ฤทธิ์ต้านอนุมูลอิสระและการตั้งตำรับลิโพโซมชนิดเปลี่ยนรูปได้ซึ่งบรรจุซิริซินจากไหม

นางสาวนันทวรรณ พวงมาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชกรรม ภาควิชาเภสัชกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

ANTI-FREE RADICAL ACTIVITY AND FORMULATION OF DEFORMABLE LIPOSOMES LOADED WITH SILK SERICIN

Miss Nantawan Puangmalai

A Thesis Submitted Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmaceutics
Department of Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2007

Thesis Title	ANTI-FREE RADICAL ACTIVITY AND FORMULATION OF DEFORMABLE LIPOSOMES LOADED WITH SILK SERICIN
By	Miss Nantawan Puangmalai
Field of Study	Pharmaceutics
Thesis Advisor	Associate Professor Suchada Chutimaworapan, Ph.D.
Thesis Co-Advisor	Associate Professor Duangdeun Meksuriyen, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

Pornpen Pramyothin ..... Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pornpen Pramyothin, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor Uthai Suvanakoot, Ph.D.)

Suchada Chutimaworapan ..... Thesis Advisor

(Associate Professor Suchada Chutimaworapan, Ph.D.)

D. Meksuriyen ..... Thesis Co-advisor

(Associate Professor Duangdeun Meksuriyen, Ph.D.)

Waraporn Suwakul ..... Member

(Associate Professor Waraporn Suwakul, Ph.D.)

..... Member

(Assistant Professor Chamnan Patarapanich, Ph.D.)

นันทวรรณ พวงมาลัย: ฤทธิ์ต้านอนุมูลอิสระและการตั้งตำรับลิโพโซมชนิดเปลี่ยนรูปได้ซึ่งบรรจุซิริซินจากไหม. (ANTI-FREE RADICAL ACTIVITY AND FORMULATION OF DEFORMABLE LIPOSOMES LOADED WITH SILK SERICIN) อ. ที่ปรึกษา: รศ.ดร.สุชาดา ชุติมาวรพันธ์, อ. ที่ปรึกษาร่วม: รศ.ดร.ดวงเดือน เมฆสุริเยนทร์, 126 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านอนุมูลอิสระของซิริซินจากไหมด้วยวิธีดีออกซีไรโบส และตั้งตำรับลิโพโซมชนิดเปลี่ยนรูปได้ซึ่งบรรจุซิริซินเพื่อให้เกิดผลชะลอการเกิดริ้วรอยก่อนวัย ด้วยวิธีระเหยแบบกลับวัตภาค ซึ่งจะศึกษาถึงปัจจัยที่มีผลต่อการเตรียมลิโพโซมชนิดเปลี่ยนรูปได้ คือ สัดส่วนของฟอสฟาทิดิลโคลีน (พีซี) และสารลดแรงตึงผิว ชนิดสารลดแรงตึงผิว และปริมาณลิพิด เพื่อหาสูตรที่มีความเหมาะสมที่สุด การทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธีดีออกซีไรโบสพบว่าซิริซินสามารถยับยั้งอนุมูลอิสระได้ โดยมีค่าการยับยั้งอนุมูลไฮดรอกซิลที่ 50% (IC_{50}) เท่ากับ 0.9 มิลลิกรัมต่อมิลลิลิตร และพบว่าสามารถเตรียมลิโพโซมชนิดเปลี่ยนรูปได้โดยวิธีระเหยแบบกลับวัตภาค อนุภาคที่ได้เป็นแบบชั้นเดียวขนาดใหญ่ (แอลยูวี) สามารถคัดเลือกสูตรตำรับที่เหมาะสม ประกอบด้วย พีซี 2% สแปน 80 เป็นสารลดแรงตึงผิว และสัดส่วนของพีซีและสารลดแรงตึงผิวเท่ากับ 85:15 โดยน้ำหนัก ได้ค่าเปอร์เซ็นต์การกักเก็บสารสำคัญเท่ากับ 89.84 ± 2.87% วัดขนาดอนุภาคและการกระจายของขนาดด้วยเทคนิคการกระเจิงแสงแบบไดนามิก ได้ขนาดในช่วง 240 นาโนเมตร และสแปนเท่ากับ 3.088 หลังจากนั้นมีการตั้งตำรับลิโพโซมชนิดเปลี่ยนรูปได้ซึ่งบรรจุซิริซินจากไหมในรูปของซีรัม ศึกษาปัจจัยของชนิดและปริมาณสารเพิ่มความหนืดที่ใช้ คือ คาร์โบพอลอัลเทรซ 10 พอล็อกซาเมอร์ 407 และไคโตซาน (ชนิดน้ำหนักโมเลกุลสูง) ผลจากการทดสอบความคงตัวทางกายภาพด้วยวิธีร้อนสลับเย็น 6 รอบ ได้พิจารณาคัดเลือกสูตรที่ใช้ 0.5% คาร์โบพอลอัลเทรซ 10 เพื่อนำไปผสมกับลิโพโซมชนิดเปลี่ยนรูปได้ เมื่อนำสูตรตำรับไปศึกษาการซึมผ่านผิวหนังโดยใช้ผิวหนังหน้าท้องลูกหมูเป็นโมเดลในการทดลอง พบว่าตำรับที่มีลิโพโซมชนิดเปลี่ยนรูปได้และลิโพโซมชนิดเติมซึ่งบรรจุซิริซินสามารถเพิ่มการซึมผ่านผิวหนังได้เมื่อเทียบกับสารละลายซิริซินซึ่งไม่ผ่านผิวหนัง แสดงให้เห็นว่ารูปแบบการนำส่งด้วยลิโพโซมชนิดเปลี่ยนรูปได้และลิโพโซมชนิดเติมสามารถเพิ่มการนำส่งซิริซินผ่านผิวหนังได้ และการซึมผ่านของลิโพโซมชนิดเปลี่ยนรูปได้ซึ่งบรรจุซิริซินเมื่อผสมกับสารเพิ่มความหนืดแล้ว จะมีการซึมผ่านได้ช้าลง แต่มีค่าสัมประสิทธิ์การซึมผ่านมากกว่ารูปแบบลิโพโซมที่ไม่ได้ผสมเป็นซีรัมอย่างมีนัยสำคัญทางสถิติที่ระดับความเชื่อมั่นที่ 95%

ภาควิชา	เภสัชกรรม	ลายมือชื่อนิสิต.....
สาขาวิชา	เภสัชกรรม	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2550	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4876572033 : MAJOR PHARMACEUTICS
KEY WORD: SERICIN / DEFORMABLE LIPOSOMES / ANTI-FREE RADICAL ACTIVITY / ANTI-WRINKLE SERUM / SKIN PERMEATION

NANTAWAN PUANGMALAI: ANTI-FREE RADICAL ACTIVITY AND FORMULATION OF DEFORMABLE LIPOSOMES LOADED WITH SILK SERICIN. THESIS ADVISOR: ASSOC. PROF. SUCHADA CHUTIMAWORAPAN, Ph.D., THESIS CO-ADVISOR: DUANGDEUN MEKSURIYEN, Ph.D., 126 pp.

The purposes of this study were to investigate anti-free radical activity of sericin from Thai silk by deoxyribose assay and to formulate deformable liposomes (DL) loaded with sericin for anti-wrinkle effects by reverse phase evaporation method. Effects of the ratios of phosphatidyl choline (PC) and surfactant, types of surfactants and lipid content on DL for the most appropriate formulation were investigated. The deoxyribose assay demonstrated the capability of scavenging hydroxyl radical of sericin with IC_{50} at 0.9 mg/ml. It was found that the formation of DL could be achieved by reverse phase evaporation method and obtained large unilamellar vesicles (LUVs). The optimum formulation comprised of 2% PC, Span 80 as surfactant and the ratio of PC:Span 80 as 85:15 by weight. Entrapment efficiency was 89.84 ± 2.87 %. Size and size distribution determined by dynamic light scattering technique was 240 nm and span 3.088. The formulation of serum with DL loaded with silk sericin was carried out with types of viscosity enhancers: Carbopol ultrez-10, Poloxamer 407 and chitosan (high molecular weight). The results of physical stability test by heating and cooling method for 6 cycles revealed that the appropriate viscosity enhancer was 0.5% Carbopol ultrez-10, which was mixed with DL. Afterwards, skin permeation study using newborn abdominal pig skin as skin model revealed that deformable liposomes and conventional liposomes (CL) loaded with sericin could enhance the skin penetration of sericin through pig skin as compared with sericin solution which showed no permeation. It was demonstrated that DL and CL could enhance the delivery of sericin through skin. In the formulation of DL loaded with sericin containing in serum, sericin permeated through skin slower, but the permeability coefficient was significantly higher than DL without containing in serum ($P<0.05$).

Department	:	Pharmacy	Student's Signature: Nantawan Puangmalai
Field of Study	:	Pharmaceutics	Advisor's Signature: Suchada Chutimaworapan
Academic Year	:	2007	Advisor's Signature: D. Meksuriyen

ACKNOWLEDGEMENTS

This thesis has been succeeded with the great supports from several people. I shall remind in great kindness for their helps, supports and advices.

First of all, I would like to express my gratitude for the invaluable advice, guidance and enthusiastic encouragement throughout my research study to my advisor, Associate Professor Suchada Chutimaworapan, Ph.D. Her understanding, kindness and patience are honestly appreciated.

I would like to express deep appreciation and grateful thanks to my co-advisor, Associate Professor Duangdeun Meksuriyen, Ph.D. for her valuable suggestion, kindness and encouragement during the biochemical study

I would like to express my great gratitude to Associate Professor Uthai Suvanakoot, Chairman of my thesis examination committee, as well as other committee members, Associate Professor Waraporn Suwakul, Ph.D. and Assistant Professor Chamnan Patarapanich, Ph.D. I would like to express grateful thanks for their kind advice and make this thesis complete.

My gratitude is given to the Chulalongkorn University research fund for partially financial support to my thesis work, The Queen Sirikit Institute of Sericulture, Thailand for sericin contribution. I am very grateful to Mr. Prateep Meesilp, the director of Institute of Sericulture at Srisakate for spending his valuable time and suggestion.

And sincere thanks Assistant Professor Nontima Vanabhuti, Ph.D. for contributing phosphatidyl choline, Professor Garnpimol Ritthidej, Ph.D. for allowance of using Mastersizer, The Government of Pharmaceutical Organization for allowance of using Zetasizer, and Petchaburi farm, for giving the death newborn pigs.

Sincere thanks are also given to all staff members of the Department of Pharmacy and Department of Biochemistry for their assistance and great helpful support and other people whose names have not been mentioned here.

Ultimately, I would like to express my sincere and deepest gratitude to my family for their endless love, understanding and encouragement throughout this study.

CONTENTS

Page

LIST OF TABLES

Table **Page**

Table **Page**

Table **Page**

Table **Page**

LIST OF FIGURES

Figure **Page**

LISTS OF ABBREVIATIONS

ANOVA	=	analysis of variance
BSA	=	bovine serum albumin
°C	=	degree Celsius
C_d	=	concentration of drug in donor
CL	=	conventional liposomes
CL_Ser	=	conventional liposomes (pure phosphatidyl choline) loaded with sericin
CLchol_Ser	=	conventional liposomes (phosphatidyl choline:cholesterol) loaded with sericin
cm	=	centimeter
CSLM	=	confocal scanning laser microscopy
C.V.	=	coefficient of variation
Cu^+	=	copper ion
D	=	diffusion coefficient
Da	=	dalton
DL	=	deformable liposomes
DLSp	=	deformable liposomes preparing with Span 80
DLSp10_Ser	=	deformable liposomes preparing with 10% of Span 80 loaded with sericin
DLSp15_Ser	=	deformable liposomes preparing with 15% of Span 80 loaded with sericin
DLSp15_Ser equi 24 h	=	deformable liposomes preparing with 15% of Span 80 loaded with sericin, skin equilibrate 24 hours
DLSp15_Sercb	=	deformable liposomes preparing with 15% of Span 80 loaded with sericin, containing in Carbopol ultrez-10
DLSp20_Ser	=	deformable liposomes preparing with 20% of Span 80 loaded with sericin
DLTw	=	deformable liposomes preparing with Tween 80
DLTw10_Ser	=	deformable liposomes preparing with 10% of Tween 80 loaded with sericin
DLTw15_Ser	=	deformable liposomes preparing with 15%

		of Tween 80 loaded with sericin
DLTw20_Ser	=	deformable liposomes preparing with 20% of Tween 80 loaded with sericin
EDTA	=	ethylenediamine tetraacetic acid
et al.	=	*et alii,* 'and others'
Fe^{2+}	=	ferrous ion
Fe^{3+}	=	ferric ion
$FeCl_3$	=	ferric chloride
g	=	gram
GSH	=	glutathione
h	=	hour
h	=	thickness of membrane
$HO_2^{\cdot-}$	=	hydroperoxyl radical
H_2O_2	=	hydrogen peroxide
HOCl	=	hypochlorous acid
HNO_2	=	nitrous acid
K	=	partition coefficient
kDA	=	kilodalton
kg	=	kilogram
$L^{\cdot}$	=	lipid radical
$LH^{\cdot}$	=	lipid unsaturated fatty acyl chain
$LOO^{\cdot}$	=	lipid peroxyl radical
LUVs	=	large unilamellar vesicles
M	=	cumulative amount of sericin
MDA	=	malondialdehyde
mg	=	milligram
min	=	minute
ml	=	milliliter
MLVs	=	multilamellar vesicles
mm	=	millimeter
mM	=	millimolar
mPas	=	millipascal

MW	=	molecular weight
n	=	sample size
nm	=	nanometer
no.	=	number
$NO\cdot$	=	nitric oxide
$NO_2\cdot$	=	nitrogen peroxide
NO_2^+	=	nitronium cation
N_2O_4	=	dinitrogen tetroxide
N_2O_3	=	dinitrogen trioxide
O_2	=	oxygen
$O_2^{\cdot -}$	=	superoxide anion
O_3	=	ozone
$^{\cdot}OH$	=	hydroxyl radical
$ONOO^-$	=	peroxynitrite
ONOOH	=	peroxynitrous acid
P	=	permeability coefficient
P	=	permeability coefficient
PBS	=	phosphate buffer saline
PC	=	phosphatidyl choline
PEG	=	polyethylene glycol
R^2	=	coefficient of determination
REVs	=	reverse phase evaporation vesicles
RNS	=	reactive nitrogen species
$RO^{\cdot}$	=	alkoxyl radical
$ROO^{\cdot}$	=	peroxyl radical
ROONO	=	alkyl peroxynitrates
ROS	=	reactive oxygen species
rpm	=	revolution per minute
S	=	surface area
SC	=	stratum corneum
SD	=	standard deviation
SDS	=	sodium dodecyl sulphate

SDS-PAGE	=	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	=	scanning electron microscope
Ser N	=	sericin new lot.
Ser O	=	sericin old lot.
SPSS	=	statistical package for the social science
SUVs	=	small unilamellar vesicles
t	=	time
TBA	=	thiobarbituric acid
TBARS	=	thiobarbituric acid reactive substances
TCA	=	trichloroacetic acid
µg	=	microgram
µm	=	micrometer
UV	=	ultraviolet
w/v	=	weight by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

Silk worms (*Bombyx mori* Linn.) are insects that its cocoons are useful in silk processing. Cocoons contain two main substances; sericin and fibroin. Usually they contain 20-30% of sericin and 70-80% of fibroin. During silk production, sericin is discarded in waste water. In Thailand, the Queen Sirikit Institute of Sericulture can derive and purify sericin from waste water. Sericin is useful in various fields such as biodegradable materials, membrane materials, functional biomaterials, medical biomaterials, functional fibers and fabrics (Zhang, 2002). Sericin is used for cryoprotectant substances (Tsujimoto et al., 2001), enzyme immobilization (Zhang et al., 2004) and wound healing (Zhang, 2002). Several researchers have studied in anti-free radical activity of sericin both in vitro and in vivo. The opportunity of colon tumorigenesis was inhibited by using sericin (Zhaorigetu et al., 2001) and the risk of skin cancer was decreased by reducing oxidative stress (Zhaorigetu et al., 2003b).

Moreover, sericin was applied in the air conditioner for inhibiting oxidation or free radical formation which was toxic for cells (Sarovat, 2003). However, there are few studies which determine anti-free radical activity of sericin derived from wastewater in Thai silk production. Innovative approaches on a new application of sericin possessing anti-free radical activity are still needed in order to increase its value. Then it needs to formulate sericin to be anti-wrinkle products from anti-free radical activity.

Recently deformable liposomes or Transfersomes® have been a new interesting drug delivery system for skin administration. There are many drugs which are using this system to enhance delivery macromolecules such as gap junction protein (Paul at al., 1998), insulin (Guo et al., 2000), antioxidant enzymes: superoxide dismutase and catalase (Simoes et al., 2004), and Interleukin-2 (Hofer et al., 2004). The researchers proposed that deformable liposomes; which were more elastic, could squeeze themselves passing 5-folds smaller pores (Cevc, Schatzlein, and Blume, 1995). Although deformable liposomes are an efficient drug delivery system, compositions in their formula had an effect on their properties. To date, there is no study that investigated the effect of deformable liposomes loaded with sericin on its

physicochemical properties. Therefore, we need to investigate formulation factors which had affect the properties of deformable liposomes loaded with sericin.

The main purposes of this present study were to find out anti-free radical activity and to develop a new application of sericin from Thai silk processing in system of deformable liposomes. Furthermore, this study was to assess the possibility of producing deformable liposomes loaded with silk sericin as an anti-wrinkle serum.

Objectives :

1. To investigate anti-free radical activity of silk sericin in vitro.
2. To formulate and investigate the effects of types of edge activators and ratios of phosphatidyl choline and edge activators on deformable liposomes.
3. To formulate dispersion systems containing deformable liposomes loaded with silk sericin as anti-wrinkle serum.
4. To investigate anti-free radical activity of deformable liposomes loaded with silk sericin in vitro.
5. To investigate and compare skin permeation of deformable liposomes loaded with silk sericin with those dispersed in anti-wrinkle serum.

CHAPTER II

LITERATURE REVIEWS

A. SILK SERICIN

1. Sources of sericin

Silk is classified by source that one is Mulberry silkworms (*Bombyx mori* Linn.) and the other one is Wild silkworms such as *Antheraea pernyi* (Chinese tussah), *Antheraea assama*, *Antheraea mylitta* (India Tussah), *Samia cynthia ricini*. Silk is a linear sequence of amino acid as the primary structure: Ser-Gly-(Ala-Gly)$_n$ (Lamoolphak, 2005). The structure of silk is shown in Figure 1.

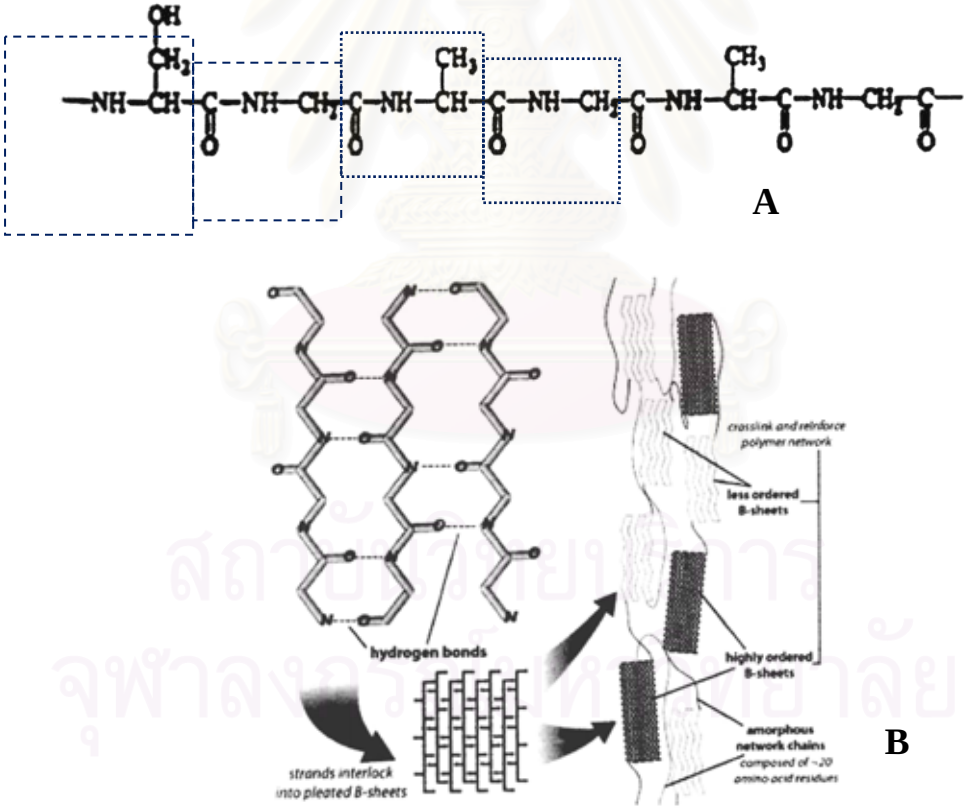


Figure 1. Structure of silk. Key to refer: A, primary structure of crystalline-coding region of silk: Ser-Gly-(Ala-Gly)$_n$; B, secondary structure of a strand of silk (Modified from Lamoolphak, 2005; http://www. Scq.ubc.ca/)

Secondary structure of silk is categorized in three groups. First, random coils refers to the totally disordered and rapidly fluctuating set of conformations assumed by denatured proteins and others polymers in solution. Second, α-helix conformation is only one helical peptide conformation which has simultaneous allowed angles and a favorable hydrogen bonding pattern. The structure of polypeptide chains arrange in themselves into helical segments. It takes place when the carbonyl of amino acid and the amide of adjacent amino acid formed H-bond. Third, β-sheet conformation or β-pleated sheets, hydrogen bonding occurs between neighboring polypeptide chains rather than within one as in α-helices (Lamoolphak, 2005).

In silk industry, large amounts of silk waste are produced. These wastes have properties similar to silk cocoons, which contain two main proteins; sericin and fibroin. Sericin is recovered from the waste water after silk degumming. Silk degumming takes place in various processes such as by hot water, soap, detergent, acid, and enzymes (Fabiani et al., 1996; Freddi, Mossotti and Innocenti, 2003; Lamoolphak, 2005; Kurioka, Kurioka F., and Yamazaki, 2004). Generally, silk cocoons contain 20-30% of sericin, which is globular protein that is soluble in water. The component of silk is shown in Figure 2.

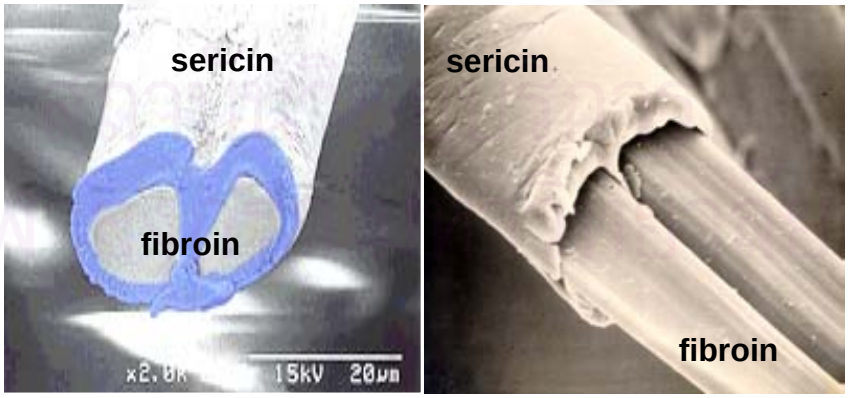


Figure 2. Fibroin is wrapped up in sericin.

2. Physicochemical properties of sericin.

Structure of sericin.

Sericin is made of 18 amino acids which the major amino acids are polar such as serine, aspartic acid and glycine. Amino acid compositions of silk sericin (grams of amino acid/100 grams of protein) are demonstrated in Table 1. Sericin takes on a globular shape, which there is three conformations in the secondary structures of silk protein; random coils, α-helix and ß-sheet (Lamoolphak, 2005).

Table 1. Amino acid compositions of silk sericin and fibroin (grams of amino acid/100 grams of protein) (โมโตอิ มินากาวะ, อีอีชิ คาวาอิ และ เข็มชัย เหมะจันทร, 2530).

Amino acid		**sericin**	**fibroin**
Non-polar	Glycine	8.66	41.25
Amino acid	Alanine	3.51	28.87
	Valine	3.14	2.63
	Leucine	1.02	0.32
	Isoleucine	0.77	0.44
	Proline	0.66	-
	Phenylalanine	0.50	0.58
Acid amino acid	Aspartic acid	17.03	0.78
	Glutamic acid	7.46	0.69
Basic amino acid	Arginine	6.07	0.86
	Histidine	1.88	-
	Lysine	4.95	0.17
Oxy amino acid	Serine	27.32	13.22
	Threonine	7.48	0.81
	Tyrosine	4.43	10.96
Sulfur-complex	Methionine	-	-
amino acid	Cystein	0.20	-
	Total	95.08	101.56

Molecular weight.

Size of molecules depends on temperature, pH and processing time (Zhang, 2002). Generally it is ranged from 10-300 kDa.

Description.

Sericin is fine yellow powder.

Solubility.

Sericin is a water-soluble protein, which is dissolved in a polar solvent. Size of sericin molecules has effects on solubility. Small peptides are soluble in cold water and large molecules are soluble in hot water (Zhang, 2002). Interaction between sericin and water is shown in Figure 3.

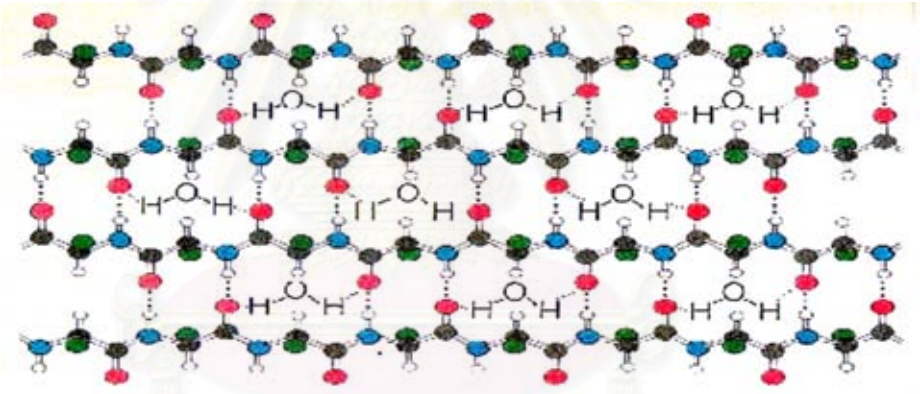


Figure 3. Interaction between sericin and H_2O molecule.

Protein content.

Sericin is approximately 95 % protein purity.

Stability

Sericin is hydrolyzed in acid or alkaline solutions, or degraded by the protease enzyme (Zhang, 2002).

3. Application of sericin

Sericin, derived from silkworm *Bombyx mori*, is a macromolecular protein and has been applied for many advantages such as antioxidant, antimicrobial, UV resistant, and moisturizer. Molecular weight of sericin was found to be related to its functions. Sericin in range of lower molecular weight ($\leq$ 20 kDa) or sericin hydrolyzate is used in cosmetics but sericin in range of higher molecular weight ($\geq$ 20 kDa) is modified as a coating materials, degradable biomaterials, functional membranes, hydrogels, fibers and fabrics. Sericin can be cross-linked, copolymerized and blended with other artificial materials (Zhang, 2002).

In 1998 the first evidence for antioxidant activity of sericin was reported by Kato et al. for its effect in suppression in vitro lipid peroxidation using the thiobarbituric acid reactive substances (TBARS). Sericin was found to decrease TBARS at the concentration of 0.3 %. Furthermore they were found that sericin could inhibit tyrosinase activity at the concentration of 0.5 and 1.0 %. Antioxidant activity of sericin might be due to a remarkably high content of hydroxyl amino acid. The hydroxy group of amino acid such as serine might be responsible the action by chelating trace elements such as copper and iron.

The followed research was done to discover the mechanism of the inhibitory effect of sericin against colon tumorigenesis. In 2001, Zhaorigetu et al. investigated the oxidative stress and cell proliferation of mice after they treated them to the state of colon tumerigenesis. They compared the reducing oxidative stress and the reducing cell proliferation of two groups, of which one was added sericin supplement but another one was not (control). The supplemental sericin was found to reduce cell proliferation in colon epithelium and to suppress colonic oxidative stress marker, which was related to the advance of tumorigenesis.

Furthermore, Zhaorigetu et al. (2003a) investigated the protective effect of sericin on UVB-induced acute damage and tumor promotion in mouse skin. The formation of skin lesions by UVB light was significantly inhibited by sericin

treatment. The topical application of sericin inhibited the tumor incidence for 94%. This study showed that topical application of sericin has an inhibitory effect on UVB-induced sunburn lesions and tumor promotion in mouse skin. Then sericin could be a useful photoprotective agent against UVB-induced acute damage and tumor promotion. Moreover, the researchers hypothesized that this mechanism may involve inhibition of oxidative stress.

The protective effect of sericin on tumor promotion revealed suppressing oxidative stress and inflammatory responses. The results showed the strong antitumor-promoting effect of sericin in the mouse skin tumorigenesis model. Sericin was suggested to use for cancer chemoprotective agent against skin cancer. Sericin suppressed an oxidative stress marker (4-HNE), the protein expression of proliferation-related genes (*c-myc* and *c-fos*), an important pro-inflammatory mediator (COX-2) and a pro-inflammatory cytokines and effected tumor promoter (TNF-α). These finding suggest that sericin has suppressive activity against both chemical-induced skin tumorigenesis and UV-radiation- induced skin tumorigenesis by reducing oxidative stress (Zhaorigetu et al., 2003b).

Nevertheless, sericin was studied the properties of air filters by coating the filters with sericin, which revealed not only the antioxidant activities against hydroxyl radical ($^{\cdot}OH$) but also the antimicrobial activities in silk sericin from different species of waste cocoons. The findings showed that sericin clearly reduced the concentration of the hydroxyl radicals and had fungal and Micrococcus inhibition capacity (Sarovart, 2003).

Sericin was registered in the patent of Thailand in categories of the invention from using as wound dressing which the main constituents was silk fibroin and sericin (Patent of Thailand A61L 27/00, 2000). Two years later Zhang showed the review publication of applications of natural silk protein sericin in biomaterials. This publications reviewed that sericin was used in cosmetics, degradable biomaterials, functional membranes, hydrogels, coated films, wound dressing, cross-

linking agents, suppression lipid peroxidation, inhibition of tyrosinase, cryoprotectant, fibers and fabrics (Zhang, 2002).

Cho et al. (2003) prepared sericin in nanoparticles forms by diafiltration method. They used moisture-retaining properties of sericin to react with poly ethylene glycol (PEG) to obtain self-assembled sericin nanoparticles. Furthermore, sericin was prepared to microparticles and was used as immobilized enzyme (Zhang et al., 2004). Next, the moisturizer properties of sericin were studied in vivo (Padamwar, 2005). Nevertheless, Teramoto, Nakajima and Takabayashi (2005) also prepared elastic silk sericin hydrogel. These findings suggested that sericin hydrogel, which was prepared without both chemicals cross-linking agent and irradiation, might be a drug carrier or scaffold for tissue engineering.

B. ANTI-FREE RADICAL ACTIVITY

Free radicals are atoms or molecules having an unpaired electron which were unstable and very reactive (Fang, Yang, and Wu, 2002). They adapted themselves stable by scavenging electrons from the others. A free radical is easily formed when a covalent bond between entities is broken and one electron remains with each newly formed atom.

By definition, a free radical is any atom such as oxygen and nitrogen with at least one unpaired electron, and is capable of existing independently. In human and many organisms, the use of oxygen in oxidative phosphorylation yields a system of energy transfer by production of the transferable energy component or ATP. Although oxygen is necessity for many living organisms, the most of the damage caused by oxygen could be produced free radical oxygen (Mcanalley et al., 2003).

Any free radical involving oxygen can be referred as a reactive oxygen species (ROS) or reactive nitrogen species (RNS) if nitrogen is involved. Both are shown in Table 2. Reactive oxygen species (ROS) are generated under normal conditions as aerobic metabolism. Their reactive properties make them can react with DNA,

proteins, carbohydrates and lipids in a destructive manner. Biological systems have developed antioxidant, which referred to any substance delays or prevents oxidation of substrate, to combat the effects of radicals (Halliwell, 1997). Oxidative stress is implicated in the pathogenesis of several diseases including AIDS, Parkinson, sclerosis and Alzheimer (Curtin, Donovan, and Cotter, 2002).

Table 2. Reactive oxygen and nitrogen species (Halliwell, 1997).

Radicals	*Nonradicals*
Reactive oxygen species	
Superoxide anion, $O2^{\cdot-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $^{\cdot}OH$	Hypochlorous acid, HOCl
Peroxyl, $ROO^{\cdot}$	Ozone, O_3
Alkoxyl, $RO^{\cdot}$	Singlet oxygen, $^1\Delta_g$
Hydroperoxyl, $HO_2^{\cdot-}$	
Reactive nitrogen species	
Nitric oxide, NO·	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\cdot}$	Dinitrogen tetroxide, N_2O_4
	Dinirogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium cation, NO_2^+
	Alkyl peroxynitrates, ROONO

The mitochondria are a major site of generation of free radicals. These free radicals are initiated by electron transport pathway in respiration, UV light, smoking and poisoning agent etc. During respiration, mitochondria generates superoxide anion ($O_2^{\cdot-}$), which is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) both in mitochondria and cytosol. H_2O_2 is diminished by glutathione (GSH) and catalase. Excess H_2O_2 can react with Fe^{2+} to form hydroxyl radicals ($^{\cdot}OH$) via Fenton reaction. Nevertheless, Haber-Weiss reaction can also be generated $^{\cdot}OH$. Hydroxyl radical is highly toxic to cells. It continues to react with lipid unsaturated

fatty acyl chain ($LH^{\cdot}$), lipid radical ($L^{\cdot}$) is produced. This is followed by addition of oxygen to $L^{\cdot}$ to yield a lipid peroxyl radical ($LOO^{\cdot}$), which further propagates the peroxidation reaction. The mechanism of oxidation in cell is shown in Figure 4.

