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การพัฒนาเควอร์ซิตินนิโอโซมโดยวิธีโปรนิโอโซมเจล

DEVELOPMENT OF QUERCETIN NIOSOMES BY PRONIOSOME GEL METHOD

PATHAMAPORN CHUETEE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHARMACY (PHARMACEUTICAL TECHNOLOGY)
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บทคัดย่อ

วัตถุประสงค์ของการศึกษานี้เพื่อคัดเลือกสารลดแรงตึงผิวชนิดไม่มีประจุที่มีศักยภาพในการเตรียม และศึกษาปัจจัยที่เหมาะสมที่มีผลต่อลักษณะและความคงตัวของเควอร์ซิตินนิโอโซม เมื่อเตรียมโดยวิธีโปรนิโอโซมเจล สารลดแรงตึงผิวไม่มีประจุที่ใช้ศึกษาขั้นตอนคัดเลือกได้แก่ Span 60, Span 80, Tween 20, Tween 80, Brij 52, Brij 58, Brij 93, Brij 97 และ Brij 98 พบว่าสารในกลุ่ม Brij มีความสามารถในการสร้างอนุภาคฤงกลมที่ดีที่สุด Brij 98 เป็นสารลดแรงตึงผิวชนิดแรกที่เลือกมาทดสอบหาปัจจัยที่ส่งผลดีต่อขนาดอนุภาคและความคงตัวโดยใช้การออกแบบการทดลองเชิงแฟคทอเรียลสองยกกำลังสาม พบว่าปัจจัยที่เหมาะสมได้แก่ การเติมไดเฮกซาเด็กซิลฟอสเฟต (DCP) 0.005 กรัม และเควอร์ซิติน 0.02 กรัม ต่อ 1 กรัมของไขมันผสม และใช้ฟอสเฟตบัฟเฟอร์ พีเอช 6.0 เป็นสารเจือจาง สภาวะเหล่านี้ได้ใช้ในการศึกษาต่อ ๆ มาถึงผลของสัดส่วนต่อโมลาร์ของคอเลสเตอรอลต่อลักษณะของนิโอโซมจากโพลีออกซีเอธิลีนอัลคิลอีเธอร์ 6 ชนิด (กลุ่มชื่อการค้า Brij: Brij 30, Brij 52, Brij 58, Bri 93, Brij 97 และ Brij 98) ถูกนำมาศึกษาโดยปรับเปลี่ยนสัดส่วนสารลดแรงตึงผิวชนิดไม่มีประจุต่อคอเลสเตอรอลที่สัดส่วนโมลาร์ 1:1, 2:1 และ 3:1 พบว่า Brij 30 ที่สัดส่วนต่อโมลาร์ของสารลดแรงตึงผิวต่อคอเลสเตอรอลที่ 3:1 ให้ผลนิโอโซมที่มีลักษณะที่ดี ตำรับเควอร์ซิตินนิโอโซมนี้ได้ถูกนำไปทดสอบความคงตัวโดยการเก็บเป็นเวลา 90 วัน ที่อุณหภูมิ 4 องศาเซลเซียส 25 องศาเซลเซียส25 องศาเซลเซียสในที่มืดและ 45 องศาเซลเซียส และทำการติดตามดูการเปลี่ยนแปลงลักษณะทางกายภาพ ทดสอบร้อยละการกักเก็บและประสิทธิภาพการยับยั้งอนุมูลอิสระดีพีพีเอช โดยคิดเป็นร้อยละการยับยั้ง พบว่าตำรับมีความคงตัวดีที่อุณหภูมิ25 องศาเซลเซียสโดยสรุปการศึกษานี้แสดงให้เห็นว่า เควอร์ซิตินนิโอโซมที่มีความคงตัวสูงสามารถเตรียมได้สำเร็จโดยใช้วิธีโปรนิโอโซมเจล และระบบนำส่งนี้สามารถนำไปประยุกต์ใช้กับสารที่ละลายน้ำยากชนิดอื่น ๆ ได้

คำสำคัญ: โปรนิโอโซมเจล นิโอโซม เควอร์ซิติน โพลีออกซีเอธิลีนอัลคิลอีเธอร์ บริจ

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ABSTRACT

The objectives of this study were to screen the potential of non-ionic surfactants and to optimize the factors affecting the characteristics and the stability of quercetin niosomes prepared by proniosome gel method. Nonionic surfactants used in the screening were Span 60, Span 80, Tween 20, Tween 80, Brij 52, Brij 58, Bri 93, Brij 97 and Brij 98. It was found that Brij series showed the highest ability to form vesicles. Brij®98 was firstly selected to study the factors promoting the good size and stability by 2^3 factorial design. The optimal factors are the adding of DCP 0.005 g, the amount of quercetin at 0.02 g/g of lipid mixture and the pH 6.0 phosphate buffer as hydration medium. These conditions were used in subsequent study of the effect of the molar ratio of cholesterol on niosome characteristic. Six polyoxyethylene alkyl ether (POAE, Brij series: Brij 30, Brij 52, Brij 58, Bri 93, Brij 97 and Brij 98) were studied by varying the non-ionic surfactant: cholesterol at 1:1, 2:1 and 3:1 molar ratios. It was found that Brij 30 with the molar ratio of surfactant: cholesterol at 3:1 provided the good characteristics. The resulted quercetin niosome was tested for its stability after storing for 90 days at 4°C, 25°C, 25°C in dark place and 45°C by monitoring for physical change, % EE and DPPH-scavenging activity as % inhibition. The niosome demonstrated the high stability at 25°C. In conclusion, this study shows that stable quercetin niosome can be successfully prepared by proniosome gel method. Such a promising delivery system can be applied to other water insoluble drug.

Keywords: Proniosome gel, niosome, quercetin**,** polyoxyethylene alkyl ether, Brij

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Pathamaporn Chuetee

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LIST OF ABBREVIATIONS

% EE	Percent entrapment efficiency
°C	Degree Celsius
µg	Microgram
µm	Micrometer
Abs	Absorbance
CHO	Cholesterol
Coef	Coefficient
DCP	Dihexadecyl phosphate
DF	Degree of freedom
g	Gram
HLB	Hidrophile-lipophile balance
min	Minute
ml	Milliliter
MS	Mean of square
MW	Molecular weight
nm	Nanometer
P	P value
SA	Stearyl amine
SE Coef	Standard error coefficient
SEM	Scanning electron microscopy
SS	Sum of square
TEM	Transmission electron microscopy

CHAPTER 1
INTRODUCTION

Drug delivery system using colloidal particulate carriers such as liposome and niosome are famous and have divergent advantages over conventional dosage forms. They are useful vehicles for drug delivery of both hydrophobic and hydrophilic drugs. The liposomes contain natural or synthetic phospholipids, assemble into bilayers and like niosomes, they always contain cholesterol constitute as membrane modifier. However, liposomes have many problems such as degradation by hydrolysis or oxidation, they need special storage and handling. They undergo sedimentation, aggregation or fusion on storage. Moreover, of the purity of natural phospholipids is also variable, problematic in sterilization, transportation and distribution. The method of preparation and the uniformity of dose are hard to scale up. The used of unacceptable organic solvents are also problematic as well as the incomplete of hydration step of the thin film during processing. (1)

Therefore niosome preparation by proniosome gel method should be another good alternative. The proniosome is usually refered a dry product or a liquid crystalline gel that can be hydrated immediately before use. The advantages of this method are the unuse of harmful solvents as well as easy to prepare. Quercetin is an active ingredient to be used in this research because it is one of the most abundant flavoniod in plants commonly found in apples, onions, teas, berries and brassica vegetables, as well as in many seeds, nuts, flowers, barks and leaves. (2) Quercetin is stable in human urine, human plasma, acetonitrile and water at 4°C, -20°C and -80°C. (2) It is insoluble in water and soluble in ethanol (2 mg/ml) and 30 mg/ml in dimethyl sulfoxide. Quercetin appears to provide many beneficial effects on human health including cardiovascular protection, anticancer activity, anti ulcer, antiviral acitivity, anti-inflammatory effects due to its antioxidant activity. (2) The present study, quercetin niosome was prepared by proniosome gel method. The factors affecting quercetin niosome characteristics and stability were studied. The non-ionic surfactants used in the screening study were Span 60, Span 80, Tween 20, Tween 80, Brij 52, Brij 58, Brij 93, Brij 97, Brij 98, Myrj 45 and Myrj 59. Blank niosomes

from those non-ionic surfactants were firstly formulated to investigate the assembling ability into vesicles by proniosome gel method. Finally, the most appropriate formula those were selected for optimization study.

Quercetin niosome made from Brij 30 was prepared for tentative that was for topical application as skin antioxidant and antiaging. Physical characteristics, entrapment efficient and stability upon various storage conditions were studied.

1.1 Objectives

1. To study how to prepare niosome by proniosome gel method.
2. To fabricate a good quercetin niosome dispersion for topical used aspect.
3. To study the stability of the quercetin niosome formulated from the selected non-ionic surfactant

1.2 Scope of the study

1. Ten non-ionic surfactants were studied by preparing blank niosome to find out the good surfactant that forms the vesicles by proniosome gel method.
2. The appropriate non-ionic surfactant from the previous study was selected for optimization study using factorial design to find out the factors that affects on physical characteristics of niosomes.
3. Quercetin niosome was prepared from the most appropriate non-ionic surfactant and was evaluated for the characteristics, entrapment efficiency and stability by DPPH method.

1.3 Expected outcome of research

1. The understanding in new method of niosome preparation which is easy, simple and free from hazardous organic solvent.
2. A good quercetin niosome formula which can be developed for tentative uses for skin as antiaging.
3. A methodology that can be applied to formulate other low solubility substances.

CHAPTER 2
LITERATURE REVIEWS

Niosomes are non-ionic surfactant based vesicles that has been developed alternative to liposomes to overcome the problems using phospholipids. Niosomes was first developed and reported by L'Oreal in 1975. Niosomes similar to liposomes that can be used to carry of both hydrophilic and lipophilic drugs. These liposomes like vesicles are formed from the hydrate mixture of non-ionic surfactant, cholesterol and change inducing substance. The stable vesicles form only with the presence of proper mixture of the lipids and do not form spontaneously. (3) Niosomes have been studied as drug delivery system to carry drugs, bioactive substances for various aspected uses.

