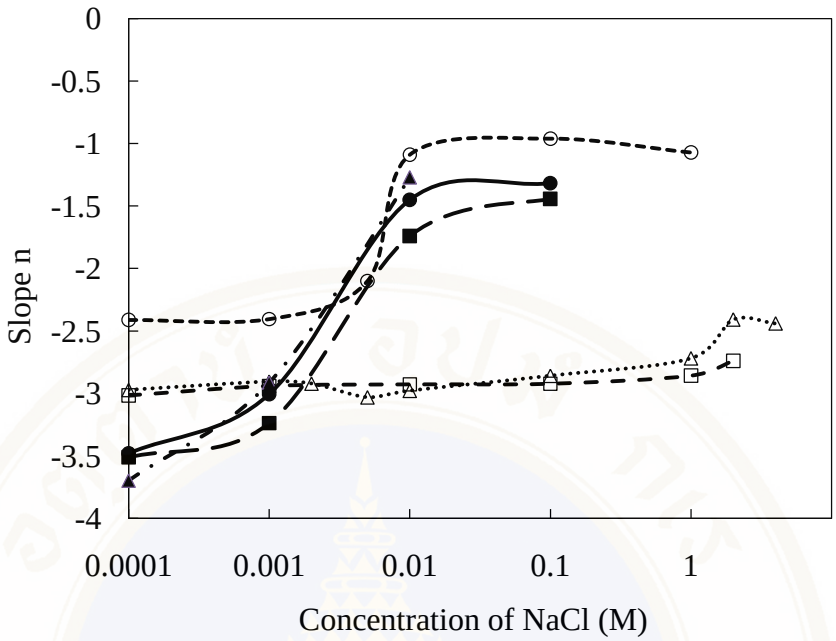


Figure 4.22: Slope *n* as a function of NaCl concentration for bare (closed symbols) and polymeric surfactant-covered (open symbols) particles obtained from dextran derivatives with DS of 25%(circles), 80%(squares) and 150% modified dextran (triangles). The polymeric surfactant was a dextran ester with DS = 15 %.

These results underline the increase of colloidal stability of nanoparticles by using 15% modified dextran as surfactant. It can be explained by steric stabilization, due to osmetic and elastic compression of the surfactant layer at the surface of the particle. No improvement of colloidal stability by surfactant was observed for nanoparticles obtained from 25% modified dextran. A lower amount of adsorbed surfactant on their surface, due to their higher hydrophilicity (hydrophilic units represent about 60wt% in the polymer), may be responsible for the low colloidal stability of these nanoparticles [65]. To improve colloidal stability of nanoparticle obtained from 25% modified dextran, nanoprecipitation was performed in the presence of 1 g/L gum arabic or in 0.01 M NaCl. No significant improvement of colloidal stability was observed in both cases.

4.4 Enzymatic degradation of modified dextran

4.4.1 Degradation by dextranase

The presence of dextranase in difference tissues such as kidney, colon, spleen and lung but not in blood makes dextran derivatives ideal candidate for targeted delivery of therapeutic agents [158-160]. As well demonstrated in previous studies, degradability of dextran derivatives by dextranase is influenced by the type of functional groups and DS of modified dextran [151, 157]. Degradation of dextran modified with ethylglycidyl ether, epoxyoctane or epoxydodecane decreased with increasing DS [151].

In this work, degradations of modified dextran by dextranase were evaluated for various time of incubation (4, 24, 48 and 168 h). The cleavage of dextran by dextranase led to oligosaccharide reducing chain ends which were titrated by Sumner reagent. The number of glucose units in the final fragments could be calculated from the following equation.

$$\textit{Number of glucose unit in fragment} = \frac{\textit{Total amount of glucose unit}}{\textit{Amount of reducing end}} \qquad (13)$$

As shown in Figure 4.23, native dextran was completely degraded in 4 h; the final product of degradation being isomaltose. It was found that modified dextrans with DS lower than 25% were completely degraded after 168 h. On the contrary, the degradation of highly modified dextran was very slow. The reason might be the insolubility in water of these dextran derivatives. To verify the degradability of highly modified dextran nanoparticles, those polymers were used to follow lipase degradation in the following section.

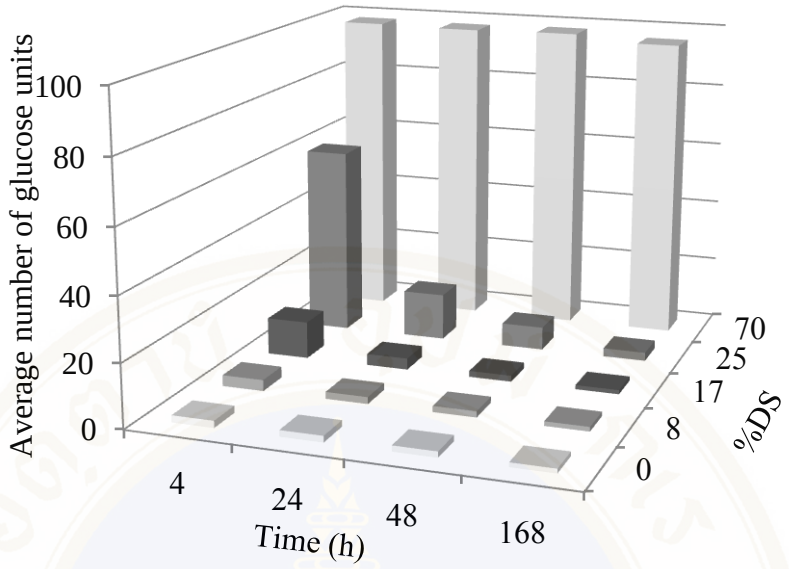


Figure 4.23: Enzymatic degradation by dextranase of native and modified dextran with various DS as a function of time.

4.4.2 Degradation by porcine pancreatic lipase

Pancreatic lipase, present in human digestive system, is primary enzyme that hydrolyze ester bond. It has been used to evaluate degradability of biomaterial such as polyesters used for biomedical applications [161]. Nevertheless, to the best of our knowledge, the enzymatic degradation by lipase of dextran esters with DS as high as 80 and 150% has never been reported. Recent results are available for DS = 21% [153]. The hydrolysis of DexT40-VD was presented in Figure 4.24.

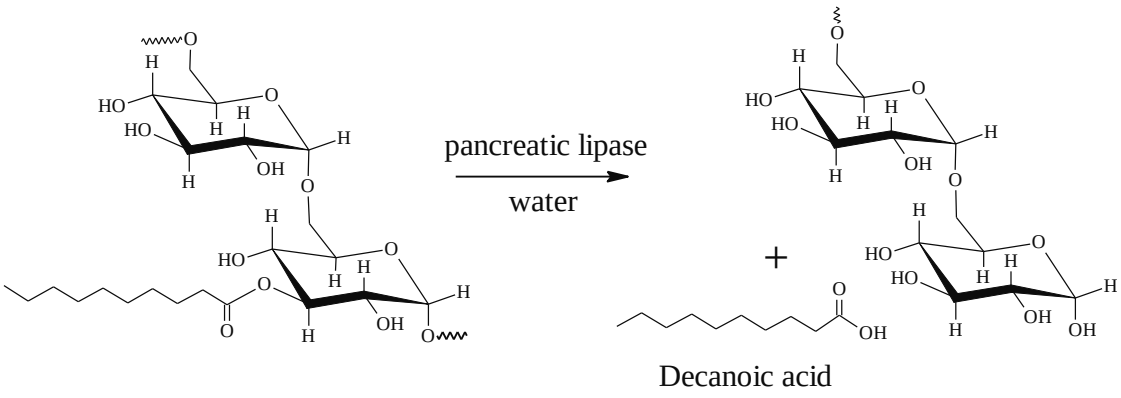


Figure 4.24: Hydrolysis of dextran decanoate by pancreatic lipase.