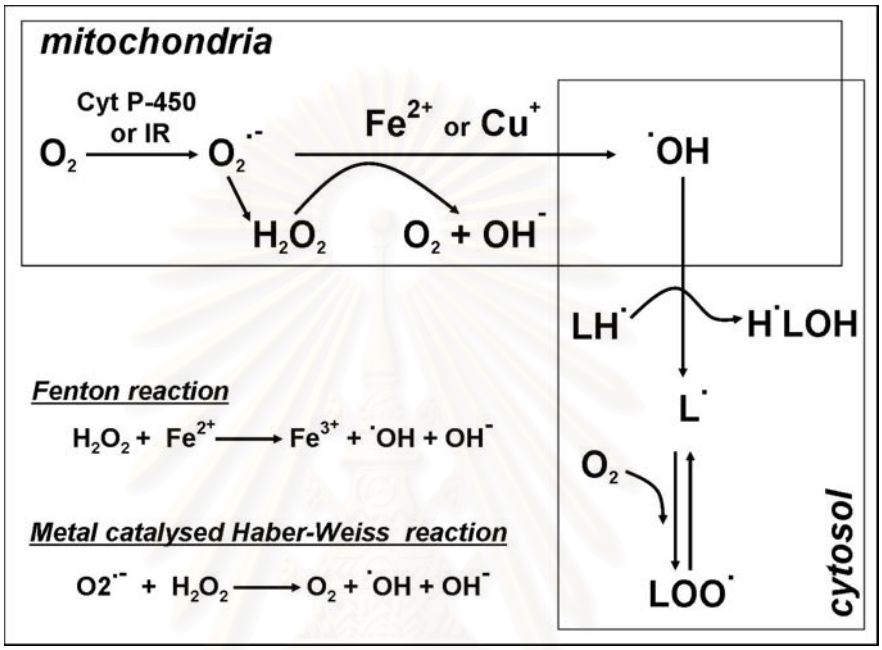


Figure 4. The mechanism of oxidation in cells. (Modified from Curtin, Donovan, and Cotter, 2002; Fang, Yang, and Wu, 2002).

Moreover, the skin region which always contact with oxygen and increasingly exposed to ultraviolet (UV) irradiation. Thus, skin damage induced by reactive oxygen species (ROS) has increased from the risk of photo-oxidation. The resulting oxidative stress causes cellular damage, changes the pattern of gene expression, leads pathology to body such as skin cancers, phototoxicity, and photoaging.

The anti-oxidation test was categorized on two bases. One was based on free radical scavenging or anti-free radical activity and another was based on lipid peroxidation (Sanchez, 2002).

1. Evaluation of anti-free radical activity

Due to there are many species of free radicals such as $O_2^{\cdot-}$, H_2O_2, $^{\cdot}OH$, HOCl, $ONOO^-$ and $LOO^{\cdot}$, they have different substrates for taking reaction testing their anti-free radical activity or their scavenging activity (Sanchez, 2002). Hydroxyl radical hydroxyl radical is very energetic, short-lived and toxic oxygen species. Its strong reactivity with biomolecules influencing it is probably capable of making more damage to biological system than other ROS (Wachiranuntasin, 2005). Hydroxyl radical scavenging activity can often be calculated using the deoxyribose assay (Halliwell, 1997; Sanchez, 2002).

The deoxyribose assay allows determinations of rate constants of reactions with hydroxyl radicals ($^{\cdot}OH$). It can assess abilities to exert pro-oxidant action and to chelate metal. In this test, for abilities to scavenge $^{\cdot}OH$, the reaction of this method is initiated by mixture of ferric chloride ($FeCl_3$) and ethylenediamine tetraacetic acid (EDTA) in presence of ascorbate to form iron(II)-EDTA (Fe^{2+}-EDTA) and oxidized ascorbate. In the other words, it can use $Fe(NH_4)_2(SO_4)_2$, which follow by reacting with EDTA to form Fe^{2+}-EDTA (Togashi et al., 2002). Then Fe^{2+}-EDTA reacts with H_2O_2 to generate $^{\cdot}OH$ and Fe^{3+}-EDTA in the Fenton reaction. Afterwards, these radicals attack the sugar deoxyribose which is degraded into fragments of malondialdehyde (MDA). MDA reacts with thiobarbituric acid (TBA) on heating at low pH to give a pink chromogen (Sanchez, 2002).

Thus $^{\cdot}OH$ scavenging activity of substance added to the reaction is measured on the inhibition of degradation of deoxyribose. Antioxidants which can scavenge $^{\cdot}OH$, inhibit the formation of a pink chromogen or resulting in the reducing of an absorption at 532 nm.

2. Evaluation of lipid peroxidation

Lipid antioxidant activity directly tests the ability of an antioxidant to inhibit the peroxidation of lipoprotein, tissue, fatty acid and membrane. Lipid

peroxidation is often initiated by adding the metal ions, e.g., as $CuSO_4$, $FeSO_4$, $FeCl_3$ plus ascorbic acid. In these cases, an antioxidant effect could occur not only by peroxyl radical scavenging, but also by metal ion chelation. The popular method is microsomal peroxidation assay (Halliwell, 1997). They contain endogenous antioxidants, such as α-tocopherol, which is the main membrane antioxidant effective in inhibiting peroxynitrite ($ONOO^-$) and can detoxify nitric oxide (NO_2) (Curtin, Donovan, and Cotter, 2002).

C. DEFORMABLE LIPOSOMES

Deformable liposomes, known as elastic vesicles, flexible liposomes or Transfersomes®, are the delivery system, which transports drugs or active ingredients through the skin. They penetrate through the intact stratum corneum (SC) under the influence of transepidermal osmotic gradient, which is shown in Figure 5 (Barry, 2001). Deformable liposomes squeeze themselves through skin pores less than one-tenth the liposomes diameter (Benson, 2005).

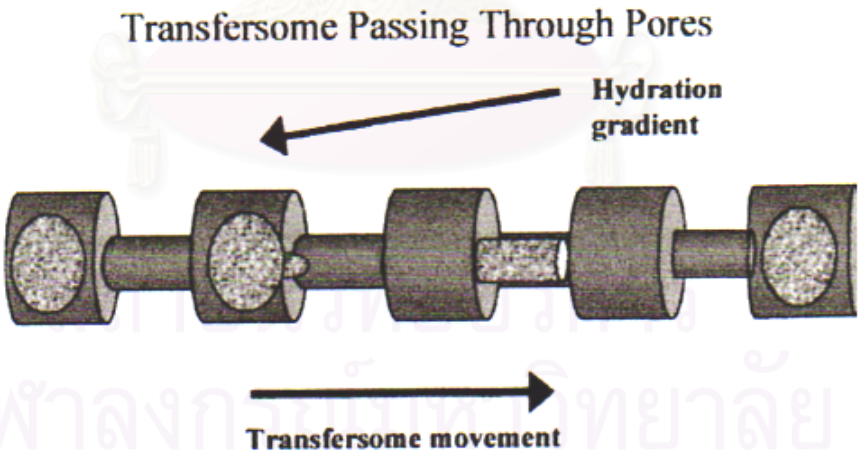


Figure 5. Deformable liposomes are driven by the water concentration gradient through minute pores of the stratum corneum (Barry, 2001).

Commonly, deformable liposomes contain phospholipids such as soya phosphatidyl choline and egg phosphatidyl choline , surfactant or edge activator, solvent such as chloroform, ethanol and methanol, buffering agent such as phosphate

buffer saline pH 7.4 and aqueous compartment which was surrounded by lipid bilayer. Favorite surfactants were sodium cholate, sodium deoxycholate, Span 80, Tween 80 and oleic acid (El Maghraby, Williams, and Barry, 1999; El Maghraby, Williams and Barry, 2000a, 2000b; Cevc and Blume, 2003; Simoes, Tapadas et al., 2004; Lee et al., 2005). Types of surfactants affect to the efficiency of delivery of deformable liposomes. Moreover, this effect of surfactants differs depending on the entrapped drugs. Thus, it is important to select an appropriate surfactant for optimal drug delivery.

Furthermore, the concentration of surfactant or the ratio between phospholipids and surfactant is very important in the formation of deformable liposomes. It is related to the flexibility or deformability of vesicle membranes. However, higher concentration of surfactant may cause a destruction of vesicles.

Deformable liposomes were more effective than the conventional liposomes because deformable liposomes delivered both small and large molecules through intact skin. Deformable liposomes had been used as a carrier for protein and peptides such as insulin, bovine serum albumin, vaccines, etc. Deformable liposomes also prolonged the release and improved the biological activity in vivo. And they were reported to increase transdermal delivery of drugs when applied without occlusion.

Deformable liposomes were characterized for different physical properties such as vesicle diameter which was measured by using photon correlation spectroscopy or dynamic light scattering method. Other characterizations were entrapment efficiency, degree of deformability or permeability, in vitro drug release, and confocal scanning laser microscopy (CSLM) study for investigating the mechanism of penetration of deformable liposomes across the skin (Biju et al., 2006).

1. Materials in formulating the deformable liposomes

1.1 Phospholipids

Phospholipids are the major structural components of biological membranes, so they are the main constituent of liposomes. The most common phospholipids are phosphatidyl choline molecules (PC) (New, 1990). Phosphatidyl choline known as lecithin is amphipathic molecules which a glycerol bridge links in a pair of hydrophobic acyl hydrocarbon chains, with a hydrophilic polar head group. The structure of soybean phosphatidyl choline is represented in Figure 6.

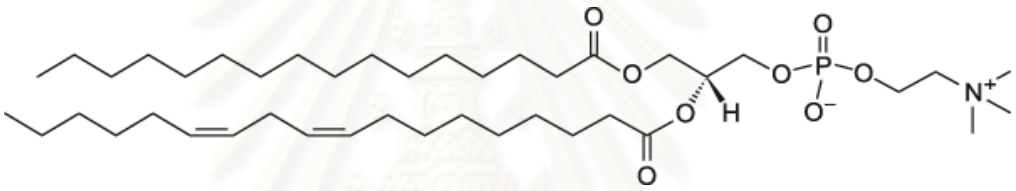


Figure 6. Structure of soybean phosphatidyl choline.

Lecithin can be derived from both natural and synthetic sources. Lecithin from natural source is different in the length and degree of saturation. Plant sourced lecithin is less saturated than mammalian sourced that it influences to be less the transition temperature. Membranes made from egg yolk lecithin have a phase transition temperature (T_c) from -15 ºC to -70 ºC compare with membrane from mammalian sources which are usually in the range 0 ºC to 40 ºC. The transition temperature is the temperature which membrane passes from a tightly ordered in gel or solid phase to a liquid crystalline phase which the molecules are more freedom of movement. Liposomes forms completely when they are prepared at temperature higher than the transition temperature ($T>T_c$). Soy lecithin is usually used in preparing liposomes because most drugs degrade at high temperature.

1.2 Edge activator

Edge activator is often a single chain surfactant, having a high radius of curvature, which destabilize lipid bilayers of the vesicles and increase deformability of the bilayers (Cevc et al., 1996; Elsayed et al., 2007). Sodium cholate, sodium deoxycholate, sorbitan monooleate (Span 80), polyoxyethylene sorbitan monooleate (Tween 80) and oleic acid are used as edge activators (El Maghraby, Williams, and Barry, 1999; El Maghraby, Williams, and Barry, 2000; Cevc and Blume, 2003; Lee et al., 2005). Structures of surfactant are shown in Figure 7.

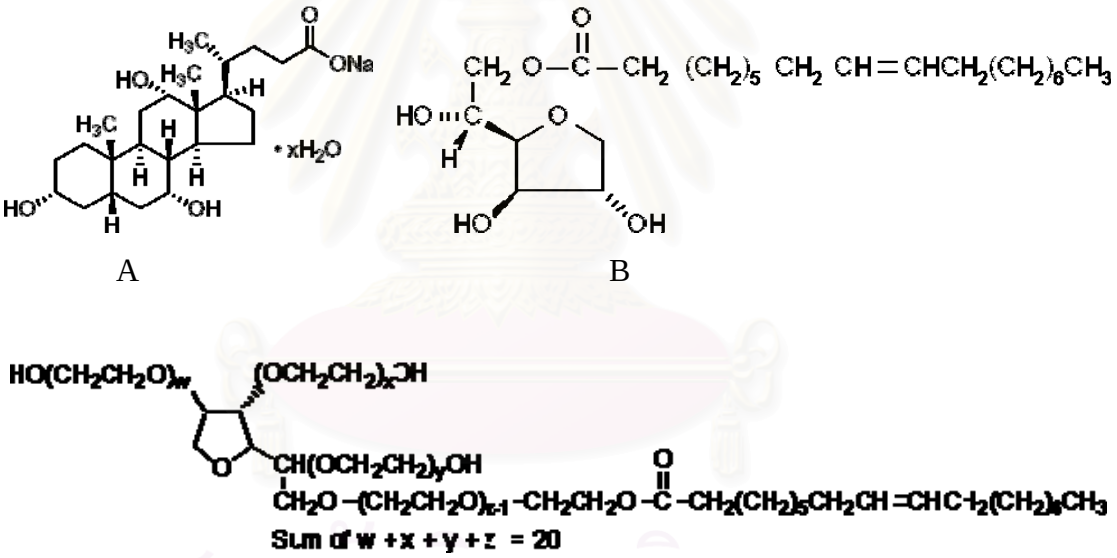


Figure 7. The structure of edge activator such as sodium cholate (A), Span 80 (B) and Tween 80 (C).

Edge activator influences to characteristics of deformable liposomes. The effect of edge activator is different depending on drug, type of edge activator and ratio between PC and surfactant. It is important to select type of surfactant and PC:surfactant ratio for optimal drug delivery.

1.3 Organic solvent

In general, methanol, ethanol, ether (diethyl, diisopropyl, petroleum), hexane, benzene, chloroform, dichloromethane, and other hydrocarbons can be used for preparation of liposomes (Arpornpattanapong, 1996). Organic solvent are used for dissolving phospholipids before continuing the next procedure in preparing liposomes.

1.4 Aqueous compartment

Because of phospholipids are amphiphilic substances which they form bilayer spontaneously when dispersed in an excess of aqueous solution. Thus, they must compose of aqueous compartment which may be only distilled water or dissolved hydrophilic materials in it. Aqueous phase can be buffered but it may disturb forming liposomes at high concentration.

2. Method for preparing the deformable liposomes

2.1 Mechanical dispersion

This method is the simplest in concept, the lipids are dried down onto the glass container vessel to be film and then dispersed by addition of the aqueous medium, followed by shaking. Upon hydration, the lipids swell and peel off the glass container in sheets, generally to form multilamellar vesicles (New, 1990). Four of the basic methods of mechanical dispersion are hand-shaken multilamellar vesicles (MLVs), non-shaken vesicles, pro-liposomes and freeze dry.

2.2 Solvent dispersion

In this class of methods, the lipids are first dissolved in organic solvent, before it was brought into contact with the aqueous phase. At this step, the phospholipids align themselves into a monolayer and then they forms liposomes after

removing the organic solvent (New, 1990). Methods in these groups are ethanol injection, ether injection and water in organic phase that they divided in techniques for formation of droplets, double emulsion vesicles, multilamellar liposomes, reverse-phase evaporation vesicles, stable plurilamellar vesicles. Liposomes prepared by solvent dispersion methods have a higher encapsulation efficiency of aqueous phase than other methods.

Especially, liposomes formed by reverse-phase evaporation techniques entrap the aqueous phase up to 65% at low salt concentrations and under optimal conditions and encapsulate even large macromolecules with high efficiency (Torchilin and Weissig, 2003).

Szoka and Papahadjopoulos developed this method in 1978. Firstly the w/o emulsions was prepared and reversed to o/w emulsions during the preparing processes. After the droplets were formed by bath sonication, the solvents in the preparation were removed by rotary evaporator. The emulsion was dried to a semisolid gel in evaporator under vacuum. The next step was to vigorous vortex the gel in order to bring about the collapse of the water droplets. The lipid monolayer which enclosed the collapses vesicles initiate to adjacent and intact vesicles, formations of the outer leaflet of bilayer of a large unilamellar vesicles (LUVs).

The vesicles were unilamellar and had a diameter of 0.5 μm. The percentage of encapsulation usually was greater than 50%. But excess lipid might disturb the collapse of gel. In conditions of more than 100 mg of lipid and 300 μl of PBS, the collapse occurred when adding the aqueous phase and it made multilamellar vesicles (MLVs) (New, 1990). The main drawback of this method is the exposure of the material to be encapsulated to an organic solvent, which may lead to denature some drugs. Thus, this method should aware of stability of entrapped material when contacting with organic solvent.

2.3 Detergent solubilization

In this group of method, phospholipids are brought into contact with detergent to form mixed micelles. The basis feature is to remove the detergent forms pre-form mixed micelles containing phospholipids, where upon unilamellar vesicle form spontaneously. Although molecules of detergent can be easily removed from the mixed micelles by dialysis, this technique are not very efficient in term of percentage entrapment values attainable (New, 1990).

3. Properties of the deformable liposomes

3.1 Types of vesicles

Deformable liposomes made by reverse phase evaporation are both unilamellar and multilamellar vesicles depending on concentration of phospholipid and ratio of aqueous phase and organic phase. At concentration of 2% PC and ratio of aqueous and organic phase at 1:3 forms unilamellar vesicles, and have heterogeneous size distribution between 100-1000 nm, which their sizes range in large unilamellar vesicles (LUVs) (New, 1990; Torchilin and Weissig, 2003).

3.2 Deformability

Deformability is used to describe capacity of deformable liposomes to penetrate through pore sizes which smaller than their sizes. Cevc, Schatzlein, and Blume (1995) reported that a suspension of Transfersomes® or deformable liposomes with an average diameter of 500 nm can be transported through the pores 5 times smaller nearly as rapidly and efficiently as pure water. Furthermore, the size of deformale liposomes before and after the pore passage is nearly the same (Cevc, Schatzlein, and Blume, 1995). This property of deformable liposomes is claimed that they have more advantages for transdermal delivery of macromolecules. The choice of edge activators may play an important role in the transdermal delivery of their loaded materials (Lee et al., 2005).

3.3 Encapsulation

Generally drug entrapment depends on preparation method, PC concentration and property of entrapped material. For encapsulation of deformable liposomes also depends on type of edge activator and PC:surfactant ratio (El Maghraby, William and Barry, 2000).

3.4 Leakage

Liposomes membranes are semi-permeable membranes, so the entrapped material can generate leakage by osmotic pressure due to its difference concentration between each side of membrane. Leakage can occur during the manufacture and storage conditions. Cholesterol is used to reduce permeability of membrane to water soluble molecules, stabilizing and increasing rigidity of vesicles (Elsayed et al., 2007). Deformable liposomes are composed of edge activator which increasing deformability of membranes. Thus, deformable liposomes should be awared of leakage, especially in case of entrapping water soluble molecules.

D. STABILITY OF LIPOSOMES FORMULATION

Liposomes change in two ways: chemical degradation and physical degradation. Chemical changes involving oxidation of PC can be minimized by these following precautions. Preparation of PC should start with freshly purified PC, freshly distilled solvent, avoid high temperature, carryout process in the absence of oxygen, deoxygenate with nitrogen, store suspension in an inert atmosphere and include an anti-oxidant into lipid membrane (New, 1990).

Physical changes can take place in liposomes with the passage of time. Liposomes may aggregate, fuse, or leak their inside drug contents. Leakage and fusion of vesicles can take place from lattice defects in membranes. These particularly occur in small unilamellar vesicles (SUVs) when it was prepared below the phase transition temperature (T_c), which it can minimize by annealing. On the

other hand, aggregation and sedimentation of neutral liposomes is brought about by Van der Waals interactions, which it tends to be take place in large vesicles. It can minimize by including a small amount of negative charge in the lipid mixtures (New, 1990). Moreover, cholesterol is used for stabilizing and increasing rigidity of membrane for prevention leakage. Methods of investigation problems of liposome instability fall in two ways. First is calculation the entrapment efficiency for investigating leakage and second is examination the sizes of liposomes after storage.

E. SKIN PERMEABILITY

The skin epidermis consists of three main layers- the stratum corneum, the granular layer and the basal layer. The most important barrier for transdermal delivery is the stratum corneum. It forms by keratinized cells, protein-rich cells and intercellular lipid layers which this layer is nonliving structure. Generally, drug administration moved through skin studies in topic of in vitro skin permeation for screening and comparing the capacity of formulation to across skin.

The passage of skin penetration involves skin structures which it is demonstrated in Figure 8 (Sinko, 2006). The important factors influencing the penetration of drug into skin are concentration of dissolved drug (C_s), the partition coefficient between the skin and the vehicle (K), diffusion coefficients which represent the resistance of drug molecule movement through vehicle (D_v) or skin barrier (D_s).

In vitro skin permeation is usually studied by vertical Franz diffusion cell. The donor compartment contains the applied drug to contact with the stratum corneum. After passing through skin, drug enters to the receptor compartment. Permeated drug is analyzed and calculated before plotting versus time to compare capacity of skin permeation.

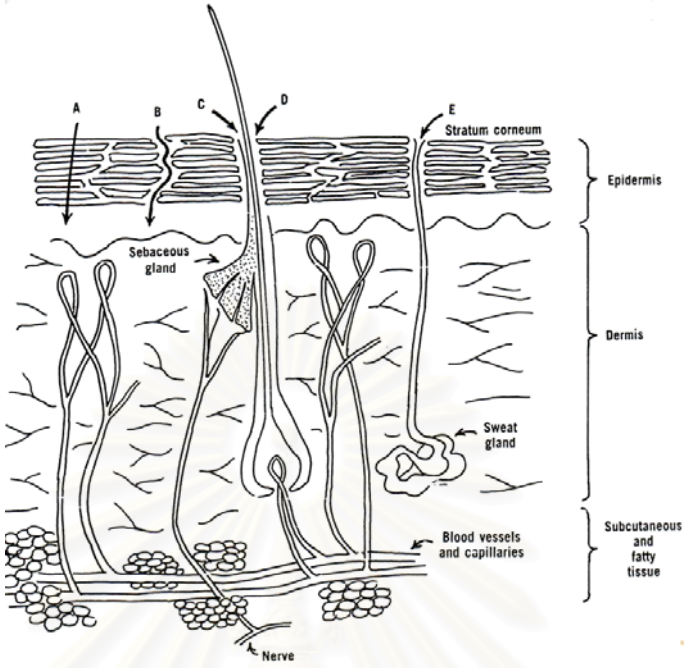


Figure 8. Skin structures and the passage of penetration. Key to sites of percutaneous penetration: A, transcellular; B, diffusion through channel between cells; C, through sebaceous ducts; D, transfollicular; E, through sweat ducts (Sinko, 2006).

In vitro permeability through animal skin has been determined in several types of animals. Generally, skin permeation studies in rabbit skin, pig skin, shed snake skin, hairless mouse skin (Itoh et al., 1990; Rigg and Barry, 1990; Jacobi et al., 2007). The previous study demonstrated that percutaneous absorptions of ten compounds through pig skins and human skin were statistically significant correlated. However skin from different body regions of pig skin was used, abdominal pig skin is particularly used.

CHAPTER III

MATERIALS AND METHODS

The experiments were divided into six parts:

PART A: CHARACTERIZATION OF SILK SERICIN PROTEIN

1. Evaluation of molecular mass of sericin by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)
2. Quantification of protein by Lowry method

PART B: DETECTION OF ANTI-FREE RADICAL ACTIVITY OF SERICIN BY DEOXYRIBOSE ASSAY

PART C: PREPARATION AND EVALUATION OF DEFORMABLE LIPOSOMES VERIFIED IN COMPOSITIONS

PART D: FORMULATION OF SERUM OR DISPERSION SYSTEM CONTAINING DEFORMABLE LIPOSOMES AND EVALUATION OF PHYSICAL STABILITY

1. Formulation of dispersion systems
2. Preparation of deformable liposomes in dispersion systems
3. Evaluation of physical stability of liposomes formulation

PART E: DETECTION OF ANTI-FREE RADICAL ACTIVITY OF DISPERSION SYSTEM CONTAINING DEFORMABLE LIPOSOMES LOADED WITH SERICIN

PART F: SKIN PERMEATION STUDY OF DEFORMABLE LIPOSOMES LOADED WITH SERICIN COMPARING WITH SERICIN IN OTHER SYSTEMS

MATERIALS

Acetic acid (Merck, Germany, lot no. 422)

Acrylamide (Sigma-Aldrich, Germany, lot no. 20K0164)

Albumin from bovine serum, electrophoresis grade (Sigma-Aldrich, Germany, lot no. 045K0676)

Ammonium persulfate (APS Finechem, Australia, lot no. FOJ138)

Bis-acrylamide (Sigma-Aldrich, Germany)

Bromophenol blue (Merck, Germany, lot no.8122)

Carbopol ultrez-10 (Noveon, Belgium, lot no. EC481ZG863)

Chitosan, 95% deacetylation, MW 550,000 Da (Seafresh chitosan lab company, Thailand)

Chloroform, AR grade (Labscan Asia, Thailand)

Cholesterol (Sigma-Aldrich, Germany, lot no. 084K0136)

Coomassie brilliant blue R-250 (Merck, Germany, lot no.617)

Copper (II) sulphate hexahydrate (Ajax Finechem, Australia, lot no. AF502341)

2-Deoxyribose (Fluka, South Korea, lot no. 1170194)

Disodium ethylene diamine tetraacetic acid (T Chemical, Thailand)

Disodium hydrogen phosphate (Merck, Germany, lot no. 125)

Ethanol (Merck, Germany)

Ferrous ammonium sulphate (Merck, Germany)

Folin-ciocalteu's phenol reagent (Merck, Germany)

Glycerol (May & Baker, England, lot no. 07162)

Hydrogen peroxide (Merck, Germany, lot no. 602)

L-ascorbic acid (Roche, Germany, lot no. TL00405864)

Mannitol (Merck, Germany)

2-Mercaptoethanol (Sigma-Aldrich, Germany, lot no. 58H0066)

Methanol (Merck, Germany)

Poloxamer 407 (BASF, Germany, lot no. WPM2527C)

Potassium dihydrogen phosphate (Merck, Germany, lot no.127)

Sericin (generous gift from the Queen Sirikit Institute of Sericulture, Thailand)

Sodium carbonate (Ajax Finechem, Australia, lot no. AF405220)

Sodium chloride (Merck, Germany, lot no. 324)
Sodium dodecyl sulfate (Ajax chemicals, Australia)
Sodium hydroxide (Merck, Germany, lot no. 630)
Sodium potassium tartrate (Farmitalia Carlo Erba, Italy)
Soy bean lecithin, Phospholipon 90 (Rhodia, Germany)
Span 80 (East Asiatic Company, Thailand, lot no. 56648)
TEMED (Sigma-Aldrich, Germany)
2-Thiobarbituric acid (Sigma-Aldrich, Germany, lot no. 066K0750)
Trichloroacetic acid (Fluka, Germany, lot no. 1222401)
Tris HCl (Sigma-Aldrich, Germany, lot no. 116K5422)
Triton® X-100 (Sigma-Aldrich, USA, lot no. 065K0122)
Tween 80 (East Asiatic Company, Thailand, lot no. 55055)

APPARATUSES

Analytical balance (AG 285, Mettler Toledo, Switzerland)
Analytical balance (PG403-S, Mettler Toledo, Switzerland)
Centrifuge bottles polycarbonate (10.4 ml) (Beckman Instruments, USA)
Centrifuge (ALC® centrifugette, Biomed group, Thailand)
Heating bath (Neslab RTE 7, Becthai, Thailand)
Hot air oven (UL50, Memmert, Germany)
Electrophoresis set (BioRad, USA)
Extruder (Lipex™, Northern Lipids, Canada)
Light microscope (Nikon Eclipse E200, Japan)
Micropipette (Biohit, Finland)
Modified Franz diffusion cells (Crown glass, USA)
Particle size analyzer (Mastersizer 2000 Ver 5.1, Malvern Instruments, UK)
Pear-shaped flask 500 ml (Schott Duran, Germany)
pH meter (Model 420A, Orion, USA)
Quartz cell (Starna, UK)
Refrigerator incubator, (FOC 225i, Chatcharee holding, Thailand)
Rotary evaporator (Rotavapor R-215, Buchi, Switzerland)

Scanning electron microscope (Model JSM-5410LV, JEOL®, Japan)
Sonicator (Transsonic digitals, Elma, Germany)
Syringe needle guage No. 23 (Nipro Corporation, Japan)
Transmission electron microscope (Model 1230, JOEL®, Japan)
Ultracentrifuge (L80, Beckman, USA)
Ultrasonic bath (Transsonic digitals T900/H, Elma, Germany)
UV-visible spectrophotometer (UV-1601, Shimadzu, Japan)
Vortex mixer (Vortex Genie-2, Scientific Industries, USA)
Water bath (WB 22, Memmert, Germany)

METHODS

PART A
CHARACTERIZATION OF SILK SERICIN PROTEIN

1. Evaluation of molecular mass of sericin by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (Walker, 2002)

There were 2 lots of sericin donated and used in this study. Sericin O (Ser O) was used in the preliminary study while sericin N (Ser N) was used in all other topics of the investigation.

Firstly, sericin-first lot (Ser O), sericin-second lot (Ser N) and bovine serum albumin (BSA) were dissolved in phosphate buffer saline (PBS) to be solutions at 2 mg/ml and then diluted for desired concentrations, 30-80 µg and 5-10 µg per one loading; 10 µl, respectively. Secondly, stock solution of acrylamide was prepared by weighing 49.2 g of acrylamide mixed with 0.8 g of bis-acrylamide. Thirdly, the other stock solutions were prepared as belowed section:

a) 2 M Tris HCl (pH 6.8, 8.8).
b) 0.1, 4 and 10% sodium dodecyl sulfate.
c) 0.1% bromophenol blue.

d) Loading dye 4x was prepared by mixing 4 g of SDS, 1.6 g of Tris HCl (pH 6.8) and 25 ml of deionized water, followed by stirring and adding 0.02 g of bromophenol blue, stirring again and adding 5 ml of glycerol.

e) Staining solution was prepared by mixing 0.25% coomassie blue R-250, 50% methanol and 10% acetic acid.

f) Destaining solution was prepared by mixing 5% methanol and 7.5% acetic acid.

Then, separating buffer was prepared by mixing 75 ml of 2 M Tris HCl (pH 8.8), 4 ml of 10% SDS and 21 ml of deionized water. Moreover, stacking buffer was prepared by mixing 25 ml of 2 M Tris HCl (pH 6.8), 4 ml of 10% SDS and 71 ml of deionized water. Lastly, electrophoresis buffer was prepared by mixing 3 g of Tris HCl, 14.4 g of glycerol and 1 g of SDS.

After preparing stock solution, it was step of running electrophoresis. First of all, samples were denatured in separating buffer by heating to 90 °C for 10 min. Secondly, main gel was freshly prepared at 10% acrylamide by adding 5.5 ml of the deionized water, 2.5 ml of 4% separating buffer, 2 ml of 50% acrylamide, 50 µl of 10% ammonium persulfate and 10 µl of TEMED. The main gel was rapidly loaded into gel plates for 4 in 5 parts. The procedure is demonstrated in Figure 9. Then the mixture was added by 0.1% SDS above the gel for preventing it from contacting with air and waited for 20 min; resulting gel. Excess SDS was removed before adding the top gel.

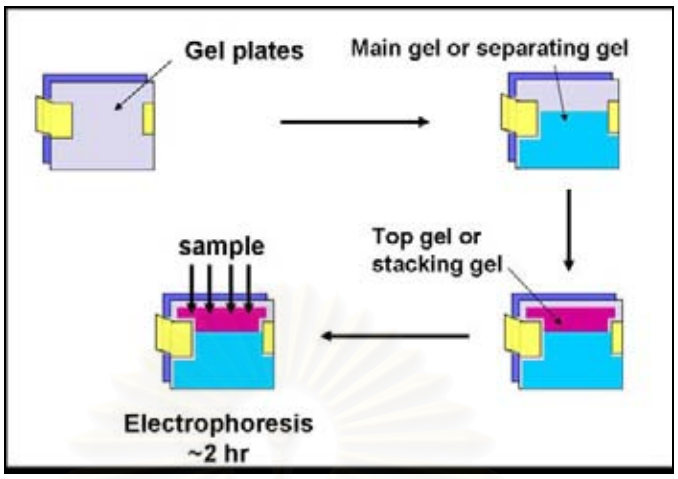


Figure 9. Procedure of setting gel plates.

Afterwards, the top gel was freshly and rapidly prepared by mixing 2.6 ml of the deionized water, 1 ml of 4% stacking buffer, 400 µl of 50% acrylamide, 30 µl of 10% ammonium persulfate and 5 µl of TEMED. The top gel was rapidly added upside the main gel until reaching the front, suddenly closed and left it about 15-20 min.

When gel had already formed, electrophoresis buffer was poured approximately half part height. Moreover, freshly mixing 28 µl of 2-mercaptoethanol with 1 ml of loading dye 4x before mixed each sample with 2.5 µl of loading dye 4x, except the marker. Then, the mixture was heated at 95 °C for 5 min before it was cooled in the ice and was brought centrifugation. Carefully loading each sample into each lane, slowly added for preventing blown it out.

Next, electrophoresis buffer was added all over the electrophoresis set. The conditions during running electrophoresis were 100 V, about 2 h and monitored color of bromophenol blue. When the color reached the front, the current turned off.

Carefully removed the gel from gel plates, stained the gel by coomassie brilliant blue R-250 and washed by the destaining solution for many times with gently shaken.

When the edge of the gel was clear, it meant completely destaining so washed by purified water and kept the gel in plastic and wrapped it by the foil until it dried. It was important that the main gel and the top gel must be prepared rapidly. If it had formed gel before loading, it could not be added. Be careful to avoid bubble formation, it made incomplete separation. Duplicated test for checking precision of the method and ran samples compared with the marker for determining the molecular masses.

2. Quantification of protein by Lowry method (Walker, 2002)

The protein contents of both lots of sericin were detected by Lowry method comparing to BSA. Firstly, dissolving sericin and BSA in distilled water to be solutions at 2 mg/ml and diluted for desired concentrations. Then, stock solutions of complex-forming reagent such as 2% Na_2CO_3, 1% $CuSO_4.5H_2O$ and 2% sodium potassium tartrate was prepared. This complexing-forming reagent should be fresh-prepared in the ratio 100:1:1, respectively. Moreover, 2 N NaOH was prepared. Next, it was step for testing which started by mixing samples in various concentrations of 1 ml with 2 N NaOH 200 µl, heated at 90 °C 20 min and let it cool down to room temperature. Then, 2 ml of complex-forming reagent was added and left it for 10 min before adding 200 µl of Folin Ciocaltau's phenol reagent. Samples were kept at room temperature for 45 min before measuring the absorption at 550 nm with distilled water as blank.

2.1 Validation of Lowry method

The analytical parameters used for validation were linearity, accuracy and precision.

2.1.1 Linearity

Three sets of eight concentrations of BSA and both lots of sericin solutions ranging from 0.05 to 0.40 mg/ml were prepared and analyzed.

Linear regression analysis of the absorbances versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

2.1.2 Accuracy

Five sets of three concentrations of Ser O at 0.10, 0.20 and 0.40 mg/ml were prepared and analyzed. The absorbance of each concentration was inversely calculated from the standard curve to obtain the estimated concentration. The accuracy of the method was determined from the percentage of recovery. The percentage of recovery was the ratios of the estimated concentrations and the real added concentrations multiplied by 100.