2.1 Niosome preparation methods

The method to prepare niosome is based on liposome technology. The basic process is the hydration of thin film composed of surfactant mixture. The bioactive or drug to be entrapped are dissolved in the aqueous phase or organic phase. The typical methods of preparation of niosome are as follows:

2.1.1 Thin film hydration technique (1)

This method is socalled "hand shaking". Mixture of the vesicle components was dissolved in diethyl ether or chloroform in a round bottom flask. The organic solvent is removed at room temperature using a rotary evaporator. The dried surfactant mixture is left and deposited on the wall of the flask. This thin film can be hydrated with the aqueous phase containing drug with gentle shaking to yield large multilamellar niosomes. The multilamellar are further processed to yield smaller unilamellar niosomes using sonication, microfluidization technique or extrusion through membrane.

2.1.2 Reverse phase evaporation technique (1)

This method is the removal of the solvent from an emulsion. Water in oil emulsion of surfactant mixtures in ether and chloroform is first formed by sonication in ice bath. The drug to be loaded is placed in any appropriate phase. The emulsion

is then dried to a semi-solid clear gel in a rotary evaporator under reduced pressure to remove the organic solvent. Next step is to collapse the gel to niosome by adding aqueous phase (phosphate buffer) drop by drop and vigorously shaking to yield niosome dispersion. Fig 1 and Fig 2 are liposome production that can be modified to prepare niosome by change phospholipid to non-ionic surfactant.

Figure 1 Liposome productions by thin film method (4)

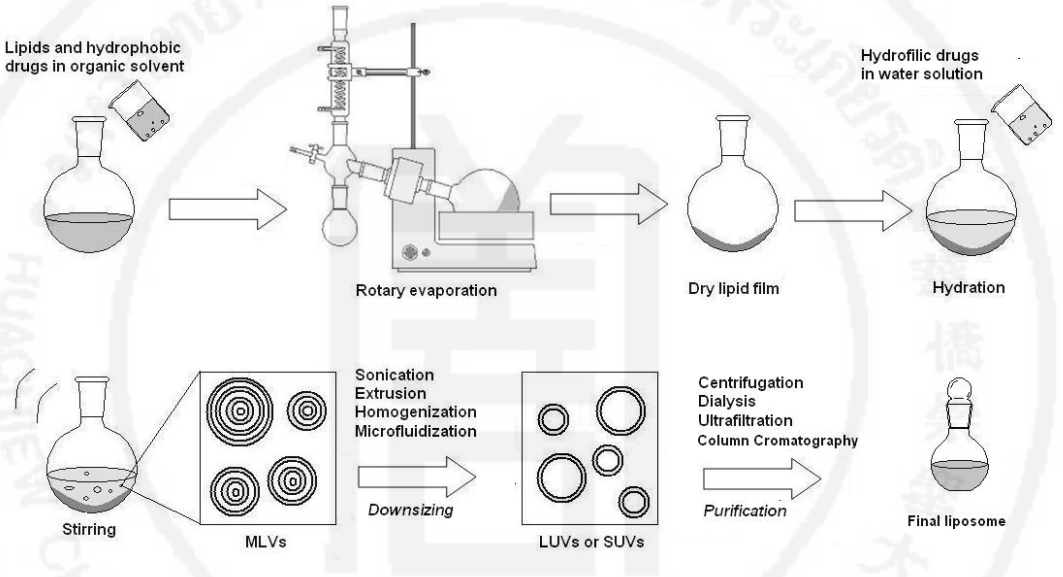
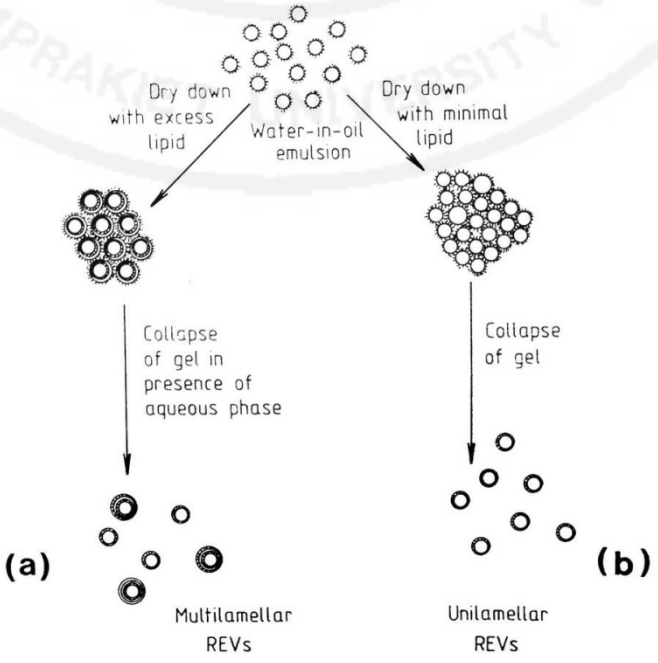


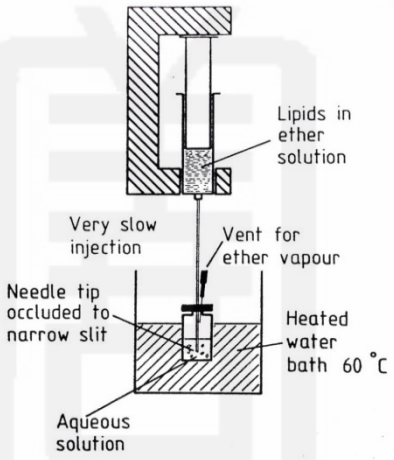
Figure 2 Liposome preparations using the reverse phase evaporation technique (5)



2.1.3 Ether injection method (1)

This method was reported by Deamer and Bangham in 1976. The lipid solution is first dissolved in diethyl ether. This solvent is then injected into warm water or aqueous media containing the drug maintained at 55-60°C under reduced pressure using 14 gauge needles. Vaporization of the ether leads to form single layered vesicles (SLVs). The vesicle sizes vary depend on the condition used. Fig 3 is liposome preparation method that can be used to prepare niosome.

Figure 3 Liposome preparations using the ether injection method (5)



2.1.4 The bubble method (1)

This is a novel technique recently developed to prepare niosomes without the use of organic solvents. The apparatus consists of a round bottomed flask with three necks positioned in water bath to control temperature. Water cooled reflux and thermometer is positioned in the first and second neck while the third neck is used to supply nitrogen gas. Surfactant mixture is dispersed in a buffer medium at 70°C and mixed with speed homogenizer and immediately afterward bubbled with nitrogen gas to yield noisome at 70°C.

2.1.5 Formation of niosomes from proniosomes (6)

Proniosome is called for a preparation consists of a water soluble carrier such as sorbitol or lactose that coated with surfactant/lipid mixture. The coating is done by dissolving the surfactant/lipid mixture with a volatile organic solvent such as chloroform or ethanol, which is sprayed onto the carrier powder under the reduced

pressure to remove the organic solvent. The noisome will form when an aqueous phase is added at temperature higher than surfactant/lipid phase transition temperature with a brief agitation. The term proniosomes now is used to describe another method of niosome preparation which is proniosome gel. The term proniosome gel will be described in the consecutive paragraph.

2.2 Comparison of niosome and liposome

Niosomes are different from liposomes in that their main vesicle component of non ionic surfactant vs phospholipids. Niosome offers certain advantages over liposomes. Liposomes compost of phospholipid which most of them are more expensive than non-ionic surfactants. The phospholipids are chemical unstable because of oxidation reaction thus, they require special formulation methods, storage and handling as show in Table 1.

Niosomes seem to have no any much problem. However, niosomes and liposomes are similar in functionality. They can be used for increasing bioavailability of the drug from the clearance, controlling the release, enhancing the skin penetration of certain drug and improving the drug therapeutic performance by protecting it from the biological environment and restricting effects to target. Like liposomes, niosomes can be used for oral, parenteral as well as topical uses cells.

Table 1 Comparison of liposomes and niosomes (6)

	Liposome	Niosome
1	Vesicle component of phospholipids	Vesicle component of non-ionic surfactant with or without cholesterol
2	Size ranges 10-3000nm	Size ranges 10-1000nm
3	Comparatively expensive	Inexpensive
4	Unstable from component of phospholipids	Stable from component of non-ionic surfactant
5	Some phospholipids have toxic	Non-ionic surfactant less toxic
6	Special storage condition are required	No special requirement

2.3 Application of niosomes

The use of niosome in cosmetics was first done by L'Oreal. (3) The cosmetic product is an anti-aging. Various advantages are found in niosome as the vesicle suspension in water based offers greater user compliance than oil based systems. The vesicles can act as a depot of hydrophilic, lipophilic or amphiphilic moieties and offer controlled release. The vesicle characteristics such as sizes and lamellarity can be varied depending on the requirement.

For pharmaceutical aspect, the application of niosomal technology to trade a number of diseases emphasizes on of drug targeting ability. Niosome can be used to target drug to the reticular-endothelial system (RES). Since the RES preferentially takes up niosome (like in liposome) vesicle. The uptake of niosomes is controlled by opsonins; serum factors. The opsonins always mask and fix the niosome for clearance by RES in liver and spleen. Such localization of drugs is utilized to treat tumer in liver and spleen, or parasitic infection to the liver. For the organ other than RES, niosome can be attached by antibodies to target them to specific organ or to particular cells.

One of the most useful aspects of niosomes is that they can enhance the uptake of drugs through the skin. For other application niosome can also be utilized for sustained drug release and localized drug to increase safety and efficacy of many drugs.

2.4 Factors affecting the formulation of niosomes

2.4.1 Nature of the drug

The hydrophilic and lipophilic of the drug affects degree of entrapment and stability. The position of the amphiphilic drug in the vesicle membrane is more stable stabile. In comparison to the lipophilic drug, hydrophilic drug trend to leak out from the vesicle more easily as shown in Table 2.