In this study, the degradations of modified dextran at various DS by porcine pancreatic lipase were measured after 4 h of incubation at 37°C. The hydrolysis of ester bond was calculated from the amount of liberated fatty acid determined automatically by titration. Results are gathered in Table 4.13**.**

Table 4.13: Hydrolysis of modified dextran by porcine pancreatic lipase after 4 h incubation.

Modified dextran (%DS)	Theoretical fatty acid amount for 100 mg polymer (mmole)	Free fatty acid after hydrolysis (mmole)	% hydrolysis
150	0.382	0.021	5.5
89	0.297	0.028	9.4
21	0.108	ND	ND

It was found that degradation of modified dextran with 80 and 150% DS was respectively equal to 9.4 and 5.5% after 4 h of incubation. No degradation was observed for the 21%DS modified dextran probably because of the low initial amount of ester attached to dextran backbone. The low activity of porcine pancreatic lipase toward highly modified dextran might be due to the gathering of hydrophobic tails inside the insoluble core or modified dextran. Consequently, the ester bonds were not accessible to lipase. The degradation of dextran backbones prior to lipase catalyzed hydrolysis might be helpful to accelerate the degradation of modified dextran. Although the hydrolysis activity of lipase toward modified dextran was still low. Nevertheless, the capacity porcine pancreatic lipase to degrade ester bonds of highly modified dextran offers the possibility to use them to prepare nanoparticles for drug delivery systems.

4.5 Encapsulation of lidocaine into nanoparticles

Encapsulation ability of modified dextran-based nanoparticles was examined by using lidocaine as a model for hydrophobic drug. Lidocaine was added at various concentrations (0.5, 1, 2.5 and 5 g/L) to a solution of modified dextran (5 g/L). The amount of lidocaine loaded in nanoparticles was determined by indirect method (determination of free lidocaine remaining in supernatant after centrifugation) and direct method (determination of loaded lidocaine after acid degradation of nanoparticles). Encapsulation ratio was calculated by following equation;

$$\%Encapsulation\ Ratio = \frac{Mass\ of\ loaded\ lidocaine}{Initial\ mass\ of\ lidocaine} \times 100 \quad (14)$$

% ratio of incorporated drug to mass of nanoparticle was calculated by following equation;

$$\%Loading\ Efficiency = \frac{Mass\ of\ loaded\ lidocaine}{Mass\ of\ polymer} \times 100 \quad (15)$$

The results were summarized in Table 4.14. The loading efficiency of lidocaine (18-29% w/w) was incorporated into the nanoparticles obtained from dextran with different DS, both with and without surfactant in the aqueous phase. Loading efficiency was not influenced by initial amount of lidocaine for the fixed concentration of polymer. This result is in a good agreement with the report published earlier [162]. In any cases, nanoparticle size increased with increasing concentration of lidocaine. When 15% modified dextran was added in the aqueous phase as stabilizer, sizes of loaded nanoparticles slightly increased except for nanoparticles obtained from 21% modified dextran. In that case, sizes of loaded nanoparticles strongly increased with the concentration of lidocaine in the absence of surfactant. This might be due to a lower compatibility of lidocaine with the loose hydrophobic core of nanoparticles formed from this biopolymer.

Table 4.14: Particle size and lidocaine encapsulation ratio for nanoparticles prepared from different dextran derivatives, with or without surfactant in the aqueous phase using 5 g/L in 5 mL THF or THF/water. The stabilizer used for certain experiments was a 15% modified dextran.

Nanoparticles	Initial ratio of lidocaine/ modified dextran (%)	Encapsulation Ratio (%)	Loading efficiency (%)	Size (nm)
150%	10	2.4	24.0	127
	20	4.0	20.4	136
	50	14.4	28.8	140
	100	21.8	21.8	147
150% With surfactant	10	1.1	18.0	129
	20	4.3	21.3	140
	50	11.0	22.0	162
	100	22.6	22.6	171
89%	10	2.0	20.0	104
	20	3.2	19.2	109
	50	13.4	19.4	116
	100	18.5	18.5	120
89% With surfactant	10	1.8	17.6	130
	20	3.8	18.9	142
	50	10.4	21.0	152
	100	19.2	19.2	167
21%	10	2.2	21.4	241
	20	4.4	22.0	495
	50	12.8	25.5	1077
	100	26.2	26.2	1604
21% With surfactant	10	2.0	19.6	222
	20	4.2	20.8	265
	50	12.1	24.2	360
	100	24.7	24.7	590

CHAPTER V
CONCLUSION

Dextran decanoate macromolecules (DexT40-VD) were synthesized in DMSO at 50^{o}C using lipase AY from *Candida rugosa* for catalyzing transesterification between polysaccharide and vinyl fatty esters. Enzymatic synthesis enables several ways to increase the DS of dextran by enzyme-catalyzed transesterification. The remarkable enhancement of dextran modification was obtained by pH-adjusted- and 50 molar equivalent of 18-crown-6 ether pretreated pH-adjusted lipase AY. Finally, the best way to reach high DS was to use stepwise addition of lipase and vinyl ester to the reaction medium. Acceleration of transesterification of DexT40 by lipase strongly increased compare with the non-catalyzed system. Moreover, the positional selectivity of dextran transesterification was detailed and related to the chemical structure of the acyl donor regioselectivity equaled between 2- and 3-OH with saturated fatty acyl donors. The 2-OH was favored for unsaturated fatty acyl donors, while sterically hindered acyl donors oriented modification toward 3-OH position.

The properties of the enzymatically modified dextrans were studied by solvent fractionation with increasing polarity (ethyl acetate, methanol and water). Samples of modified polymers obtained from the enzyme catalyzed transesterification covered a wide range of DS (10 to 150%) with distribution depending on the initial amounts of reactants and lipase. The increase solvent polarity exhibited the decrease of DS. In comparison, modified dextrans prepared by uncatalyzed transesterification (even over much longer times) exhibited DS lower than 15% with much narrower distributions. The viscosity in DMSO of modified dextran was found to decrease with the increase of DS. The adsorption of modified dextrans at oil/water and air/water interface was examined by surface pressure and interfacial tension measurements for DS varying from 10 to 150%. Water soluble polymers were effective surfactant for lowering the interfacial tension of oil/water. Water insoluble polymer exhibits the stable monolayer

on aqueous subphase. Type of solvent was important parameter for the folding conformation of polymer in surface pressure experiment.

Nanoparticle preparation of dextran decanoate obtained from catalysis was formulated by nanoprecipitation method. The core-shell nanoparticles were constructed. Water soluble modified dextrans with DS at least 21% were used as a core of nanoparticles by nanoprecipitation methods. Optimization of nanoparticles preparation was performed by using 5 ml of 5 g/L polymer in THF and 10 ml of 1 g/L of water soluble modified dextran aqueous solution. Particle size and stability of nanoparticles was influenced by polymer concentration and degree of substitution. The colloidal stability and particle size distribution of nanoparticle suspensions in sodium chloride solution was improved by using water-soluble modified dextrans as a surface coverage. The complete degradation of low modified dextran (DS up to 25%) by dextranase was observed in 7 days. Enzymatic hydrolysis of ester bond in highly modified dextran by porcine pancreatic lipase was demonstrated. Finally, the certain amount of lidocaine was encapsulated into nanoparticles prepared from all polymers. Degradability of modified dextrans by dextranase and pancreatic lipase enables the possibility of using this polymer for biomedical application. The encapsulation of lidocaine provides the feasibility of loading nonpolar substances for potential applications.