2.1.3 Precision

a) Within run precision

Five sets of three concentrations of Ser O at 0.10, 0.20 and 0.30 mg/ml in the same day were determined. The percent coefficient of variation (% CV) of each concentration was determined.

b) Between run precision

Five sets of three concentrations of Ser O at 0.10, 0.20 and 0.40 mg/ml on five different days. The between run precision was determined by analyzing the percent coefficient of variation (% CV) of each concentration.

Acceptance criteria

For accuracy, the percentage of recovery should be within 85-115% of each concentration, whereas the percent coefficient of variation for both within run and between run precision should be less than 15% (EMEA, 1995; EMEA, 1999; U.S. FDA, 2001).

PART B

DETECTION OF ANTI-FREE RADICAL ACTIVITY OF SERICIN BY DEOXYRIBOSE ASSAY (Togachi et al., 2002)

Step of preparing the stock solution started firstly by dissolving sericin, ascorbic acid and mannitol in distilled water to be solutions at 6.4 mg/ml. And they were diluted by 2-fold serial dilution to concentrations at 3.2, 1.6, 0.8, 0.4, 0.2 and 0.1 mg/ml. Secondly, stock solutions were prepared such as 150 mM NaCl, 5 mM deoxyribose, 2 mM $Fe(NH_4)_2(SO_4)_2.6H_2O$, 2 mM EDTA, 0.03% H_2O_2 and 2.8% trichloroacetic acid in distilled water. Lastly, 1% thiobarbituric acid was prepared in 50 mM NaOH. Afterwards, first of the reaction, 1 ml of 150 mM NaCl was pipetted in test tubes, mixed with 0.4 ml of 5 mM deoxyribose and 0.4 ml of each sample. Then, iron (II)-EDTA was fresh-prepared by mixing 2 mM $Fe(NH_4)_2(SO_4)_2.6H_2O$ with 2 mM EDTA in 1:1 ratio about 5 min before using. And, 0.4 ml of iron (II)-EDTA and H_2O_2, were added, mixed and incubated at 37 °C for 1 h. Followed by 1ml of 1% thiobarbituric acid in 50 mM NaOH was added before adding 1 ml of 2.8% trichloroacetic acid in the mixture aboved. Heating at 90 °C for 15 min, then ice-cooling it by ice for 15 min before placing it about 45 min to room temperature. Lastly, the absorption was measured at 532 nm for both each sample and distilled water sample as blank. Mannitol was used as positive control and the activity was compared with ascorbic acid.

1. Validation of deoxyribose assay

The analytical parameters used for validation were linearity, accuracy and precision.

1.1 Linearity

Three sets of eight concentrations of mannitol ranging from 0.10 to 6.40 mg/ml were prepared and analyzed. Linear regression analysis of the 1/absorbances versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

1.2 Accuracy

Five sets of three concentrations of mannitol at 0.40, 0.80 and 3.20 mg/ml were prepared and analyzed. The absorbance of each concentration was inversely calculated from standard curve to known the estimated concentration. The accuracy of the method was determined from the percentage of recovery. The percentage of recovery was the ratios of the estimated concentrations and the real added concentrations multiplied by 100.

1.3 Precision

a) Within run precision

Five sets of three concentrations of mannitol at 0.20, 0.80 and 3.20 mg/ml in the same day were determined. The percent coefficient of variation (% CV) of each concentration was determined.

b) Between run precision

Five sets of three concentrations of mannitol at 0.20, 0.80 and 3.20 mg/ml on five different days. The between run precision was determined by analyzing the percent coefficient of variation (% CV) of each concentration.

Acceptance criteria

For accuracy, the percentage of recovery should be within 85-115% of each concentration, whereas the percent coefficient of variation for both within run and between run precision should be less than 15% (EMEA, 1995; EMEA, 1999; U.S. FDA, 2001).

PART C

PREPARATION AND EVALUATION OF DEFORMABLE LIPOSOMES VERIFIED IN COMPOSITIONS

1. Preparation of deformable liposomes (New, 1990; Torchilin and Weissig, 2003)

Deformable liposomes (DL) were prepared by reverse phase evaporation and verified in types of surfactant and ratios of PC and surfactant at appropriate concentration of PC. LUVs were proposed from this technique. The preliminary study was investigated DL at 3.5% PC. Ser N was used for loading in liposomes.

Preparation of stock solutions was first step that started by dissolving sericin in distilled water to be stock solution at 50 mg/ml or 5% sericin. Then, stock solution of phosphatidyl choline (PC) or soybean lecithin was prepared at concentration of 50 mg/ml or 5% PC in chloroform both with and without surfactant; at 90:10, 85:15 and 80:20 as the ratio of PC:surfactant.

Afterwards, soybean PC was calculated to get final lipid concentration at 3.5% in 30 ml of final volume liposome preparation. Secondly, 12 ml of stock solution of 5% PC was mixed with 33 ml of chloroform in case of conventional liposomes (CL), or was added with various amounts of surfactants and 33 ml of chloroform in case of deformable liposomes (Table 3). Then, added it in the 500 ml pear shaped flask. Introduced 15 ml of distilled water into the lipid solution by rapidly injecting through a needle gauge No.23 from a 5 ml syringe followed by closing the system and sonication in bath sonicator for 5 min. Then, the flask was transferred directly to the rotary evaporator. Dry it down gently at 37 °C and vacuum (300 mbar) about 30 min until gel forming.

Released the vacuum, removed the flask, and subjected it to vigorous agitation by vortex mixer until gel collapsed and was transformed into viscous fluid or suspension liposome. Afterwards, 15 ml of 5% sericin was added to hydrate lipid at

the same temperature without reduce pressure until causing homogeneous liposomes suspension obtained (New, 1990). Removed the flask, replaced the stopper and reduced size by sonication for 60 min.

Table 3. Compositions of CL and DL in various ratios of PC and surfactant*.

Type of liposomes	**surfactant**	**PC:surfactant (by weight)**
CL	-	100:0
DL	Span 80	90:10
		85:15
		80:20
	Tween 80	90:10
		85:15
		80:20

*Each formulation was fixed % PC, loaded with 5 % sericin and prepared in triplicate.

2. Evaluation of deformable liposomes

The characteristics such as appearance, size and size distribution, the percentage of entrapment efficiency, deformability index and anti-free radical activity of each formulation were investigated.

2.1 Microscopic appearances

The appearances of liposomes and deformable liposomes were observed in shape, size, aggregation and type of liposomes. They were observed and photographed under the microscope with magnification of 10×100.

2.2 Scanning electron microscopy (SEM)

Surface characteristics of CL and DL were observed by scanning electron microscope. Samples were adhered on the glass slide for preparing by a specific fixation technique. Samples were fixed, dehydrated, dried mounted, and coated with gold. The scanning electron microscopy observed and demonstrated in photographs.

2.3 Transmission electron microscopy (TEM)

The bilayers of CL and DL were investigated by TEM. Samples were examined by negative staining technique and observed the results in photographs.

2.4 Particle size analysis

Laser particle sizer (Mastersizer 2000 Ver 5.1) was used to observe particle size of liposomes and deformable liposomes.

2.5 Entrapment efficiency

The volume of 2 ml of liposomes suspension was pipetted into the microcentrifuge assembly. The encapsulated protein was separated by ultracentrifugation at 62,000 rpm at 4 °C for 360 min. The precipitates containing with sericin liposomes were lyzed by adding 3.5 ml of 1% Triton X-100. After sonicated and incubated at 37 °C for 30 min, the clear solution was assayed by Lowry method. The absorbances were monitored at 550 nm and compared with the standard curve of sericin solution. The supernatants were also analyzed by Lowry method. All analyses were determined in triplicate and calculated the percentage of recovery. The entrapment efficiency was calculated by equation below (Peltonen et al., 2004; Dubey et al., 2006).

$$\text{Entrapment efficiency (\%)} = \frac{\text{Total protein content in vesicle} \times 100}{\text{Initial protein content added}}$$

Statistical analysis of differences in the Entrapment efficiency was performed by using Analysis of Variance (ANOVA). A *P*-value of 0.05 was taken as the level of significance.

2.6 Deformability index

Deformability of vesicles was evaluated by using an extruder (Lipex™, Northern Lipids, Canada), which this method modified from Song and Kim (2006). Conventional liposomes and deformable liposomes were diluted fives times with PBS. Five hundred microliters of the diluted samples was extruded through a sandwich of two sheets of 200 and 50 nm polycarbonate membrane (Nuclepore®, Whatman, USA) under 5 mbar (300 kPa) of nitrogen pressure. The weight of passed samples was monitored and calculated to deformability index by following equation (Gupta et al., 2005). Size of vesicles was determined by Zetasizer before and after extrusion. The deformability indexes of deformable liposomes were compared with conventional liposomes.

$$\text{Deformability index} = j\,(r_v/r_p)^2$$

where:

j = weight of dispersion which passed the membrane within 10 min
r_v = size of vesicles (after passes)
r_p = size of membrane pores

2.7 Hydroxyl radical scavenging activity

Samples were detected hydroxyl radical scavenging activity by modified deoxyribose assay which was described above in Part B.

2.8 Leakage

The initial preparations were diluted with PBS pH 7.4. The volume of 200 µl of diluted samples was pipetted into the microcentrifuge assembly, capped and sonicated at 37 °C for 30 min. Efflux of protein was assayed by periodic ultracentrifugation at 62,000 rpm at 4 °C for 360 min. The precipitates were lyzed by dissolving in 1 ml of 1% Triton X-100. Both precipitates and supernatants were tested by Lowry method for observing the remaining protein. Percentage of recovery was calculated for approving the accuracy of analytical method. Leakage was determined from entrapment efficiency after storage for 4 weeks in refrigerator. Statistical analysis of differences in the entrapment efficiency was performed by using Analysis of variance (ANOVA). A *P*-value of 0.05 was taken as the level of significance.

PART D
FORMULATION OF SERUM OR DISPERSION SYSTEM CONTAINING DEFORMABLE LIPOSOMES AND EVALUATION OF PHYSICAL STABILITY OF LIPOSOMES FORMULATION

1. Formulation of dispersion systems

Dispersion system was formulated by using hydrophilic polymers. Three types of polymers were chitosan (high molecular weight), Poloxamer 407 and Carbopol ultrez-10; which gave pH as base, neutral and acid, respectively. Each polymer was determined the appropriate amount to form gel with various concentrations; 1-4% chitosan, 15-30% Poloxamer 407 and 0.5-1% Carbopol ultrez-10.

2. Preparation of deformable liposomes in dispersion systems

The mixing ratio between gel and deformable liposomes was 1:1. Consequently, the physical appearances were investigated such as color, clarity, odor, spreadability, smoothness, pH and viscosity.

3. Evaluation of physical stability of liposomes formulation

After mixing for 24 h, dispersion system which loaded with deformable liposomes was kept in the refrigerator at 4-8 °C for 24 h and heated at 30 °C for 24 h (Carstensen, 1990). Each formulation was run for 6 heating-cooling cycles and physical appearances were investigated after passing 6 heating-cooling cycles. The most stable formulation was selected for further study. Moreover, sericin content in the dispersion system was investigated by Lowry method.

PART E
DETECTION OF ANTI-FREE RADICAL ACTIVITY OF DISPERSION SYSTEM CONTAINING DEFORMABLE LIPOSOMES LOADED WITH SERICIN

Samples were detected hydroxyl radical scavenging activity by modified deoxyribose assay which was described in Part B.

PART F
SKIN PERMEATION STUDY OF DEFORMABLE LIPOSOMES LOADED WITH SERICIN COMPARING WITH SERICIN IN OTHER SYSTEMS

The abdominal skin of a new born pig was excised and inspected for any defects. The underlying fat was carefully removed from the stratum corneum. The excised skin was cleaned, wrapped with aluminium foil, and stored in a freezer. In vitro skin permeation was determined by using Franz vertical diffusion cell modified from Song and Kim (2006). Each skin surfaces should be more 2.271 cm^2 which was

a diffusion cell area. The frozen skin had been thawed and soaked with PBS (pH 7.4) for 1 h before equilibrated. It was set in place with the stratum corneum facing the donor compartment and the dermal side facing the receptor compartment. The receptor medium was approximately 14 ml of PBS (pH 7.4), stirred with magnetic bar in a circulating water bath which controlled temperature at 37 ±0.5 °C. The skin was equilibrated for 1 h and gently rocking the cell for repelling the bubbles. One milliliter of samples was applied at donor compartment non-occlusive. The volume of 1 ml of samples from the receptor compartment were taken periodically and replaced with PBS to keep constant volume in receptor compartment. Samples were analyzed for amount of sericin which permeated through pig skin by Lowry method. When the cumulative amount (M) was plotted versus time, slope was observed. Then the value of slope was divided by concentration of drug in donor (C_d) for gaining the permeability coefficient (P) as shown below (Sinko, 2006). Notably, P was determined from the diffusion coefficient (D), the partition coefficient (K) and thickness of membrane (h) which influenced to the permeability.

$$M = PSC_d t$$

$$P = \frac{DK}{h}$$

where:

M = cumulative amount of sericin (mg)

P = permeability coefficient (cm/h)

S = surface area (cm^2)

C_d = concentration of drug in donor (mg/ml)

t = time (h)

D = diffusion coefficient

K = partition coefficient

h = thickness of membrane

CHAPTER IV

RESULTS AND DISCUSSION

PART A
CHARACTERIZATION OF SILK SERICIN PROTEIN

1. Evaluation of molecular masses of sericin by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis was run by using 5-10 µg BSA, 30-80 µg Ser N and 30-80 µg Ser O per 10 µl, respectively. BSA was used for evaluating reliability of the method and checking molecular masses from the marker. The results showed in Figure 10 which samples were in 13% acryamide. It was found that the molecular mass of BSA, 67 kDa (Terada et al., 2002), was truly run electrophoresis between 50-75 kDa. This result suggested that the method for running SDS-PAGE was reliable.

Both lots of sericin were run in gel, the molecular mass of them were in range 10-250 kDa. The result was close to the previous studies that SDS-PAGE of silk sericin protein was in range 20-300 kDa (Terada et al., 2002) and in range 30-200 kDa (Zhang, 2004). Although both of samples were sericin in range 10-250 kDa, their patterns were clearly different. The main of sericin O was in the high molecular mass part, but the main of sericin N was in the lower part. These results might occur from the duration of storage and the different processes. Due to sericin N was fresh-prepared and passed radiation for long preservation. Nevertheless, it might take place from the different batch. Sericin was removed from silk cocoon which was natural product. It had a high variation in sources of silk. In other point, the intensity of color also differed, which referred to quantity of molecules in that range. Intensity of sericin N was lighter than sericin O. But at the top of gel of sericin N had a blue color, which it might be from high molecular masses more than 250 kDa were stacking above. It seemed necessary to choose another marker which was in range from 10 kDa to upper than 250 kDa for proving the higher molecular mass. Moreover, the results showed the different appearance between bands of BSA and

sericin. Sericin had no clearly band, it run along the gel. Opposite to sericin, BSA had the distinctly band in 50-75 kDa. This finding might occur because of the varied molecular mass of crude sericin.

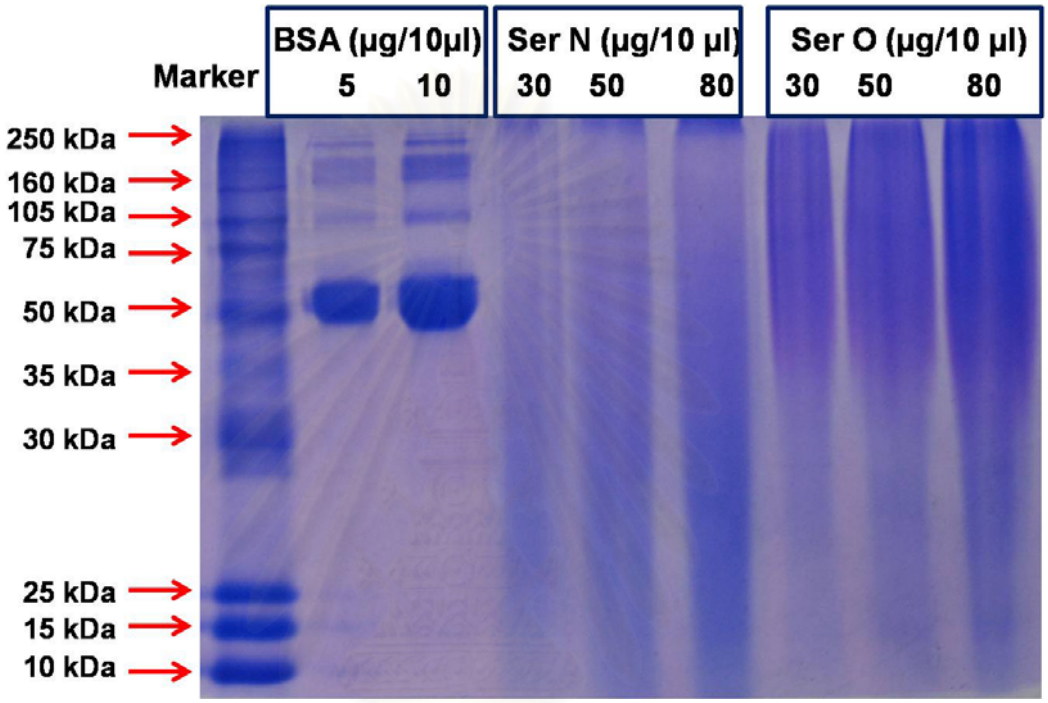


Figure 10. SDS-PAGE (13%) of BSA, Ser N and Ser O, compared with the rainbow marker.

2. Quantification of protein by Lowry method

2.1 Validation of Lowry method

2.1.1 Linearity

First of the validation, BSA, sericin O and sericin N were analyzed for protein quantities by Lowry method in eight concentrations ranging from 0.05-0.40 mg/ml for validation of linearity. Linear regression analysis of the absorbances at 550 nm versus the corresponding concentrations was performed in Figures 11, 12, and 13. The coefficients of determination (R^2) were calculated as

0.9960, 0.9980 and 0.9929, respectively. The calibration data were found to be linear with good R^2. These results indicated that UV spectrophotometric method was acceptable for quantitative analysis of sericin by Lowry method in the range of 0.05-0.40 mg/ml.

The calibration curve of standard protein (BSA) and silk sericin protein were linear in the concentration range of 0.05-0.40 mg/ml. From the data obtained, slope and y-intercept of their samples were very close. The amounts of protein in Ser O and Ser N were 99.44 and 98.65% of BSA (Data showed in appendix).

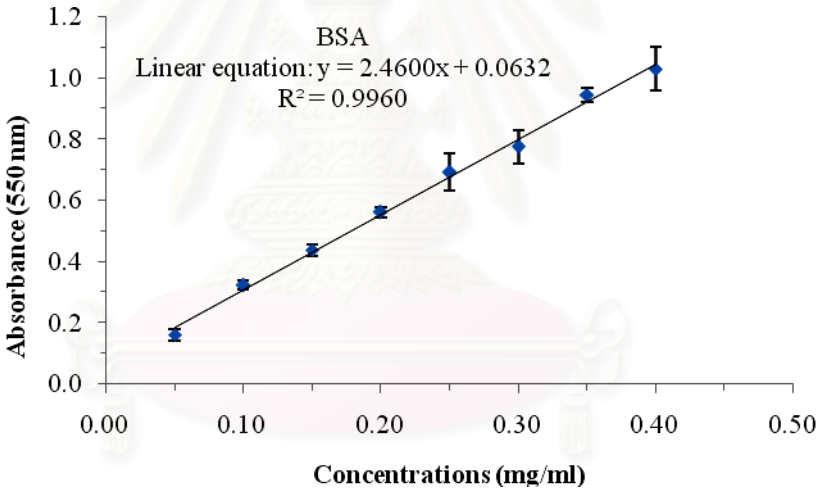


Figure 11. The standard curve of BSA, n=3.

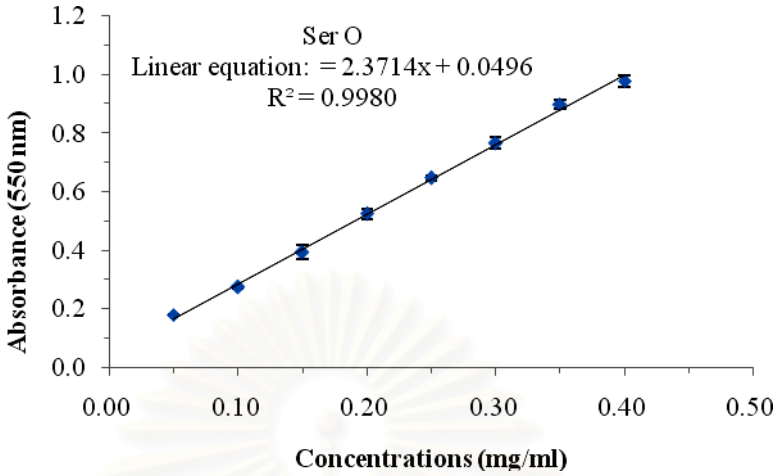


Figure 12. The standard curve of Ser O, n=3.

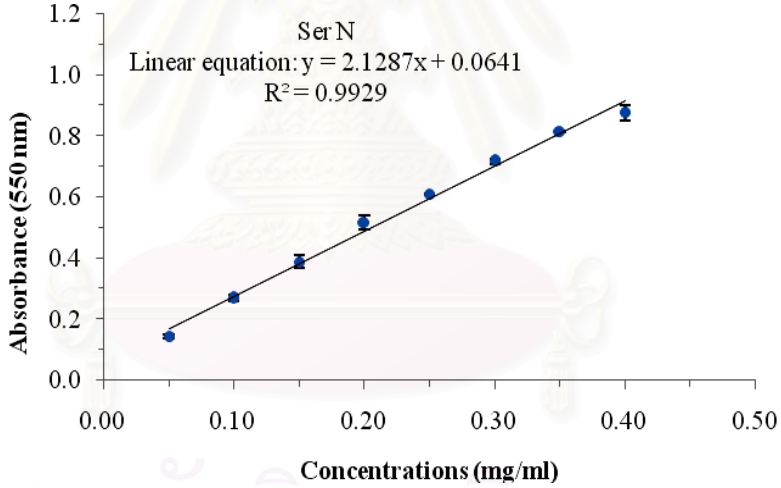


Figure 13. The standard curve of Ser N, n=3.

2.1.2 Accuracy

Ser O was prepared at the concentrations of 0.08, 0.16 and 0.32 mg/ml in five sets. Samples were analyzed by Lowry method. The absorbances at 550 nm of each samples are showed in Table 4. Moreover, the estimated concentrations and percentages of analytical recovery of each sericin concentrations are shown in Table 5 and Table 6, respectively. All percentages of analytical

recovery were in the range of 89.12-101.00%, which indicated the high accuracy of this method. Thus, it could be used for analysis of sericin in the range of study.

Table 4. The absorbance of sericin at 550 nm by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.08	0.2293	0.2160	0.2164	0.2164	0.2230	0.2202 ± 0.006
0.16	0.3941	0.4147	0.4281	0.4468	0.4215	0.4211 ± 0.019
0.32	0.8039	0.8191	0.8075	0.8112	0.8635	0.8210 ± 0.024

Table 5. The estimated concentrations of sericin by Lowry method.

Concentration (mg/ml)	Estimated concentration from standard curve (mg/ml)					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.08	0.0758	0.0701	0.0703	0.0701	0.0702	0.0713 ± 0.002
0.16	0.1453	0.1540	0.1540	0.1540	0.1540	0.1522 ± 0.004
0.32	0.3181	0.3244	0.3245	0.3246	0.3245	0.3232 ± 0.003

Table 6. The percentage of analytical recovery of sericin by Lowry method.

Concentration (mg/ml)	Percentage of analytical recovery					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.08	94.70	87.66	87.87	87.66	87.73	89.12 ± 3.121
0.16	90.80	96.22	96.22	96.22	96.22	95.14 ± 2.424
0.32	99.40	101.36	101.40	101.43	101.40	101.00 ± 0.894

2.1.3 Precision

The precision of Lowry method for quantitation of protein in sericin was determined both within run and between run as showed in Tables 7 and 8, respectively. Both of the coefficients of variation values of them were low, as 1.06-2.01% and 4.47-7.84% respectively. The coefficient of variation of the biological

analytical method should generally be less than 15%. Therefore, the Lowry method was precise for quantitative analysis of sericin in the range of study.

The analysis of sericin by Lowry method developed in this study demonstrated good linearity, accuracy and precision. Thus this method was used for the determination of the content of sericin protein.

Table 7. Data of within run precision by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm					Mean	SD	% CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
0.08	0.3077	0.3002	0.2947	0.3060	0.3091	0.3035	0.006	1.97
0.20	0.6733	0.6643	0.6643	0.6621	0.6786	0.6685	0.007	1.06
0.40	1.1861	1.2076	1.2361	1.2476	1.2097	1.2174	0.024	2.01

Table 8. Data of between run precision by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm					Mean	SD	% CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
0.10	0.2734	0.3086	0.3202	0.2702	0.2781	0.2901	0.023	7.84
0.20	0.5235	0.5501	0.5774	0.5158	0.5448	0.5423	0.024	4.47
0.30	0.7101	0.7897	0.8189	0.7441	0.7830	0.7692	0.042	5.52

Moreover, sericin in this experiment was entrapped in liposomes. Therefore, the specificity of Lowry method for sericin in liposomes formulation which might disturb the method should be validated. From the reason of the absorption curves of this method were almost span, it can not detect the interference of the peak. Thus, the results would apply from the percentage of recovery instead.

Validation of accuracy in formulation was assessed in various conditions: CL, DLSp and DLTw, which all of them were spiked with sericin, then it was ruptured by Triton-X in H_2O before testing. Another condition was sericin in

PBS. Data in appendix showed the percentage of recovery of sericin in CL, DLSp, DLTw and PBS conditions which were in range 99.35-113.55%, 100.15-108.92%, 93.14-103.59% and 90.65-106.42%, respectively (Data showed in appendix). It indicated that the conditions of CL, DLSp, DLTw, which spiked with sericin and lyzed by Triton-X did not disturb the accuracy of Lowry method. Additionally, PBS used in the present experiment of skin permeation did not disturb the Lowry method.

PART B

DETECTION OF ANTI-FREE RADICAL ACTIVITY OF SERICIN BY DEOXYRIBOSE ASSAY

1. Validation of deoxyribose assay

1.1 Linearity

The addition of Fe^{2+} to a solution of deoxyribose in the Fenton reation, the hydroxyl radical reacted to the sugar 2-deoxy-D-ribose. The product reacts with thiobarbituric acid to generate a chromogen. Any added scavenger of hydroxyl radical will compete with deoxyribose for the hydroxyl radical generated and decrease chromogen formation (Rehman, Whiteman, and Halliwell, 1997). Although mannitol is not anti-free radical substance, it generally employed as a positive control.

Samples were tested in this method were sericin and L-ascorbic acid. The results can be represented in two types. One was the plot of the proportion of absorbance at 532 nm versus the concentration. Linear regression analysis of mannitol was performed in Figure 14. Analysis of the concentration dependence of the competition allows the determination of the second-order rate constant for reaction of the scavenging compound with hydroxyl radical. The coefficient of determination (R^2) was 0.9994. The calibration data was found to be linear with the excellent coefficient of determination. These results indicated that deoxyribose assay

was acceptable for quantitative analysis of anti-free radical activity in the range of study.

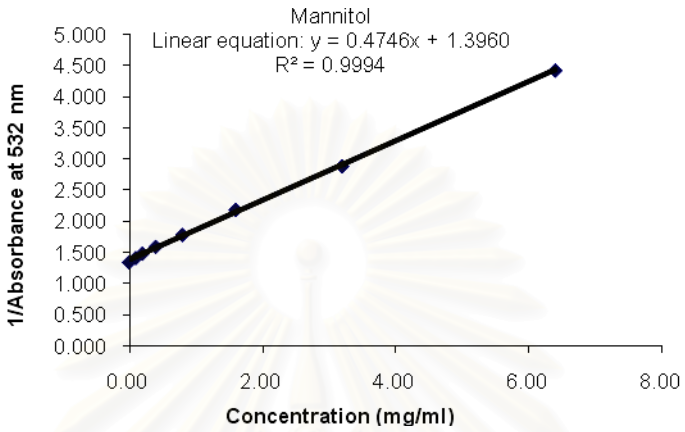


Figure 14. The standard curve of mannitol by deoxyribose assay, n=3.

From the results above, the rate constant of anti-free radical substances could be calculated from the belowed equation (Aruoma, 1989).

$$\frac{1}{A} = \frac{1}{A_0} \times \frac{(1 + k_s[S])}{k_{DR}[DR]} \quad \ldots\ldots(1)$$

$$k_s = \text{slope} \times k_{DR} \times [DR] \times A_0 \quad \ldots\ldots(2)$$

where :

A = absorbance with scavenger at concentration [S]

A_0 = absorbance without scavenger

k_s = rate constant of scavenging of S

k_{DR} = rate constant of degradation of deoxyribose

[S] = concentration of scavenger

[DR] = concentration of deoxyribose

From cited in Aruoma (1989), k_{DR} is 3.1×10^9 $M^{-1}s^{-1}$and the molecular weight of mannitol is 182.18. The slope was from the plot of the proportion of absorbance versus concentrations in molar, shown in Figure 15.

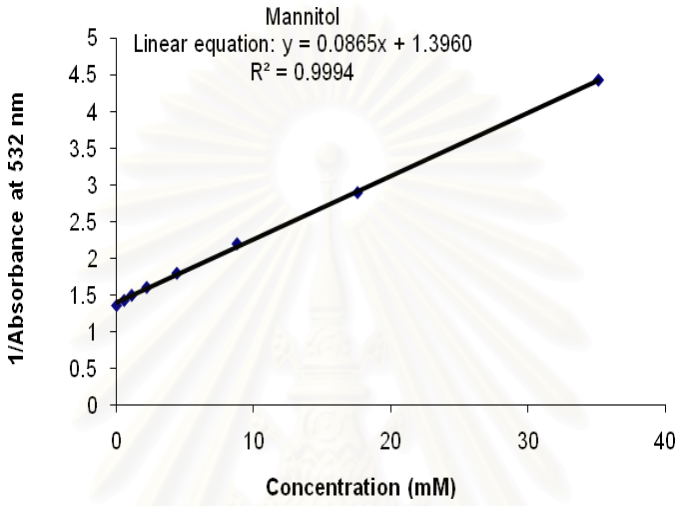


Figure 15. The 1/absorbance versus concentrations plot of sericin by deoxyribose assay, n=3.

From the equation (2), rate constant of mannitol can be observed such below (slope from Figure 15).

$$k_s = 0.0865 \times 3.1 \times 10^9 \times 0.833 \times (1/1.3960)$$

$$k_s = 1.92 \times 10^8 \ M^{-1}s^{-1} \ ; \text{from our test}$$

The value of k_s from calculation was the rate constant of scavenging of mannitol at 0.833 mM deoxyribose when heated 15 min. This finding was close to previous study if calculated the k_s to be at the same condition.

From cited in Rehman, Whiteman, and Halliwell (1997) rate constant is 1.9×10^9 $M^{-1}s^{-1}$ when used 2.8 mM deoxyribose and heated 37 ºC for 60 min. Then we can inversely calculate to obtain slope, then calculate to estimate k_s.

$1.9 \times 10^9 = \text{Slope} \times 3.1 \times 10^9 \times 2.8 \times (1/1.3960)$

$\text{Slope} = 0.3056$

$k_s = 0.3056 \times 3.1 \times 10^9 \times 0.833 \times (1/1.3960) \times 15/60$

$k_s = 1.41 \times 10^8 \ M^{-1}s^{-1}$; calculated from Rehmen et al. (1997)

The estimated value of k_s was close to the rate constant of scavenging of mannitol in this study, which condition was 0.833 mM deoxyribose and heated 15 min. This finding suggested that the initial quantity of deoxyribose and the duration of reaction played the rule to the rate constant of scavenging of anti-free radical substances.

Because the rate constant of mannitol which calculated is near k_s Rehman observed. It was found that the reaction in this study was reliable for detecting scavenging activity or anti-free radical activity of anti-free radical substances.

The result in Figure 16 was shown that the hydroxyl radical scavenging activity was dependent to concentration of sericin only at limited concentration of sericin. The competition was not the second-order rate constant. The anti-free radical activity had a plateau curve for reaction. Although the rate constant of scavenging of sericin can not be calculated for comparing with mannitol, the curve showed the higher slope in 0-0.1 mg/ml of sericin. The results assumed that sericin might have higher k_s, which referred to be a good anti-free radical substance.

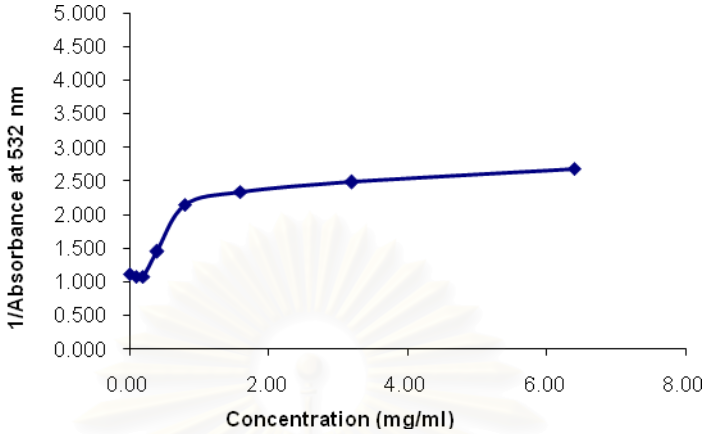


Figure 16. The 1/absorbance versus concentrations plot of sericin by deoxyribose assay, n=3.

The other one for representing the results was the plot of the percentage inhibition of MDA versus the concentrations. Figure 17 shows the percentage of inhibition of MDA by scavenger. IC_{50} of mannitol was approximately 2.6 mg/ml which represented the concentration of scavenger inhibits 50% MDA (Data showed in appendix).

Sericin was found to be good as the anti-free radical substance, which IC_{50} was 0.9 mg/ml (Figure 18). The scavenging activity of sericin was plateau and maximum about 60% at 1.5 mg/ml in approximately. This finding suggested that the higher than 1.5 mg/ml of sericin gave the activity equal to at 1.5 mg/ml. Therefore, it was unnecessary to use higher concentration for using as anti-free radical activity.