Table 2 Effect of drug on vesicular of niosomes (1)

Nature of drug	Leakage from the vesicle	Stability	Other properties
Hydrophobic drug	Decreased	Increased	Improved transdermal delivery
Hydrophilic drug	Increased	Decreased	-
Amphiphilic drug	Decreased	-	Increased encapsulation, altered electrophoretic mobility
Macromolecular drug	Decreased	Increased	-

2.4.2 Nature of surfactant

Type of surfactants influences entrapment efficiency, toxicity and stability. (7-8) The prior researches reported that the sorbitan monostearate (Span) surfactant with HLB values between 4-8 were found to be compatible with vesicle formation. Etheric surfactants and ester type surfactants are both frequently used to prepare niosomes. Etheric surfactants such as polyoxyethylene alkyl ether are more stable than ester type surfactants. Mahale et al. reported the impact of HLB values of the nonionic surfactants on niosome formation as shown in the Table 3.

Table 3 HLB value of surfactant and their impact in niosome formation (6)

HLB	Niosome formation
Low value	Needs to add CHO to increase stability
>6	Needs to add CHO in formation of bilayer vesicle
1.7-8.6	Decrease entrapment efficiency
8.6	Increase entrapment efficiency
14-16	Does not form niosome

Thus, HLB can be used as indicator of the vesicle formation. The higher HLB value, the higher of CHO is needed to form and stabilize the vesicle. And the hydrophilic or hydrophobic of non-ionic surfactant may be used to synthesis form vesicle as shown in the Table 4.

Table 4 The effect of niosome forming surfactant on the niosome dispersion (9)

	Increased hydrophobicity	Increased hydrophilicity
1	High phase transition	Low phase transition
2	Decreased leakage of low molecular weight drugs from the aqueous compartment	Increased leakage of low molecular weight drugs from the aqueous compartment
3	Increased stability of the niosome suspension	Decreased stability of the niosome suspension
4	Increased encapsulation	Improved transdermal delivery of hydrophobic molecules
5	Decreased toxicity	

2.4.3 Vesicle membrane additive

The most common additives in niosome membrane alter the vesicle formation. The parameter for self assembly of the vesicle proposed by Isaraelachvili et al. in 1985. (9) is the critical packing parameter (CCP) and CPP = V/l_cA_v

Where V = hydrophobic group volume

Lc = critical hydrophobic group length

Av = area of hydrophilic head group

A CPP of between 0.5-1 indicates that the vesicle is likely to form. While CPP < 0.5 is tended to give spherical micelles and CPP > 1 should produce inverted micelles. The surfactants with alkyl chain length between C12-C18 are suitable to prepare niosomes.

CHO influences the physical properties and structure of niosome by abolishing the gel to liquid phase transition. (10) After adding CHO usually up to 1:1 molar ratio, the instrinsic phase transition of the membrane is changed and influence the membrane permeability, encapsulation efficiency and increase bilayer rigidity. (9) The amount of CHO to be added depends on HLB value of the surfactants. High HLB values which come from large hydrophilic head group, higher amount of CHO is needed to compensate. This may increase encapsulation efficiency of hydrophobic drug and increase stability by decrease the leakage of drug from vesicle. To stabilize the niosomal vesicle from aggregation, charged lipid such as DCP, SA have been used to produce charge in niosome formulations. DCP provides negative charge and SA provides positive charge to vesicle. Adding DCP, usually reduce drug entrapment efficiency.

2.5 Temperature of hydration

The hydrating temperature should be above the gel to liquid transition temperature of the system. However the hydration medium and time of hydration of niosome are also critical factors.

Post preparation process (11)

After preparation of niosome, size reduction of niosomes is performed using one of the methods given following

1. Probe sonication to produce in the nanosize range (100-140 nanometer).

2. Extrusion through the defined pore size filter using some certain type of equipment such as LipoFast.

3. Microfluidization to yield niosome in sub-50 micrometer size.

4. High pressure homogenization to yield niosome in size below 100 nm.

5. Combination of sonication and filtration.

2.6 Entrapment efficiency evaluation

In most case, niosomal vesicles cannot be prepared to encapsulate 100% of active agent. However, this may provide an advantage in giving an initial burst to initiate therapy followed by a sustained maintenance dose. (11) The methods commonly used to separate unentrapped material from niosomes are:

1. Dialysis
2. Gel filtration
3. Centrifugation
4. Ultracentrifugation

Both the yield and the entrapment efficiency of liposome and niosome depend on the method of preparation. The additions of cholesterol usually increase the lipophilic drug entrapment efficiency. Niosome prepared by the film method and subsequence sonication result in less entrapment efficiency than by ether injection. (11)

The advantages and disadvantages of the different separation method are described as below. (9)

1. Dialysis method is suitable for vesicle larger than 10 μm and for highly viscous system. This is also not expensive method but take a long time from 5-24 hours. The resulted niosome will be diluted and need to be concentrated by appropriate instrument.

2. Centrifugation at least 7000xg can be used to gather the large niosome dispersion. This method is quick and inexpensive but it is fail to sediment the sub micron niosomes and may lead to the destruction of fragile systems.

3. Ultracentrifugation at 100000 – 150000 xg up to 1 hour is used to sediment all size of vesicles. This method is useful to separate and concentrate the niosome.

The most disadvantage of this method is the need of the expensive instrumentation. The processing time is quite long and the resulted niosome may lead to aggregation and bring to the destruction of the fragile system.

4. Gel filtration using Sephadex G50 or other appropriate gel bases is a method to separate niosome with not much advantage to other methods from which the slow processing up to many hours, the resulted niosome from the elution process is diluted. The gel bases are expensive and the method is not suitable for highly niosome formulations and the vesicle sizes larger than 10 μm. As mention above, the separation step may not be necessary to perform if the entrapment of the drug loaded is high enough in which the entrapped drug serves as a specific primary dose of the formulation.

2.7 Stability study of the niosome preparations (11)

- The superior advantages of niosomes compared to liposomes or some microencapsulation technologies are summarized as follows;

- The surfactants used in niosomes are more stable than phospholipid in liposomes thus niosomes are more stable at room temperature and less susceptible to light.

- Simple methods are required for manufacturing and the large scale production is possible and cost effective due to simple instruments used. Moreover, niosomes can be produced without the use of hazardous solvents.

As dispersed systems, niosomes are stabilized based upon formation of four different forces as van der Waals' forces among surfactant molecules, repulsive forces from charged group of surfactant molecules, entropic repulsive forces of the surfactant head groups, and short-acting repulsive forces from charged lipids adding to the double layers. (1) As mention above, the physical stability of niosome preparation may be due to the prevention of aggregation caused by steric interactions among large polar head groups of surfactant which depended on surfactant type, Other factors which affecting the stability of niosome which are nature of encapsulate drug, charged lipids or membrane spanning lipid (DCP, SA), and storage temperature. Many strategies have been developed which are the

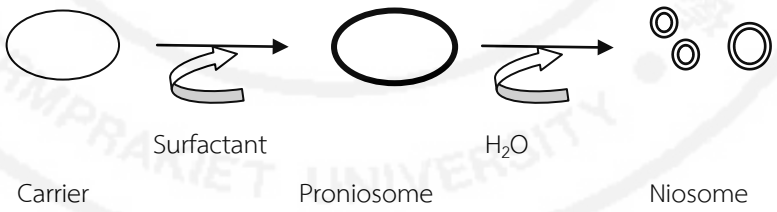
lyophilization of niosome to a reconstituted powder, proniosome preparation by coating a lipid mixture on an appropriate solid phase that readily dissolves in aqueous medium to form niosomal preparation. (6) Fig. 4 show the mechanism of noisome formation from coated carrier.

Recent niosomal preparation methods that have been in the interest of many researcher is proniosome gel method or coacervation-phase separation method. (12-19)

Perrett et al. seem to be an initial research team who described this simple method for liposome preparation. (20) This method avoids the use of unacceptable inorganic solvent and energy-expensive procedures such as sonication or extrusion. The method is nowadays called coacervation-phase separation technique which is based on the initial formation of a proliposome mixture containing lipid, ethanol and water which is converted to liposomes by a simple dilution step.

A sonication and extrusion are the most common size reduction method. Extrusion can produce nanodisperse unilamellar vesicle suspension as a result from repeat high shearing forces through polymer membrane containing well-defined pore size.

Figure 4 Niosome formations from solid carrier (6)



2.8 Membrane modifiers.

Stable niosomes can be prepared by adding of membrane modifiers along with surfactant and drugs. Polyoxyethylene alkyl ether is generally stable than ester type from esterase to triglycerides and fatty acid thus they are more toxic than ester type from this reason. (21,22)

The usual instability of niosomes are the leakage and fusion of vesicle. Both occur as a result of lattice defects in the membrane. Vesicle aggregation and sedimentation of neutral niosome is cause by Vander Waals interaction, this tends to

be more in larger vesicles from which the greater of membrane area. The simplest way to overcome it is to add a small quantity of charged induce into the lipid membrane. (3) The assembly of non-ionic surfactants to form a bilayer vesicle is characterized by an x-cross formation under light polarization microscopy.

Cholesterol is often incorporated into niosome or liposome formulations to give rigidity to the bilayer that improve the membrane stability thus enhance retention of entrapment solute. (23) Cholesterol also increase the phase transition temperature thus it reduces the leakage charge lipid usually added in the lipid membrane are DCP or SA.

Cholesterol can be incorporated in high concentration upto 1:1. It is a amphipathic molecule thus it inserts into bilayer membrane with it hydroxyl group oriented towards the aqueous surface and the aliphatic chain align parallel to the acyl chain (hydrocarbon chain) in the bilayer centre.

2.9 Proniosome gel

The term niosome is mentioned in the variety of subtypes such as proniosome, elastic niosome, surfactant ethosome and discomes. (6) Proniosome may be mentioned into two types of nonionic forming vesicles. The first is proniosome in solid carriers such as maltodextrin, mannitol or lactose which the mixture of lipids are dissolved in organic solvent and are sprayed onto the solid microparticle to result in a dry, free flowing, granular product which forms a multilamellar niosome suspension upon addition of water. (7) The second meaning of proniosome is proniosome gel. (16) Proniosome gel is basically mixture of many phases of liquid crystal which on hydration tend to form unilarmellar or multilamellar vesicles. Rawat et al. (17) described the method of preparation step by step named as coacervation phase separation method as followed

1. The surfactant, cholesterol, phosphatidyl choline and drug are mixed with the adding of minimal amount of alcohol to prevent the micelle formation.