The results from this study indicate that nanoparticles based on dextran ester appear to be a promising carrier for drug delivery system. However, examination of drug release and *in vivo* test for immune response are necessary to implement these nanoparticles as potential drug carriers. Another challenge of nanoparticles for drug delivery system is targeted delivery. Thanks to the remaining hydroxyl groups both in the core and at the surface, the attachment of antibodies or ligands can be made to develop nanoparticles for specific drug delivery.

REFERENCES

1. Jeanes A, Haynes WC, Wilham CA, Rankin JC, Melvin EH, Austin MJ, et al. Characterization and Classification of Dextrans from Ninety-six Strains of Bacteria1b. J. Am. Chem. Soc. 1954;76(20):5041-52.
2. Larm O, Lindberg B, Svensson S. Studies on the length of the side chains of the dextran elaborated by Leuconostoc mesenteroides NRRL B-512. Carbohydr. Res. 1971;20(1):39-48.
3. Belder AN, Norrman B. The distribution of substituents in partially acetylated dextran. Carbohydr. Res. 1968;8(1):1-6.
4. Kirsti AG. Solution properties of branched dextrans. J. Colloid Sci. 1958;13(4):308-28.
5. Jeanes A, Schieltz NC, Wilham CA. Molecular association in dextran and in branched amylaceous carbohydrates. J. Biol. Chem. 1984;176:617-27.
6. Koster K, Sele V, Schwartz M, Sindrup E. Blood-volume changes after infusion of dextran solutions. Lancet. 1957;273(6989):262-5.
7. Gibbons RJ, Keyes PH. Inhibition of insoluble dextran synthesis, plaque formation and dental caries in hamsters by low molecular weight dextran. Arch. Oral Biol. 1969;14(6):721-4, IN23.
8. Rotureau E, Chassenieux C, Dellacherie E, Durand A. Neutral Polymeric Surfactants Derived from Dextran: A Study of Their Aqueous Solution Behavior. Macromol. Chem. Physic. 2005;206(20):2038-46.
9. Ferreira L, Rafael A, Lamghari M, Barbosa MA, Gil MH, Cabrita AMS, et al. Biocompatibility of chemoenzymatically derived dextran-acrylate hydrogels. Int. J. Biomed. Mater. Res. 2004;68A:584-96.
10. Aumelas A, Serrero A, Durand A, Dellacherie E, Leonard M. Nanoparticles of hydrophobically modified dextrans as potential drug carrier systems. Colloid. Surface. B. 2007;59(1):74-80.

11. Gref R, Rodrigues J, Couvreur P. Polysaccharides Grafted with Polyesters: Novel Amphiphilic Copolymers for Biomedical Applications. Macromolecules. 2002;35(27):9861-7.
12. Nouvel C, Raynaud J, Marie E, Dellacherie E, Six JL, Durand A. Biodegradable nanoparticles made from polylactide-grafted dextran copolymers. J. Colloid. Interf. Sci. 2009;330(2):337-43.
13. Onishi Y, Maruno S, Kamiya S, Hokkoku S, Hasegawa M. Preparation and characteristics of dextran-methyl methacrylate graft copolymer. Polymer. 1978;19(11):1325-8.
14. Alan H H. Relative Reactivities of Hydroxyl Groups in Carbohydrates. In: Tipson RS, Derek H, editors. Adv. Carbohydr. Chem. Biochem.: Academic Press; 1976. p. 11-109.
15. Larm O, Larsson K, Scholander E, Meyer B, Thiem J. Analysis of bromine-oxidized dextran by carbon-13 NMR spectroscopy. Carbohydr. Res. 1981;91(1):13-20.
16. De Belder AN, Lindberg B, Svensson S. Synthesis of ketodextrans. Acta. Chem. Scand. 1968;22(3):949-52.
17. Arranz F, Sanchez-Chaves M. Carbon-13 nuclear magnetic resonance spectral study on the distribution of substituents in relation to the preparation method of partially acetylated dextrans. Polymer. 1988;29:507-12.
18. Ferreira L, Gil MH, Dordick JS. Enzymatic synthesis of dextran-containing hydrogels. Biomaterials. 2002;23:3957-67.
19. Brown RG, Lindberg B. Enzymic hydrolysis of ketodextran. Carbohydr. Res. 1974;38:369-73.
20. Ferreira L, Gil MH, Cabrita AMS, Dordick JS. Biocatalytic synthesis of highly ordered degradable dextran-based hydrogels. Biomaterials. 2005;26:4707-16.
21. Inprakhon P, Lalot T, Brigodiot M, Marechal E. Head and tail modification of methyl acrylate telomers by enzyme catalysis. Part 1. Reaction on the first adducts as models for longer chains. Des. Monomers Polym. 2001;4:83-93.

22. Inprakhon P, Lalot T, Brigodiot M, Marechal E. Regioselective modification of methyl acrylate telomers by enzyme catalysis. Part 2. Influence of the telogen segment on the selectivity. Des. Monomers Polym. 2001;4:95-106.
23. Inprakhon P, Lalot T. Regioselectivity of enzymatic modification of poly(methyl acrylate). J. Biotechnol. 2007;131(4):418-28.
24. Lu M, Johansson G, Albertsson P, Tjerneld F. Partitioning of proteins in dextran/hydrophobically modified dextran aqueous two-phase systems. Bioseparation. 1995;5(6):351-8.
25. Kitagawa M, Fan H, Raku T, Shibatani S, Maekawa Y, Hiraguri Y, et al. Selective enzymatic preparation of vinyl sugar esters using DMSO as a denaturing co-solvent. Biotechnol. Lett. 1999;21(4):355-9.
26. Zhang J, Pelton R, Wågberg L. Aqueous biphase formation by mixtures of dextran and hydrophobically modified dextran. Colloid Polym. Sci. 1998;276(6):476-82.
27. Bauer KH, Reinhart T, inventors; Bauer, Kurt H, assignee. Water-soluble dextran fatty acid esters and their use as solubilizers. United States patent 5750678. 1998.
28. Beesh M, Majewska P, Vandamme TF. Synthesis and characterization of dextran esters as coating or matrix systems for oral delivery of drugs targeted to the colon. Int. J. Drug Delivery. 2010;2:22-31.
29. Schehlmann V, Bauer KH, Kesselhut J-F, inventors; BASF Aktiengesellschaft, assignee. Dextran esters, the preparation thereof and the use thereof for coating or embedding drugs. United States patent 5739122 1998.
30. Bauer KH, Reinhart T, Stenz R. Dextran fatty acid esters as polymeric surfactants with low hemolytic activity. Macromol. Symp. 1997;120(1):39-45.
31. Vieira NAB, Moscardini MS, Tiera VAdO, Tiera MJ. Aggregation behavior of hydrophobically modified dextran in aqueous solution: a fluorescence probe study. Carbohydr. Polym. 2003;53(2):137-43.
32. Fournier C, Leonard M, Le Coq-Leonard I, Dellacherie E. Coating Polystyrene Particles by Adsorption of Hydrophobically Modified Dextran. Langmuir. 1995;11(7):2344-7.