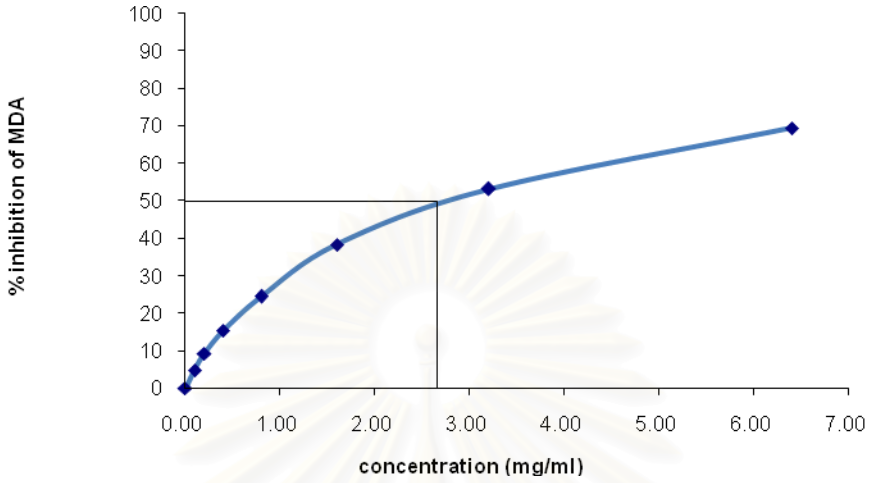


Figure 17. The plot represented the percentage of inhibition of MDA by mannitol, n=3.

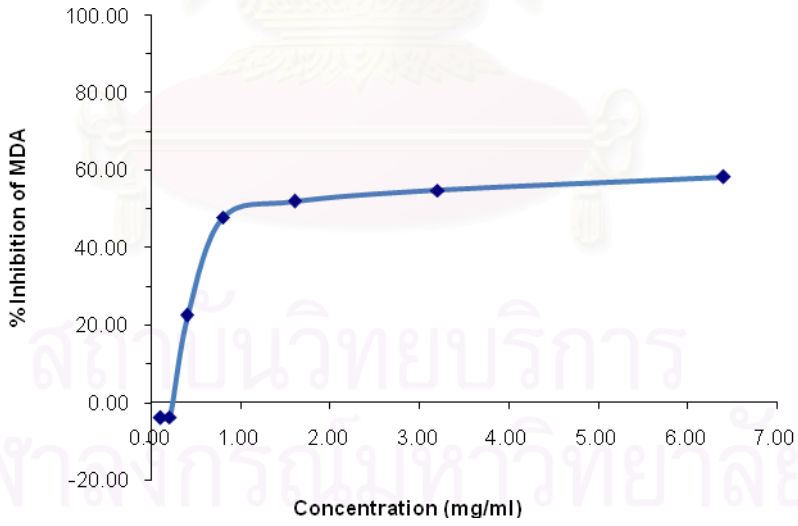


Figure 18. The plot represented the percentage of inhibition of MDA by sericin, n=3.

In Figure 19, at low concentration of L-ascorbic acid showed the pro-oxidant property because it elevated MDA while at high concentration of ascorbic

acid can inhibit the production of MDA (Carr and Frei, 1999; Ivanova and Ivanov, 2000; Aruoma, 2003).

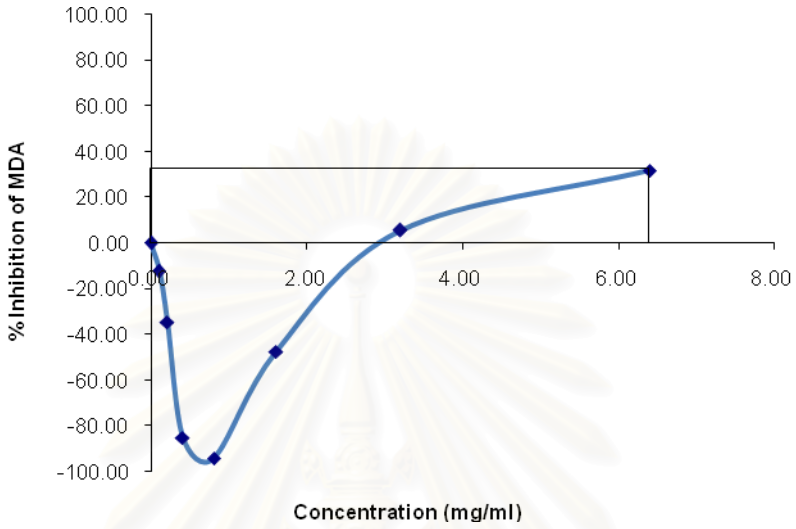


Figure 19. The plot represented the percentage of inhibition of MDA by L-ascorbic acid, n=3.

The absorbance at 532 nm was increased, so the percentage of inhibition of MDA was in negative section. as showed in Figure 19. This finding might take place leading to the reduction of Fe^{3+} by ascorbic acid to be Fe^{2+}. Then, Fe^{2+} could increasingly generate the hydroxyl radical as depicted in Figure 20. Nevertheless, the curve showed that the percentage of inhibition MDA was approximately 37% at 6.4 mg/ml of ascorbic acid. It might assume that its IC_{50} was higher than sericin. Therefore, this result referred that sericin had a stronger anti-free radical activity than ascorbic acid. However, sericin at low concentration exhibited the higher anti-free radical activity in this study.

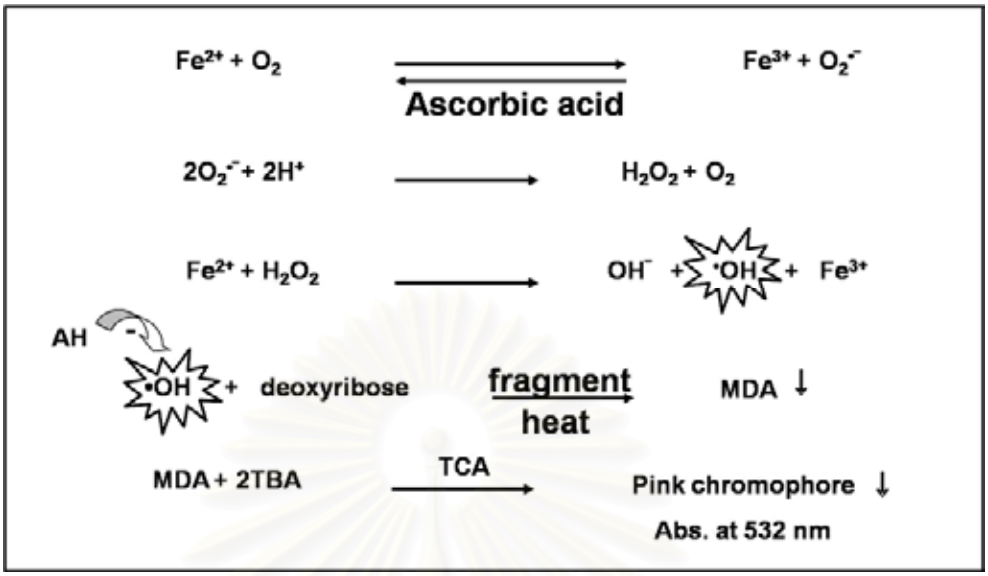


Figure 20. The reaction of deoxyribose assay (modified from Sanchez, 2002).

1.2 Accuracy

Mannitol was prepared at the concentrations of 0.02, 0.08 and 0.32 mg/ml in five sets. Samples were analyzed by deoxyribose assay and finally were detected by UV spectrophotometer. The absorbance at 532 nm of each samples and the proportion of absorbance are shown in Tables 9 and 10, respectively. Moreover, the estimated concentrations and percentages of analytical recovery of each sericin concentrations are shown in Tables 11 and 12, respectively. All percentages of analytical recovery were in the range of 89.09-109.78 %, which indicated the high accuracy of this method. Thus, it could be used for analysis of anti-free radical substances in the range of study.

Table 9. The absorbance of mannitol at 532 nm by deoxyribose assay.

Concentration (mg/ml)	**Absorbance at 532 nm**					**Mean ± SD**
	Set 1	**Set 2**	**Set 3**	**Set 4**	**Set 5**	
0.40	0.6849	0.6899	0.6878	0.6852	0.6842	0.6864 ± 0.002
0.80	0.5897	0.5968	0.5861	0.5849	0.5845	0.5884 ± 0.005
3.20	0.3387	0.3296	0.3326	0.3331	0.3257	0.3319 ± 0.005

Table 10. The 1/absorbance of mannitol at 532 nm by deoxyribose assay.

Concentration (mg/ml)	1/Absorbance at 532 nm					Mean
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.40	1.4601	1.4495	1.4539	1.4594	1.4616	1.4569
0.80	1.6959	1.6757	1.7062	1.7098	1.7109	1.6997
3.20	2.9525	3.0340	3.0066	3.0021	3.0703	3.0131

Table 11. The estimated concentrations of mannitol at 532 nm by deoxyribose assay.

Concentration (mg/ml)	Estimated concentration from standard curve (mg/ml)					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.40	0.3628	0.3413	0.3503	0.3615	0.3658	0.3564 ± 0.010
0.80	0.8411	0.8002	0.8621	0.8694	0.8715	0.8489 ± 0.030
3.20	3.3900	3.5553	3.4998	3.4907	3.6290	3.5130 ± 0.088

Linear equation: $y = 0.4930x + 1.2812$; $R^2 = 0.9973$

Table 12. The percentage of analytical recovery of mannitol at 532 nm by deoxyribose assay.

Concentration (mg/ml)	Percentage of analytical recovery					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.40	90.70	85.34	87.58	90.38	91.46	89.09 ± 2.561
0.80	105.14	100.02	107.76	108.67	108.94	106.11 ± 3.717
3.20	105.94	111.10	109.37	109.08	113.41	109.78 ± 2.754

1.3 Precision

The precision of mannitol by deoxyribose assay was determined both within run and between run as showed in Tables 13 and 14, respectively. Both coefficients of variation values of them were low, as 0.16-1.34% and 2.58-6.47% respectively. The coefficient of variation of the biological analytical method should

generally be less than 15%. Therefore, the deoxyribose assay was precise for quantitative analysis of anti-free radical substances.

Table 13. Data of within run precision of mannitol by deoxyribose assay.

Concentration (mg/ml)	Absorbance at 532 nm					Mean	SD	% CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
0.20	0.7651	0.7600	0.7580	0.7710	0.7582	0.7625	0.006	0.73
0.80	0.6096	0.6089	0.6124	0.5954	0.5962	0.6045	0.008	1.33
3.20	0.3326	0.3317	0.3311	0.3318	0.3317	0.3318	0.001	0.16

Table 14. Data of between run precision of mannitol by deoxyribose assay.

Concentration (mg/ml)	Absorbance at 532 nm					Mean	SD	% CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
0.2	0.6807	0.6863	0.6727	0.7600	0.7710	0.7141	0.047	6.62
0.8	0.5554	0.5533	0.5563	0.6089	0.5954	0.5739	0.026	4.58
3.2	0.3446	0.3476	0.3280	0.3317	0.3318	0.3367	0.009	2.60

From the results had been shown above, the analysis of by deoxyribose assay developed in this study showed good linearity, accuracy and precision. Thus this method was used for the determination of the scavenging capability of anti-free radical substances of sericin.

The reaction of deoxyribose assay should be determined the accuracy of the method when being used in various conditions in preparing deformable liposomes. The study was investigate the percentage of inhibition of MDA by sericin in water, hot water (60 ºC, 1 h), chloroform and phospholipid condition (PC, Span in $CHCl_3$). The results are shown in Table 15 and Figure 21. It was found that the percentage of inhibition of MDA by sericin in phospholipid condition clearly decreased. Statistical analysis used was ANOVA and Turkey's Honesty Significant Difference test at 5% significant level. There was a significant difference of mean of % inhibion of MDA (P<0.05). Sericin in PC condition (Ser_PC) was significant

different from sericin in other conditions such as in H_2O (Ser_H_2O), hot water (Ser_heat) and chloroform (Ser_$CHCl_3$). The result showed that Ser_PC had a lower anti-free radical activity. Additionally, temperature at 60 °C and chloroform did not interfere analysis of anti-free radical activity of sericin. Conversely, phospholipids highly interfered the deoxyribose assay. It seemed that the deoxyribose assay of anti-free radical activity in formulations containing PC should concern about the interfering effect of PC.

Table 15. The percentage of inhibition of 0.5 mg/ml of sericin in various conditions, which may interfere deoxyribose assay, n=3.

Conditions	**% Inhibition of MDA (mean ± SD)**
Ser_H_2O	46.95 ± 4.46
Ser_heat	47.72 ± 4.61
Ser_$CHCl_3$	50.73 ± 6.57
Ser_PC	19.27 ± 10.92*

*****The mean values are statistically different at α=0.05.

The results was similar to the previous report indicating that the antioxidant activity of the extract added in formulations could not be assessd using the deoxyribose assay (Georgetti et al., 2006). It might occur from PC interfered to the development of the reactions. Soybean PC, an unsaturated phospholipid, can initiate the free radical reaction (New, 1990). Oxidation of polyunsaturated fatty acid involves the processes of lipid peroxidation (Camejo, Wallin, and Enojarvi, 1998). Oxidized product may generate thiobarbituric acid reactive substances (TBARS) in system of analysis. The deoxyribose assay developed the reaction assessed the chromogen of TBA-MDA, which also is TBARS (Halliwell and Gutteridge, 1981). Therefore, the increasing of TBARS by PC appears to decrease the percentage of inhibition of MDA.

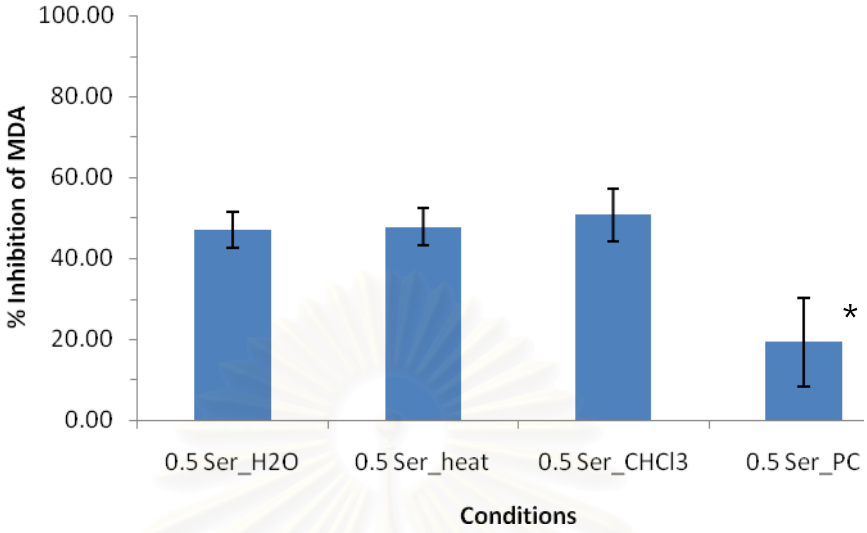


Figure 21. The interference of the percentage of inhibition of sericin in water, heat 60 ºC, chroloform and phospholipids by deoxyribose assay, n=3.

PART C

PREPARATION AND EVALUATION OF DEFORMABLE LIPOSOMES VERIFIED IN COMPOSITIONS

1. Preparation of deformable liposomes

In this study, CL and DL could be prepared by reverse phase evaporation method. Conditions for forming w/o emulsion were the ratio of oil in solvent to aqueous phase at 3:1 and sonication time 5 min in the bath sonicator. Evaporation conditions were 37 °C, 300 mbar for 30 min. After w/o emulsion was evaporated about 10 min, it would dry down to be gel attach to the round bottom glass. Gel collapsed would be occurred about 30 min after starting evaporation.

This stage of preparation had influenced from the concentration of PC. At the high concentration of PC, 3.5%, the gel looked like film attached to the flask. It did not collapse by itself. The vesicles occurred when adding water to hydrate them. It might occur because the concentration of lipid employed was increased, it should be add water at the gel stage (New, 1990). The preparation of DL at 3.5% PC gave large

vesicles, 13.84 ± 0.10 μm diameters determined by DLS. Scanning electron microscopy (SEM) showed that the spherical vesicles were heterogeneous sizes, approximate in range 2-6 μm diameters. The difference of both methods might be from the attachment of vesicles. Figure 22 shows electron micrograph of DLSp20 at 3.5% PC. It was found that it had some attachment of vesicles.

It was noticed that the investigation by dynamic light scattering, the sizes were larger than by SEM. Because of if it had aggregation of vesicles, the light scattering was their packing, not only a particle. Moreover, the finding suggested that the deformable liposomes at 3.5% PC were multilamellar vesicles (MLVs). The reason of this finding could be from the excess lipid was present, so it will be dried down and coated with multiples layers of phospholipid membrane (New, 1990).

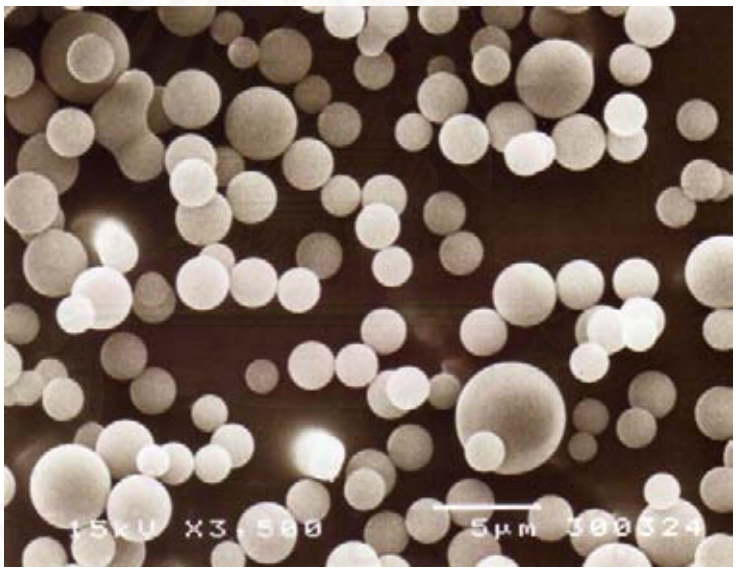


Figure 22. Scanning electron micrograph of DLSp20, PC:Span 80 of 80:20 by weight at 3.5% PC (3,500×).

Since the aim of the study would like to obtain large unilamellar vesicles (LUVs), the concentration of PC would be study for the optimal to gain LUVs. Thus the PC concentration at 2% as reported in the previous study was investigated (New, 1990; Torchillin and Weissig, 2003). It was found that the gel rapidly collapsed

without adding water to hydrate as showed in Figure 23. It might due to the concentration of lipid employed was optimum and the amount of water was sufficient. However, it was unnecessary to add water for collapse, the addition of water to hydrate would help the completion of the formation of liposomes. The preparation of deformable liposomes at 2% PC gave a smaller vesicles, 0.10 ± 0.00 μm diameter determined by DLS, whereas scanning electron microscopy (SEM) could not observed the particles. This results was consistent with that the size of LUVs, which was not more than 1000 nm or 1 μm. The finding suggested that the deformable liposomes at 2% PC were large unilamellar vesicles (LUVs). The reason of this finding could be from the optimum lipid and the volume of water was presented (New, 1990).

Figure 23. Gel collapsed occurred and formed unilamellar vesicles.

After sericin collapsed, it was hydrated at the same temperature without reducing pressure for 150 min. Sericin could be entrapped in the CL and DL. The conditions of preparation of liposomes were crucial for characteristic of vesicles. The reproducibility of vesicles would be another topic, which should be examined. The study of reproducibility of liposomes was done triplicate in conventional formulation. Conventional liposomes (CL) with 2% PC in the formulation was detected their sizes by light scattering. It was found that sizes of three batches were 0.10 ± 0.00, 0.11 ±

0.00 and 0.11 ± 0.00 μm, respectively. This finding demonstrated the good reproducibility of formulation and the preparation method.

Moreover, deformable liposomes, which possessed special property of deformability and could penetrate through the smaller pore size, could be demonstrated by the formulation of DLSp15 that was extruded through 0.2 μm. The results revealed that the DLSp15, which had two peaks of mode diameter- small size and big size. The small sizes were about 0.1 μm and the big sizes were about 10 μm. It was shown that they were able to be extruded through 0.2 μm of polycarbonate membrane. Although, the result demonstrated that mean sizes D (v, 0.5) after extrusion were close, the pattern of peaks were different. The big sizes (10 μm) peak decreased. The vesicles sizes after extrusion were less than 0.78 μm that equaled to 95.26 ±0.12 % (Table 16). It depicted that the vesicles could pass through the 4 times smaller pore size.

The results demonstrated the particles could not all but some deform and pass through 0.2 μm pore sizes. It referred to the vesicles, about 10 μm, were reduced as showed in Figure 24 and 25. Although deformable liposomes could penetrate through 0.2 μm pore size, it could not pass through greater than 4 times smaller pore sizes. It was similar to the previous study that reported the capability of deformable liposomes to pass through 2-5 times smaller pore sizes (Cevc, Schatzlein, and Blume, 1995; Gallarate et al., 2006; Chirio et al., 2007; Mishra et al.,2007).

It might due to deformability of DLSp15, however it might due to the aggregation of vesicles after extrusion. These results could not conclude the deformability of DL, thus it should be investigated and calculated the deformability index to confirm the property of DL related to their elasticity.

Table 16. The percentage of vesicles (DLSp15) in various sizes before and after extrusion.

Sizes range	Before extrusion	After extrusion
	Mean ± SD (%)	
< 0.20 µm	63.61 ± 0.44	82.08 ± 0.58
0.20 - 0.78 µm	3.61 ± 0.15	13.18 ± 0.46
< 0.78 µm	67.21 ± 0.47	95.26 ± 0.12

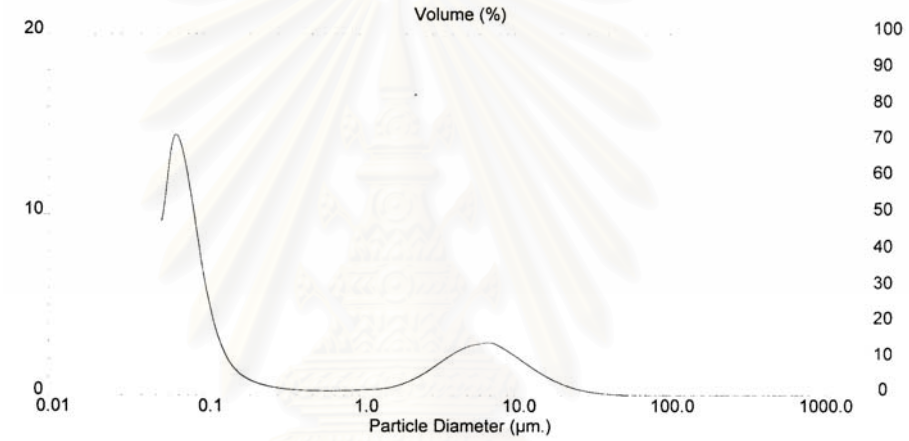


Figure 24. DLSp15 before extrusion determined by dynamic light scattering.

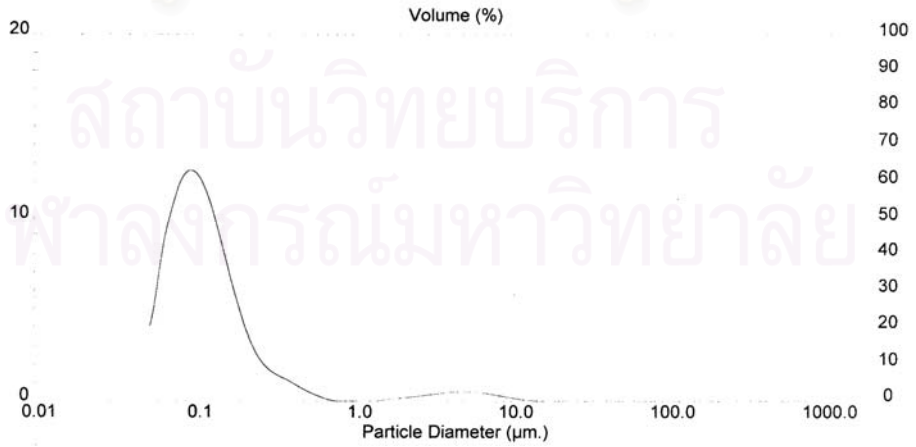


Figure 25. DLSp15 after extrusion pass through 0.2 µm polycarbonate membrane determined by dynamic light scattering.

2. Evaluation of deformable liposomes

This study investigated the effect of formulation such as CL, DL, type of surfactant using as edge activator and the ratio of PC and surfactant. All formulations were investigated in many properties that the results were classified follows:

2.1 Microscopic appearances

Physical examination by light microscopy of all vesicles revealed they were smaller than 1 μm diameter, thus vesicles could not depict the accurate sizes.

2.2 Scanning electron microscopy (SEM)

It was found that scanning electron microscopy (SEM) could not exhibit the image of vesicles. It might occur if the particles were smaller than 300 nm.

2.3 Transmission electron microscopy (TEM)

Since the SEM was not capable to visualize the DL particles, the TEM was applied instead. It was showed that TEM could visualize the images of DL. The DLSp15 particles appeared as round shaped. Some agglomerated vesicles could be observed. Approximate size of DLSp15 was in the range of 230-365 nm, which was close to the sizes determined by DLS (240 nm). Figure 26 showed the electron micrograph of deformable liposomes (DLSp15).

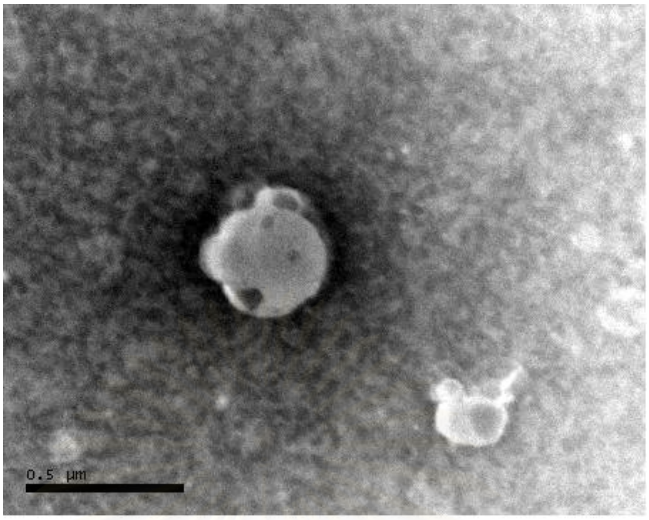


Figure 26. Transmission electron micrograph of DLSp15_Ser.

2.4 Particle size analysis

Conventional liposomes and deformable liposomes prepared in this study were found to have mean vesicle size approximately 200 nm, except DLSp20 was about 400 nm (Table 17). The results showed that size of vesicles were correlated with the method of preparation. Moreover, size increased at the higher concentration of surfactant. It might occur from the partition of surfactant into lipid bilayers (Simoes et al., 2004). However, the difference of size was due to type of surfactant. Span 80 was clearly different, but Tween 80 was little different. It might occur from the different of intercalation of surfactant (Bouwstra, van den Bergh, and Suhonen, 2001). Or it might be from type of surfactant because it was reported the different trend from previous studies (Gallarate et al., 2006; Chirio et al., 2007). Nevertheless, all of them were quite similar in the size distribution. It was observed that it had narrow size distribution at the low concentration of surfactant. This result might be from the higher ratios of surfactant seemed to increase quantity in lipid membrane. In the stage of adding water phase for hydrating vesicles, the higher lipid

phase was hardly complete. Thus the heterogeneous size might occur during hydrating the vesicles.

Table 17. Size and uniformity of various formulations of vesicles by dynamic light scattering.

Formulations	**PC:surfactant (by weight) size (μm) and span**			
	100:0	**90:10**	**85:15**	**80:20**
CL_Ser	0.192, 4.171	-	-	-
DLSp_Ser	-	0.165, 0.482	0.240, 3.088	0.404, 9.787
DLTw_Ser	-	0.167, 0.749	0.188, 3.280	0.185, 2.620

2.5 Entrapment efficiency

The entrapment efficiencies of CL and DL were in range 49.20-102.12% (Table 18). The results showed that the preparation by reverse phase evaporation gave high entrapment efficiency of sericin. The finding was consistent with previous studies that claimed for higher encapsulation efficiency of macromolecules such as antigens and peptides by the same method (New, 1990; Guo, Ping, and Zhang, 2000; Gupta et al., 2005). The entrapment efficiency seemed to be increased in concentration of 10-15% Span 80. And at higher concentration of 20% Span 80, the entrapment efficiency was decreased. This result is consistent with Mishra et al. (2007) that could explained that it due to the possible coexistence of mixed micelles and vesicles at higher concentration of surfactant, which mix micelles had lower entrapment of sericin. Notably, the entrapment efficiency of DLTw was increased in range of 10-20% Tween 80. It might be from it was still in the optimum concentration for forming vesicles. The reason should be investigate for more understanding in the future.

After statistical analysis with one-way ANOVA and Turkey's Honesty Significant Difference test at 5% significant level, there were significant differences between CL and all DL formulations.

Table 18. Entrapment efficiency of CL and DL when storage in refrigerator (4-8 ºC) for 4 weeks.

Rx	PC: surfactant	%EE (SD)				
		wk 0	**wk 1**	**wk 2**	**wk 3**	**wk 4**
CL_Ser	100:0	72.27 (2.18)	61.77 (2.92)	105.85 (0.43)	93.42 (1.64)	104.82 (14.47)
DLSp10_Ser	90:10	71.34 (3.59)	74.32 (0.79)	84.24 (10.43)	68.99 (5.01)	84.98 (13.43)
DLSp15_Ser	85:15	89.84 (2.87)	93.56 (7.32)	81.49 (1.33)	85.69 (8.17)	92.10 (1.84)
DLSp20_Ser	80:20	49.20 (5.27)	84.69 (11.63)	64.26 (6.71)	79.03 (6.98)	93.71 (14.30)
DLTw10_Ser	90:10	76.74 (8.67)	58.97 (9.72)	75.96 (2.00)	81.52 (1.95)	65.32 (10.07)
DLTw15_Ser	85:15	95.66 (7.14)	92.77 (6.76)	61.35 (6.73)	91.75 (3.11)	84.02 (4.06)
DLTw20_Ser	80:20	102.12 (9.32)	85.04 (5.63)	64.79 (3.46)	90.65 (6.20)	90.80 (10.72)

Statistic data showed that different efficiency group, which were higher the entrapment were DLSp15_Ser, DLTw15_Ser and DLTw20_Ser (Table 19). It could be assumed that three formulations were good entrapment. For selection only one formulation, the followed study should be examined other properties such as leakage and deformability.

Table 19. Analysis the entrapment efficiency of formulation by one-way ANOVA and Turkey's HSD.

Tukey HSD

		Subset for alpha = .05			
formulation	N	1	2	3	4
DLSp20_Ser	3	49.2033			
DLSp10_Ser	3		71.3400		
CL_Ser	3		72.2733		
DLTw10_Ser	3		76.7400	76.7400	
DLSp15_Ser	3			89.8400	89.8400
DLTw15_Ser	3				95.6567
DLTw20_Ser	3				102.1167
Sig.		1.000	.927	.198	.254

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

2.6 Leakage

In this study, the leakage of the DL, which correlated with the ability of entrapment of sericin, was investigated. The leakage assumed from the ability of retaining the entrapped sericin. The leakage of vesicles was due to the arrangement of surfactant was not appropriate. Because of there were many types of the intercalation of surfactant in lipid membranes (Bouwstra, van den Bergh, and Suhonen, 2001). And there were the reports about the effects of types and ratios of PC:surfactant on the cabability of entrapment of DL (El Maghraby, William, and Barry, 2000a; Mishra et al., 2007). The data was calculated from entrapment efficiency at 0, 1, 2, 3, and 4 weeks. The results of entrapment efficiency of all formulations were showed in Table 18. Unfortunately, the fluctuations of entrapment efficiency was noted, thus the conclusion can be hardly stated. It might occur from the variation during sampling for determined the entrapment efficiency by ultracentrifugation.

From the selection of the highest entrapment group, the entrapment efficiencies of DLSp15_Ser, DLTw15_Ser and DLTw20_Ser during study of the leakage were evaluated by one-ANOVA at 5% significant level.

The comparisons with in each formulation were shown in Table 20, 21 and 22. It was found that the entrapment efficiencies of DLSp15_Ser during storage 4 weeks were non-significant different. Conversely, DLTw15_Ser and DLTw20_Ser were significant different (P<0.05). The results showed that it was the good entrapment efficiency of DLSp15_Ser after storage in refrigerator for 4 weeks. Then, it was a rational for selection this formulation to contain in serum. Because of DLSp15_Ser seemed to be the best formulation from high entrapment and low leakage. It might occur from the arrangement of surfactant in lipid bilayers was tightly fitted and appropriated, thus limited the leakage of sericin from the lipid bilayers of vesicles.

Table 20. Analysis the entrapment efficiency of DLSp15_Ser by one-way ANOVA during storage 4 weeks in refrigerator.

ANOVA

Sp15_EElk

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	292.678	4	73.169	2.738	.089
Within Groups	267.217	10	26.722		
Total	559.895	14			

Table 21. Analysis the entrapment efficiency of DLTw15_Ser by one-way ANOVA during storage 4 weeks in refrigerator.

ANOVA

Tw15_EElk

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2339.799	4	584.950	17.385	.000
Within Groups	336.473	10	33.647		
Total	2676.272	14			

Table 22. Analysis the entrapment efficiency of DLTw20_Ser by one-way ANOVA during storage 4 weeks in refrigerator.

ANOVA

Tw20_EElk

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2258.551	4	564.638	9.937	.002
Within Groups	568.189	10	56.819		
Total	2826.740	14			

2.7 Deformability index

Another one topic, which was crucial for preparation of DL, was deformability. Deformability index was shown in Table 23. In the present study the deformability of both CLchol_Ser and CL_Ser were compared with DL. From the results, CLchol_Ser could not pass through pores smaller than its diameters showing no deformability. Incontrast, CL_Ser could pass, but in the lower rate than DL. The result might occur from increasing rigidity of cholesterol in liposomes (Benson, 2005; Gupta et al., 2005). In support of Hiruta et al. (2006), they conclude that addition of lipophilic compound, such as cholesterol, tended to decrease the elasticity.

Table 23. Deformability index of CL and DL.

Rx	PC: Surfactant (or Chol)	Sizes before (nm)	Sizes after (nm)	Deform-ability index
CLchol_Ser	7:3	254±14	0±0	0.00±0.00
CL_Ser	100:0	523±28	162±9	0.21±0.08
DLSp10_Ser	90:10	217±4	200±10	2.74±0.34
DLSp15_Ser	85:15	355±52	332±14	7.20±0.78
DLSp20_Ser	80:20	350±20	287±64	6.79±0.88
DLTw10_Ser	90:10	260±2	161±0	2.58±0.22
DLTw15_Ser	85:15	192±2	27±10	0.01±0.00
DLTw20_Ser	80:20	251±8	68±29	0.05±0.00

Values represent Mean ± SD

Moreover, this finding reported that deformable liposomes had elasticity comparing with CL. DL could pass through pore smaller than itself. The best formulation selected above, DLSp15_Ser, also had the highest deformability index. Thus, it might assume that PC:Span 80 (85:15) was the optimum formulation of loaded sericin. These results indicated that the elasticity of vesicles depended on both PC:surfactant ratio and type of surfactant. This finding agreed with the previous study by Mishra et al. (2007). They reported that increasing the level of Span 80 in vesicles brought no advantages. The high concentration of surfactant decreased the elasticity of liposomes. Although Tween 80 was used as edge activator at optimum concentration approximately 15% in many research, this study discovered that it had no deformability when using more than 10%. Therefore, these results showed that type of surfactant and PC:surfactant ratios affected to the deformability (El Maghraby, Williams, and Barry, 2000; Mishra, et al. 2007). Biju et al. (2006) reported that the concentration of surfactant was crucial in the formation of DL because at sublytic concentration, it provided flexibility to vesicles and at the higher concentration, it caused destruction of vesicles. Nevertheless, Mishra et al. (2007) indicated that high concentration of surfactant could be concern about vesicular solubilization of liposomes to mixed micelles which are less deformable and decreased skin permeation.