2. The mixture was kept at 60-70°C on water bath with lid to prevent the loss of solvent.

3. Glycerol solution (1%) or buffer solution is added in limited amount to form gel and not form the dispersion. The gel is warmed again at 60-70 °C on water bath and the proniosome gel is formed.

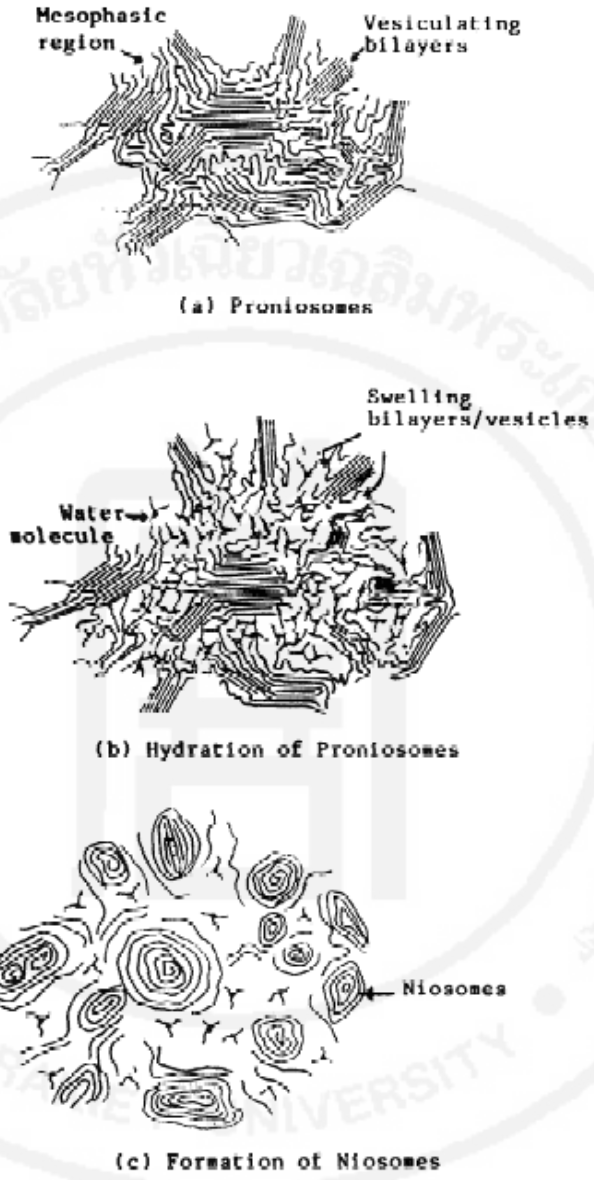
4. Water, as the concentration above the limited value is added into proniosome gel to form bilayer vesicle called niosome.

Kakkar et al. prepared valsartan proniosome form proniosome gels (the mixtures composed of Span 60, Span 40, lecithin and cholesterol. The % entrapment efficiency (% EE) and drug release after hydration with phosphate buffer were studied and found to be high values. The stability of proniosome gel was studied by investigating the % EE after storage at 4 °C and 37°C. It was found to be quite stable at 4-8°C over one month period. (18)

As proniosome gel is a dry formulation, it provides more advantages in ease to produce, higher stability of vesicle and drug compared to suspension niosome from leaking and aggregation. Moreover proniosome gel is convenience for transportation, distribution and storage. Proniosome gel can be used as drug delivery system known as provesicular system (19,24) after applying on the skin, the proniosome gel can be converted into niosome in situ by absorbing water from skin. Mechanism of drug delivery through skin of the niosome gel is still not clear.

However, many scientists proposed two types of vesicle-skin interaction. One, vesicle contacts and adheres with stratum corneum and the drug penetrates across the stratum corneum. Two, the interaction involves the ultra structure change in the intercellular lipid regions of the deeper layer of skin. Vora et al. proposed the mechanism of niosomes formation from proniosome gel as shown in the Fig. 5.

Figure 5 Mechanism of niosomes formation after hydration (21)



2.10 Nonionic surfactant

Surfactants are organic compounds containing both hydrophobic groups and hydrophilic groups by which they called amphiphilics. Surfactants usually diffuse in water and absorb at interface between air and water or at the border between oil and water. When surfactants are used in low concentration they can greatly reduce surface tension at two liquid interfaces. Nonionic surfactant means surfactant that is not ionizing in aqueous solution since it has no charged group on its head. Nonionic

surfactants do not react with other ions. As a result, they do not form insoluble salts, can be used in strong acidic solutions and tend to have low toxicity profiles. (25)

The most common type of nonionic surfactant used in industry including in pharmaceuticals are ethoxylated alcohols. Alcohol ethoxylated are known for their advantages such as low toxicity, high biodegradability, varying ethoxylation ranges and admirable cleaning performance. A typical fatty alcohol ethoxylate structure will appear as the following:

$$\mathbf{R\ (OCH_2CH_2)_nOH}$$

R	= oil soluble part of the surfactant (hydrocarbon chain)
OCH_2CH_2	= ethylene oxide, water soluble part
n	= mole of ethylene oxide

The HLB values of the surfactant are from mole of ethylene oxide, higher in n will heighten the water solubility or HLB values. Nonionic surfactant can be classified in many subtypes which are the following: (26)

- Polyoxyethylene glycol alkyl ethers (Brij): CH_3–$(CH_2)_{10-16}$–$(O\text{-}C_2H_4)_{1-25}$–OH:
  - Octaethylene glycol monododecyl ether
  - Pentaethylene glycol monododecyl ether
- Polyoxypropylene glycol alkyl ethers: CH_3–$(CH_2)_{10-16}$–$(O\text{-}C_3H_6)_{1-25}$–OH
- Glucoside alkyl ethers: CH_3–$(CH_2)_{10-16}$–(O-Glucoside)$_{1-3}$–OH:
  - Decyl glucoside,
  - Lauryl glucoside
  - Octyl glucoside
- Polyoxyethylene glycol octylphenol ethers: C_8H_{17}–(C_6H_4)–$(O\text{-}C_2H_4)_{1-25}$–OH:
  - Triton X-100
- Polyoxyethylene glycol alkylphenol ethers: C_9H_{19}–(C_6H_4)–$(O\text{-}C_2H_4)_{1-25}$–OH:
  - Nonoxynol-9
- Glycerol alkyl esters:
  - Glyceryl laurate

- Polyoxyethylene glycol sorbitan alkyl esters: Polysorbate
- Sorbitan alkyl esters: Spans
- Cocamide MEA, cocamide DEA
- Dodecyldimethylamine oxide
- Block copolymers of polyethylene glycol and polypropylene glycol: Poloxamers
- Polyethoxylated tallow amine

Table 5 Polyoxyethylene alkyl ether used in this study (26)

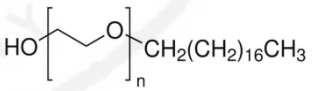
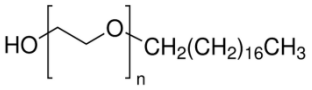
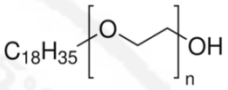
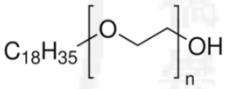
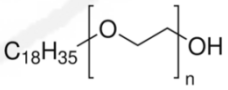
Name Brij 30 : polyoxyethylene (4) lauryl ether; Polyethylene glycol dodecyl ether **Structure** **Chemical information** MW : 362 MF : $C_{12}H_{25}(OCH_2CH_2)_4OH$ HLB : 9 Density : 0.95 g/mL at 25° C $C_{12}H_{25}(OCH_2CH_2)_4OH$
Name Brij 52 : Polyethylene glycol hexadecyl ether, Polyoxyethylene (2) cetyl ether **Structure** **Chemical information** MW : 330 MF : $C_{16}H_{33}(OCH_2CH_2)_nOH$, n~2 HLB : 5 Density : 0.978 g/mL at 25° C 
Name Brij 58 : Polyethylene glycol hexadecyl ether, Polyoxyethylene (20) cetyl ether **Chemical information** **Structure** MW : 1124 MF : $C_{16}H_{33}(OCH_2CH_2)_nOH$, n~20 HLB : 16 Density : g/mL at 25° C 

Table 5 (continued)

Name Brij 93 : Polyethylene glycol oleyl ether, Polyoxyethylene (2) oleyl ether **Chemical information** **Structure** MW : 356.58 MF : $C_{18}H_{35}(OCH_2CH_2)_nOH$, n~2 HLB : 4 Density : 0.912 g/mL at 25° C 
Name Brij 97 : Polyoxyethylene (10) oleyl ether **Chemical information** **Structure** MW : 709 MF : $C_{18}H_{35}(OCH_2CH_2)_nOH$, n~10 HLB : 12.4 Density : 1 g/mL at 25° C 
Name Brij 98 : Polyoxyethylene (20) oleyl ether **Chemical information** **Structure** MW : 1149.53 MF : $C_{18}H_{35}(OCH_2CH_2)_nOH$, n~20 HLB : 15 Density : 1.07 g/mL at 25° C 

2.11 Free radical or oxidant (27)

Free radicals are reactive molecules due to the presence of one or more unpaired electron. Its reactive species, that can either oxidize other compounds or easily form radicals, will arise. This partly reactive oxygen are collectively described as reactive oxygen species (ROS) and reactive nitrogen species (RNS). In aerobic life forms, the reduction of oxygen is of special interest. Its comprises binding of most of

the oxygen to hydrogen to give water. The ROS include singlet oxygen ($^1O_3$), superoxide ($O_2^{-\bullet}$) radical, hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\bullet$), ozone (O_3) and hypochlorous acid (HOCl). Examples of RNS are nitric oxide ($NO^\bullet$) radical and nitrogen species is given in Table 6. In the human body ROS and RNS are produced. It important physiology functions, including smooth muscle relaxation, metabolism of xenobiotics and the respiratory burst.