33. Ramírez JC, Sánchez-Chaves M, Arránz F. Dextran functionalized by cyclic carbonate groups as supports of bioactive compounds. Angew. Makromol. Chem. 1993;206(1):77-85.
34. Ramirez JC, Sanchez-Chaves M, Arranz F. Functionalization of dextran with chloroacetate groups: immobilization of bioactive carboxylic acids. Polymer. 1994;35(12):2651-5.
35. Ramirez JC, Sánchez-Chaves M, Arranz F. Dextran functionalized by 4-nitrophenyl carbonate groups. Aminolysis reactions. Angew. Makromol. Chem. 1995;225(1):123-30.
36. Nichifor M, Lopes A, Carpov A, Melo E. Aggregation in Water of Dextran Hydrophobically Modified with Bile Acids. Macromolecules. 1999;32(21):7078-85.
37. Usmanov TI, Karimova UG. NMR -spectroscopic study of substituent distribution in polysaccharide acetates. Vysokomol Soedin A. 1990;32(9):1871-81.
38. Sanchez-Chaves M, Arranz F. Preparation of dextran-bioactive compound adducts by the direct esterification of dextran with bioactive carboxylic acids. Polymer. 1997;38(10):2501-5.
39. van Dijk-Wolthuis WNE, Kettenes-van den Bosch JJ, van der Kerk-van Hoof A, Hennink WE. Reaction of dextran with glycidyl methacrylate: An unexpected transesterification. Macromolecules. 1997;30:3411-3.
40. De Belder AN, Norman B. Substitution patterns of O-(2-hydroxyethyl) starch and O-(2-hydroxyethyl) dextran. Carbohydr. Res. 1969;10(3):391-4.
41. Arranz F, San Roman J, Sanchez-Chaves M. 13C NMR study of the selectivity of the modification of dextran with ethyl chloroformate. Macromolecules. 1987;20:801-6.
42. Mahner C, Lechner MD, Nordmeier E. Synthesis and characterisation of dextran and pullulan sulphate. Carbohydr. Res. 2001;331:203-8.
43. Sanchez-Chaves M, Arranz F. Synthesis of amidoxime-containing modified dextran. Polymer. 1996;37(19):4403-7.

44. Nouvel C, Dubois P, Dellacherie E, Six J-L. Silylation reaction of dextran: effect of experimental conditions on silylation yield, regioselectivity and chemical stability of silylated dextrans. Biomacromolecules. 2003;4:1443-50.

45. Usmanov TI, Karimova UG, Sarymsakov A. 13C NMR study of substituent distribution in carboxymethyl ethers of polysaccharides. Vysokomol Soedin A. 1990;32(6):1176-83.

46. Krentsel L, Chaubet F, Rebrov A, Champion J, Ermakov I, Bittoun P, et al. Anticoagulant activity of functionalized dextrans. Structure analysis of carboxymethylated dextran ans first Monte Carlo simulations. Carbohydr. Polym. 1997;33:63-71.

47. Vollmer A, Voiges K, Bork C, Fiege K, Cuber K, Mischnick P. Comprehensive analysis of the substitution pattern in dextran ethers with respect to the reaction conditions. Anal. Bioanal. Chem. 2009;395(6):1749-68.

48. Adachi S, Ogata M, Tobita H, Hashimoto K. Effects of molecular weight of dextran and NAD+ density on coenzyme activity of high molecular weight NAD+ derivative covalently bound to dextran. Enzym. Microb. Tech. 1984;6(6):259-62.

49. Pugazhendhy S, Shrivastava P, Sinha S, Shrivastava S. Lamotrigine–dextran conjugates-synthesis, characterization, and biological evaluation. Med. Chem. Res. 2011;20(5):595-600.

50. Axen R, Porath J, Ernback S. Chemical Coupling of Peptides and Proteins to Polysaccharides by Means of Cyanogen Halides. Nature. 1967;214(5095):1302-4.

51. Sundberg L, Porath J. Preparation of adsorbents for biospecific affinity chromatography : I. Attachment of group-containing ligands to insoluble polymers by means of bifunctional oxiranes. J. Chromatogr. A. 1974;90(1):87-98.

52. Shrivastava KS, Jain DK, Shrivastava KP, Trivedi P. Flurbiprofen- and Suprofen-Dextran Conjugates: Synthesis, Characterization and Biological Evaluation. Trop. J. Pharm. Res. 2009;8(3):221-9.

53. Larsen C, Jensen BH, Olesen HP. Stability of ketoprofen-dextran ester prodrugs in homogenates of various segments of the pig GI tract. Acta Pharm. Nordica. 1991;3(1):41-4.

54. Mehvar R. Dextrans for targeted and sustained delivery of therapeutic and imaging agents. J. Control. Release. 2000;69:1-25.

55. Lee KY, Mooney DJ. Hydrogels for Tissue Engineering. Chem. Rev. 2001;101(7):1869-80.

56. Jen AC, Wake MC, Mikos AG. Review: Hydrogels for cell immobilization. Biotechnol. Bioeng. 1996;50(4):357-64.

57. Wang K, Burban J, Cussler E. Hydrogels as separation agents. In: Dušek K, editor. Responsive Gels: Volume Transitions II: Springer Berlin / Heidelberg; 1993. p. 67-79.

58. Hennink WE, Talsma H, Borchert JCH, De Smedt SC, Demeester J. Controlled release of proteins from dextran hydrogels. J. Control. Release. 1996;39(1):47-55.

59. Hoare TR, Kohane DS. Hydrogels in drug delivery: Progress and challenges. Polymer. 2008;49(8):1993-2007.

60. Hovgaard L, Brøndsted H. Dextran hydrogels for colon-specific drug delivery. J. Control. Release. 1995;36(1-2):159-66.

61. De Sousa Delgado A, Leonard M, Dellacherie E. Surface properties of polystyrene nanoparticles coats with dextran-PEO copolymer architecture on protein adsortion. Langmuir. 2001;17:4386-91.

62. Coombes AGA, Tasker S, Lindblad M, Holmgren J, Hoste K, Toncheva V, et al. Biodegradable polymeric microparticles for drug delivery and vaccine formulation: the surface attachment of hydrophilic species using the concept of poly(ethylene glycol) anchoring segments. Biomaterials. 1997;18(17):1153-61.

63. Rouzes C, Durand A, Leonard M, Dellacherie E. Influence of polymeric surfactants on the properties of drug loaded PLA nanospheres. Colloid. Surface. B. 2003;32:125-35.

64. Rotureau E, Leonard M, Marie E, Dellacherie E, Camesano TA, Durand A. From polymeric surfactants to colloidal systems (1): Amphiphilic dextrans for emulsion preparation. Colloid. Surface. A. 2006;288(1-3):131-7.
65. Gavory C, Durand A, Six J-L, Nouvel C, Marie E, Leonard M. Polysaccharide-covered nanoparticles prepared by nanoprecipitation. Carbohydr. Polym. 2011;84(1):133-40.
66. Hornig S, Heinze T. Nanoscale structures of dextran esters. Carbohydr. Polym. 2007;68(2):280-6.
67. Hornig S, Bunjes H, Heinze T. Preparation and characterization of nanoparticles based on dextran-drug conjugates. J. Colloid Interf. Sci. 2009;338(1):56-62.
68. Ge J, Lu D, Yang C, Liu Z. A Lipase-Responsive Vehicle Using Amphipathic Polymer Synthesized with the Lipase as Catalyst. Macromol. Rapid Comm. 2011;32(6):546-50.
69. Ferreira L, Ramos MA, Gil MH, Dordick JS. Exquisite regioselectivity and increased transesterification activity of an immobilized Bacillus subtilis protease. Biotechnol. Progr. 2002;18:986-93.
70. Klibanov AM. Enzymes that work in organic solvents. CHEMTECH. 1986:354-9.
71. Klibanov AM. Asymmetric transformations catalyzed by enzymes in organic solvents. Accounts Chem. Res. 1990;23(4):114-20.
72. Okahata Y, Mori T. Lipid-coated enzymes as efficient catalysts in organic media. Trends Biotechnol. 1997;15:50-4.
73. Jones JB, Beck JF. Application of Biochemical Systems in Organic Chemistry. Jones JB, Sih JC, Parlman D, editors. New York: Wiley; 1986.
74. Kaewprapan K, Tuchinda P, Marie E, Durand A, Inprakhon P. pH-imprinted lipase catalyzed synthesis of dextran fatty acid ester. J. Mol. Catal. B-Enzym. 2007;47(3-4):135-42.
75. Janssen AEM, Lefferts AG, van't Riet K. Enzymatic synthesis of carbohydrate esters in aqueous media. Biotechnol. Lett. 1990;12:711-6.