PART D

FORMULATION OF SERUM OR DISPERSION SYSTEM CONTAINING DEFORMABLE LIPOSOMES AND EVALUATION OF PHYSICAL STABILITY

1. Formulation of dispersion systems

From the preliminary study, the concentration of viscosity enhancer were compared such as 1-4% chitosan, 15-30% Poloxamer 407 and 0.5-1% Carbopol ultrez-10. It was determined by physical appearance of serum after mixing with liposomes at 1:1 ratio. The optimum concentrations of viscosity enhancers were 3% chitosan, 20% Poloxamer 407 and 1% Carbopol ultrez-10. The proposed formulation,

serum, which was defined as a dispersion system that should be as viscous liquids, which it was not too condense and should be as a drop after pumping from the bottle. The present study had only evaluated the optimum by visual observation. Afterwards, it was mixed with DLSp15_Ser in the next topic for selecting the best formulation.

2. Preparation of deformable liposomes in dispersion systems

The optimum formulation of deformable liposomes, DLSp15_Ser was mixed with dispersion systems with 1:1 mixing ratio. This might be named as serum. The final concentrations of gelling agent in serum were 1.5% chitosan, 10% Poloxamer 407 or 0.5% Carbopol ultrez-10. The physical characteristics of serum were shown in Table 24.

Table 24. Physical characteristics of serum at room temperature.

	Gel bases for mixing with DLSp15_Ser		
	Carbopol ultrez-10	**Poloxamer 407**	**Chitosan**
Color	yellowish	yellowish	yellowish
Clarity	+++	++++	++
Odor	-	-	Pungent odor
Spreadability	++++	+++	+
Smoothness	++++	+++	+
pH	6.75 ± 0.04	8.84 ± 0.02	5.70 ± 0.20
Viscosity	++++	+	++++

where,

- \- = not being that characteristics
- \+ = little
- ++ = moderate
- +++ = much
- ++++ = very much

The results showed that chitosan was not appropriate for using as gelling agent for formulation of the serum. Because it had a pungent odor of acid and had a sticky appearance, looked like a rubber after storage for 1 week. Consequently, Carbopol ultrez-10 and Poloxamer 407 were the gelling agent which gave good physical appearances. But it also should be evaluated the results of their stability.

3. Evaluation of physical stability

After passing the heating-cooling 6 cycles, it was found that the system of Carbopol ultrez-10 was the best formulation in physical appearances. It was found that sedimentation of DL was found in the serum prepared from Poloxamer 407 (Figure 27). Turbidity and yellowish particles were settling down. This result might be occurred from the thermoreversibility of Poloxamer 407 that forming gel upon the rising of the temperature. When Poloxamer 407 was in the refrigerator, cooling, it became sol and had less viscosity, and became gel when it was heated. Thus, sedimentation of DL was observed. In contrast, Carbopol ultrez-10 was higher viscosity and had no thermoreversible property. Thus, Carbopol ultrez-10 had the stable formulation for containing DLSp15_Ser.

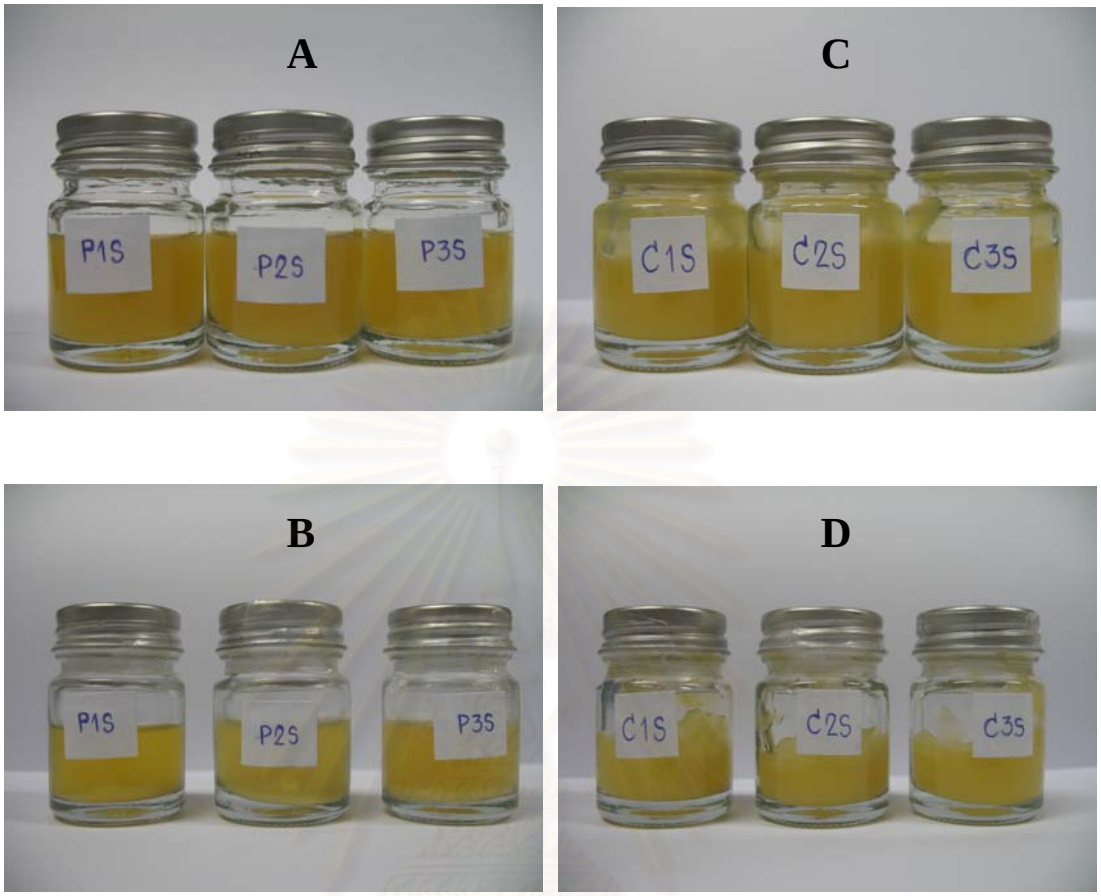


Figure 27. DLSp15_Ser in various gelling agents both before and after passing the heating-cooling 6 cycles; Poloxamer 407 before (A), Poloxamer 407 after (B), Carbopol ultrez-10 before (C) and Carbopol ultrez-10 after (D), (which P: Poloxamer 407, C: Carbopol ultrez-10, no. 1-3: n, and S: sericin loaded), n=3.

PART E

DETECTION OF ANTI-FREE RADICAL ACTIVITY OF DISPERSION SYSTEM CONTAINING DEFORMABLE LIPOSOMES LOADED WITH SERICIN

From the study of accuracy of deoxyribose assay in determining anti-free radical activity, it demonstrated that the method was not adequate to evaluate the activity of the formulation containing PC. The interference of formulation component, PC affected the processes of the reactions of deoxyribose assay. Thus, the anti-free radical of the formulation of serum containing deformable liposomes

loaded with sericin assumed by detection of the activity of sericin in conditions of preparing deformable by reverse phase evaporation as showed in Figure 21 (Part B).

PART F

SKIN PERMEATION STUDY OF DEFORMABLE LIPOSOMES LOADED WITH SERICIN COMPARING WITH SERICIN IN OTHER SYSTEMS

The permeation of sericin from different systems is depicted in Figures 28 and 29. The plot showed the relation between cumulative amount of sericin permeated through abdominal skin of a newborn pig versus time for 24 h. It exhibited that sericin solution could not permeated through skin. The result from the present study was consistent to the previous study reported by Gupta et al. (2005). It concerned about molecular mass of bioactive molecules which normally could not permeate across the skin if it was larger than 500 Da. From the Figure 28, the cumulative amount of sericin solution could not be detected.

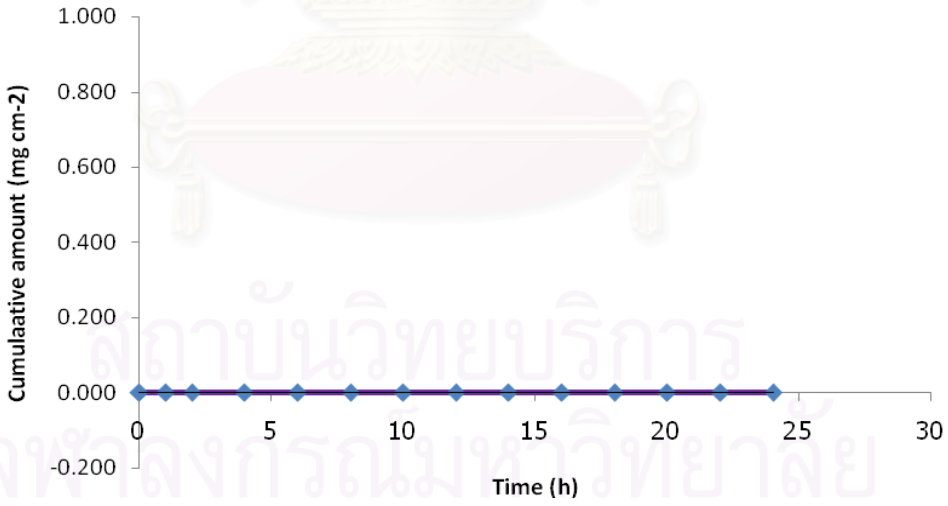


Figure 28. Cumulative amount of sericin from sericin solution in permeation study in vitro.

The comparative results of DL and CL are shown in Figure 29. This finding demonstrated that the plot of cumulative amount of sericin versus time from both CL and DL had a linear profile. It was noticed that permeability of sericin from DL and

CL were close. This finding demonstrated that CL and DL could enhance the permeation of sericin. The discovered result might be attributed from the mechanism of penetration of vesicles. Elsayed et al. (2006) revealed that DL could improve skin delivery of drug by two mechanisms. First, drug carrier or intact vesicles concerned with deformability of DL and hydration gradient. Second, the penetration enhancer involved to the modification of lipid lamellae, which this mechanism also explained the skin delivery of CL.

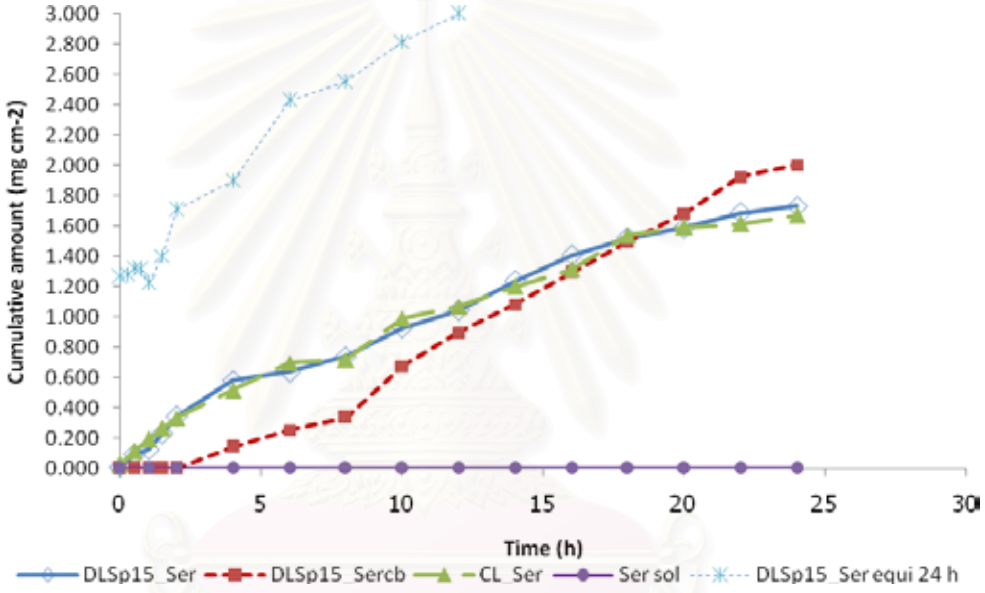


Figure 29. Cumulative amount of sericin from various formulations in permeation study in vitro.

For further comparing and understanding the membrane permeability of these formulations, the membrane permeability coefficients were calculated. These coefficients were calculated from the slope, which obtained from the linear plot as in Figures 30. The slopes were divided by the concentration in the donor phase (C_d) (Sinko, 2006).

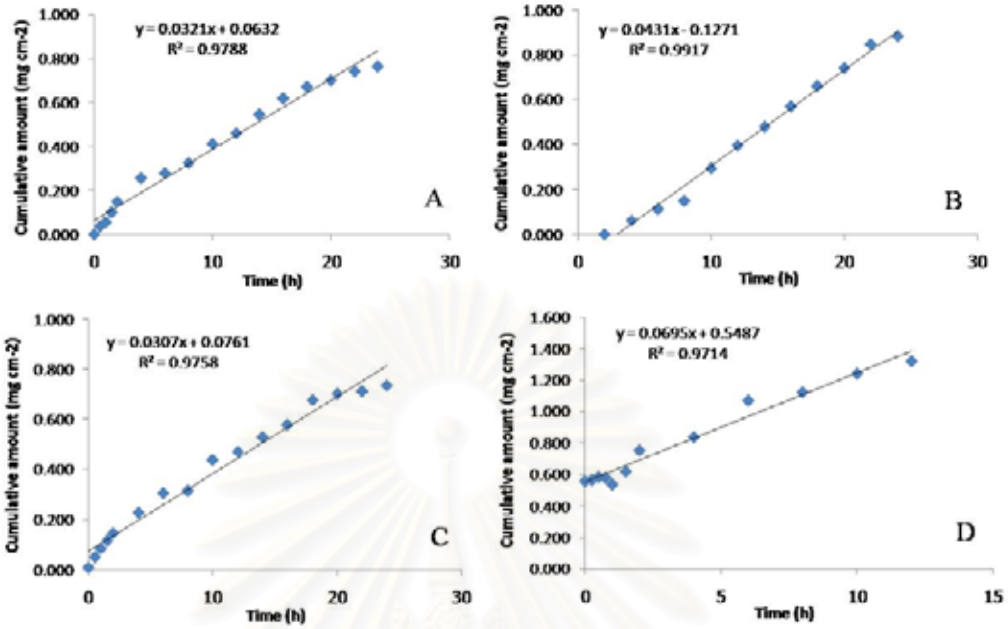


Figure 30. Cumulative amount of sericin from sericin in various systems in permeation study in vitro for obtained slope, key; A: DLSp15_Ser, B: DLSp15_Sercb, C: CL_Ser, D: DLSp15_Ser equi 24 h.

Table 25 shows the membrane permeability coefficients (P) of various formulations. From the results, the permeability of DL was non-significantly higher than CL. The permeability coefficients were closed. It might occur from their penetration enhancer mechanism (El Maghraby, Williams, and Barry, 2004; Elsayed et al., 2006). This mechanism was penetration enhancers by modifying the intercellular lipid lamellar (El Maghraby, Williams, and Barry, 2004; Elsayed et al., 2006). Because of the surfactant that was used as edge activator may disturbing the arrangement of lipid membrane. And soybean lecithin had T_m about -15 ± 5 ºC, thus at the body temperature vesicles was melted and fused with lipid membrane (Benson, 2005).

Another mechanism as drug carrier was still occurred, but it might be less than penetration enhancer effect. It was clearly depicted from the permeability of the intact vesicles of DLSp15_Sercb, which was DL in serum. The permeability coefficient of DLSp15_Sercb was significant different from DL without loading in gel and CL (P<0.05) (Data was shown in Appendix).

In the part of DLSp15_Sercb, which was DL dispersing in Carbopol ultrez-10 dispersion, showed its lag time at 3 h. It might be attributed that at the initial stage the sericin needed the time for diffusing or releasing from the serum. Moreover, the result showed that DLSp15_Sercb was slower permeated at the initial stage at first 16 h. After 16 h of study, trend of permeation of DLSp15_Sercb was increased. It might occur from the suspension of DLSp15_Ser was faster evaporated at the initial stage. Contrast with DLSp15_Sercb, the Carbopol ultrez-10 became dried or dehydrated after 16 h and hence enhanced the hydration gradient. Therefore, the permeation of DLSp15_Sercb was increased after loading samples for 16 h. In this stage vesicles might be delivery from the drug carrier or intact vesicles mechanism as claimed for DL. This mechanism explained about the intact vesicles could enter the stratum corneum. This mechanism depends on the transcutaneous hydration gradient, which many researches referred to non-occlusion application (Barry, 2001; El Maghraby, Williams, and Barry, 2001a; Elsayed et al., 2006). DL was penetrated through pores and followed the hydration gradient.

Table 25. The membrane permeability coefficients of various formulation of sericin.

formulation	slope	C_d (mg/ml)	P (cm/hr)	P (cm/sec)
Ser sol	0	25.0	0	0
DLSp15_Ser	0.0321	25.4	1.26×10^{-3}	3.51×10^{-7}
DLSp15_Sercb	0.0431	13.5	3.19×10^{-3}	$8.87 \times 10^{-7*}$
CL_ser	0.0307	26.7	1.15×10^{-3}	3.20×10^{-7}
DLSp15_Ser equi 24 h**	0.0695	25.4	2.73×10^{-3}	7.59×10^{-7}

All experiments were investigated in triplicate (n=3), except DLSp15_Ser equi 24 (n=1)

*The mean values are statistically different at α=0.05.

**not included in statistical analysis

The method of other research involving investigation the mechanism of intact vesicles, it was equilibrated skin for long period about 24 h (El Maghraby, William, Barry, 2001b; Dubey et al., 2006; Elsayed et al., 2006; Bendas and Tadros, 2007). Therefore the effect of dehydration gradient of the skin on the permeation was studied by dehydrating an abdominal newborn pig skin for 24 h before loading samples. It was found that DLSp15_Ser equi 24 h had the higher membrane permeability

coefficient. It might occur from the hydration gradient. When the skin was left exposed to air for 24 h, it had more hydration gradient between upper and lower part of skin. DL avoided dehydration, thus it penetrated through deeper tissues via pores in the stratum corneum (Benson, 2005). Other part of this study was equilibrated membrane only one hour for keeping viability of skin. When comparing DLSp15 by different equilibration time, they were different (El Maghraby, Williams, and Barry, 2001a). There was a biological change of the skin. Due to the smell exerted in the solution, that it might be occurred from tissue degeneration. Then the study was discontinued to 24 h.

Therefore, DL might deliver sericin by synergistic mechanism of penetration enhancer and intact vesicles. Then, the further studies the evaluation for specific the mechanism of DL and CL and the influence of hydration gradient should be performed.

CHAPTER V

CONCLUSIONS

The present study was to investigate anti-free radical activity of sericin from Thai silk. The new delivery system as deformable liposomes was applied for silk sericin. The outcome of the investigation can be concluded as follows.

1. The investigation of the anti-free radical activity of silk sericin by deoxyribose assay reveals that sericin is capable to inhibit hydroxyl radical. The IC_{50} is approximately 0.9 mg/ml. The percentage of inhibition of MDA by sericin is high in comparison with L-ascorbic acid at the same concentration.

2. Deformable liposomes can be prepared by reverse phase evaporation and collapse by themselves at the concentration of 2% of soybean phospholipid. Most formulations from this method have high entrapment efficiency of above 70%, exception DLSp20. The composition of PC:Span 80 equals 85:15 (DLSp15), is the optimum preparation conditions of deformable liposomes. This formulation seems to be the best formulation for loading sericin because it exhibits advantages than others in topics of entrapment efficiency, stability and deformability. Moreover, selected deformable liposomes are LUVs, which their sizes are quite small as 240 nm.

3. The formulation of serum suggests that 0.5% of carbopol is the optimum and appropriate in using as viscosity enhancer

4. The percentage of inhibition of MDA can not be observed directly by deoxyribose assay. The applied method gives the comment that the condition of reverse phase evaporation did not destroy the anti-free radical activity of sericin.

5. The vesicles of deformable liposomes and conventional liposomes can enhance the skin penetration of sericin through pig skin comparing with sericin solution which no penetration. Moreover, deformable liposomes loaded in serum has lag time of 3 h, but it has the higher permeability coefficient than DL and CL.

However, the further studies regarding other analytical method for detection activity of inhibition all of reactive oxygen species (ROS), the mechanism of delivery and the influence of transdermal gradient should be performed.

REFERENCES

Thai

โมโตอิ มินากาวะ, อีอีชิ คาวาอิ และเข็มชัย เหมะจันทร. 2530. วิทยาการไหม เล่ม 1. คณะกรรมการส่งเสริมสินค้าไหมไทย กรมส่งเสริมอุตสาหกรรม.

โคโซ ทสึโมยูชิ. 2000. วัสดุแต่งบาดแผล ซึ่งประกอบด้วยไหมไฟโบรอินและซีริซินเป็นส่วนประกอบหลัก และวิธีการสำหรับการเตรียมสิ่งเดียวกัน. Patents of Thailand No. 37266 [Online]. Avaliable from: http://www.ipic.moc.go.th/ [2006, April 21].

English

Arpornpattanapong, S. 1996. Preparation of liposomes containing protein extract from *Pasteurella multocida*. Master's Thesis, Graduate School, Chulalongkorn University.

Aruoma, O. I., Laughton, M., and Halliwell, B. 1989. Carnosine, homocarnosine and anserine: Could they act as antioxidants in vivo?. Biochemistry Journal 264: 863-869.

Aruoma, O. I. 2003. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutation Research 523-524: 9-20.

Barry, B. W. 2001. Novel mechanisms and devices to enable successful transdermal drug delivery. European Journal of Pharmaceutical Sciences 14: 101-114.

Bendas, E. R., and Tadros, M. I. 2007. Enhanced transdermal delivery of salbutamol sulfate via ethosomes. American Association of Pharmaceutical Scientists Technology 8(4), article 107: 1-8.

Benson, H. A. E. 2005. Transdermal drug delivery: Penetration enhancement techniques. Current Drug Delivery 2: 23-33.

Biju, S. S., Sushama, T., Mishra P.R., Khar, R. K. 2006. Vesicular system: An overview. Indian Journal of Pharmaceutical Sciences 68: 141-153.

Bouwstra, J. A., van den Berge, B. A. I., and Suhonen, M. 2001. Topical Application of drugs: Mechanisms involved in chemical enhancement. Journal of Receptor & Signal Transduction Research 21(2&3): 259-286.

Camejo, G., Wallin, B., and Enojarvi, M. 1998. Analysis of oxidation and antioxidants using microtiter plates. In Donald Armstrong ed., Free radical and antioxidant protocols, pp.377-378. New Jersey: Humana Press.

Carr, A., and Frei, B. 1999. Does vitamin C act as a pro-oxidant under physiological conditions?. Journal of the Federation of American Societies for Experimental Biology 13: 1007-1023.

Carstensen, J. T. 1990. Drug stability principle and pratices. Vol. 43. New York: Marcel Dekker, pp. 425-426.

Cevc, G., Schatzlein, A., and Blume, G. 1995. Transdermal drug carriers: Basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides. Journal of Controlled Release 36: 3-16.

Cevc, G., and Blume, G. 2003. Biological activity and characteristics of triamcinolone-acetonide formulated with the self- regulating drug carriers, Transfersomes®. Biochimica et Biophysica Acta 1614(2): 156-164.

Chirio, D., Cavalli, R., Trotta, F., Carlotti, M. E., and Trotta, M. 2007. Deformable liposomes containing alkylcarbonates of Y- cyclodextrins for dermal applications. Journal of Inclusion Phenomena Macrocyclic Chemistry 57: 645-649.

Cho, K. Y., Moon, J. Y., Lee, Y. W., Lee, K. G., Yeo, J. H., Kweon, H. Y., Kim, K. H., and Cho, C. S. 2003. Preparation of self-assembled silk sericin nanoparticles. International Journal of Biological Macromolecules 32: 36-42.

Curtin, J. F., Donovan, M., and Cottet, T.G. 2002. Regulation and measurement of oxidative stress in apoptosis. Journal of Immunological Methods 265: 49-72.

Dubey, V., Mishra, D., Asthana, A., and Jain, K. N. 2006. Transdermal delivery of a pineal hormone: Melatonin via elastic liposomes. Biomaterials 27: 3491-3496.

El Maghraby, G. M. M., Williams, A. C., and Barry, B. W. 1999. Skin delivery of oestradiol from deformable and traditional liposomes: Mechanistic studies. Journal of Pharmacology 51: 1123-1134.

El Maghraby, G. M. M., Williams, A. C., and Barry, B. W. 2000a. Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration. International Journal of Pharmaceutics 196: 63-74.

El Maghraby, G. M. M., Williams, A. C., and Barry, B. W. 2000b. Skin delivery of oestradiol from lipid vesicles: importance of liposome structure. International Journal of Pharmaceutics 204: 159-169.

El Maghraby, G. M. M., Williams, A. C., and Barry, B. W. 2001a. Skin hydration and possible shunt route penetration in controlled estradiol delivery from ultradeformable and standard liposomes. Journal of Pharmacy and Pharmacology 53: 1311-1322.

El Maghraby, G. M. M., Williams, A. C., and Barry, B. W. 2001b. Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in vitro. Journal of Pharmacy and Pharmacology 53:1069-1077.

El Maghraby, G. M. M., Williams, A. C., and Barry, B. W. 2004. Interactions of surfactants (edge activators) and skin penetration enhancers with liposomes. International Journal of Pharmaceutics. 276: 143-161.

Elsayed, M. M. A., Abdallah, O. Y., Naggar, V. F., and Khalafallah, N. M. 2006. Deformable liposomes and ethosomes: Mechanism of enhanced skin delivery. International Journal of Pharmaceutics 332: 60-66.

Elsayed, M. M. A., Abdallah, O. Y., Naggar, V. F., and Khalafallah, N. M. 2007. Lipid vesicles for skin delivery of drugs: Reviewing three decades of research. International Journal of Pharmaceutics 332: 1-16.

EMEA. 1995. ICH Topic Q 2 (R1) Validation of analytical procedures: Text and methodology [Online]. Available from: http://www.emea.eu.int [2007, September 10].

EMEA. 1999. ICH Topic Q 6 B Specifications: Test Procedures and acceptance criteria for biotechnological/biological products [Online]. Available from: http://www.emea.eu.int [2007, September 10].

Fabiani, C., Pizzichini, M., Spadoni, M., and Zeddita, G. 1996. Treatment of waste water from silk degumming processes for protein recovery and water reuse. Desalination 105: 1-9.

Fang, Y.Z., Yang, S., and Wu, G. 2002. Free radicals, antioxidant, and nutrition. Nutrition 18: 872-879.

Freddi, G., Mossotti, R. and Innocenti, R. 2003. Degumming of silk fabric with several proteases. Journal of Biotechology 106: 101-112.

Gallarate, M., Chirio, D., Trotta, M., Carlotti, M. E. 2006. Deformable liposomes as topical formulations containing α-Tocopherol. Journal of Dispersion Science and Technology 27: 703-713.

Georgetti, S. R., Casagrande, R., Vincentini, F. T. M. C., Verri, W. A. Jr., and Fonseca, M. J. V. 2006. Evaluation of the antioxidant activity of soybean extract by different in vitro methods and investigation of this activity after its incorporation in topical formulations. European Journal of Phamaceutics 64 99-106.

Guo, J., Ping, Q., and Zhang, L. 2000. Transdermal delivery of insulin in mice by using lecithin vesicle as a carrier. Drug Delivery 7: 113-116.

Gupta, P. N., Mishra, V., Rawat, A., Dubey, P., Mahor, S., Jain, S., Chatterji, D. P., and Vyas, S. P. 2005. Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: A comparative study. International Journal of Pharmaceutics 293: 73-82.

Halliwell, B., and Gutteridge, J. M. C. 1981. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts. Federation of European Biochemical Societies Letters 128(2): 347-351.

Halliwell, B. 1997. Antioxidants: The basic-what they are and how to evaluate them. In Helmet Sies (ed.), Antioxidants in disease mechanisms and therapy, pp. 3-21. San Diego: Academic Press.

Hiruta, Y., Hattori, Y., Kawano, K., Obata, Y., and Maitani, Y. 2006. Novel ultra-deformable vesicles entrapped with bleomycin and enhanced to penetrate rat skin. Journal of Controlled Release 113 146-154.

Hofer, C., van Randenborgh, H., Lehmer, A., Hartung, R., and Breul, J. 2004. Transcutaneous IL-2 uptake mediated by Transfersomes® depends on concentration and fractionated application. Cytokine 25: 141-146.

Itoh, T., Magavi, R., Casady, R. L., Nishihata, T., and Rytting, J. H. 1990. A method to predict the percutaneous permeability of various componds: Shed snake skin as a model membrane. Pharmaceutical Research 7(12): 1302-1306.

Ivanova, E., and Ivanov, B. 2000. Mechanism of the extracellular antioxidant defend. Experimental pathology and parasitology 4:49-59.

Jacobi, U., Kaiser, M., Toll, R., Mangelsdorf, S., Audring, H., Otberg, N., Sterry, W., and Lademann, J. 2007. Porcine ear skin: An invite model for human skin. Skin Research and Technology 13: 19-24.

Kato, N., Sato, S., Yamanaka, A., Yamada, H., Fuwa, N., and Nomura, M. 1998. Inhibits lipid peroxidation and tyrosinase activity. Bioscience, Biotechnology, and Biochemistry 62(1): 145-147.

Kurioka, A., Kurioka, F. and Yamazaki, M. 2004. Characterization of sericin powder prepared from citric acid-degraded sericin polypeptides of the silkworm, *Bombyx Mori*. Bioscience, Biotechnology, and Biochemistry 68(4): 774-780.

Lamoolphak, W. 2005. Production of protein solution from silk waste by subcritical water hydrolysis for preparation of sericin and fibroin microparticles. Master's Thesis, Faculty of Engineering, Chulalongkorn University.

Lee, E. H., Kim, A., Oh, Y. K., and Kim, C. K. 2005. Effect of edge activators on the formation and transfection efficiency of ultradeformable liposomes. Biomaterials 26: 205-210.

Mcanalley, S., Koepke, M., Le, L., Nennum, E., RAC, and Mcanalley, B. 2003. In vitro methods for testing antioxidant potential: A review. Glycoscience & Nutrition 4(2): 1-9.

Mishra, D., Garg, M., Dubey, V., Jain, S., and Jain, N. K. 2007. Elastic liposomes mediated transdermal delivery of an anti-hypertensive agent: Propranolol hydrochloride. Journal of Pharmaceutical Sciences, 96(1): 145-155.

New, R.C.C. 1990. Liposomes: A practical approach. United States: Oxford University Press.

Padamwar, M. N., Pawar, A. P., Daithankar, A. V. and Mahadik, K. R. 2005. Silk sericin as a moisturizer: an in vivo study. Journal of Cosmetic Dermatology 4: 250-257.

Paul, A., Cevc, G., and Bachhawat, B. K. 1998. Transdermal immunization with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes. Vaccine 16 (2/3): 188-195.

Peltonen, L., Aitta, J., Hyvonen, S., Karjalainen, M., and Hirvonen, J. 2004. Improved entrapment efficiency of hydrophilic drug substance during nanoprecipitation of poly(l)lactide nanoparticles. American Association of Pharmaceutical Scientists Technology 5(1): article 16.

Rehman, A., Whiteman, M., and Halliwell, B. 1997. Scavenging of hydroxyl radicals but not of peroxynitite by inhibitors and substrates of nitric oxide syntheses. British Journal of Pharmacology 122: 1702-1706.

Rigg, P. C., and Barry, B. W. 1990. Shed snake skin and hairless mouse skin as model membranes for human skin during permeation studies. Journal of Investigative Dermatology 94: 235-240.

Sanchez, M. 2002. Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Science and Technology International 8(3): 121-137.

Sarovat, S. 2003. The use of sericin as an antioxidant and antimicrobial for polluted air treatment. Master's Thesis, Graduate School, Chulalongkorn University.

Simoes, S. I., Marques, C. M., Cruz, M. E. M., Cevc, G., and Martin, M. B. F. 2004. The effect of cholate on solubilisation and permeability of simple and protein-loaded phosphatidylcholine/sodium cholate mixed aggregates designed to mediate transdermal delivery of macromolecules. European Journal of Pharmaceutics and Biopharmaceutics 58: 509-519.

Simoes, S. I., Tapadas, J. M., Marques, C. M., Cruz, M. E. M., Martin, M. B. F., and Cevc, G. 2004. Permeabilisation and solubilisation of soybean phosphatidylcholine bilayer, as membrane models, by polysorbate, Tween 80. European Journal of Pharmaceutics and Biopharmaceutics 26: 307-317.

Sinko, P. J. 2006. Martin's physical pharmacy and pharmaceutical sciences 5 th ed. Philadelphia: Lippincott Williams & Wilkins.

Song, Y. K., and Kim, C. K. 2006. Topical delivery of low-molecular-weight heparin with surface-charged flexible liposomes. Biomaterials 27: 271-280.

Terada, S., Nishimura, T., Sasaki, M., Yamada, H., and Miki, M. 2002. Sericin, a protein derived from silkworms, accelerates the proliferation of several mammalian cell lines including a hybridoma. Cytotechnology 40: 3-12.

Teramoto, H., Nakajima, and Takabayashi, C. 2005. Preparation of elastic silk sericin hydrogel. Bioscience, Biotechnology, and Biochemistry 69(4): 845-847.

Togashi, S. I., Takahashi, N., Iwama, M., Watanabe, S., Tamagawa, K., and Fukui, T. 2002. Antioxidative collagen-derived peptides in human-placenta extract. Placenta 23: 497-502.

Torchilin, V., and Weissig, V. 2003. Liposomes: A practical approach. 2 nd ed. United States: Oxford University Press.

Tsujimoto, K., Takagi, H., Takahashi, M., Yamada, H. and Nakamori, S. 2001. Cryoprotective effect of the serine-rich repetitive sequence in silk protein sericin. Journal of Biochemistry 129: 979-986.

U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, and Center for Veterinary Medicine. 2001. Guidance for industry bioanalytical method validation [Online]. Available from: http://www.fda.gov/cvm [2007, September 10].

Wachiranuntasin, K. 2005. Evaluation of stability, antioxidative and free radical scavenging activities of *Artocarpus lakoocha* heartwood extract. Master's Thesis, The Faculty of Pharmaceutical Science, Chulalongkorn University.