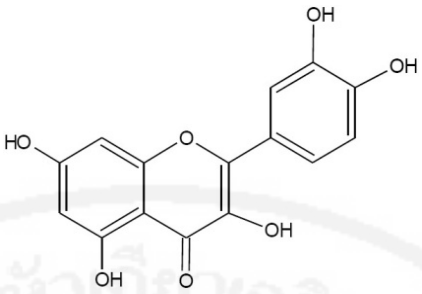
Table 6 Typical physiological reactive oxygen and nitrogen species (27)

Radicals	Non-radicals
Reactive oxygen species	
Superoxide, $O_2^{-\bullet}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $HO^\bullet$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^\bullet$	Ozone, O_3
Alloxyl, $RO^\bullet$	Singlet oxygen, $^1O_3$
Reactive nitrogen species (RNS)	
Nitric oxide, $NO^\bullet$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^\bullet$	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Peroxynitrite, $ONOO^-$
	Alkyl peroxynitrite, $ROONO^-$

2.12 Quercetin (2)

Quercetin is a flavonoid compound found in the bark and rinds of many plants and fruits. It is supplied as a crystalline solid. Quercetin is insoluble in water and soluble in ethanol (2mg/ml) and 30 mg/ml in DMSO. As flavonoid, quercetin is the aglycone which mean minus the sugar molecule of a glycoside. The chemical name of quercetin is 3,3',4',5,7-pentahydroxyflavone. The molecular weight is 362.2 and appears in a yellowish crystalline solid. Quercetin has an octanol-water partition coefficient about 1.82 ± 0.32.

Figure 6 Structure of quercetin (2)



Harwood et al. (28) review the scientific literature association on the safety of quercetin for food application. Recently, quercetin has been marketed in the United State primarily as a dietary supplement. (29)

The typical uses in clinical practice, an oral dose of quercetin is 400-500 mg, three time a day. Quercetin appears to provide many beneficial effects on human health including cardiovascular protection, anticancer activity, antiulcer, antiviral acitivity, anti-inflammatory effects due to its antioxidant activity. (2) Nowadays, many of recent studies have shown quercetin to be an anticancer agent in various cancer cell types such as human breast adenocarcinoma cells (MCF-7) (30), U87-MG human glioblastoma and U251 and SHG44 human glioma cells. (31) The anticancer activity of quercetin has been attributed to various mechanisms including antioxidative activity, the inhibition of enzymes that activate carcinogen.

Various studies have demonstrated that the intake of quercetin as food supplement offers benefit effects for human health. Quercetin provides ethnopharmacological meaning, as many plants that contain quercetin do.

For topical uses, the quercetin effects appear to be due to its antioxidants activity, including the scavenging of oxygen radicals. The limitations of water solubility of quercetin make it difficult to permeate through skin or membrane. Many strategies have been used to improve permeability of quercetin for transdermal delivery. Vicentini et al studies the protection mechanism of quercetin on UV irradiation stimulate inflammatory which is the causes of photoaging and skin damage. UV irradiation leads to the activation of two major pathways which are nuclear factor kappa B (NF-KB) and activator protein-1 (AP-1). The experiment held on primary human keratinocytes subjected to solar UV irradiation. They found that

quercetin mediated at least in part in inhibition on NF-KB activation and cytokine production. (32) Bose et al used SLN nanoparticle as delivery system for permeation study through human skin in vitro. They found that SLN in nanoscale showed higher degree in localization within the skin compared to SLN in the micrometer range. The advantage of the accumulation of quercetin in the skin help to delay UV radiation mediated epidermal cell damage. (33)

Other nanoparticle delivery system of quercetin have been studies made from poly-D, L-lactide (PLA). Quercetin loaded PLA nanoparticles have been successfully formulated by Kumari et al. with the size ranges of 130 ± 30 nm. The kinetic study showed that it had burst release followed by slow and sustained release with the retaining of the antioxidant activity. (34)

Nonionic surfactant based nanovesicles that have been evaluated for transdermal or topical application including tretinoin (35), 5-fluorouracil (36), gallidermin (37), and benzoyl peroxide. (38) The non-ionic surfactants used in most of those research works were span series such as Span 20, Span 40, Span 60 and Span 80. For POAE, they are not much to be used when compared to Span series. Most of niosomes were prepared by thin film method.

CHAPTER 3

EXPERIMENTAL

3.1 Materials

1. Quercetin hydrate (Aldrich lot STBC5253V, Belgium)
2. Cholesterol (Merck lot K25236272 849, Germany)
3. Dihexadecyl phosphate (Aldrich lot 0001384652, USA)
4. Brij 30 (Aldrich lot MKBB9080, USA)
5. Brij 52 (Aldrich lot 0001384652, USA)
6. Brij 58 (Aldrich lot 019K0032, USA)
7. Brij 93 (Aldrich lot MKBG5253V, USA)
8. Brij 97 (Aldrich lot D00074369, USA)
9. Brij 98 (Aldrich lot 04915LEV, USA)
10. Span 60 (Srijun lot 30328B, Thailand)
11. Span 80 (Fluka lot 0001384683, USA)
12. Tween 20 (Merck lot S5371684 927, Germany)
13. Tween 80 (Qrec lot 100109-0113, New Zealand)
14. Myrj 45 (Aldrich lot 082H0304, USA)
15. Myrj 59 (Aldrich lot 082H0728, USA)
16. Isopropyl alcohol (CarboErba lot K45219834 403V6C995246E)
17. Monobasic potassium phosphate (QRec lot S5158-1-1000, New Zealand)
18. Sodium hydroxide (QRec lot P5104-1-1000 130804-0216, New Zealand)
19. Aluminum nitrate (QRec lot A4018-1-0500 110118-0114, New Zealand)
20. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Aldrich lot STBB0510, Germany)
21. Sodium acetate (UNIVAR Ajax lot 0801100, New Zealand)

3.2 Equipments

1. Transmission electron microscopy (TEM), (TECNAI 20, Philips, Japan)
2. Cryoscanning electron microscopy (Cryo-SEM) (JEOL, JSM-6010LV)
3. Scanning electron microscopy (SEM) (HITACHI SEM S-2500)
4. Nanosizer (Delsa^{TM}Nano C, Beckman Coulter, USA)

5. UV/visible spectrophotometer (Cary 1E, Varian, Australia)
6. Stereomicroscope (Nikon Eclip 5oi)
7. Centrifuge (รุ่น D-78532, Hettich, Germany)
8. pH meter (Shott, Germany)
9. Ultrasonic Cleaner (D200H, Taiwan)

3.3 Methods

3.3.1 Screening study of non-ionic surfactants

This study was performed to investigate the potential of the vesicle assembling ability of various non-ionic surfactant in prepared by proniosome gel method. The method and surfactant:cholesterol molar ratio modified from the method of Fang, et al. (14) The compositions of the membrane are listed in Table 7. The molar ratio of non-ionic surfactant and cholesterol was 3:1 and the batch size of the total lipid mixture was one gram. Non-ionic surfactant and cholesterol were weighed as stated in the Table 7 and were mixed with 1.5 ml of isopropyl alcohol in a narrow shaped beaker and warmed in a water bath up to 60±5°C for 5 min. Then 1.5 ml of pH 6.0 phosphate buffer was added and mixed on the water bath for 2 min till the clear gel was observed. The mixture was allowed to cool down till the dispersion was converted to proniosomal gel. Niosome preparation was formed by adding 10 ml of pH 6.0 phosphate buffer into gel. The mixture was mixed by gentle stirring to avoid a formally bubble and sonicated for 3 rounds of 3 min interval. The dispersion was kept in well closed tube bottle at 4°C until characterized.

Table 7 The compositions of proniosome gels in the screening experiment

Non-ionic surfactant	Non-ionic surfactant (g)	Cholesterol (g)
Span 60	0.82	0.18
Span 80	0.82	0.18
Tween 20	0.93	0.07
Tween 80	0.93	0.07
Brij 52	0.78	0.22
Brij 58	0.92	0.08
Brij 93	0.78	0.22
Brij 98	0.92	0.08
Myrj 45	0.87	0.13
Myrj 59	0.98	0.02

3.3.2 Factors affecting quercetin niosome formulation

This study was to investigate factors affecting the characteristics and stability of quercetin niosome prepared by proniosome gel method. The optimization study was held on a total experiments designed form 2^3 factorial design composed of 3 variables each was set at 2 levels. The non-ionic surfactant used as main bilayer component is Brij 98 since it showed appropriate result in the previous study. The factors of variables were pH of phosphate buffer, the amount of quercetin and the adding of DCP. For each factor, the lower and higher values are stated in Table 8

pH of phosphate buffer	(A) : (-) = pH 6.0	(+) = pH 7.4
Quercetin (g)	(B) : (-) = 0.01	(+) = 0.02
Dihexadecyl phosphate (g)	(C) : (-) = 0.000	(+) = 0.005

Table 8 The factors and levels of quercetin niosome formulations

Factors / Level	pH of phosphate buffer (A)	Quercetin (B) (g)	DCP (C) (g)
Low (-1)	6.0	0.01	0.000
High (+1)	7.4	0.02	0.005

The factorial design was applied to optimize the niosome preparation with proper size and size distribution. The data were analyzed using Minitab 14 (Free trial version) software.

3.3.2.1 Preparation of quercetin niosomes

Proniosomes were prepared by proniosome gel method modified from the method of Fang et al. (14) The compositions of vesicle membrane are listed in Table 9 The molar ratio of non-ionic surfactant and cholesterol are 3:1. The total weight of each experiment is 1 g of lipid mixture. A contain amount of quercetin, non-ionic surfactant, cholesterol and 0.005 g of dihexadecyl phosphate (DCP) (if necessary) were mixed with 1.5 ml isopropyl alcohol in a beaker and warmed in a water bath upto 60±5°C for 5 min. A 1.5 ml of pH 6.0 or pH 7.4 phosphate buffer was added and still warmed on the water bath for 2 min till the clear gel was observed.