76. Janssen AEM, Klabbers C, Franssen MCR, van't Riet K. Enzymatic synthesis of carbohydrate esters in 2-pyrrolidone. Enzyme Microb. Tech. 1991;13(7):565-72.

77. Córdova A, Hult K, Iversen T. Esterification of methyl glycoside mixtures by lipase catalysis. Biotechnol. Lett. 1997;19(1):15-8.

78. Chamouleau F, Coulon D, Girardin M, Ghoul M. Influence of water activity and water content on sugar esters lipase-catalyzed synthesis in organic media. J. Mol. Catal. B-Enzym. 2001;11(4-6):949-54.

79. MacManus DA, Vulfson EN. Reversal of regioselectivity in the enzymatic acylation of secondary hydroxyl groups mediated by organic solvent. Enzyme Microb. Tech. 1997;20:225-8.

80. Secundo F, Barletta GL, Dumitriu E, Carrea G. Can an inactivating agent increase enzyme activity in organic solvent? Effects of 18-crown-6 on lipase activity, enantioselectivity, and conformation. Biotechnol. Bioeng. 2007;97(1):12-8.

81. Kuntz Jr ID, Kauzmann W. Hydration of Proteins and Polypeptides. In: C.B. Anfinsen JTE, Frederic MR, editors. Advances in Protein Chemistry: Academic Press; 1974. p. 239-345.

82. Klibanov AM. Improving enzymes by using them in organic solvents. Nature. 2001;409:241-6.

83. Xu K, Griebenow K, Klibanov AM. Correlation between catalytic activity and secondary structure of subtilisin dissolved in organic solvents. Biotechnol. Bioeng. 1997;56(5):485-91.

84. Knubovets T, Osterhout JJ, Klibanov AM. Structure of lysozyme dissolved in neat organic solvents as assessed by NMR and CD spectroscopies. Biotechnol. Bioeng. 1999;63(2):242-8.

85. Yang L, Dordick JS, Garde S. Hydration of Enzyme in Nonaqueous Media Is Consistent with Solvent Dependence of Its Activity. Biophys. J. 2004;87(2):812-21.

86. Dai L, Klibanov AM. Striking activation of oxidative enzymes suspended in nonaqueous media. P. NATL. ACAD. SCI. USA. 1999;96:9475-8.

87. Griebenow K, Klibanov AM. Lyophilization-induced reversible changes in secondary structure of proteins. P. Natl. Acad. Sci. USA. 1995;92:10969-76.

88. Dong A, Meyer DJ, Kendrick SB, Manning CM, Carpenter FJ. Effect of secondary structure on the activity of enzymes suspended in organic solvents. Arch. Biochem. Biophys. 1996;334(2):406-14.

89. Wu J-C, Song B-D, Xing A-H, Hayashi Y, Talukder MMR, Wang S-C. Esterification reactions catalyzed by surfactant-coated *Candida rugosa* lipase in organic solvents. Process Biochem. 2002;37(11):1229-33.

90. Dabulis K, Klibanov AM. Dramatic enhancement of enzymatic activity in organic solvents by lyoprotectants. Biotechnol. Bioeng. 1993;41:566–71.

91. Ru MT, Dordick JS, Reimer JA, Clark DS. Optimizing the salt-induced activation of enzymes in organic solvents: Effects of lyophilization time and water content. Biotechnol. Bioeng. 1999;63(2):233-41.

92. Broos J, Sakodinskaya IK, Engbersen JFJ, Verboom W, Reinhoudt DN. Large Activation of Serine Proteases by Pretreatment with Crown Ethers. J. Chem. Soc. Chem. Comm. 1995:255-6.

93. van Unen D-J, Engbersen JFJ, Reinhoudt DN. Why do crown ethers activate enzyme in organic solvents? Biotechnol. Bioeng. 2002;77(3):248-55.

94. Santos AM, Montañez Clemente I, Barletta G, Griebenow K. Activation of serine protease subtilisin Carlsberg in organic solvents: combined effect of methyl-β-cyclodextrin and water. Biotechnol. Lett. 1999;21(12):1113-8.

95. Rich JO, Dordick JS. Imprinting Enzymes for Use in Organic Media. In: Vulfson EN, Halling PJ, Holland HL, editors. Enzymes in Nonaqueous Solvents: Humana Press; 2001. p. 13-7.

96. Plou FJ, Cruces MA, Ferrer M, Fuentes G, Pastor E, Bernabe M, et al. Enzymatic acylation of di- and trisaccharides with fatty acids: choosing the appropriate enzyme, support and solvent. J. Biotechnol. 2002;96(1):55-66.

97. Ferrer M, Cruces MA, Bernabe M, Ballesteros A, Plou FJ. Lipase-Catalyzed Regioselective Acylation of Sucrose in Two-Solvent Mixtures. Biotechnol. Bioeng. 1999;65(10-16):10.

98. Ferrer M, Soliveri J, Plou FJ, Lopez-Cortes N, Reyes-Duarte D, Christensen M, et al. Synthesis of sugar esters in solvent mixtures by lipases from *Thermomyces lanuginosus* and *Candida antarctica* B, and their antimicrobial properties. Enzyme Microb. Tech. 2005;36:391–8.

99. Ge J, Lu D, Wang J, Liu Z. Lipase Nanogel Catalyzed Transesterification in Anhydrous Dimethyl Sulfoxide. Biomacromolecules. 2009;10(6):1612-8.

100. Therisod M, Klibanov AM. Regioselective acylation of secondary hydroxyl groups in sugars catalyzed by lipases in organic solvents. J. Am. Chem. Soc. 1987;109(13):3977-81.

101. MacManus DA, Vulfson EN. Substituent effects on the regioselectivity of enzymatic acylation of 6-O-alkylglycopyranosides using *Pseudomonas cepacia* lipase. Carbohydr. Res. 1995;279:281-91.

102. Rich JO, Bedell BA, Dordick JS. Controlling enzyme-catalyzed regioselectivity in sugar ester synthesis. Biotechnol. Bioeng. 1995;45(5):426-34.

103. Wang YF, Lalonde JJ, Momongan M, Bergbreiter DE, Wong CH. Lipase catalyzed irreversible transesterifications using enol esters as acylating reagents: preparative enantio- and regioselective syntheses of alcohols, glycerol derivatives, sugars, and organometallics. J. Am. Chem. Soc. 1988;110:7200-5.

104. Kaewprapan K. Lipase catalyzed transesterification of dextran vinyl ester. Bangkok: Mahidol University; 2007.

105. Ema T, Maeno S, Takaya Y, Sakai T, Utaka M. Significant effect of acyl groups on enantioselectivity in lipase-catalyzed transesterifications. Tetrahedron: Asymmetry. 1996;7(3):625-8.

106. Lee JS, Jung YJ, Doh MJ, Kim YM. Synthesis and Properties of Dextran–Nalidixic Acid Ester as a Colon-Specific Prodrug of Nalidixic Acid. Drug Dev. Ind. Pharm. 2001;27(4):331-6.