Walker, J. M. 2002. The protein protocol handbook. 2 nd ed. New Jersey: Humana Press.

Zhang, Y. Q. 2002. Applications of natural silk protein sericin in biomaterials. Biotechnology Advances 20: 91-100.

Zhang, Y. Q., Tao, M. L., Shen, W. D., Zhou, Y. Z., Ding, Y., Ma, Y. and Zhou, W. L. 2004. Immobilization of L-asparaginase on the microparticles of the natural silk sericin protein and its characters. Biomaterials 25: 3751-3759.

Zhaorigetu, S., Sasaki, M. Watanabe, H. and Kato, N. 2001. Supplemental silk protein, sericin suppresses colon tumorigenesis in 1,2-dimethylhydrazine-treated mice by reducing oxidative stress and cell proliferation. Bioscience, Biotechnology, and Biochemistry 65(10): 2181-2186.

Zhaorigetu, S., Yanaka, N., Sasaki, M. Watanabe, H. and Kato, N. 2003a. Inhibitory effects of silk protein, sericin on UVB-induced acute damage and tumor promotion by reducing oxidative stress in the skin of hairless mouse. Journal of Photochemistry and Photobiology B: Biology 71: 11-17.

Zhaorigetu, S., Yanaka, N., Sasaki, M. Watanabe, H. and Kato, N. 2003b. Silk protein, sericin, suppresses DMBA-TPA-induced mouse skin tumorigenesis by reducing oxidative stress, inflammatory responses and endogeneous tumor promoter TNF-α. Oncology Reports 10: 537-543.

APPENDICES

Linearity of Lowry method

Table 26. Absorbances of BSA by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm				SD	%CV	% protein
	n_1	n_2	n_3	mean			
0.05	0.1500	0.1770	0.1567	0.1612	0.019	11.84	79.70
0.10	0.3026	0.3239	0.3466	0.3244	0.015	4.64	106.17
0.15	0.4230	0.4498	0.4464	0.4397	0.019	4.31	102.04
0.20	0.5447	0.5677	0.5750	0.5625	0.016	2.89	101.48
0.25	0.7263	0.6400	0.7128	0.6930	0.061	8.81	102.41
0.30	0.7394	0.8188	0.7699	0.7760	0.056	7.23	96.59
0.35	0.9502	0.9164	0.9709	0.9458	0.024	2.53	102.51
0.40	0.9507	1.0520	1.0890	1.0306	0.072	6.95	98.31
					average		**98.65**

Table 27. Absorbances of Ser O by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm				SD	%CV	% protein
	n_1	n_2	n_3	mean			
0.05	0.1676	0.1576	0.1700	0.1650	0.007	4.30	82.80
0.10	0.3086	0.3231	0.3202	0.3173	0.010	3.24	103.29
0.15	0.4269	0.4547	0.4512	0.4443	0.020	4.44	103.27
0.20	0.5501	0.5817	0.5774	0.5697	0.022	3.93	102.95
0.25	0.7005	0.7057	0.7083	0.7048	0.004	0.52	104.33
0.30	0.7897	0.8006	0.8189	0.8031	0.008	0.97	100.25
0.35	0.9058	0.9087	0.9610	0.9252	0.002	0.22	100.11
0.40	0.9997	1.0354	1.0627	1.0326	0.025	2.44	98.52
					average		**99.44**

Table 28. Absorbances of Ser N by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm				SD	%CV	% protein
	n_1	n_2	n_3	mean			
0.05	0.1423	0.1437	0.1506	0.1455	0.004	3.05	66.94
0.10	0.2690	0.2712	0.2678	0.2693	0.002	0.64	83.79
0.15	0.3789	0.3871	0.4012	0.3891	0.011	2.90	88.31
0.20	0.5161	0.5037	0.5354	0.5184	0.016	3.08	92.52
0.25	0.6227	0.5995	0.6075	0.6099	0.012	1.93	88.89
0.30	0.7385	0.7076	0.7061	0.7174	0.018	2.55	88.64
0.35	0.8315	0.8101	0.8081	0.8166	0.013	1.59	87.50
0.40	0.8831	0.8712	0.8799	0.8781	0.006	0.70	82.81
					average		**84.93**

Accuracy of CL_Ser of Lowry method

Table 29. The absorbance of sericin in CL at 550 nm by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	0.2797	0.2803	0.2716	0.2772	0.2804	0.2778 ± 0.004
0.20	0.4939	0.5098	0.5001	0.4812	0.5088	0.4988 ± 0.012
0.40	0.8315	0.8221	0.9087	0.8251	0.8369	0.8449 ± 0.036

Table 30. The estimated concentrations of sericin in CL by Lowry method.

Concentration (mg/ml)	Estimated concentration from standard curve (mg/ml)					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	0.1145	0.1148	0.1104	0.1132	0.1148	0.1136 ± 0.002
0.20	0.2217	0.2297	0.2248	0.2153	0.2292	0.2241 ± 0.016
0.40	0.3907	0.3860	0.4293	0.3875	0.3934	0.3974 ± 0.018

Table 31. The percentage of analytical recovery of sericin in CL by Lowry method.

Concentration (mg/ml)	Percentage of analytical recovery					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	114.48	114.78	110.43	113.23	114.83	113.55 ± 1.863
0.20	110.85	114.83	112.40	107.67	114.58	112.07 ± 2.952
0.40	97.67	96.50	107.34	96.87	98.35	99.35 ± 4.523

Accuracy of DLSp_Ser of Lowry method

Table 32. The absorbance of sericin in DLSp at 550 nm by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	0.2891	0.2789	0.2893	0.2997	0.3103	0.2935 ± 0.012
0.20	0.5016	0.5126	0.5122	0.5040	0.5254	0.5112 ± 0.009
0.40	0.8596	0.8696	0.8708	0.8669	0.8696	0.8673 ± 0.005

Table 33. The estimated concentrations of sericin in DLSp by Lowry method.

Concentration (mg/ml)	Estimated concentration from standard curve (mg/ml)					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	0.1039	0.0986	0.1040	0.1093	0.1148	0.1061 ± 0.006
0.20	0.2129	0.2186	0.2184	0.2142	0.2251	0.2178 ± 0.005
0.40	0.3967	0.4018	0.4024	0.4004	0.4018	0.4006 ± 0.002

Table 34. The percentage of analytical recovery of sericin in DLSp by Lowry method.

Concentration (mg/ml)	Percentage of analytical recovery					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	103.87	98.64	103.98	109.31	114.75	106.11 ± 6.131
0.20	106.47	109.29	109.19	107.08	112.57	108.92 ± 2.396
0.40	99.17	100.45	100.60	100.10	100.45	100.15 ± 0.582

Accuracy of DLTw_Ser of Lowry method

Table 35. The absorbance of sericin in DLTw at 550 nm by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	0.2832	0.2933	0.2949	0.2800	0.2864	0.2876 ± 0.006
0.20	0.4634	0.4742	0.4775	0.4799	0.4740	0.4738 ± 0.006
0.40	0.8142	0.8304	0.8286	0.8097	0.8208	0.8207 ± 0.009

Table 36. The estimated concentrations of sericin in DLTw by Lowry method.

Concentration (mg/ml)	Estimated concentration from standard curve (mg/ml)					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	0.1014	0.1065	0.1073	0.0998	0.1030	0.1036 ± 0.003
0.20	0.1923	0.1977	0.1994	0.2006	0.1976	0.1975 ± 0.003
0.40	0.3692	0.3774	0.3765	0.3670	0.3726	0.3725 ± 0.005

Table 37. The percentage of analytical recovery of sericin in DLTw by Lowry method.

Concentration (mg/ml)	Percentage of analytical recovery					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	101.39	106.49	107.29	99.78	103.01	103.59 ± 3.233
0.20	96.15	98.87	99.70	100.31	98.82	98.77 ± 1.591
0.40	92.31	94.36	94.13	91.74	93.14	93.14 ± 1.128

Accuracy of Ser_PBS of Lowry method

Table 38. The absorbance of sericin in PBS at 550 nm by Lowry method.

Concentration (mg/ml)	Absorbance at 550 nm					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	0.2488	0.2533	0.2574	0.2511	0.2596	0.2540 ± 0.004
0.20	0.4413	0.4346	0.4430	0.4648	0.4657	0.4499 ± 0.014
0.40	0.8585	0.7815	0.7961	0.8063	0.7909	0.8067 ± 0.030

Table 39. The estimated concentrations of sericin in PBS by Lowry method.

Concentration (mg/ml)	Estimated concentration from standard curve (mg/ml)					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	0.1040	0.1061	0.1080	0.1051	0.1090	0.1064 ± 0.002
0.20	0.1932	0.1901	0.1940	0.2041	0.2045	0.1972 ± 0.007
0.40	0.3866	0.3510	0.3577	0.3624	0.3553	0.3626 ± 0.014

Table 40. The percentage of analytical recovery of sericin in PBS by Lowry method.

Concentration (mg/ml)	Percentage of analytical recovery					Mean ± SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
0.10	103.99	106.07	107.97	105.05	108.99	106.42 ± 2.058
0.20	96.62	95.06	97.01	102.06	102.27	98.60 ± 3.334
0.40	96.66	87.74	89.43	90.61	88.83	90.65 ± 3.515

Linearity of deoxyribose assay

Table 41. Absorbances of mannitol by deoxyribose assay.

Concentration	Absorbance at 532 nm					
(mg/ml)	n_1	n_2	n_3	mean	SD	%CV
0	0.7291	0.7333	0.7513	0.7379	0.01	1.60
0.10	0.6779	0.7158	0.7126	0.7021	0.02	2.99
0.20	0.6579	0.6745	0.6750	0.6691	0.01	1.45
0.40	0.6070	0.6376	0.6280	0.6242	0.02	2.51
0.80	0.5328	0.5671	0.5726	0.5575	0.02	3.87
1.60	0.4484	0.4532	0.4641	0.4552	0.01	1.77
3.20	0.3383	0.3513	0.3468	0.3455	0.01	1.91
6.40	0.2281	0.2295	0.2199	0.2258	0.01	2.30

Table 42. 1/Absorbances of mannitol by deoxyribose assay.

Concentration	1/Absorbance at 532 nm					
(mM)	n_1	n_2	n_3	mean	SD	%CV
0.00	1.3716	1.3637	1.3310	1.3554	0.02	1.59
0.55	1.4751	1.3970	1.4033	1.4252	0.04	3.05
1.10	1.5200	1.4826	1.4815	1.4947	0.02	1.47
2.20	1.6474	1.5684	1.5924	1.6027	0.04	2.53
4.39	1.8769	1.7634	1.7464	1.7956	0.07	3.95
8.78	2.2302	2.2065	2.1547	2.1971	0.04	1.76
17.57	2.9560	2.8466	2.8835	2.8953	0.06	1.92
35.13	4.3840	4.3573	4.5475	4.4296	0.10	2.32

Molecular weight of mannitol is 182.18.

Table 43. The percentage of inhibition of MDA by deoxyribose assay using mannitol as antioxidants.

Concen-tration (mg/ml)	% inhibition of MDA				SD
	n_1	n_2	n_3	mean	
0.00	7.02	2.39	5.15	4.85	2.33
0.10	9.77	8.02	10.16	9.31	1.14
0.20	16.75	13.05	16.41	15.40	2.04
0.40	26.92	22.66	23.79	24.46	2.21
0.80	38.50	38.20	38.23	38.31	0.17
1.60	53.60	52.09	53.84	53.18	0.95
3.20	68.71	68.70	70.73	69.38	1.17

Table 44. 1/Absorbances of sericin by deoxyribose assay.

Concen-tration (mg/ml)	1/Absorbance at 532 nm				SD	%CV
	n_1	n_2	n_3	mean		
0.00	1.11	1.12	1.13	1.12	0.01	1.05
0.10	1.06	1.07	1.09	1.08	0.02	1.50
0.20	1.06	1.08	1.10	1.08	0.02	1.79
0.40	1.47	1.49	1.39	1.45	0.05	3.62
0.80	2.14	2.14	2.15	2.14	0.00	0.19
1.60	2.34	2.36	2.30	2.33	0.03	1.15
3.20	2.49	2.48	2.46	2.48	0.01	0.57
6.40	2.65	2.77	2.63	2.68	0.07	2.77

Table 45. The percentage of inhibition of MDA by deoxyribose assay using sericin as antioxidants.

Concen-tration (mg/ml)	% inhibition of MDA				SD
	n_1	n_2	n_3	mean	
0.10	-4.48	-4.16	-3.55	-4.06	0.47
0.20	-4.88	-3.13	-3.29	-3.76	0.97
0.40	24.35	24.86	18.34	22.52	3.63
0.80	48.19	47.80	47.23	47.74	0.48
1.60	52.57	52.60	50.82	52.00	1.02
3.20	55.45	54.95	54.00	54.80	0.73
6.40	58.07	59.66	56.96	58.23	1.36

Table 46. 1/Absorbances of ascorbic acid by deoxyribose assay.

Concen-tration (mg/ml)	1/Absorbance at 532 nm				SD	%CV
	n_1	n_2	n_3	mean		
0.00	1.16	1.14	1.06	1.12	0.05	4.71
0.10	1.11	0.92	0.97	1.00	0.10	9.62
0.20	0.84	0.85	0.80	0.83	0.03	3.17
0.40	0.62	0.62	0.57	0.60	0.03	4.36
0.80	0.55	0.59	0.59	0.58	0.02	3.50
1.60	0.79	0.77	0.71	0.76	0.04	5.66
3.20	1.21	1.22	1.13	1.18	0.05	4.24
6.40	1.81	1.43	1.71	1.65	0.20	11.88

Table 47. The percentage of inhibition of MDA by deoxyribose assay using ascorbic acid as antioxidants.

Concen-tration (mg/ml)	% inhibition of MDA				SD
	n_1	n_2	n_3	mean	
0.10	-4.52	-23.74	-8.41	-12.22	10.16
0.20	-38.23	-33.45	-32.01	-34.56	3.26
0.40	-86.09	-84.57	-84.35	-85.00	0.95
0.80	-109.19	-93.59	-79.71	-94.16	14.75
1.60	-46.40	-47.02	-49.14	-47.52	1.44
3.20	4.42	6.51	6.14	5.69	1.12
6.40	36.31	20.77	38.17	31.75	9.55

Accuracy of deoxyribose assay

Table 48. Absorbances of sericin in various conditions, which may interfere deoxyribose assay.

Conditions	Absorbance at 532 nm					
	n_1	n_2	n_3	mean	SD	%CV
0.5 Ser_H_2O	2.40	2.42	2.54	2.46	0.08	3.18
0.5 Ser_heat	2.42	2.45	2.61	2.49	0.10	4.17
0.5 Ser_CHCl3	2.13	2.22	2.22	2.19	0.05	2.38
0.5 Ser_PC	1.64	1.50	1.71	1.62	0.11	6.57

Table 49. The percentage of inhibition of sericin in various conditions, which may interfere deoxyribose assay.

Conditions	% inhibition of MDA				
	n_1	n_2	n_3	mean	SD
0.5 Ser_H_2O	48.22	41.99	50.63	46.95	4.46
0.5 Ser_heat	48.51	42.77	51.88	47.72	4.61
0.5 Ser_CHCl3	53.14	55.75	43.30	50.73	6.57
0.5 Ser_PC	24.39	6.72	26.69	19.27	10.92

Table 50. Analysis the percentage of inhibition of sericin in various conditions, which may interfere deoxyribose assay by one-way ANOVA.

ANOVA

pInh_MDA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1942.266	3	647.422	12.720	.002
Within Groups	407.176	8	50.897		
Total	2349.441	11			

Multiple Comparisons

Dependent Variable: pInh_MDA
Tukey HSD

(I) conditions	(J) conditions	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Ser_H2O	Ser_heat	-.77333	5.82506	.999	-19.4272	17.8806
	Ser_CHCl3	-3.78333	5.82506	.913	-22.4372	14.8706
	Ser_PC	27.68000(*)	5.82506	.006	9.0261	46.3339
Ser_heat	Ser_H2O	.77333	5.82506	.999	-17.8806	19.4272
	Ser_CHCl3	-3.01000	5.82506	.953	-21.6639	15.6439
	Ser_PC	28.45333(*)	5.82506	.005	9.7994	47.1072
Ser_CHCl3	Ser_H2O	3.78333	5.82506	.913	-14.8706	22.4372
	Ser_heat	3.01000	5.82506	.953	-15.6439	21.6639
	Ser_PC	31.46333(*)	5.82506	.003	12.8094	50.1172
Ser_PC	Ser_H2O	-27.68000(*)	5.82506	.006	-46.3339	-9.0261
	Ser_heat	-28.45333(*)	5.82506	.005	-47.1072	-9.7994
	Ser_CHCl3	-31.46333(*)	5.82506	.003	-50.1172	-12.8094

* The mean difference is significant at the .05 level.

pInh_MDA

Tukey HSD

		Subset for alpha = .05	
conditions	N	1	2
Ser_PC	3	19.2667	
Ser_H2O	3		46.9467
Ser_heat	3		47.7200
Ser_CHCl3	3		50.7300
Sig.		1.000	.913

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Preparation of deformable liposomes

Table 51. Size and size distribution of DL-MLVs at 3.5% PC, using Span 80 as edge activator (n_1).

Result Statistics

Distribution Type: Volume	Concentration = 0.0066 %Vol	Density = 1.000 g / cub. cm	Specific S.A. = 0.8924 sq. m / g
Mean Diameters:	D (v, 0.1) = 2.98 um	D (v, 0.5) = 13.96 um	D (v, 0.9) = 35.25 um
D [4, 3] = 16.79 um	D [3, 2] = 6.72 um	Span = 2.312E+00	Uniformity = 7.466E-01

Size_Low (um)	In %	Size_High (um)	Under%	Size_Low (um)	In %	Size_High (um)	Under%
0.05	0.00	0.06	0.00	6.63	3.94	7.72	32.95
0.06	0.00	0.07	0.00	7.72	3.98	9.00	36.93
0.07	0.00	0.08	0.00	9.00	4.16	10.48	41.09
0.08	0.00	0.09	0.00	10.48	4.52	12.21	45.61
0.09	0.00	0.11	0.00	12.21	5.05	14.22	50.66
0.11	0.00	0.13	0.00	14.22	5.73	16.57	56.39
0.13	0.00	0.15	0.00	16.57	6.48	19.31	62.87
0.15	0.00	0.17	0.00	19.31	7.24	22.49	70.10
0.17	0.00	0.20	0.00	22.49	7.39	26.20	77.50
0.20	0.00	0.23	0.00	26.20	6.93	30.53	84.43
0.23	0.04	0.27	0.04	30.53	5.88	35.56	90.31
0.27	0.06	0.31	0.09	35.56	4.46	41.43	94.77
0.31	0.07	0.36	0.16	41.43	2.96	48.27	97.73
0.36	0.08	0.42	0.24	48.27	1.63	56.23	99.36
0.42	0.08	0.49	0.32	56.23	0.64	65.51	100.00
0.49	0.09	0.58	0.40	65.51	0.00	76.32	100.00
0.58	0.10	0.67	0.50	76.32	0.00	88.91	100.00
0.67	0.11	0.78	0.61	88.91	0.00	103.58	100.00
0.78	0.18	0.91	0.79	103.58	0.00	120.67	100.00
0.91	0.27	1.06	1.06	120.67	0.00	140.58	100.00
1.06	0.41	1.24	1.47	140.58	0.00	163.77	100.00
1.24	0.60	1.44	2.07	163.77	0.00	190.80	100.00
1.44	0.87	1.68	2.94	190.80	0.00	222.28	100.00
1.68	1.24	1.95	4.18	222.28	0.00	258.95	100.00
1.95	1.67	2.28	5.84	258.95	0.00	301.68	100.00
2.28	2.15	2.65	8.00	301.68	0.00	351.46	100.00
2.65	2.67	3.09	10.67	351.46	0.00	409.45	100.00
3.09	3.14	3.60	13.81	409.45	0.00	477.01	100.00
3.60	3.53	4.19	17.34	477.01	0.00	555.71	100.00
4.19	3.80	4.88	21.14	555.71	0.00	647.41	100.00
4.88	3.92	5.69	25.06	647.41	0.00	754.23	100.00
5.69	3.95	6.63	29.01	754.23	0.00	878.67	100.00

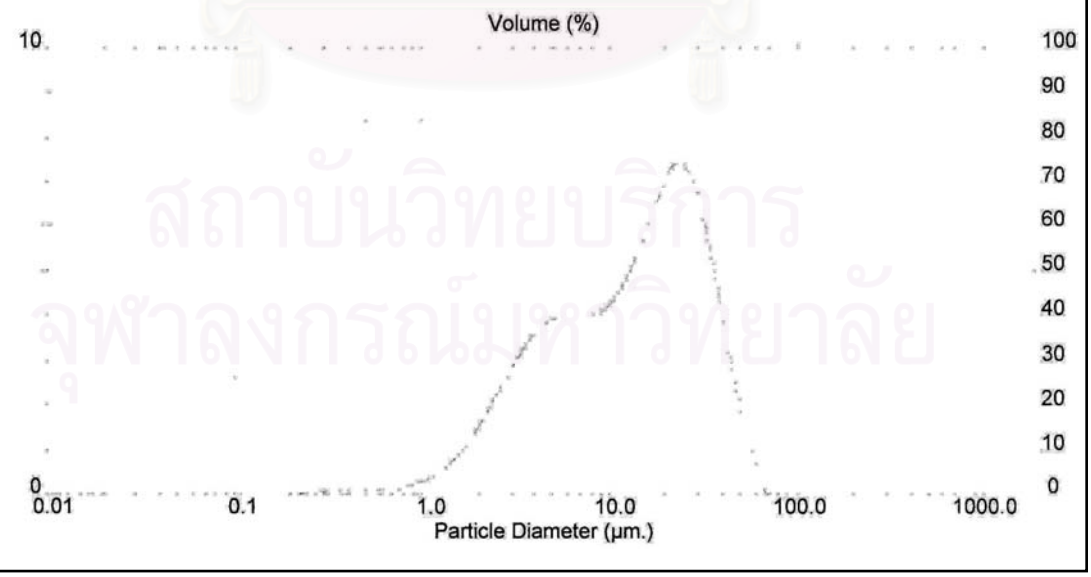


Table 52. Size and size distribution of DL-MLVs at 3.5% PC, using Span 80 as edge activator (n_2).

Result Statistics

Distribution Type: Volume
Mean Diameters:
D [4, 3] = 16.66 um

Concentration = 0.0065 %Vol
D (v, 0.1) = 2.97 um
D [3, 2] = 6.69 um

Density = 1.000 g / cub. cm
D (v, 0.5) = 13.79 um
Span = 2.325E+00

Specific S.A. = 0.8970 sq. m / g
D (v, 0.9) = 35.03 um
Uniformity = 7.503E-01

Size_Low (um)	In %	Size_High (um)	Under%	Size_Low (um)	In %	Size_High (um)	Under%
0.05	0.00	0.06	0.00	6.63	3.98	7.72	33.21
0.06	0.00	0.07	0.00	7.72	4.02	9.00	37.23
0.07	0.00	0.08	0.00	9.00	4.21	10.48	41.44
0.08	0.00	0.09	0.00	10.48	4.56	12.21	46.00
0.09	0.00	0.11	0.00	12.21	5.09	14.22	51.09
0.11	0.00	0.13	0.00	14.22	5.75	16.57	56.84
0.13	0.00	0.15	0.00	16.57	6.48	19.31	63.32
0.15	0.00	0.17	0.00	19.31	7.21	22.49	70.53
0.17	0.00	0.20	0.00	22.49	7.34	26.20	77.87
0.20	0.00	0.23	0.00	26.20	6.85	30.53	84.72
0.23	0.04	0.27	0.04	30.53	5.80	35.56	90.52
0.27	0.05	0.31	0.09	35.56	4.38	41.43	94.90
0.31	0.06	0.36	0.16	41.43	2.89	48.27	97.80
0.36	0.08	0.42	0.23	48.27	1.59	56.23	99.38
0.42	0.08	0.49	0.31	56.23	0.62	65.51	100.00
0.49	0.09	0.58	0.40	65.51	0.00	76.32	100.00
0.58	0.10	0.67	0.50	76.32	0.00	88.91	100.00
0.67	0.11	0.78	0.61	88.91	0.00	103.58	100.00
0.78	0.18	0.91	0.79	103.58	0.00	120.67	100.00
0.91	0.27	1.06	1.06	120.67	0.00	140.58	100.00
1.06	0.41	1.24	1.47	140.58	0.00	163.77	100.00
1.24	0.61	1.44	2.08	163.77	0.00	190.80	100.00
1.44	0.88	1.68	2.96	190.80	0.00	222.28	100.00
1.68	1.25	1.95	4.21	222.28	0.00	258.95	100.00
1.95	1.68	2.28	5.89	258.95	0.00	301.68	100.00
2.28	2.17	2.65	8.06	301.68	0.00	351.46	100.00
2.65	2.69	3.09	10.75	351.46	0.00	409.45	100.00
3.09	3.16	3.60	13.91	409.45	0.00	477.01	100.00
3.60	3.56	4.19	17.46	477.01	0.00	555.71	100.00
4.19	3.82	4.88	21.29	555.71	0.00	647.41	100.00
4.88	3.95	5.69	25.24	647.41	0.00	754.23	100.00
5.69	3.98	6.63	29.23	754.23	0.00	878.67	100.00

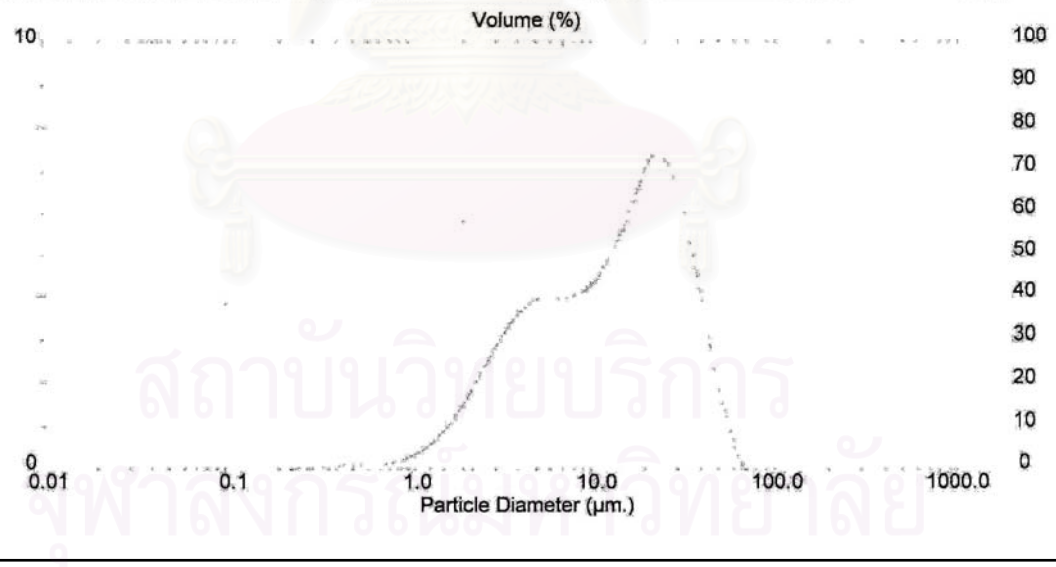


Table 53. Size and size distribution of DL-MLVs at 3.5% PC, using Span 80 as edge activator (n_3).

Result Statistics

Distribution Type: Volume
Concentration = 0.0063 %Vol
Density = 1.000 g / cub. cm
Specific S.A. = 0.8938 sq. m / g
Mean Diameters:
D (v, 0.1) = 2.98 um
D (v, 0.5) = 13.78 um
D (v, 0.9) = 34.97 um
D [4, 3] = 16.64 um
D [3, 2] = 6.71 um
Span = 2.323E+00
Uniformity = 7.493E-01

Size_Low (um)	In %	Size_High (um)	Under%	Size_Low (um)	In %	Size_High (um)	Under%
0.05	0.00	0.06	0.00	6.63	3.99	7.72	33.18
0.06	0.00	0.07	0.00	7.72	4.04	9.00	37.22
0.07	0.00	0.08	0.00	9.00	4.22	10.48	41.44
0.08	0.00	0.09	0.00	10.48	4.58	12.21	46.02
0.09	0.00	0.11	0.00	12.21	5.10	14.22	51.12
0.11	0.00	0.13	0.00	14.22	5.76	16.57	56.88
0.13	0.00	0.15	0.00	16.57	6.49	19.31	63.37
0.15	0.00	0.17	0.00	19.31	7.22	22.49	70.58
0.17	0.00	0.20	0.00	22.49	7.34	26.20	77.93
0.20	0.00	0.23	0.00	26.20	6.85	30.53	84.78
0.23	0.03	0.27	0.03	30.53	5.79	35.56	90.57
0.27	0.05	0.31	0.09	35.56	4.38	41.43	94.95
0.31	0.06	0.36	0.15	41.43	2.88	48.27	97.83
0.36	0.07	0.42	0.22	48.27	1.57	56.23	99.40
0.42	0.08	0.49	0.30	56.23	0.60	65.51	100.00
0.49	0.09	0.58	0.39	65.51	0.00	76.32	100.00
0.58	0.10	0.67	0.49	76.32	0.00	88.91	100.00
0.67	0.11	0.78	0.59	88.91	0.00	103.58	100.00
0.78	0.18	0.91	0.77	103.58	0.00	120.67	100.00
0.91	0.27	1.06	1.04	120.67	0.00	140.58	100.00
1.06	0.41	1.24	1.45	140.58	0.00	163.77	100.00
1.24	0.60	1.44	2.05	163.77	0.00	190.80	100.00
1.44	0.88	1.68	2.93	190.80	0.00	222.28	100.00
1.68	1.24	1.95	4.17	222.28	0.00	258.95	100.00
1.95	1.68	2.28	5.85	258.95	0.00	301.68	100.00
2.28	2.17	2.65	8.02	301.68	0.00	351.46	100.00
2.65	2.69	3.09	10.70	351.46	0.00	409.45	100.00
3.09	3.16	3.60	13.86	409.45	0.00	477.01	100.00
3.60	3.55	4.19	17.42	477.01	0.00	555.71	100.00
4.19	3.83	4.88	21.24	555.71	0.00	647.41	100.00
4.88	3.96	5.69	25.20	647.41	0.00	754.23	100.00
5.69	3.99	6.63	29.19	754.23	0.00	878.67	100.00

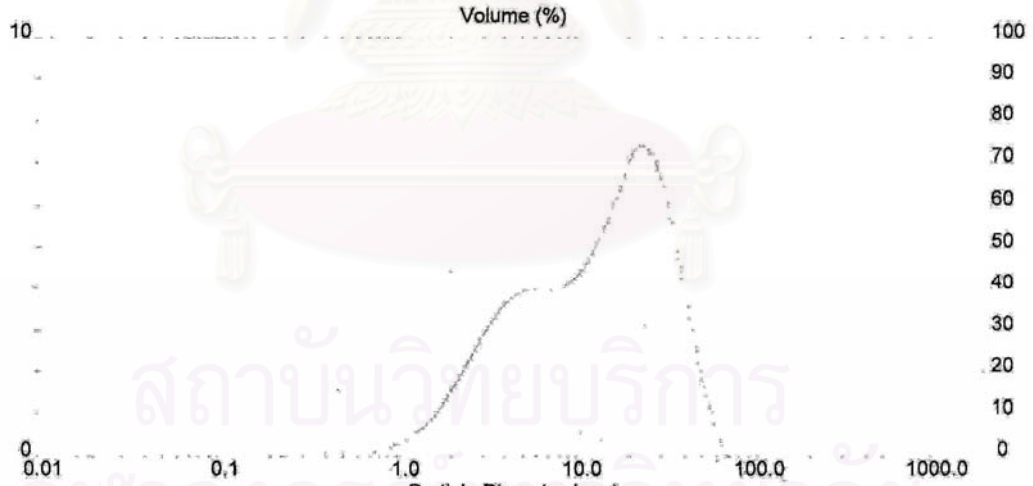


Table 54. Size and size distribution of DL-LUVs at 2% PC, using Span 80 as edge activator (n_1).

Result Statistics

Distribution Type: Volume	Concentration = 0.0040 %Vol	Density = 1.000 g / cub. cm	Specific S.A. = 51.9399 sq. m / g
Mean Diameters:	D (v, 0.1) = 0.06 um	D (v, 0.5) = 0.10 um	D (v, 0.9) = 8.66 um
D [4, 3] = 2.77 um	D [3, 2] = 0.12 um	Span = 8.928E+01	Uniformity = 2.799E+01

Size_Low (um)	In %	Size_High (um)	Under%	Size_Low (um)	In %	Size_High (um)	Under%
0.05	9.54	0.06	9.54	6.63	2.91	7.72	87.99
0.06	13.92	0.07	23.45	7.72	2.64	9.00	90.63
0.07	13.60	0.08	37.05	9.00	2.29	10.48	92.92
0.08	10.55	0.09	47.61	10.48	1.89	12.21	94.81
0.09	6.97	0.11	54.58	12.21	1.50	14.22	96.31
0.11	4.20	0.13	58.78	14.22	1.14	16.57	97.45
0.13	2.49	0.15	61.27	16.57	0.84	19.31	98.28
0.15	1.53	0.17	62.80	19.31	0.59	22.49	98.87
0.17	1.02	0.20	63.82	22.49	0.40	26.20	99.27
0.20	0.73	0.23	64.55	26.20	0.26	30.53	99.52
0.23	0.56	0.27	65.11	30.53	0.16	35.56	99.68
0.27	0.44	0.31	65.55	35.56	0.09	41.43	99.77
0.31	0.36	0.36	65.90	41.43	0.05	48.27	99.83
0.36	0.31	0.42	66.21	48.27	0.03	56.23	99.85
0.42	0.28	0.49	66.49	56.23	0.02	65.51	99.87
0.49	0.27	0.58	66.75	65.51	0.01	76.32	99.88
0.58	0.26	0.67	67.01	76.32	0.01	88.91	99.89
0.67	0.26	0.78	67.28	88.91	0.01	103.58	99.90
0.78	0.28	0.91	67.56	103.58	0.02	120.67	99.92
0.91	0.31	1.06	67.87	120.67	0.02	140.58	99.94
1.06	0.33	1.24	68.19	140.58	0.02	163.77	99.95
1.24	0.36	1.44	68.55	163.77	0.02	190.80	99.97
1.44	0.43	1.68	68.98	190.80	0.01	222.28	99.99
1.68	0.58	1.95	69.56	222.28	0.01	258.95	100.00
1.95	0.79	2.28	70.35	258.95	0.00	301.68	100.00
2.28	1.08	2.65	71.43	301.68	0.00	351.46	100.00
2.65	1.43	3.09	72.86	351.46	0.00	409.45	100.00
3.09	1.83	3.60	74.69	409.45	0.00	477.01	100.00
3.60	2.22	4.19	76.91	477.01	0.00	555.71	100.00
4.19	2.54	4.88	79.44	555.71	0.00	647.41	100.00
4.88	2.76	5.69	82.20	647.41	0.00	754.23	100.00
5.69	2.88	6.63	85.08	754.23	0.00	878.67	100.00

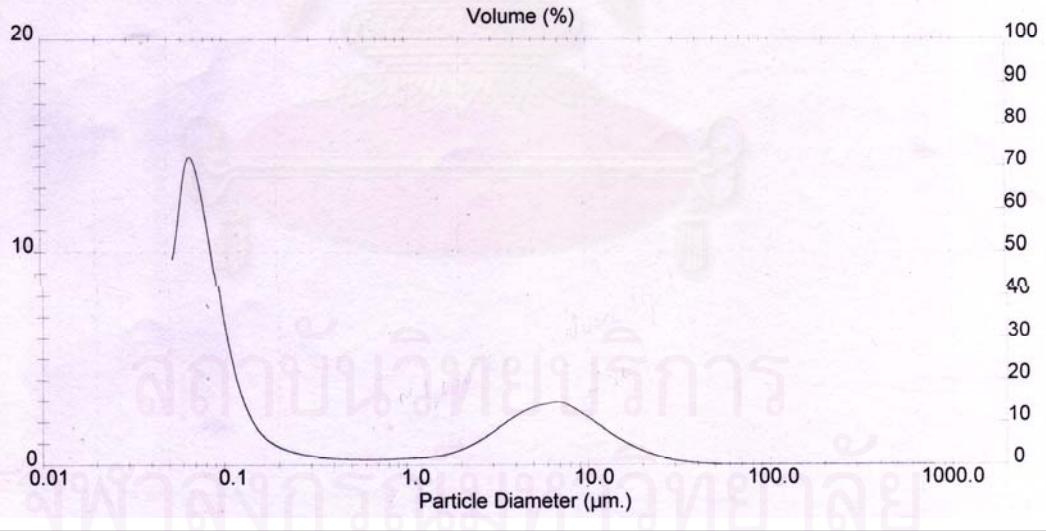


Table 55. Size and size distribution of DL-LUVs at 2% PC, using Span 80 as edge activator (n_2).