The mixture was allowed to cool down till the mixture was converted to some proniosome gel. Niosome preparation was formed by adding 10 ml of the same buffer and sonicated 3 round of 3 min interval. The obtained niosomes were used for subsequent characteristic determination.

Table 9 The composition of proniosome gel formulations

formulation	Brij 98 (g)	Cholesterol (g)	pH of phosphate buffer	Quercetin (g)	DCP (g)
1	0.92	0.08	6.0	0.01	-
2	0.92	0.08	7.4	0.01	-
3	0.92	0.08	6.0	0.02	-
4	0.92	0.08	7.4	0.02	-
5	0.92	0.08	6.0	0.01	0.005
6	0.92	0.08	7.4	0.01	0.005
7	0.92	0.08	6.0	0.02	0.005
8	0.92	0.08	7.4	0.02	0.005

3.3.2.2 Characterization of quercectin niosome and stability.

The sizes of niosomal vesicles were measured using a Delsa Nano™ C particle size analyzer (Beckman Coulter, USA). A sample was placed into a glass cuvette which was slowly shaked before placing into the sample holder of the instrument to obtain the particle sizes and polydispersity index (PI). All the vesicle size measurements were performed in triplicate using scattering angle of 90° and at 25°C. All the preparations were characterized for size at initial time and the end of a month. The appearance of the dispersion was also observed. The stability of the niosomes were monitored from the change of sizes and size distribution after storage.

3.3.2.3 Effect of dilution volume sizes and entrapment efficiency

The best formulation from the optimization study was selected and further studied by varying the dilution volumes of phosphate buffer form 10 ml, 20 and 30 ml per gram of lipid mixture.

The entrapment efficiency of each diluted volume of phosphate buffer was examined by membrane filtration method. (The analytical method was finely described in the next part.

3.3.3 Preparation and evaluation of niosomes from polyoxyethylene alkyl ether prepared by proniosome gel method

According to the results of the previous optimization experiments of the proniosome gel formulation, the 0.02 g of quercetin and 0.005 g of DCP with pH 6.0 phosphate buffer were selected to use in this study. The optimum variables which had significant effect (p-value < 0.05) on good characteristic evaluated form previous experiment showed the formulation design of polyoxyethylene alkyl ethers used in the study. The surfactants to CHO ratio were varied as indicated in the Table 10.

Table 10 Formulation design of polyoxyethylene alkyl ether niosome gel preparation with pH 6.0 phosphate buffer

Surfactants (code)	DCP (g)	Drug (g)	Surfactant:CHO 1 : 1 (g)	Surfactant:CHO 2 : 1 (g)	Surfactant:CHO 3 : 1 (g)
Brij 30 (B30)	0.005	0.02	0.48:0.52	0.65:0.35	0.74:0.26
Brij 52 (B52)	0.005	0.02	0.46:0.54	0.63:0.37	0.72:0.28
Brij 58 (B58)	0.005	0.02	0.74:0.26	0.85:0.15	0.90:0.10
Brij 93 (B93)	0.005	0.02	0.48:0.52	0.65:0.35	0.73:0.27
Brij 97 (B97)	0.005	0.02	0.65:0.35	0.79:0.21	0.85:0.15
Brij 98 (B98)	0.005	0.02	0.75:0.25	0.86:0.14	0.90:0.10

3.3.3.1 Preparation method

A 0.02 g of quercetin with non-ionic surfactant, cholesterol and 0.005 g of dihexadecyl phosphate were mixed with 1.5 ml isopropyl alcohol in a beaker and warmed in a water bath upto 60±5°C for 5 min. A 1.5 ml of 6.0 pH phosphate buffer

was added and still warmed on the water bath for 2 min till the clear gel was observed. The mixture was allowed to cool down till the dispersion was converted to proniosomal gel. Niosomal dispersion was formed by adding 10 ml of pH 6.0 phosphate buffer previously wormed at 60±5°C and gentlely mixed. The mixture was sonicated for 3 rounds of 3 min interval.

The resulted niosomal disperstions were kept in the well closed glass tubes in dark place at 4°C for further characterization.

3.3.3.2 Selection of niosome formulation

Six types of polyoxyethylene alkyl ethers were formulated by proniosome gel method as described in Table 3.4 The resulted niosomes were characterized for size and size distribution at the initial time and after 1 month concomitantly with the observation under microscope to monitor. The criteria used to select the best formula based on the completion of vesicle formation and the lack of quercetin crystals observe under microscope. The most appropriate formulation was selected and studied in the following experiment in 3.3.

3.3.3.3 Physical examinations of the selected niosome preparation

1) Optical microscopic examination and surface morphology

An optical microscope with a camera attachment was used to observe the shape of the niosomal preparations at the initial time and at 2 and 4 weeks. The morphology of a certain formula was confirmed using cryo-scanning electron microcope. The image was captured at a desired magnification.

2) Vesicle size determination

The sizes of niosomal vesicles prepared from different non-ionic surfactants and different molar ratios of surfactant to cholesterol were measured using a Delsa Nano™ C particle size analyzer (Beckman Coulter, USA). The sample was placed into a glass cuvette which was slowly shaked before placing into the sample holder of the instrument to obtain the particle size and polydispersity index (PI). All the vesicle size measurements were performed in triplicate.

3) Zeta potential measurement

To determine the surface charge of the quercetin niosomes, the zeta potential was measured using a Delsa Nano™ C after diluting with distilled water as necessary.

4) % Encapsulation efficiency

The quercetin-containing niosomes were separated from unentrapped drug by centrifuge tube membrane with molecular weight cut off of 100 KDa at 6500 rpm for 20 min. A 0.2 ml of the clear filtrate was determined for the free drug. A 1 ml of total drug loaded in niosome was determined by centrifuge tube membrane. The filtrate or total drug taking 0.2 ml and incubated in the dark condition for 40 min with 9.0 ml of color reagent of the aluminium chloride method. The color reagent was prepared by mixing 86 ml of 80% ethanol with 2 ml of 10% w/v aluminium chloride and 2 ml of 1 M sodium acetate. A set of quercetin standard solutions in 80% ethanol was prepared ranging from 10-40 µg/ml. A 0.2 ml of each concentration of standard solution was incubated with 9.0 ml of color reagent in the same manner to determine the linearity of the detection. After 40 min of incubation, the resulted mixtures were then analyzed spectrophotometrically at 430 nm. The percentage of drug encapsulation was calculated by the following equation:

$$\text{Entrapment efficiency (\% EE)} = \frac{Abs_{(total)} - Abs_{(free)}}{Abs_{(total)}} \times 100$$

Where, A_{total} = absorbance of total drug in noisome dispersion, A_{free} = absorbance of free drug in niosome dispersion filtrate. The percentages of quercetin encapsulation measurement were carried out in triplicate.

3.3.4 Stability of quercetin niosomes during storage conditions

Four aliquots of quercetin niosome prepared from Brij 30 and cholesterol at 3:1 molar ratio were placed in 30-ml screw-capped test tubes and stored at 4°C,

45°C/75%RH, 25°C with and without sunlight exposure. The stability of the quercetin was monitored using the % scavenging activity at various time intervals which were 1 day, 15 days, 30 days and 90 days. The storage samples were evaluated using DPPH assay. The preparation were prepared and evaluated of stability in triplicate.

3.3.4.1 DPPH – Scarvenging activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) was dissolved in 80% ethanol at a final concentration of about 8.37×10^{-5} M (0.0033 g/100 ml). A 1000 microgram/ml of pure quercetin solution in 80% ethanol was prepared and was diluted into 5 different concentrations ranging from 20-100 microgram/ml. The calibration curve of quercetin was performed by incubating a 200 microlitre of each standard solution with 7.0 ml of DPPH solution and kept in dark at room temperature for 40 min. The absorbance was taken at 520 nm in UV-vis spectrophotometer againt control solution (200 microlitre of DI water and 7.0 ml DPPH solution). The antioxidant activity of quercetin niosome samples was performed by incubating 200 microlitre of each sample in the same manner with 7.0 ml of DPPH solution. The results were expressed in average means with standard deviation of triplicate experimental setup. The free radical scavenging activity was calculated using the following equation:

$$\% \text{ Inhibition} = \left(\frac{Abs_{(control)} - Abs_{(sample)}}{Abs_{(control)}} \right) \times 100$$

The calibration curve was plotted with concentration of quercetin standard solution vs % scavenging activity to determine the linearity of the detection.

3.3.4.2 % Entrapment efficiency

%EE of niosome after storing at various conditions at various time interval were measured the %EE using the method described in 4).

3.3.5 Formulation variables affecting the characteristics of quercetin niosome prepared from Brij 30

This study was performed by varying the dilution volumes from 10, 30 and 50 ml of pH 6.0 phosphate buffer per 1 gram of lipid mixtures composed of Brij 30 to CHO at 3:1 molar ratio, 0.005 g of DCP with the quercetin loaded of 0.02 g per 1 gram of lipid mixture. The study was to investigated the reproducibility of the proniosome gel method on the sizes and size distribution, zeta potential and %EE upon the dilution volume. The experiments were held in triplicate. The stability of the storage on size, zeta potential was also monitored. Using method described in 2) and 3)

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Screening of various nonionic surfactants to form niosomes by proniosome gel (coacervation-phase separation) method

This experiment was held to screen the ability in constructing of vesicle by proniosome gel method. Ten types of non-ionic surfactants were used as listed in Table 11. The method used in this study was modified from Fang et al (16) by which niosome was prepared by proniosome gel method. In this study one gram of lipid mixture was coacervation as a gel and then diluted with pH 7.4 or pH 6.0 phosphate buffer. After diluting with pH 7.4 or pH 6.0 phosphate buffer, the size and size distribution were investigated. The results showed that most of them could form vesicles with different range of vesicle size and size distribution. Under microscopic observation, two types of them which are Myrj 45 and Myrj 59 could not form vesicles at all. This might be due to the condition used was not appropriate.