107. Jung Y, Lee J, Kim H, Kim Y, Kim Y. Synthesis and properties of dextran-5-aminosalicylic acid ester as a potential colon-specific prodrug of 5-aminosalicylic acid. Arch. Pharm. Res. 1998;21(2):179-86.

108. Varshosaz J, Emami J, Tavakoli N, Fassihi A, Minaiyan M, Ahmadi F, et al. Synthesis and evaluation of dextran-budesonide conjugates as colon specific prodrugs for treatment of ulcerative colitis. Int. J. Pharm. 2009;365(1-2):69-76.

109. Franssen O, Vos OP, Hennink WE. Delayed release of a model protein from enzymatically-degrading dextran hydrogels. J. Control. Release. 1997;44(2-3):237-45.

110. Ebert KH, Ederer HJ, Emmert J. Oligomer formation in the chemical degradation of dextran. Polym. Bull. 1982;6(8):471-6.

111. Liu L, Xu X, Guo S, Han W. Synthesis and self-assembly of chitosan-based copolymer with a pair of hydrophobic/hydrophilic grafts of polycaprolactone and poly(ethylene glycol). Carbohydr. Polym. 2009;75(3):401-7.

112. Huang Y, Li L, Fang Ye. Preparation of size-tunable, highly monodisperse particles by self-assembly of N-phthaloylchitosan-g-polycaprolactone molecular bottle brushes. Mater. Lett. 2009;63(16):1416-8.

113. Yu H, Wang W, Chen X, Deng C, Jing X. Synthesis and characterization of the biodegradable polycaprolactone-graft-chitosan amphiphilic copolymers. Biopolymers. 2006;83(3):233-42.

114. Rodrigues JS, Santos-Magalhães NS, Coelho LCBB, Couvreur P, Ponchel G, Gref R. Novel core(polyester)-shell(polysaccharide) nanoparticles: protein loading and surface modification with lectins. J. Control. Release. 2003;92(1-2):103-12.

115. Prabu P, Chaudhari A, Aryal S, Dharmaraj N, Park S, Kim W, et al. In vitro evaluation of poly(caporlactone) grafted dextran (PGD) nanoparticles with cancer cell. J. Mater. Sci: Mater. M. 2008;19(5):2157-63.

116. Akoh C, Swanson B. Synthesis and Properties of Alkyl Glycoside and Stachyose Fatty Acid Polyesters. J. Am. Oil Chem. Soc. 1989;66(9):1295-301.

117. Osvaldo N, Oliveira J. Langmuir-Blodgett Films - Properties and Possible Applications. Braz. J. Phys. 1992;22(2):60-9.

118. Marie E, Rotureau E, Dellacherie E, Durand A. From polymeric surfactants to colloidal systems: 4. Neutral and anionic amphiphilic polysaccharides for miniemulsion stabilization and polymerization. Colloid. Surface. A. 2007;308(1-3):25-32.

119. Kaewprapan K, Tuchinda P, Marie E, Durand A, Inprakhon P. pH imprinted lipase catalyzed synthesis of dextran fatty acid ester. J Mol Catal B-Enzym. 2007;47:135-42.

120. Long JA, Osmond DWJ, Vincent B. The equilibrium aspects of weak flocculation. J. Colloid Interf. Sci. 1973;42(3):545-53.

121. Franssen O, Stenekes RJH, Hennink WE. Controlled release of a model protein from enzymatically degrading dextran microspheres. J. Control. Release. 1999;59(2):219-28.

122. Unen D-JV, Engbersen JFJ, Reinhoudt DN. Studies on the mecanism of crown-ether-induced activation of enzymes in non-aqueous media. J Mol Catal B-Enzym. 2001;11:877-82.

123. Ferrer M, Soliveri J, Plou FJ, López-Cortés N, Reyes-Duarte D, Christensen M, et al. Synthesis of sugar esters in solvent mixtures by lipases from Thermomyces lanuginisus and Candida antarctica B, and their antimicrobial properties. Enzym Microbiol Technol. 2005;36:391-8.

124. Ferrer M, Cruces AM, Bernabé M, Ballesteros A, Plou FJ. Lipase-catalyzed regioselective acylation of sucrose in two-solvent mixtures. Biotechnol Bioeng. 1999;65:10-6.

125. Çatiker E, Güner A. Unperturbed molecular dimensions and the theta temperature of dextran in dimethylsulfoxide (DMSO) solutions. Polym. Bull. 1998;41(2):223-30.

126. Unen D-JV, Engbersen JFJ, Reinhoudt DN. Why do crown ethers activate enzymes in organic solvent? Biotechnol. Bioeng. 2001;77:248-55.

127. Santos AM, Vidal M, Pacheco Y, Frontera J, Báez C, Ornellas O, et al. Effect of crown ethers on structure, stability, activity, and enantioselectivity of Subtilisin carlsberg in organic solvent. Biotechnol. Bioeng. 2001;74:295-308.

128. Engbersen JFJ, Broos J, Verboom W, Reinhoudt DN. Effect of crown ethers and small amounts of cosolvent on the activity and enantioselectivity of a-chymotrypsin in organic solvents. Pure. Appl. Chem. 1996;68:2171-8.
129. Engbersen JFJ, Broos J, Verboom W, Reinhoudt DN. Effect of crown ethers and small amounts of cosolvent on the activity and enantioselectivity of a-chymotrypsin in organic solvents. Pure. Appl. Chem. 1996;68:2171-8.
130. Broos J, Martin M-N, Rouwenhorst I, Verboom W, Reinhoudt DN. Acceleration of enzyme-catalyzed reactions in organic solvents by crown ethers. Recl. Trav. Chim. Pay-B. 1991;110:222-5.
131. Ferrer M, Cruces MA, Plou FJ, Pastor E, Fuentes G, Bernabe M, et al. Chemical versus enzymatic catalysis for the regioselective synthesis of sucrose ester of fatty acids. Stud. Surf. Sci. Catal. 2000;130:509-14.
132. Jeans A, Seymour FR. The α-D-glucopyranosidic linkages of dextrans: comparison of percentages from structural analysis by periodate oxidation and by methylation. Carbohydr. Res. 1979;74(1):31-40.
133. Ferrer M, Angeles Cruces M, Plou FJ, Bernabé M, Ballesteros A. A Simple Procedure for the Regioselective Synthesis of Fatty Acid Esters of Maltose, Leucrose, Maltotriose and n-Dodecyl Maltosides. Tetrahedron. 2000;56(24):4053-61.
134. Pérez-Victoria I, Morales JC. Complementary regioselective esterification of non-reducing oligosaccharides catalyzed by different hydrolases. Tetrahedron. 2006;62:878-86.
135. Akoh CC, Lee G-C, Shaw J-F. Protein engineering and applications of *Candida rugosa* lipase isoforms. Lipids. 2004;39(6):513-26.
136. Grochulski P, Bouthillier F, Kaslauskas J, Serreqi AN, Schrag JD, Zoimek E, et al. Analogs of reaction intermediates identify a unique substrate binding site in Candida rugosa lipase. Biochemistry-US. 1994;33:3494-500.
137. Schmitt J, Brocca S, Schmid RD, Pleiss J. Blocking the tunnel: engineering of Candida rugosa lipases mutants with short chain length specificity. Protein Eng. 2002;15:595-601.

138. Kaewprapan K, Wongkongkatep J, Panbangred W, Phinyocheep P, Marie E, Durand A, et al. Lipase-catalyzed synthesis of hydrophobically modified dextrans: Activity and regioselectivity of lipse from *Candida rugosa*. J Biosci. Bioeng. 2011;112:124-9.