Result Statistics

Distribution Type: Volume	Concentration = 0.0039 %Vol	Density = 1.000 g / cub. cm	Specific S.A. = 51.2414 sq. m / g
Mean Diameters:	D (v, 0.1) = 0.06 um	D (v, 0.5) = 0.10 um	D (v, 0.9) = 8.73 um
D [4, 3] = 2.75 um	D [3, 2] = 0.12 um	Span = 8.842E+01	Uniformity = 2.737E+01

Size_Low (um)	In %	Size_High (um)	Under%	Size_Low (um)	In %	Size_High (um)	Under%
0.05	9.30	0.06	9.31	6.63	2.97	7.72	87.81
0.06	13.63	0.07	22.93	7.72	2.70	9.00	90.51
0.07	13.39	0.08	36.32	9.00	2.34	10.48	92.85
0.08	10.45	0.09	46.77	10.48	1.93	12.21	94.79
0.09	6.96	0.11	53.73	12.21	1.53	14.22	96.31
0.11	4.23	0.13	57.96	14.22	1.16	16.57	97.47
0.13	2.52	0.15	60.49	16.57	0.84	19.31	98.32
0.15	1.57	0.17	62.05	19.31	0.59	22.49	98.90
0.17	1.05	0.20	63.10	22.49	0.39	26.20	99.30
0.20	0.75	0.23	63.86	26.20	0.25	30.53	99.55
0.23	0.57	0.27	64.43	30.53	0.15	35.56	99.70
0.27	0.45	0.31	64.88	35.56	0.09	41.43	99.79
0.31	0.37	0.36	65.26	41.43	0.05	48.27	99.84
0.36	0.32	0.42	65.58	48.27	0.03	56.23	99.87
0.42	0.29	0.49	65.87	56.23	0.02	65.51	99.89
0.49	0.28	0.58	66.15	65.51	0.01	76.32	99.90
0.58	0.27	0.67	66.43	76.32	0.01	88.91	99.91
0.67	0.28	0.78	66.71	88.91	0.02	103.58	99.93
0.78	0.30	0.91	67.00	103.58	0.02	120.67	99.95
0.91	0.32	1.06	67.32	120.67	0.02	140.58	99.97
1.06	0.34	1.24	67.66	140.58	0.01	163.77	99.98
1.24	0.36	1.44	68.02	163.77	0.01	190.80	99.99
1.44	0.44	1.68	68.46	190.80	0.01	222.28	100.00
1.68	0.59	1.95	69.04	222.28	0.00	258.95	100.00
1.95	0.80	2.28	69.85	258.95	0.00	301.68	100.00
2.28	1.09	2.65	70.94	301.68	0.00	351.46	100.00
2.65	1.45	3.09	72.39	351.46	0.00	409.45	100.00
3.09	1.86	3.60	74.25	409.45	0.00	477.01	100.00
3.60	2.25	4.19	76.51	477.01	0.00	555.71	100.00
4.19	2.58	4.88	79.09	555.71	0.00	647.41	100.00
4.88	2.81	5.69	81.90	647.41	0.00	754.23	100.00
5.69	2.93	6.63	84.84	754.23	0.00	878.67	100.00

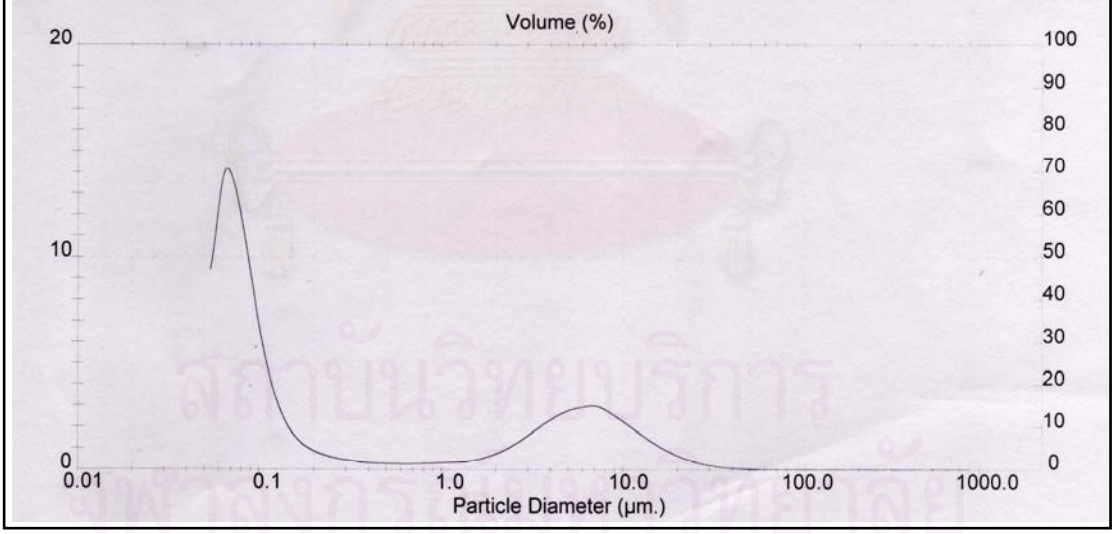


Table 56. Size and size distribution of DL-LUVs at 2% PC, using Span 80 as edge activator (n_3).

Result Statistics

Distribution Type: Volume	Concentration = 0.0055 %Vol	Density = 1.000 g / cub. cm	Specific S.A. = 52.0313 sq. m / g
Mean Diameters:	D (v, 0.1) = 0.06 um	D (v, 0.5) = 0.10 um	D (v, 0.9) = 8.63 um
D [4, 3] = 2.75 um	D [3, 2] = 0.12 um	Span = 8.894E+01	Uniformity = 2.777E+01

Size_Low (um)	In %	Size_High (um)	Under%	Size_Low (um)	In %	Size_High (um)	Under%
0.05	9.56	0.06	9.56	6.63	2.86	7.72	88.09
0.06	13.93	0.07	23.49	7.72	2.60	9.00	90.68
0.07	13.59	0.08	37.08	9.00	2.25	10.48	92.93
0.08	10.53	0.09	47.61	10.48	1.86	12.21	94.80
0.09	6.96	0.11	54.57	12.21	1.48	14.22	96.28
0.11	4.21	0.13	58.77	14.22	1.13	16.57	97.40
0.13	2.51	0.15	61.28	16.57	0.82	19.31	98.23
0.15	1.56	0.17	62.84	19.31	0.58	22.49	98.81
0.17	1.06	0.20	63.90	22.49	0.39	26.20	99.20
0.20	0.78	0.23	64.68	26.20	0.26	30.53	99.45
0.23	0.60	0.27	65.28	30.53	0.16	35.56	99.62
0.27	0.48	0.31	65.77	35.56	0.10	41.43	99.72
0.31	0.40	0.36	66.16	41.43	0.07	48.27	99.79
0.36	0.34	0.42	66.50	48.27	0.04	56.23	99.83
0.42	0.31	0.49	66.81	56.23	0.03	65.51	99.86
0.49	0.29	0.58	67.10	65.51	0.02	76.32	99.88
0.58	0.28	0.67	67.38	76.32	0.02	88.91	99.90
0.67	0.28	0.78	67.65	88.91	0.02	103.58	99.92
0.78	0.29	0.91	67.95	103.58	0.02	120.67	99.94
0.91	0.31	1.06	68.25	120.67	0.02	140.58	99.95
1.06	0.33	1.24	68.58	140.58	0.02	163.77	99.97
1.24	0.35	1.44	68.93	163.77	0.01	190.80	99.99
1.44	0.43	1.68	69.36	190.80	0.01	222.28	99.99
1.68	0.58	1.95	69.94	222.28	0.00	258.95	100.00
1.95	0.79	2.28	70.73	258.95	0.00	301.68	100.00
2.28	1.07	2.65	71.79	301.68	0.00	351.46	100.00
2.65	1.42	3.09	73.21	351.46	0.00	409.45	100.00
3.09	1.81	3.60	75.02	409.45	0.00	477.01	100.00
3.60	2.18	4.19	77.20	477.01	0.00	555.71	100.00
4.19	2.49	4.88	79.70	555.71	0.00	647.41	100.00
4.88	2.71	5.69	82.41	647.41	0.00	754.23	100.00
5.69	2.82	6.63	85.23	754.23	0.00	878.67	100.00

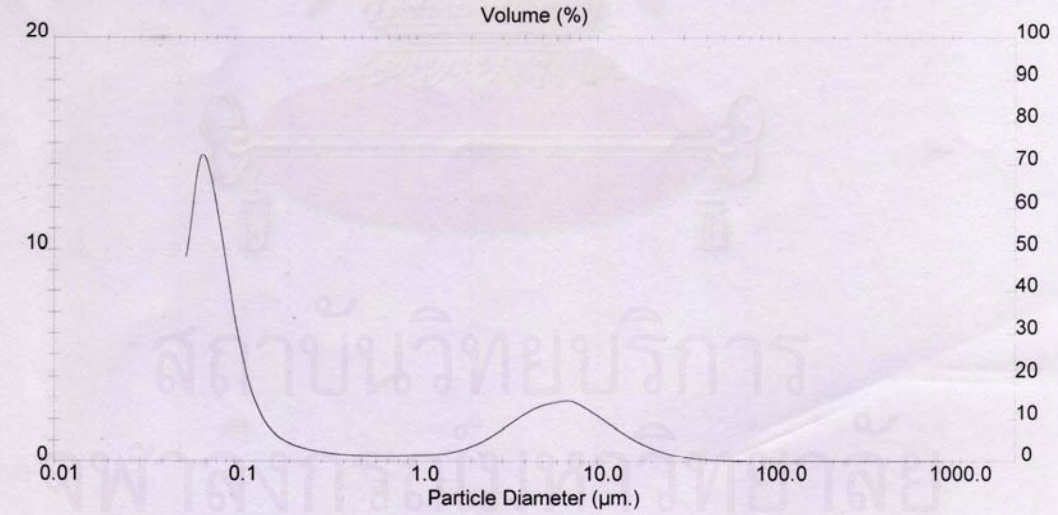
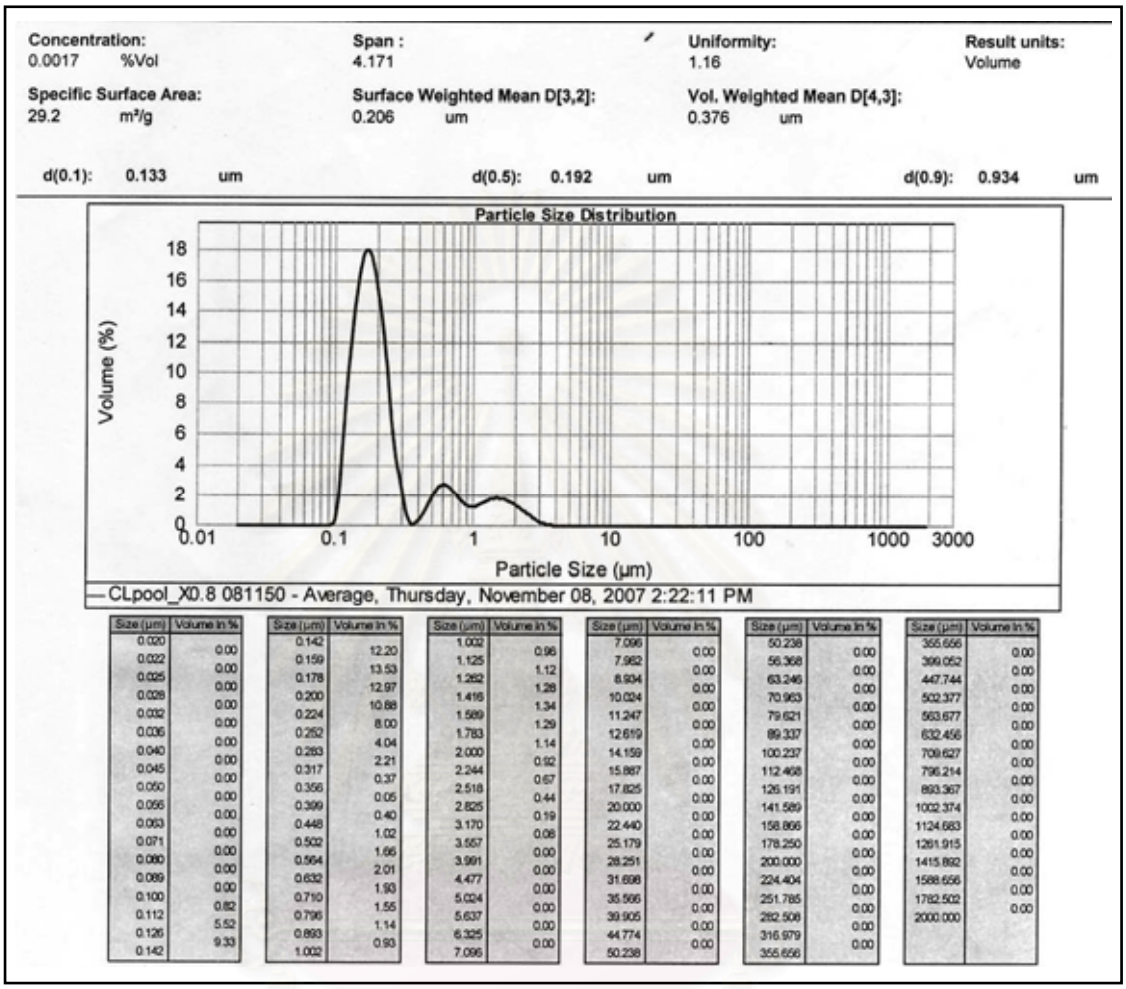


Table 57. Size and size distribution of CL at 2% PC.



Concentration:	Span :	Uniformity:	Result units:
0.0017 %Vol	4.171	1.16	Volume
Specific Surface Area:	**Surface Weighted Mean D[3,2]:**	**Vol. Weighted Mean D[4,3]:**	
29.2 m²/g	0.206 um	0.376 um	

d(0.1): 0.133 um d(0.5): 0.192 um d(0.9): 0.934 um

Size (µm)	Volume In %
0.020	0.00
0.022	0.00
0.025	0.00
0.028	0.00
0.032	0.00
0.036	0.00
0.040	0.00
0.045	0.00
0.050	0.00
0.056	0.00
0.063	0.00
0.071	0.00
0.080	0.00
0.089	0.00
0.100	0.00
0.112	0.82
0.126	5.52
0.142	9.33

Size (µm)	Volume In %
0.142	12.20
0.159	13.53
0.178	12.97
0.200	10.88
0.224	8.00
0.252	4.04
0.283	2.21
0.317	0.37
0.356	0.05
0.399	0.40
0.448	1.02
0.502	1.66
0.564	2.01
0.632	1.93
0.710	1.55
0.796	1.14
0.893	0.93
1.002	

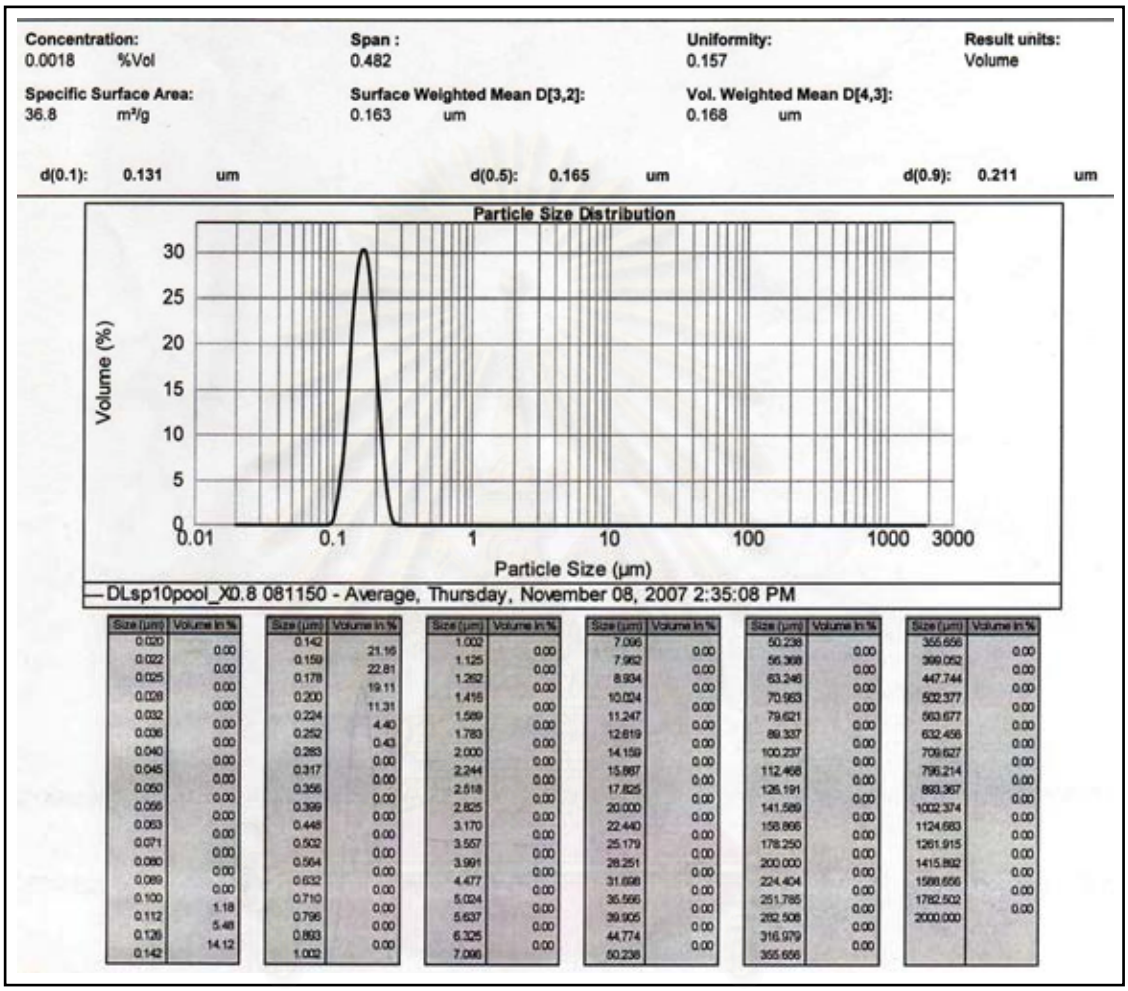
Size (µm)	Volume In %
1.002	0.96
1.125	1.12
1.262	1.28
1.416	1.34
1.589	1.29
1.783	1.14
2.000	0.92
2.244	0.67
2.518	0.44
2.825	0.19
3.170	0.08
3.557	0.00
3.991	0.00
4.477	0.00
5.024	0.00
5.637	0.00
6.325	0.00
7.096	

Size (µm)	Volume In %
7.096	0.00
7.962	0.00
8.934	0.00
10.024	0.00
11.247	0.00
12.619	0.00
14.159	0.00
15.887	0.00
17.825	0.00
20.000	0.00
22.440	0.00
25.179	0.00
28.251	0.00
31.698	0.00
35.566	0.00
39.905	0.00
44.774	0.00
50.238	

Size (µm)	Volume In %
50.238	0.00
56.368	0.00
63.246	0.00
70.963	0.00
79.621	0.00
89.337	0.00
100.237	0.00
112.468	0.00
126.191	0.00
141.589	0.00
158.866	0.00
178.250	0.00
200.000	0.00
224.404	0.00
251.785	0.00
282.506	0.00
316.979	0.00
355.656	

Size (µm)	Volume In %
355.656	0.00
399.052	0.00
447.744	0.00
502.377	0.00
563.677	0.00
632.456	0.00
709.627	0.00
796.214	0.00
893.367	0.00
1002.374	0.00
1124.683	0.00
1261.915	0.00
1415.892	0.00
1588.656	0.00
1782.502	0.00
2000.000	0.00

Table 58. Size and size distribution of DL at 2% PC, using 10% of Span 80 as edge activator.

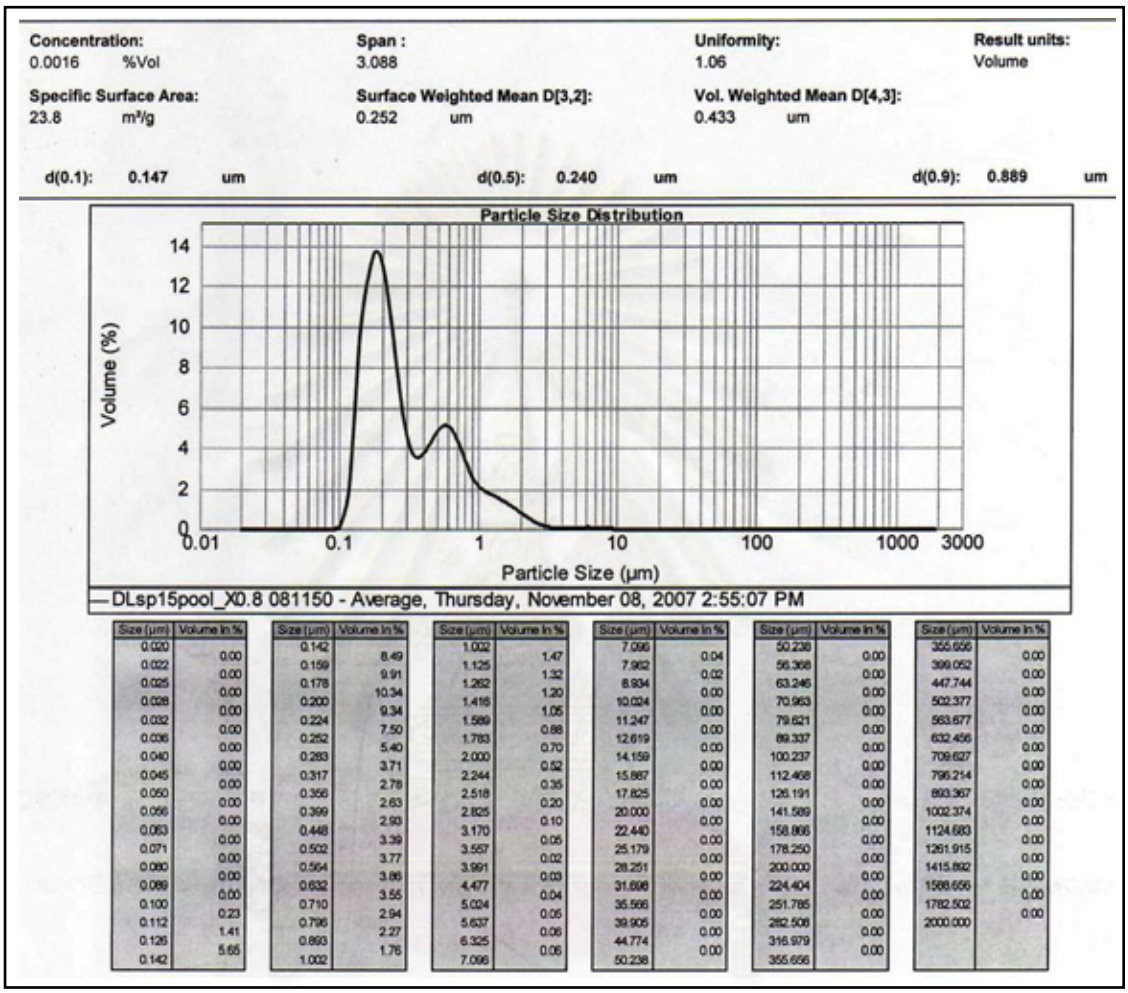


Concentration:	Span :	Uniformity:	Result units:
0.0018 %Vol	0.482	0.157	Volume
Specific Surface Area:	**Surface Weighted Mean D[3,2]:**	**Vol. Weighted Mean D[4,3]:**	
36.8 m^2/g	0.163 um	0.168 um	

d(0.1): 0.131 um d(0.5): 0.165 um d(0.9): 0.211 um

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	21.16	1.002	0.00	7.096	0.00	50.238	0.00	355.656	0.00
0.022	0.00	0.159	22.81	1.125	0.00	7.962	0.00	56.368	0.00	399.052	0.00
0.025	0.00	0.178	19.11	1.262	0.00	8.934	0.00	63.246	0.00	447.744	0.00
0.028	0.00	0.200	11.31	1.416	0.00	10.024	0.00	70.953	0.00	502.377	0.00
0.032	0.00	0.224	4.40	1.589	0.00	11.247	0.00	79.621	0.00	563.677	0.00
0.036	0.00	0.252	0.43	1.783	0.00	12.619	0.00	89.337	0.00	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.00	14.159	0.00	100.237	0.00	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.00	15.887	0.00	112.468	0.00	796.214	0.00
0.050	0.00	0.356	0.00	2.518	0.00	17.825	0.00	126.191	0.00	893.367	0.00
0.056	0.00	0.399	0.00	2.825	0.00	20.000	0.00	141.589	0.00	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	0.00	22.440	0.00	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.00	25.179	0.00	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.00	28.251	0.00	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.00	31.698	0.00	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.00	35.566	0.00	251.785	0.00	1782.502	0.00
0.112	1.18	0.796	0.00	5.637	0.00	39.905	0.00	282.508	0.00	2000.000	0.00
0.126	5.48	0.893	0.00	6.325	0.00	44.774	0.00	316.979	0.00		
0.142	14.12	1.002	0.00	7.096	0.00	50.238	0.00	355.656	0.00		

Table 59. Size and size distribution of DL at 2% PC, using 15% of Span 80 as edge activator.

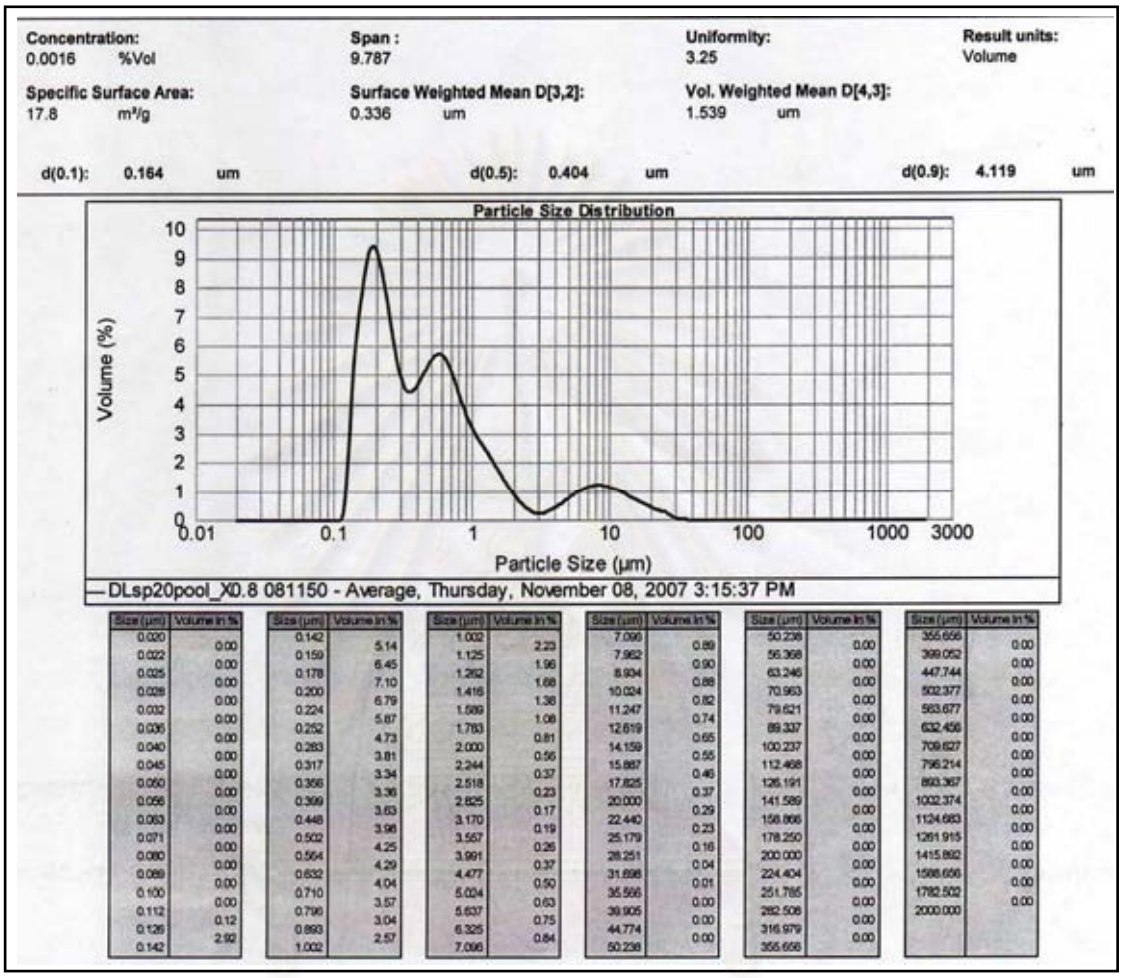


Concentration:	Span :	Uniformity:	Result units:
0.0016 %Vol	3.088	1.06	Volume
Specific Surface Area:	**Surface Weighted Mean D[3,2]:**	**Vol. Weighted Mean D[4,3]:**	
23.8 m^2/g	0.252 um	0.433 um	

d(0.1): 0.147 um d(0.5): 0.240 um d(0.9): 0.889 um

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	8.49	1.002	1.47	7.096	0.04	50.238	0.00	355.656	0.00
0.022	0.00	0.159	9.91	1.125	1.32	7.962	0.02	56.368	0.00	399.052	0.00
0.025	0.00	0.178	10.34	1.262	1.20	8.934	0.00	63.246	0.00	447.744	0.00
0.028	0.00	0.200	9.34	1.416	1.05	10.024	0.00	70.963	0.00	502.377	0.00
0.032	0.00	0.224	7.50	1.589	0.88	11.247	0.00	79.621	0.00	563.677	0.00
0.036	0.00	0.252	5.40	1.783	0.70	12.619	0.00	89.337	0.00	632.456	0.00
0.040	0.00	0.283	3.71	2.000	0.52	14.159	0.00	100.237	0.00	709.627	0.00
0.045	0.00	0.317	2.78	2.244	0.35	15.887	0.00	112.468	0.00	796.214	0.00
0.050	0.00	0.356	2.63	2.518	0.20	17.825	0.00	126.191	0.00	893.367	0.00
0.056	0.00	0.399	2.93	2.825	0.10	20.000	0.00	141.589	0.00	1002.374	0.00
0.063	0.00	0.448	3.39	3.170	0.05	22.440	0.00	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	3.77	3.557	0.02	25.179	0.00	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	3.86	3.991	0.03	28.251	0.00	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	3.55	4.477	0.04	31.698	0.00	224.404	0.00	1588.656	0.00
0.100	0.23	0.710	2.94	5.024	0.05	35.566	0.00	251.785	0.00	1782.502	0.00
0.112	1.41	0.796	2.27	5.637	0.06	39.905	0.00	282.508	0.00	2000.000	
0.126	5.65	0.893	1.76	6.325	0.05	44.774	0.00	316.979	0.00		
0.142		1.002		7.096		50.238		355.656			

Table 60. Size and size distribution of DL at 2% PC, using 20% of Span 80 as edge activator.



Concentration:	Span :	Uniformity:	Result units:
0.0016 %Vol	9.787	3.25	Volume
Specific Surface Area:	**Surface Weighted Mean D[3,2]:**	**Vol. Weighted Mean D[4,3]:**	
17.8 m²/g	0.336 um	1.539 um	

d(0.1): 0.164 um d(0.5): 0.404 um d(0.9): 4.119 um

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	5.14	1.002	2.23	7.099	0.89	50.238	0.00	355.656	0.00
0.022	0.00	0.159	6.45	1.125	1.96	7.962	0.90	56.368	0.00	399.052	0.00
0.025	0.00	0.178	7.10	1.262	1.68	8.934	0.88	63.246	0.00	447.744	0.00
0.028	0.00	0.200	6.79	1.416	1.38	10.024	0.82	70.953	0.00	502.377	0.00
0.032	0.00	0.224	5.87	1.589	1.08	11.247	0.74	79.621	0.00	563.677	0.00
0.035	0.00	0.252	4.73	1.783	0.81	12.619	0.65	89.337	0.00	632.456	0.00
0.040	0.00	0.283	3.81	2.000	0.56	14.159	0.55	100.237	0.00	709.627	0.00
0.045	0.00	0.317	3.34	2.244	0.37	15.887	0.46	112.468	0.00	796.214	0.00
0.050	0.00	0.356	3.36	2.518	0.23	17.825	0.37	126.191	0.00	893.367	0.00
0.056	0.00	0.399	3.63	2.825	0.17	20.000	0.29	141.589	0.00	1002.374	0.00
0.063	0.00	0.448	3.98	3.170	0.19	22.440	0.23	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	4.25	3.557	0.26	25.179	0.16	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	4.29	3.991	0.37	28.251	0.04	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	4.04	4.477	0.50	31.698	0.01	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	3.57	5.024	0.63	35.566	0.00	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	3.04	5.637	0.75	39.905	0.00	282.506	0.00	2000.000	0.00
0.126	0.12	0.893	2.57	6.325	0.84	44.774	0.00	316.979	0.00		
0.142	2.92	1.002		7.099		50.238	0.00	355.656	0.00		

Table 61. Size and size distribution of DL at 2% PC, using 10% of Tween 80 as edge activator.