Table 11 shows the physical characteristic by visual observation and mean size of the freshly prepared niosomes and those stored for 1 month. After storing upto 1 month niosomes constructed from Brij 58 and Brij 98 still dispersed in homogenous fashion while others showed aggregation or separation. The change in particle size was observed in all formulas upon storage except Brij 58 and Brij 98, the physical appearance also does not change.

Vesicles from Span 60 and Span 80 were very large. The niosome from Span 60 precipitated but could be redispersed by shaking. The niosome from Span 80 upwardly separated with the mean size of 3653.3 ± 217.81 nm. The mean size of niosomes from Tween 20 and Tween 80 were 513.2 ± 14.65 and 729.1 ± 45.81 nm, respectively. After standing for 24 hours, niosome from Tween 80 precipitated but was reversible.

Table 11 Physical characteristic by visual observation and mean sizes of blank niosomes from various non-ionic surfactant

Non-ionic surfactant	Stability			
	24 h		1 month	
	Mean size (nm)	Appearance (1 day)	Mean size (nm)	Appearance (30 days)
Span 60	1606. ±123.54	Aggregation	4717.9 ±359.81	Aggregation
Span 80	3653.3 ±217.81	Aggregation	23781.9 ±4180.63	Aggregation
Tween 20	513.2 ±14.65	Translucent dispersion	1791.0 ±334.01	Aggregation
Tween 80	729.1 ±45.81	Aggregation	1107.8 ±165.60	Aggregation
Brij 52	188.3 ±4.91	Aggregation	6307.0 ±1252.45	Aggregation
Brij 58	233.0 ±0.36	Translucent dispersion	432.6 ±2.05	Homogenous and milkly dispersion
Brij 93	357.3 ±12.50	Aggregation	357.7 ±13.74	Aggregation
Brij 98	210.7 ±2.57	Translucent dispersion	537.3 ±7.40	Homogenous and milkly dispersion
Myrj 45	7439.2 ±6712.68	Waxy like dispersion	8092.2 ±974.73	Aggregation
Myrj 59	956.2 ±184.71	Milkly like dispersion	1478.8 ±158.55	Aggregation

For Brij series, the mean size of all the vesicles were smaller than other series. The mean size of Brij 52, Brij 58, Brij 93 and Brij 98 were 188.3 ± 491, 233.0 ± 0.36, 357.3 ± 12.5 and 210.7 ± 2.57 nm, respectively. Among the Brijs, niosomes from Brij58 and Brij98 which the HLB are 16 and 15, respectively, resulted in the milky dispersion after preparing and they still homogeneous dispersed after stored for 1 month.

From the study showed that, the fixed temperature of 60±5°C in the gel formation step might not be appropriate to every types of nonionic surfactant used since the size of the alkyl chain alters the melting of the lipid mixture subsequently alters the completion of gel formation in the process.

The criteria used to select the best non-ionic surfactant for further study based on the smallest size, good size distribution and the best stability after storage for a month. Niosomes from both Brij 58 and Brij 98 showed the best results, but Brij 98 was finally selected because of its lower melting point and it offers the lowest size compared to Brij 58.

4.2 Optimization study: Factors affecting quercetin niosome formation

The objective of this part was to optimize the factors affecting the formation of niosome vesicles. Based on the screening study from previous 4.1, Brij 98 was selected for the optimization study using 2^3 factorial design. Three factors (independent variable), with two levels of each, including: pH of buffer (X_1), quercetin amount (X_2) and adding of DCP (X_3) were investigated the effect on the particle size (Y_1) and physical stability (aggregation) (Y_2). Multiple linear regression and ANOVA were performed to analyze the data.

Table 12 Illustrate the independent variables setting and the treatment response (dependent variable).

Run	Independent variables			Dependent variable (Y_1) Mean size (nm) ± SD
	X_1	X_2	X_3	
1	-1	-1	-1	260 ± 0.6
2	+1	-1	-1	285.6 ± 6.2
3	-1	+1	-1	245 ± 2.2
4	+1	+1	-1	294.7 ± 3.7
5	-1	-1	+1	477.5 ± 39.9
6	+1	-1	+1	392.1 ± 13.6
7	-1	+1	+1	320.5 ± 30.6
8	+1	+1	+1	371.3 ± 6.2

Table 13 A independent variables 2^3 full factorial design.

Independent variables	Low level	High level
X_1 = pH of buffer	6.0	7.4
X_2 = quercetin amount	0.01 g/g	0.02 g/g
X_3 = adding of DCP	0.00 g/g	0.005 g/g

4.2.1 Influence of various factors on quercetin niosome size and stability

As shown in Table 12, the vesicles sizes of all the quercetin niosomes were around 245-477 nm. The mean size of niosomes using the MINITAB 14 to analyze the effect of factors on the vesicles size formation, Table 14 is the resulting regression analysis to show the significant effect on the quercetin niosome size.

Table 14 Regression analysis: mean size to pH of buffer, quercetin amount and adding of DCP

Predictor	Coef	SE Coef	T	P
Constant	291.8	178.9	1.63	0.178
pH of buffer	7.23	25.23	0.29	0.789
Quercetin amount	-4593	3532	-1.30	0.263
Adding of DCP	23795	7064	3.37	0.028

S = 49.9519 R-Sq = 76.6% R-Sq (adj) = 59.1%

A quadratic model of the design is:

size (Y_1) = 292 + 7.23 pH – 4593 quercetin amount + 23795 adding of DCP ....Eq. 1

The absolute values of coefficient reflect the magnitude of size change while the factors change from low level to high level. As shown in Eq. 1, the size of niosomes were most affected by the adding of DCP. The quercetin amount and pH of the used buffer were found to cause less and no effect on size, respectively.

Further analysis using ANOVA indicates that the adding of DCP highly influence the size of niosomes ($p < 0.05$).

Table 15 ANOVA analysis: Mean size versus adding of DCP

Source	DF	SS	MS	F	P
DCP	1	28310	28310	11.79	0.014
Error	6	14404	2401		
Total	7	42714			

Figure 7 Main effect plot for mean size of quercetin niosome

Fig 7 illustrates the main effect of each factor at each level. Each spot is the mean size of the eight formulations upon each factor (independent variable). The adding of DCP shows the significant bigger in mean size of the quercetin niosomes compared to those without the adding of DCP (p = 0.028).

Figure 8 Interaction plot for mean size of quercetin niosomes

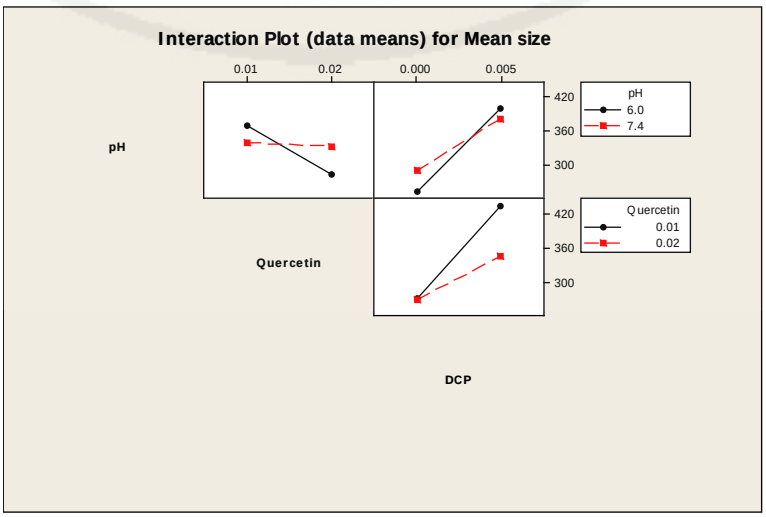


Fig 8 provides a graphical representation of the mean size of each level of three independent variables, the dependent variable (size) goes on the y–axis and the three dependent variables go along the x - axis and in the legend.

The interaction effect reveals upon the less parallel of the two lines. The more likely of the cross–intersect of the lines, shows the more significant interaction, as shown in the Fig 4.2, there is no interaction between the amount of quercetin and the adding of DCP.

From the pH–quercetin plot, there is a cross–over interaction. It appears that the size of vesicles from both low and high amount of quercetin has no effect from the pH of the used buffer. The similar result presents in the pH–DCP plot In summary, the plot shows no main effect of pH and no main effect of quercetin amount, but a cross–over interaction.

From the quercetin amount–DCP plot shows that the adding of 0.01 g/g of quercetin resulted in bigger mean size compared to those that adding of 0.02 g/g of the quercetin, but just in case of the adding of DCP of 0.005 g/g only.

Thus, the adding of DCP shows the main effect that resulted in bigger mean size than no adding of DCP.

4.2.2 Influence of factors on the quercetin niosome stability

Table 16 shows the initial vesicle size of quercetin niosomes from the optimization trail by 2^3 factorial design and those after storage for 1 month. The resulting formulations were kept at 4°C with the light protection condition. At the end of 1 month storing, samples were withdrawn and the particle size was measured to check for any aggregation of the vesicles. At the initial time formulas 1 to 4 which containing no DCP revealed the smaller size compared to the formulas 5 to 8 which containing 0.005 g of DCP per a gram of the lipid mixture. After storing, significant bigger in sizes were found in the formulas without DCP. DCP is generally added into liposome or niosome to increase the charge repulsion between adjacent bilayer. It is responsible for the increase of curvature and increase the vesicles size. (16) In the long term, it also stabilizes the vesicle size by reducing the aggregation of the vesicle due to the negative repulsion charge.

At the end of 1 month storing period, the physical appearance was observed for aggregation, separation or precipitation. Most of the formulas showed the change from translucent and well dispersed to precipitation except the formulas 6 and 7.