139. Güner A. The algorithmic calculations of solubility parameter for the determination of interactions in dextran/certain polar solvent systems. Eur. Polym. J. 2004;40:1587-94.

140. Icoz DZ, Kokini JL. Examination of the validity of the Flory-Huggins solution theory in terms of miscibility in dextran systems. Carbohydr. Polym. 2007;68:59-67.

141. Antoniou E, Tsianou M, Alexandridis P. Solution properties of polysacchairdes in water and polar organic solvents. Polym. Prepr. 2005;46:532-3.

142. Antoniou E, Themistou E, Sarkar B, Tsianou M, Alexandridis P. Structure and dynamics of dextran in binary mixtures of a good and a bad solvent. Colloid Polym. Sci. 2010;288:1301-12.

143. Antoniou E, Buitrago CF, Tsianou M, Alexandridis P. Solvent effects on polysaccharide conformation. Carbohydr. Polym. 2010;79:380-90.

144. Antoniou E, Alexandridis P. Polymer conformation in mixed aqueous-polar organic solvents. Eur. Polym. J. 2010;46:324-35.

145. Solomon OF, Gottesman RS. Considerations about the possibility of determining [η] and K by means of the "One point measurement" method. Makromol. Chem. 1969;127:153-64.

146. Deb PC, Chatterjee SR. On polynomial expansion of Log relative viscosity. Makromol. Chem. 1969;125:283-5.

147. Catiker E, Güner A. Unperturbed molecular dimensions and theta temperature of dextran in dimethylsulfoxide (DMSO) solutions. Polym. Bull. 1998;41:223-30.

148. Wu M, Dellacherie E, Durand A, Marie E. Poly(n-butyl cyanoacrylate) nanoparticles via miniemulsion polymerization (1): Dextran-based surfactants. Colloid. Surface. B. 2009;69(1):141-6.

149. Spigone E, Cho G-Y, Fuller GG, Cicuta P. Surface rheology of a polymer monolayer: effect of polymer chain length and compression rate. Langmuir. 2009;25(13):7457-64.

150. Kaewprapan K, Wongkongkatep J, Panbangred W, Phinyocheep P, Marie E, Durand A, et al. Lipase-catalyzed synthesis of hydrophobically modified dextrans: Activity and regioselectivity of lipase from Candida rugosa. J. Biosci. Bioeng.. 2011;112(2):124-9.

151. Aumelas A, Serrero A, Durand A, Dellacherie E, Léonard M. Nanoparticles of hydrophobically modified dextrans as potential drug carrier systems. Colloid. Surface. B. 2007;59:74-80.

152. Gavory C, Durand A, Six J-L, Nouvel C, Marie E, Léonard M. Polysaccharide-covered nanoparticles prepared by nanoprecipitation. Carbohydr. Polym. 2011;84(1):133-40.

153. Ge J, Lu D, Yang C, Liu Z. A lipase-responsive vehicle using amphipatic polymer synthesized with the lipase as catalyst. Macromol. Rapid. Commun. 2011;32:546-50.

154. Hornig S, Heinze T. Nanoscale structures of dextran esters. Carbohydr. Polym. 2007;68:280-6.

155. Ydens I, Degée P, Nouvel C, Dellacherie E, Six J-L, Dubois P. 'Surfactant-free' stable nanoparticles from biodegradable and amphiphilic poly(e-caprolactone)-grafted dextran copolymers. e-Polymers. 2005;046.

156. Rouzes C, Gref R, Leonard M, De Sousa Delgado A, Dellacherie E. Surface modification of poly(lactic acid) nanospheres using hydrophobically modified dextrans as stabilizers in an o/w emulsion/evaporation technique. J. Biomed. Mater. Res. 2000;50(4):557-65.

157. Frazier RA, Davies MC, Matthijs G, Roberts CJ, Schacht E, Tendler SJB, et al. In situ surface plasmon resonance analysis of dextran monolayer degradation by dextranase. Langmuir. 1997;13:7115-20.

158. Kamath KR, Park K. Study on the release of invertase from enzymatically degradable dextran hydrogels. Polym. Gels Netw. 1995;3:243-54.

159. Hovgaard L, Brondsted H. Dextran hydrogels for colon-specific drug delivery. J. Control. Release. 1995;36:159-66.

160. Mehvar R. Dextrans for targeted and sustained delivery of therapeutic and imaging agents. J. Control. Release. 2000;69:1-25.

161. Peng H, Ling J, Liu J, Zhu N, Ni X, Shen Z. Controlled enzymatic degradation of poly([var epsilon]-caprolactone)-based copolymers in the presence of porcine pancreatic lipase. Polym. Degrad. Stabil.. 2010;95(4):643-50.

162. Görner T, Gref R, Michenot D, Sommer F, Tran MN, Dellacherie E. Lidocaine-loaded biodegradable nanospheres. I. Optimization of the drug incorporation into the polymer matrix. J. Control. Release. 1999;57:259-68.

APPENDICES

APPENDIX A

CERTIFICATE OF ANALYSIS

No. 000079025
Date Feb.23,2006

Messrs.

Product: Lipase AY "Amano" 30

Lot No.: LAYE0151016 Quantity: 0.03 kg Date Assayed: Jan.17,2006

Test Items	Specification	Test Results
Loss on drying: (1g, 105°C, 4hr.)	Not more than 8.00 %	4.25 %
Heavy Metals(as Pb):	Not more than 40 μg/g	Not more than 40 μg/g
As(as Arsenic Trioxide):	Not more than 3 μg/g	Not more than 3 μg/g
Lead(Pb):	Not more than 10 μg/g	Not more than 10 μg/g
Lipase activity: (LMAP)Method pH7.0	Not less than 30,000 U/g	32,800 U/g
Total viable aerobic count:	Not more than 1,000 /g	Not more than 1,000 /g
Coliforms:	Not more than 30 /g	Not more than 30 /g
Escherichia coli:	Negative/25g	Negative/25g
Salmonella:	Negative/25g	Negative/25g
	Judgment	Pass

Remark:

SAMPLE

Quality Control Manager

Prepared by

AMANO Amano Enzyme Inc.

Head Office
2-7,1-chome,Nishiki,Naka-ku,Nagoya,Aichi 460-8630 JAPAN
Tel: +81.(0)52.211.3032 Fax: +81.(0)52.211.3054
Nagoya Plant
Kunotsubo,Nishiharu-cho,Nishikasugai-gun,Aichi 481-8533 JAPAN
Tel: +81.(0)568.21.3883 Fax: +81.(0)568.22.1954

APPENDIX B

Preparation of Sumner's reagent

For 100 ml of reagent use:

- 16 g of tartaric acid
- 1 g of 3,5 dinitrosalicylic acid
- 1 g of NaOH
- 0.2 g of phenol

Adjusted the volume with milliQ water

For 4 ml of sample, 6 ml of reagent and 0.4 ml of sodium sulfite were added, then mixed and incubated at 95 °C for 15 min. Cool down the mixture and measure the absorbance at 620 nm.

APPENDIX C

Residual activity (%±SD) of pH-imprinted lipase AY after 5 h of incubation in *tert*-amyl alcohol, DMSO and the mixture of DMSO to *tert*-amyl alcohol (7:3 v/v) at 50°C.

Time of incubation (h)	DMSO	tert-amyl alc.	DMSO:tert-amyl alc. (7:3v/v)
0	100.00	100.00	100.00
1	10.66±1.63	107.16±3.13	5.48±2.59
2	16.21±1.16	110.94±7.99	1.83±0.01
3	16.74±2.76	109.52±9.08	9.86±4.45
4	15.95±2.04	115.49±2.24	6.75±1.71
5	10.48±2.53	113.34±1.67	9.87±1.76

Effect of ratio of *tert*-amyl alcohol (5, 10, 20 and 30%) to DMSO on % DS of DexT40 by VD, reaction time 32 h at 50°C in DMSO.