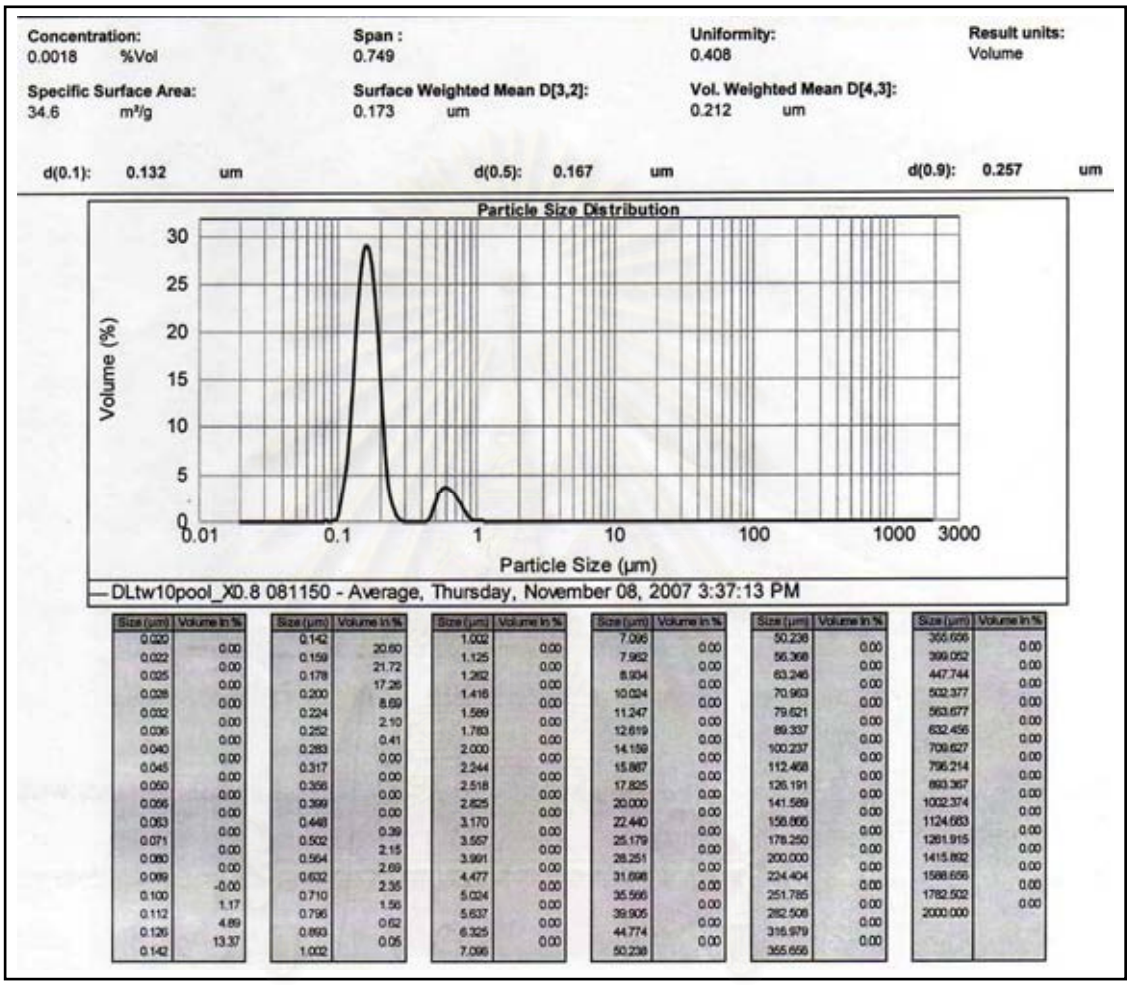
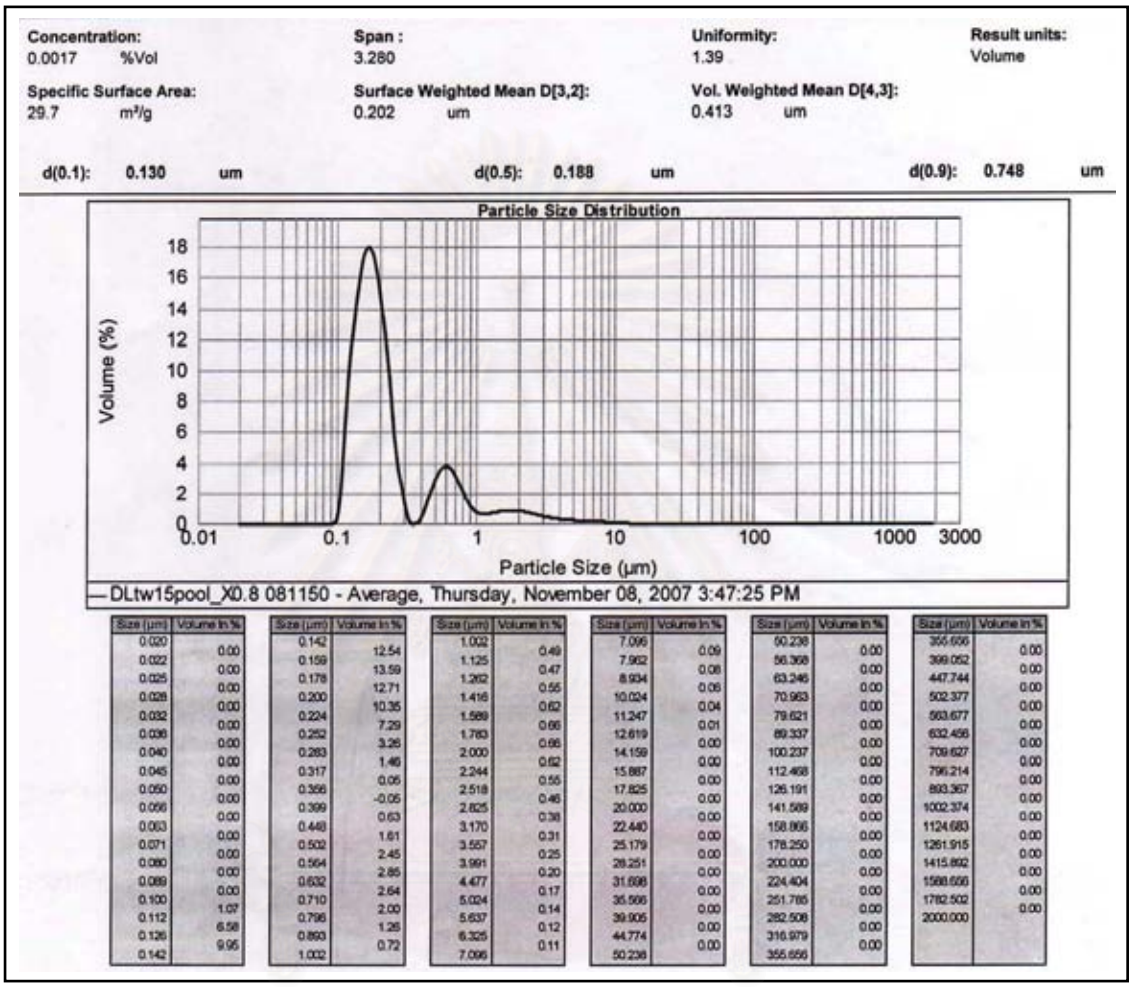


Table 62. Size and size distribution of DL at 2% PC, using 15% of Tween 80 as edge activator.

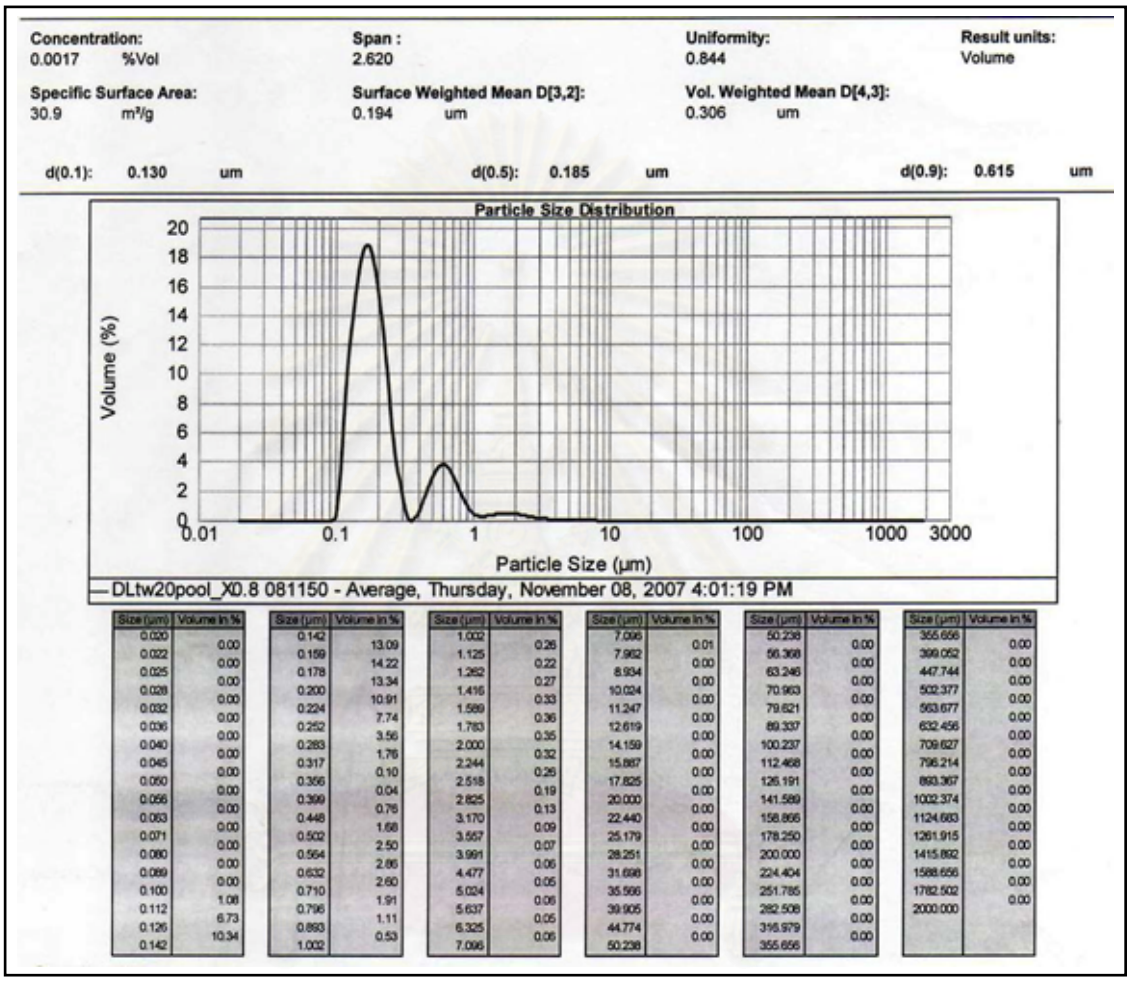


Concentration:	Span :	Uniformity:	Result units:
0.0017 %Vol	3.280	1.39	Volume
Specific Surface Area:	**Surface Weighted Mean D[3,2]:**	**Vol. Weighted Mean D[4,3]:**	
29.7 m^2/g	0.202 um	0.413 um	

d(0.1): 0.130 um d(0.5): 0.188 um d(0.9): 0.748 um

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	12.54	1.002	0.49	7.096	0.09	50.238	0.00	355.656	0.00
0.022	0.00	0.159	13.59	1.125	0.47	7.962	0.08	56.368	0.00	399.052	0.00
0.025	0.00	0.178	12.71	1.262	0.55	8.934	0.06	63.246	0.00	447.744	0.00
0.028	0.00	0.200	10.35	1.416	0.62	10.024	0.04	70.953	0.00	502.377	0.00
0.032	0.00	0.224	7.29	1.589	0.66	11.247	0.01	79.621	0.00	563.677	0.00
0.036	0.00	0.252	3.26	1.783	0.66	12.619	0.00	89.337	0.00	632.456	0.00
0.040	0.00	0.283	1.46	2.000	0.62	14.159	0.00	100.237	0.00	709.627	0.00
0.045	0.00	0.317	0.05	2.244	0.55	15.887	0.00	112.468	0.00	796.214	0.00
0.050	0.00	0.356	-0.05	2.518	0.46	17.825	0.00	126.191	0.00	893.367	0.00
0.056	0.00	0.399	0.63	2.825	0.38	20.000	0.00	141.589	0.00	1002.374	0.00
0.063	0.00	0.448	1.81	3.170	0.31	22.440	0.00	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	2.45	3.557	0.25	25.179	0.00	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	2.85	3.991	0.20	28.251	0.00	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	2.64	4.477	0.17	31.698	0.00	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	2.00	5.024	0.14	35.566	0.00	251.785	0.00	1782.502	0.00
0.112	1.07	0.796	1.26	5.637	0.12	39.905	0.00	282.508	0.00	2000.000	0.00
0.126	6.58	0.893	0.72	6.325	0.11	44.774	0.00	316.979	0.00		
0.142	9.95	1.002		7.096		50.238	0.00	355.656	0.00		

Table 63. Size and size distribution of DL at 2% PC, using 20% of Tween 80 as edge activator.



Concentration:	Span :	Uniformity:	Result units:
0.0017 %Vol	2.620	0.844	Volume
Specific Surface Area:	**Surface Weighted Mean D[3,2]:**	**Vol. Weighted Mean D[4,3]:**	
30.9 m²/g	0.194 um	0.306 um	

d(0.1): 0.130 um d(0.5): 0.185 um d(0.9): 0.615 um

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	13.09	1.002	0.26	7.096	0.01	50.238	0.00	355.656	0.00
0.022	0.00	0.159	14.22	1.125	0.22	7.962	0.00	56.368	0.00	399.052	0.00
0.025	0.00	0.178	13.34	1.262	0.27	8.934	0.00	63.246	0.00	447.744	0.00
0.028	0.00	0.200	10.91	1.416	0.33	10.024	0.00	70.963	0.00	502.377	0.00
0.032	0.00	0.224	7.74	1.589	0.36	11.247	0.00	79.621	0.00	563.677	0.00
0.036	0.00	0.252	3.56	1.783	0.35	12.619	0.00	89.337	0.00	632.456	0.00
0.040	0.00	0.283	1.76	2.000	0.32	14.159	0.00	100.237	0.00	709.627	0.00
0.045	0.00	0.317	0.10	2.244	0.26	15.887	0.00	112.468	0.00	796.214	0.00
0.050	0.00	0.356	0.04	2.518	0.19	17.825	0.00	126.191	0.00	893.367	0.00
0.056	0.00	0.399	0.76	2.825	0.13	20.000	0.00	141.589	0.00	1002.374	0.00
0.063	0.00	0.448	1.68	3.170	0.09	22.440	0.00	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	2.50	3.557	0.07	25.179	0.00	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	2.86	3.991	0.06	28.251	0.00	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	2.60	4.477	0.05	31.698	0.00	224.404	0.00	1588.656	0.00
0.100	1.08	0.710	1.91	5.024	0.06	35.566	0.00	251.785	0.00	1782.502	0.00
0.112	6.73	0.796	1.11	5.637	0.05	39.905	0.00	282.508	0.00	2000.000	
0.126	10.34	0.893	0.53	6.325	0.06	44.774	0.00	316.979	0.00		
0.142		1.002		7.096		50.238		355.656			

Table 64. The percentage of recovery of CL and DL by ultracentrifugation (week 0).

formulation	supernatant (mg)	precipitate (mg)	% recovery	mean	SD
CL_Ser 1	142.94	35.25	101.82		
CL_Ser 2	141.20	37.36	102.03	103.63	2.95
CL_Ser 3	151.52	35.80	107.04		
DLSp10_Ser 1	112.04	36.77	85.04		
DLSp10_Ser 2	115.15	33.60	85.00	85.13	0.19
DLSp10_Ser 3	112.71	36.64	85.35		
DLSp15_Ser 1	102.32	46.57	85.08		
DLSp15_Ser 2	106.27	44.19	85.98	85.54	0.45
DLSp15_Ser 3	105.72	44.00	85.55		
DLSp20_Ser 1	139.35	22.16	92.29		
DLSp20_Ser 2	158.46	27.39	106.20	101.90	8.34
DLSp20_Ser 3	163.36	24.26	107.21		
DLTw10_Ser 1	133.53	41.78	100.18		
DLTw10_Ser 2	158.07	39.85	113.09	107.33	6.57
DLTw10_Ser 3	156.78	33.49	108.72		
DLTw15_Ser 1	135.72	48.34	105.17		
DLTw15_Ser 2	152.04	51.12	116.09	108.48	6.61
DLTw15_Ser 3	138.29	44.03	104.18		
DLTw20_Ser 1	143.48	56.44	91.39		
DLTw20_Ser 2	159.24	48.51	94.97	93.76	2.05
DLTw20_Ser 3	159.37	48.23	94.90		

Table 65. The percentage of recovery of CL and DL by ultracentrifugation (week 1).

formulation	supernatant (mg)	precipitate (mg)	% recovery	mean	SD
CL_Ser 1 wk 1	146.27	29.24	100.29		
CL_Ser 2 wk 1	155.23	31.40	106.64	106.41	6.01
CL_Ser 3 wk 1	164.50	32.01	112.29		
DLSp10_Ser 1 wk 1	112.38	36.83	85.26		
DLSp10_Ser 2 wk 1	111.56	37.60	85.23	85.27	0.04
DLSp10_Ser 3 wk 1	112.23	37.06	85.31		
DLSp15_Ser 1 wk 1	106.58	42.76	85.34		
DLSp15_Ser 2 wk 1	102.60	47.67	85.87	85.53	0.30
DLSp15_Ser 3 wk 1	99.49	49.92	85.37		
DLSp20_Ser 1 wk 1	142.10	48.03	108.64		
DLSp20_Ser 2 wk 1	136.12	42.60	102.13	106.48	3.77
DLSp20_Ser 3 wk 1	153.75	36.40	108.66		
DLTw10_Ser 1 wk 1	183.50	35.09	99.93		
DLTw10_Ser 2 wk 1	173.90	26.41	91.57	96.55	4.40
DLTw10_Ser 3 wk 1	187.75	26.96	98.15		
DLTw15_Ser 1 wk 1	141.59	49.67	109.29		
DLTw15_Ser 2 wk 1	151.15	46.58	112.99	110.88	1.91
DLTw15_Ser 3 wk 1	150.21	42.91	110.36		
DLTw20_Ser 1 wk 1	161.93	40.03	92.32		
DLTw20_Ser 2 wk 1	163.57	45.57	95.61	94.30	1.74
DLTw20_Ser 3 wk 1	165.80	41.96	94.98		

Table 66. The percentage of recovery of CL and DL by ultracentrifugation (week 2).

formulation	supernatant (mg)	precipitate (mg)	% recovery	mean	SD
CL_Ser 1 wk 2	136.98	53.17	108.66		
CL_Ser 2 wk 2	136.87	52.81	108.39	109.32	1.38
CL_Ser 3 wk 2	141.29	52.80	110.91		
DLSp10_Ser 1 wk 2	110.53	47.74	90.43		
DLSp10_Ser 2 wk 2	109.20	41.19	85.94	87.19	2.83
DLSp10_Ser 3 wk 2	111.67	37.43	85.20		
DLSp15_Ser 1 wk 2	116.27	41.28	90.03		
DLSp15_Ser 2 wk 2	121.15	40.94	92.62	93.79	4.46
DLSp15_Ser 3 wk 2	132.77	40.00	98.73		
DLSp20_Ser 1 wk 2	133.72	29.95	93.53		
DLSp20_Ser 2 wk 2	157.18	35.99	110.39	106.66	11.72
DLSp20_Ser 3 wk 2	172.65	30.44	116.05		
DLTw10_Ser 1 wk 2	180.83	37.63	99.87		
DLTw10_ Ser 2 wk 2	171.26	39.11	96.17	99.22	2.79
DLTw10_Ser 3 wk 2	185.13	37.20	101.64		
DLTw15_Ser 1 wk 2	176.55	28.73	93.84		
DLTw15_Ser 2 wk 2	185.86	28.74	98.10	96.73	2.51
DLTw15_Ser 3 wk 2	180.38	34.56	98.26		
DLTw20_Ser 1 wk 2	197.91	31.48	104.86		
DLTw20_Ser 2 wk 2	181.42	31.32	97.25	101.13	3.81
DLTw20_Ser 3 wk 2	187.16	34.39	101.28		

Table 67. The percentage of recovery of CL and DL by ultracentrifugation (week 3).

formulation	supernatant (mg)	precipitate (mg)	% recovery	mean	SD
CL_Ser 1 wk 3	136.12	47.65	105.01		
CL_Ser 2 wk 3	139.59	46.34	106.24	106.70	1.95
CL_Ser 3 wk 3	144.32	46.14	108.83		
DLSp10_Ser 1 wk 3	132.96	35.87	96.47		
DLSp10_Ser 2 wk 3	161.61	36.01	112.93	103.61	8.44
DLSp10_Ser 3 wk 3	145.90	31.61	101.43		
DLSp15_Ser 1 wk 3	187.50	38.20	103.18		
DLSp15_Ser 2 wk 3	180.41	44.43	102.78	101.19	3.10
DLSp15_Ser 3 wk 3	167.64	45.89	97.62		
DLSp20_Ser 1 wk 3	174.24	43.37	99.48		
DLSp20_Ser 2 wk 3	187.53	38.62	103.38	101.92	2.13
DLSp20_Ser 3 wk 3	188.54	36.56	102.90		
DLTw10_Ser 1 wk 3	126.88	39.76	95.22		
DLTw10_Ser 2 wk 3	122.99	40.82	93.60	95.36	1.82
DLTw10_Ser 3 wk 3	128.47	41.71	97.24		
DLTw15_Ser 1 wk 3	144.12	47.59	87.64		
DLTw15_Ser 2 wk 3	161.57	45.47	94.65	91.28	3.51
DLTw15_Ser 3 wk 3	155.71	44.56	91.55		
DLTw20_Ser 1 wk 3	153.52	41.85	89.31		
DLTw20_Ser 2 wk 3	148.65	46.31	89.12	88.99	0.41
DLTw20_Ser 3 wk 3	145.83	47.81	88.52		

Table 68. The percentage of recovery of CL and DL by ultracentrifugation (week 4).

formulation	supernatant (mg)	precipitate (mg)	% recovery	mean	SD
CL_Ser 1 wk 4	149.27	60.76	96.01		
CL_Ser 2 wk 4	178.04	47.90	103.29	101.50	4.85
CL_Ser 3 wk 4	181.54	48.58	105.20		
DLSp10_Ser 1 wk 4	144.49	50.14	111.22		
DLSp10_Ser 2 wk 4	154.66	37.59	109.86	111.36	1.58
DLSp10_Ser 3 wk 4	158.04	39.73	113.01		
DLSp15_Ser 1 wk 4	155.91	47.07	92.79		
DLSp15_Ser 2 wk 4	162.51	45.30	95.00	94.24	1.25
DLSp15_Ser 3 wk 4	161.88	45.78	94.93		
DLSp20_Ser 1 wk 4	163.31	55.10	99.84		
DLSp20_Ser 2 wk 4	196.05	43.20	109.37	106.16	5.47
DLSp20_Ser 3 wk 4	196.76	42.28	109.27		
DLTw10_Ser 1 wk 4	118.10	29.96	84.61		
DLTw10_Ser 2 wk 4	125.82	38.47	93.88	87.38	5.65
DLTw10_Ser 3 wk 4	116.84	29.55	83.65		
DLTw15_Ser 1 wk 4	192.21	44.35	108.14		
DLTw15_Ser 2 wk 4	203.71	40.72	111.74	108.44	3.16
DLTw15_Ser 3 wk 4	189.71	40.95	105.44		
DLTw20_Ser 1 wk 4	195.24	39.23	107.19		
DLTw20_Ser 2 wk 4	193.73	48.92	110.92	109.21	1.89
DLTw20_Ser 3 wk 4	191.53	48.05	109.52		

Table 69. The entrapment efficiency of CL_Ser by ultracentrifugation (week 0-4).

formulation	% EE				SD
	n_1	n_2	n_3	mean	
CL_Ser wk 0	70.50	74.71	71.61	72.27	2.18
CL_Ser wk 1	58.47	62.80	64.02	61.77	2.92
CL_Ser wk 2	106.35	105.62	105.60	105.85	0.43
CL_Ser wk 3	95.30	92.68	92.27	93.42	1.64
CL_Ser wk 4	121.52	95.79	97.16	104.82	14.47

Table 70. The entrapment efficiency of DLSp10_Ser, DLSp15_Ser and DLSp20_Ser by ultracentrifugation (week 0-4).

formulation	% EE				SD
	n_1	n_2	n_3	mean	
DLSp10_Ser wk 0	73.54	67.20	73.28	71.34	3.59
DLSp10_Ser wk 1	73.66	75.19	74.11	74.32	0.79
DLSp10_Ser wk 2	95.47	82.39	74.85	84.24	10.43
DLSp10_Ser wk 3	71.74	72.02	63.21	68.99	5.01
DLSp10_Ser wk 4	100.29	75.19	79.45	84.98	13.43
DLSp15_Ser wk 0	93.14	88.39	87.99	89.84	2.87
DLSp15_Ser wk 1	85.52	95.34	99.83	93.56	7.32
DLSp15_Ser wk 2	82.57	81.88	80.00	81.49	1.33
DLSp15_Ser wk 3	76.41	88.86	91.79	85.69	8.17
DLSp15_Ser wk 4	94.15	90.59	91.57	92.10	1.84
DLSp20_Ser wk 0	44.31	54.78	48.52	49.20	5.27
DLSp20_Ser wk 1	96.06	85.20	72.81	84.69	11.63
DLSp20_Ser wk 2	59.91	71.98	60.88	64.26	6.71
DLSp20_Ser wk 3	86.73	77.23	73.11	79.03	6.98
DLSp20_Ser wk 4	110.19	86.39	84.56	93.71	14.30

Table 71. The entrapment efficiency of DLTw10_Ser, DLTw15_Ser and DLTw20_Ser by ultracentrifugation (week 0-4).

formulation	% EE				SD
	n_1	n_2	n_3	mean	
DLTw10_Ser wk 0	83.55	79.69	66.98	76.74	8.67
DLTw10_Ser wk 1	70.17	52.82	53.91	58.97	9.72
DLTw10_Ser wk 2	75.26	78.21	74.40	75.96	2.00
DLTw10_Ser wk 3	79.52	81.63	83.41	81.52	1.95
DLTw10_Ser wk 4	59.92	76.94	59.11	65.32	10.07
DLTw15_Ser wk 0	96.67	102.24	88.06	95.66	7.14
DLTw15_Ser wk 1	99.33	93.16	85.82	92.77	6.76
DLTw15_Ser wk 2	57.45	57.47	69.12	61.35	6.73
DLTw15_Ser wk 3	95.18	90.95	89.13	91.75	3.11
DLTw15_Ser wk 4	88.70	81.45	81.90	84.02	4.06
DLTw20_Ser wk 0	112.88	97.01	96.46	102.12	9.32
DLTw20_Ser wk 1	80.05	91.15	83.93	85.04	5.63
DLTw20_Ser wk 2	62.95	62.64	68.78	64.79	3.46
DLTw20_Ser wk 3	83.70	92.61	95.63	90.65	6.20
DLTw20_Ser wk 4	78.46	97.84	96.10	90.80	10.72

Table 72. Data for calculation of the deformability index of CL and DL, n=5.

formulation	size before (nm)	size after (nm)	r_v/r_p	$(r_v/r_p)^2$	j		DI	
					mean (mg)	SD	mean	SD
CLchol_Ser	254	0	0	0.00	16.0	2.55	0.00	0.00
CL_Ser	524	162	3	10.54	20.0	7.84	0.21	0.08
DLSp10_Ser	217	200	4	16.04	171.0	21.00	2.74	0.34
DLSp15_Ser	355	332	7	43.97	163.8	17.74	7.20	0.78
DLSp20_Ser	350	287	6	33.00	205.8	26.53	6.79	0.88
DLTw10_Ser	260	161	3	10.41	247.8	21.26	2.58	0.22
DLTw15_Ser	192	27	1	0.29	25.8	10.06	0.01	0.00
DLTw20_Ser	251	68	1	1.83	26.2	1.30	0.05	0.00

Skin permeation

Table 73. Cumulative amount of Ser_sol, permeated through the pig skin membrane, n=3.

Time (h)	Cumulative amount (mg)				
	n_1	n_2	n_3	mean	SD
0	-0.291	-0.204	-0.287	-0.261	0.05
1	-0.039	-0.180	0.028	-0.064	0.11
2	-0.163	0.090	-0.073	-0.049	0.13
4	-0.156	-0.111	-0.200	-0.156	0.04
6	-0.231	-0.176	-0.269	-0.226	0.05
8	-0.207	-0.211	-0.194	-0.204	0.01
10	-0.320	-0.193	-0.248	-0.254	0.06
12	-0.363	-0.203	-0.254	-0.273	0.08
14	-0.320	-0.261	-0.300	-0.294	0.03
16	-0.405	-0.255	-0.338	-0.333	0.08
18	-0.413	-0.275	-0.330	-0.339	0.07
20	-0.350	-0.234	-0.314	-0.299	0.06
22	-0.433	-0.266	-0.355	-0.351	0.08
24	-0.435	-0.297	-0.344	-0.359	0.07

Table 74. Cumulative amount of DLSp15_Ser, permeated through the pig skin membrane, n=3.

Time (h)	Cumulative amount (mg)				
	n_1	n_2	n_3	mean	SD
0	0.038	-0.029	-0.006	0.001	0.03
0.5	0.148	0.131	-0.018	0.087	0.09
1	0.215	0.122	0.044	0.127	0.09
1.5	0.286	0.291	0.116	0.231	0.10
2	0.477	0.256	0.296	0.343	0.12
4	0.712	0.545	0.492	0.583	0.12
6	0.905	0.576	0.436	0.639	0.24
8	0.979	0.650	0.594	0.741	0.21
10	1.123	0.777	0.888	0.929	0.18
12	1.341	0.914	0.878	1.044	0.26
14	1.391	1.277	1.045	1.238	0.18
16	1.629	1.249	1.342	1.407	0.20
18	1.839	1.366	1.363	1.523	0.27
20	1.744	1.474	1.548	1.589	0.14
22	1.964	1.505	1.590	1.686	0.24
24	1.959	1.561	1.683	1.734	0.20

Table 75. Cumulative amount of DLSp15_Sercb, permeated through the pig skin membrane, n=3.

	Cumulative amount (mg)				
Time (h)	**n_1**	**n_2**	**n_3**	**mean**	**SD**
0	-0.238	-0.202	-0.270	-0.237	0.03
0.5	-0.175	-0.110	-0.178	-0.154	0.04
1	-0.126	-0.098	-0.097	-0.107	0.02
1.5	-0.064	-0.029	-0.080	-0.058	0.03
2	-0.027	-0.016	-0.041	-0.028	0.01
4	0.188	0.214	0.028	0.143	0.10
6	0.338	0.314	0.111	0.254	0.12
8	0.378	0.436	0.190	0.335	0.13
10	0.617	0.956	0.449	0.674	0.26
12	0.925	1.089	0.682	0.898	0.20
14	1.130	1.329	0.789	1.083	0.27
16	1.261	1.693	0.923	1.292	0.39
18	1.450	2.096	0.947	1.498	0.58
20	1.674	2.252	1.123	1.683	0.56
22	1.845	2.629	1.302	1.925	0.67
24	1.917	2.868	1.226	2.004	0.82

Table 76. Cumulative amount of CL_Ser, permeated through the pig skin membrane, n=3.

	Cumulative amount (mg)				
Time (h)	**n_1**	**n_2**	**n_3**	**mean**	**SD**
0	-0.015	-0.003	0.087	0.023	0.06
0.5	0.087	0.149	0.108	0.115	0.03
1	0.207	0.220	0.156	0.194	0.03
1.5	0.189	0.300	0.304	0.264	0.07
2	0.246	0.414	0.343	0.334	0.08
4	0.416	0.566	0.569	0.517	0.09
6	0.571	0.702	0.817	0.697	0.12
8	0.720	0.540	0.897	0.719	0.18
10	0.946	0.786	1.242	0.991	0.23
12	1.100	0.919	1.190	1.070	0.14
14	1.194	1.044	1.368	1.202	0.16
16	1.265	1.154	1.523	1.314	0.19
18	1.378	1.403	1.837	1.539	0.26
20	1.463	1.427	1.892	1.594	0.26
22	1.506	1.485	1.856	1.616	0.21
24	1.477	1.701	1.836	1.671	0.18

Table 77. Cumulative amount of DLSp15_Ser equi 24 h, permeated through the pig skin membrane, n=1.

	Cumulative amount (mg)
Time (h)	**n_1**
0	1.265
0.25	1.283
0.5	1.326
0.75	1.315
1	1.221
1.5	1.404
2	1.706
4	1.902
6	2.428
8	2.550
10	2.819
12	2.999

Table 78. Analysis the permeability coefficient of sericin in various formulations, by one-way ANOVA.

ANOVA

Px10_7

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	60.782	2	30.391	8.620	.017
Within Groups	21.153	6	3.525		
Total	81.934	8			

Multiple Comparisons

Dependent Variable: Px10_7
Tukey HSD

(I) formulation	(J) formulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DLSp15_Ser	DLSp15_Sercb	-5.35667(*)	1.53307	.030	-10.0605	-.6528
	CL_Ser	.30000	1.53307	.979	-4.4039	5.0039
DLSp15_Sercb	DLSp15_Ser	5.35667(*)	1.53307	.030	.6528	10.0605
	CL_Ser	5.65667(*)	1.53307	.024	.9528	10.3605
CL_Ser	DLSp15_Ser	-.30000	1.53307	.979	-5.0039	4.4039
	DLSp15_Sercb	-5.65667(*)	1.53307	.024	-10.3605	-.9528

* The mean difference is significant at the .05 level.

Px10_7

Tukey HSD

formulation	N	Subset for alpha = .05	
		1	2
CL_Ser	3	3.2033	
DLSp15_Ser	3	3.5033	
DLSp15_Sercb	3		8.8600
Sig.		.979	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

VITA

Miss Nantawan Puangmalai was born on September 20, 1982 in Petchaburi, Thailand. She received her Bachelor's degree in Pharmacy from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand in 2005. Then, she entered the Master's degree program in Pharmacy at Chulalongkorn University in the same year.