	%DS	
% *tert*-amyl alcohol	Control reaction	Reaction with enzyme
0	10.0	48.0
5	1.6	27.2
10	1.2	20.6
20	0.8	2.0
30	1.4	0.9

APPENDIX D

Residual activity (%) of pH-adjusted lipase AY and pH-imprinted lipase AY co-lyophilized with 25, 50, 100 and 200 molar equivalent of 18-crown-6 at 5 h of incubation in DMSO at 50°C

Time (h)	Molar Equivalent of 18-crown-6				
	0	25	50	100	200
0	100	100	100	100	100
1	10.7±1.6	8.5±1.6	18.0±4.5	10.4±2.3	10.6±4.2
2	16.2±1.2	6.1±1.5	12.8±3.2	10.1±6.1	3.5±0.8
3	16.7±2.8	7.1±2.3	10.4±2.6	6.1±3.8	5.7±3.0
4	15.6±2.0	6.6±3.6	7.3±1.7	5.3±3.5	2.4±1.3
5	10.5±2.5	5.9±2.4	7.6±1.2	5.3±3.5	3.9±2.9

Effect of molar equivalent of 18-crown-6 co-lyophilized lipase and added to reaction media on %DS of DexT40 by VD, reaction time 32 h at 50°C in DMSO.

Molar equivalent	%DS	
	co-lyophilized with 18-crown-6	18-crown-6 in solution
0	45.0	45.0
25	52.6	13
50	63.6	11
100	38.6	9.4
200	17.6	8.16

APPENDIX E

The kinetic of transesterification of lipase co-lyophilized with 18-crown-6 on % modification of DexT40 by vinyl decanoate compared with pH-adjusted enzyme and native enzyme , reaction time 32 h at 50°C in DMSO.

Time (h)	%DS		
	co-lyophilized	pH-adjusted	native enzyme
0.5	18.11	-	-
1	24.61	8.88	2.71
1.5	31.42	-	-
2	39.66	-	2.93
3	47.2	17.81	3.3
4	54.03	-	2.58
5	55.88	25.46	-
6	-	-	1.99
7	-	26.9	-
8	-	-	1.36
9	-	31.52	-
11	59.77	40.16	1.4
19	61.11	-	-
22	62.04	-	-
26	62.29	46.76	1.62
32	66.09	48.38	1.5

APPENDIX F

Time course for the conversion percentage of transesterification of DexT40 with VD batch and 3 successive stepwise reaction.

Time of reaction (h)	%DS	
	Stepwise	Batch
4	20.42	-
8	49.28	-
16	57.6	-
24	62.64	-
32	59.02	-
48	59.04	55.6
56	68.02	-
64	77.72	-
80	78.16	-
96	81.74	61.5
104	80.46	-
112	96.95	-
128	91.63	-
144	88.07	65.2

APPENDIX G

Variation of degree of substitution as a function of reaction time for lipase-catalyzed transesterification of native dextran and already modified dextran with DS = 25%.

Time (h)	%DS	
	Modified dextran	Native DexT40
0	24.6	0
2	41.6	27.8
4	51.5	39.5
6	52.3	48.2
8	55.1	48.9
10	57.5	52.6

Enzymatic degradation by dextranase of native and modified dextran with various DS as a function of time.

%Modification	Time (h)				
	0	8	17	25	70
4	2.1	3.5	11.8	59.7	98.0
24	2.0	2.2	3.6	14.9	97.6
48	1.81	1.9	1.9	7.4	98.2
168	1.38	1.4	1.3	2.7	94.0

BIOGRAPHY

NAME Miss Kulthida Kaewprapan

DATE OF BIRTH August 29th, 1983

PLACE OF BIRTH Sakhon Nakhon, Thailand

INSTITUE ATTENDED King Mongkut's Institute of Technology Ladkrabang, 2002-2005
Bachelor of Science (Biotechnology)
Mahidol University, 2005-2007
Master of Science (Biotechnology)
Mahidol University, 2007-2011
Doctor of Philosophy (Biotechnology)
Institut National Polytechnique de Lorraine, 2007-2011
Doctor of Philosophy (Processes and Products Engineering)

RESEARCH GRANTS Center of Excellent on Agricultural Biotechnology, Science and Technology Postgraduate Eduation and Research Development Office (PERDO), Commission on Higher Education, Ministry of Education
Franco-Thai Cooperation in Higher Education and Research
Eiffel scholarship, Center of Higher Education French Ministry of Foreign and European Affairs

Research Assistant scholarship, Faculty of Graduate Studies, Mahidol University

HOME ADDRESS 19 Vibhavadi 41 Sanambin, Donmuang, Bangkok, 10210, Thailand
Tel. +66(0)25336609
Email: nan_gunner@hotmail.com

PUBLICATION

1. Kaewprapan K, Tuchinda P, Marie E, Durand A, Inprakhon P. pH-imprinted lipase catalyzed synthesis of dextran fatty acid ester. J. Molec Catalysis B: Enzymatic. 2007;47(3-4):135-42.
2. Kaewprapan K, Wongkongkatep J, Panbangred W, Phinyocheep P, Marie E, Durand A, et al. Lipase-catalyzed synthesis of hydrophobically modified dextrans: Activity and regioselectivity of lipase from *Candida rugosa*. J. Biosci. Bioeng. 2011;112(2):124-9.
3. Kaewprapan K, Baros F, Marie E, Inprakhon P, Durand A. Macromolecular surfactants synthesized by lipase-catalyzed transesterification of dextran with vinyl decanoate. Carbohydr. Polym. 2012; 88(1):313-20.
4. Kaewprapan K, Inprakhon P, Marie E, Durand A. Enzymatically degradable nanoparticles of dextran esters as potential drug delivery systems. Carbohydr. Polym. 2012: In Press.

PRESENTATION

1. Kaewprapan K, Inprakhon P. Lipase catalyzed the synthesis of biopolymeric surfactant. Poster presented at the 2nd International Conference on Advances in Petrochemicals and Polymer; 2007, June 25-28, Imperial Queen's Park Hotel, Bangkok, Thailand.
2. Kaewprapan K, Inprakhon P. Enzymatic Synthesis of Biopolymeric Surfactant. Oral presentation at the 3rd AgBiotech Graduate Conference; 2007, Dcember 7-8, Kasertsart University, Kamphaeng saen Campus, Nakhon Pathom, Thailand.
3. Kaewprapan K, Durand A, Marie E, Inprakhon P. Nanoparticles of hydrophobically modified dextran synthesized by enzymatic catalysis.

Poster presented at the 5th Asian Conference on Nanoscience & Nanotechnology; November 1-3, 2010, Miraikan, Tokyo, Japan.

4. Kaewprapan K, Durand A, Marie E, Inprakhon P. Enzymatic catalysis of hydrophobically Modified dextran for nanoparticles formation. Oral presentation at the 4th AG-BIO/PERDO Graduate Conference on Agricultural Biotechnology and UT-KU Joint Seminar; December 9-10, 2010, Kasertsart University, Kamphaeng saen Campus, Nakhon Pathom, Thailand.
5. Kaewprapan K, Durand A, Marie E, Inprakhon P. Synthesis of amphiphilic dextran by lipase form *Candida rugosa*: Strategies to enhance enzyme activity in organic solvents. Oral presentation at the 23rd Annual Meeting of TSB; February 1-2, 2012, Imperial Queen's Park Hotel, Bangkok, Thailand.