Table 16 Size and size distribution (n=3) of quercetin niosomes after hydrating with the same buffer and after storage at 4°C for a month (the measurement was performed in triplicate)

Formula	24 hours		1 month	
	Mean size (nm) (±SD)	Appearance	Mean size (nm) (±SD)	Appearance
1	260.1 ± 0.6	Translucent and well dispersed	981.6 ± 17.3	Precipitation observed
2	285.6 ± 6.2	Translucent and well dispersed	1353.4 ± 58.2	Precipitation observed
3	245.1 ± 2.2	Translucent and well dispersed	1095.4 ± 140.5	Precipitation observed
4	294.7 ± 3.7	Translucent and well dispersed	1466.9 ± 56.9	Precipitation observed
5	477.5 ± 39.9	Translucent and well dispersed	311.0 ± 16.4	Precipitation observed
6	392.1 ± 13.6	Translucent and well dispersed	210.7 ± 0.8	Translucent and well disperse
7	320.5 ± 30.6	Translucent and well dispersed	204.3 ± 2.1	Translucent and well disperse
8	371.3 ± 6.2	Translucent and well dispersed	272.5 ± 3.9	Precipitation observed

From the data analysis reveals that the incorporation of DCP increases both the niosome size and the stability. For the present method of niosome production by coacervation-phase separation or socalled proniosome gel method, these factors needed to be optimized to produce the smallest vesicle size with the best stability

upon storing. Therefore, the factors selected for next study were the pH at 6.0 and the adding of DCP. The quercetin amount was 0.02 g/g of lipid mixture to offer the high loading to additive ingredient of the formulation of the intended used. The pH 6.0 phosphate buffer was selected according to the literature review that quercetin is more stable in acid than neutral medium.

A number of studies have been carried out for finding the factor affecting the niosome size such as the percent of incorporated cholesterol, percent drug load, and the size reduction time by sonication. In this study, the nonionic surfactant to cholesterol ratio was fixed at 3: 1, and the sonication time was also fixed as a constant factor. Other factors which were the dilution volume of the medium used to prepare the niosome dispersion and the amount of cholesterol that affect the vesicle characteristics had seen studied in the consequent part of the thesis

However, the formula 7 was finally selected for next study according to the most appropriate results based on size and stability.

4.2.3 Morphology of quercetin niosome from Brij 98

Quercetin niosome (formula 7) morphology after storing for a month was observed using both transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Both of them show spherical shape in micron range (Fig 9 and Fig 10). The shape of the vesicles was consistent after storing at 4°C for 1 month. This indicates the promising good production method by dilution from proniosome gel.

Figure 9 TEM micrograph of quercetin niosome formula 7 which composed of Brij 98:CHO (3:1), 0.005 g of DCP, 0.02 g of quercetin and diluted with phosphate buffer pH 6.0.

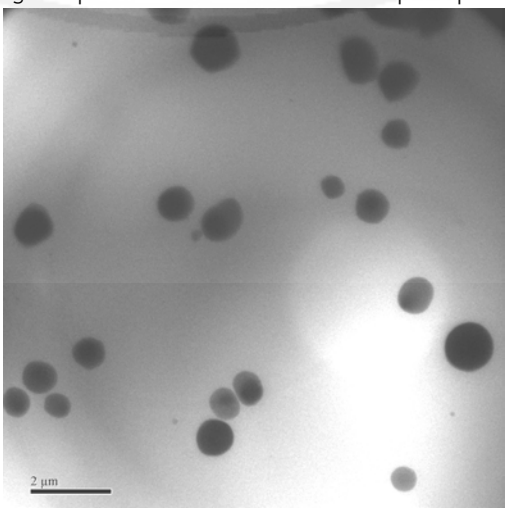
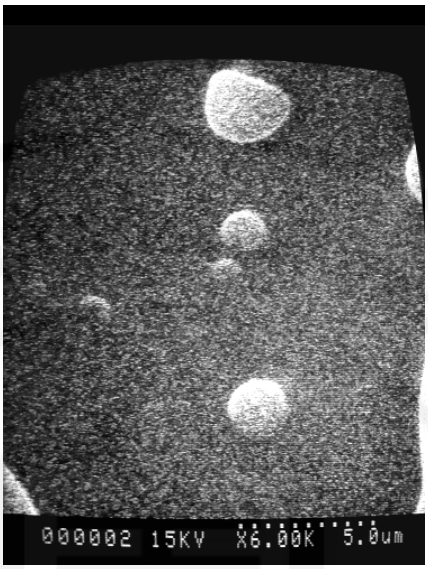


Figure 10 SEM micrograph of quercetin niosome formula 7 which composed of Brij 98:CHO (3:1), 0.005 g of DCP, 0.02 g of quercetin and diluted with phosphate buffer pH 6.0.



4.2.4 Effect of dilution on size and %EE of quercetin niosome formula 7

The effect of process-related variable like dilution volume was also studied in the formula 7 by increasing the amount of phosphate buffer from 10-30 ml/1 g. The sizes and % EE were evaluated and were shown in the Table 4.7. It was found that higher in dilution volume decrease the sizes and % EE. The higher in dilution volume of phosphate buffer, nanovesicles could be successfully generated with very high % EE (98.25-99.32%). However, 10 ml was used throughout in the study to maximize the concentration of quercetin in niosome dispersion for tentative use.

Table 17 Effect of dilution volume on size and % EE of formula 7

Volume of buffer (ml)	Mean size (nm)	% EE
10	346.4 ± 5.8	99.32
20	108.9 ± 1.6	98.74
30	134.9 ± 4.1	98.25

*Values represent mean ± SD (n = 3)

4.3 Investigation of polyoxyethylene alkyl ether (POAE) vesicles formation by proniosome gel method

Niosomes are alternative to liposomes and are important from technical viewpoint as they posses better stability and make less troublesome disadvantages of phospholipids. Niosomes also have a great possibility for controlled and targeted delivery of many drugs. (17) Nonionic surfactants have received the most attention for topical and percutaneous absorption since they have low irritancy potential and have skin permeation enhancing ability by disrupting the stratum corneum lamellae structure. Park et al studied various type of polyoxyethylene alkyl ether as enhancers of ibuprofen through rat skin. They found that polyoxyethylene alkyl ether showed high ability for enhancing the drug absorption. The surfactant containing ethylene oxide or EO chain length of 2-5, HLB value of 7-9 and alkyl chain length of C16-C18 are the very effective enhancing promotors. (22). However, the size and shape of both the alkyl chain and the POE groups were found to be important factors for the enhancement of skin permeation of ibuprofen.

The above finding was the reason why six POAE again were studied in this experiment. From the previous study, Brij 98 showed the high potential to spontaneously form good quercetin niosome dispersion with nanoscale size and very high entrapment efficiency. From previous literature reviews, HLB value of nonionic surfactant plays an important property to form the proper vesicles. Cholesterol is well known to exhibit conflict effects on size and drug entrapment (6) due to its hydrophobicity. Conversely, cholesterol improves the stability of drug by reducing of permeability of the bilayer. For hydrophobic substance like quercetin, the increasing of cholesterol content might show some benefit to the formulation.

4.3.1 Effect of cholesterol concentration on the vesicle formation and stability.

The six POAE with different HLB values were investigated for the effect of cholesterol concentration on the vesicle formation since cholesterol plays an important role on the solubility of substance in bilayer or on the other hand it increase the %EE.

Recently report studied by Wilkhu et al. (40) whose consider the organization of surfactants into niosome using thermo gravimetric analysis. They found that cholesterol plays a key role in assembling of bilayer. Without cholesterol the vesicle cannot be buit and higher concentration of cholesterol decrease the time required for niosome assembly. The studied also found that the heat enthalpy for melting of the lipid mixture decrease upon the increasing of the cholesterol while the adding of DCP increase the melting enthalpy. This study also suggests that the cholesterol did not melt but dissolved into the molten mixture upon heat therefore the melting point itself is very high (~148°c). The intercalation of cholesterol within the bilayer reduces the average area per molecule and overall critical packing parameter (CCP) of surfactant.

In general if CPP < 0.5 it indicates a large contribution from the hydrophilic head group area of the molecule then it forms spherical micelle. While the CPP of the nonionic surfactant is in between 0.5–1 (0.5 < CCP < 1), the bilayer of vesicle is formed but if CPP > 1 the invert micelle will be formed. (9)

Table 18 The physical properties of polyoxyethylene glycol alkyl ethers used in the study (26)

Trade name	Chemical information	MW	HLB	Density g/mL at 25° C	Melting point (°C)
Brij 30	polyoxyethylene (4) lauryl ether	362	9	1.07	14
Brij 52	Polyoxyethylene (2) cetyl ether	330	5	0.978	32.8
Brij 58	Polyoxyethylene (20) cetyl ether	1124	16	1.05	38
Brij 93	Polyoxyethylene (2) oleyl ether	356	4	0.912	10
Brij 97	Polyoxyethylene (10) oleyl ether	709	12.4	1.00	~10
Brij 98	Polyoxyethylene (20) oleyl ether	1149	15	1.07	30-40

Table 19 Summaries the effect of cholesterol concentration on the physical properties

Surfactant	Dispersion apperance		
	1:1	2:1	3:1
Brij 30	Incomplete formation of vesicles with the unentraped crystals was observed	Large vesicles were formed with the unentraped crystals was observed	Homogeneous vesicles were formed without the quercetin crystals was observed
Brij 52	Large irregular vesicles were formed	Large irregular vesicles were formed	Large irregular vesicles were formed
Brij 58	No formation of vesicles	No formation of vesicles	No formation of vesicles
Brij 93	Incomplete formation of vesicles	Small vesicles were formed with the unentraped crystals	Incomplete formation of vesicles with the unentraped crystals
Brij 97	No formation of vesicles	No formation of vesicles	No formation of vesicles
Brij 98	No formation of vesicles	No formation of vesicles	Formation of small and homogenous size vesicles

*Surfactant to cholesterol molar ratio

Table 19 summarizes the physical properties of the niosome containing quercetin at 0.02 g/g of lipid mixture. It is interesting to note that very high HLB value non-ionic surfactants which are Brij 58, Brij 97 and Brij 98 where HLB value are 16, 12.4 and 15, respectively cannot easily from the vesicle. In contrast to the lower HLB values like Brij 30, Brij 52 and Brij 93 the vesicle are spontaneously formed, therefore the lack of uniformity of size dues to the limited of sonication time in the process of preparation.

Figure 11 Photograph of quercetin niosome dispersions prepared from various non-ionic surfactants to cholesterol at 1:1 molar ratio after storage for 1 day (upper), 7 days (middle) and 30 days (lower)

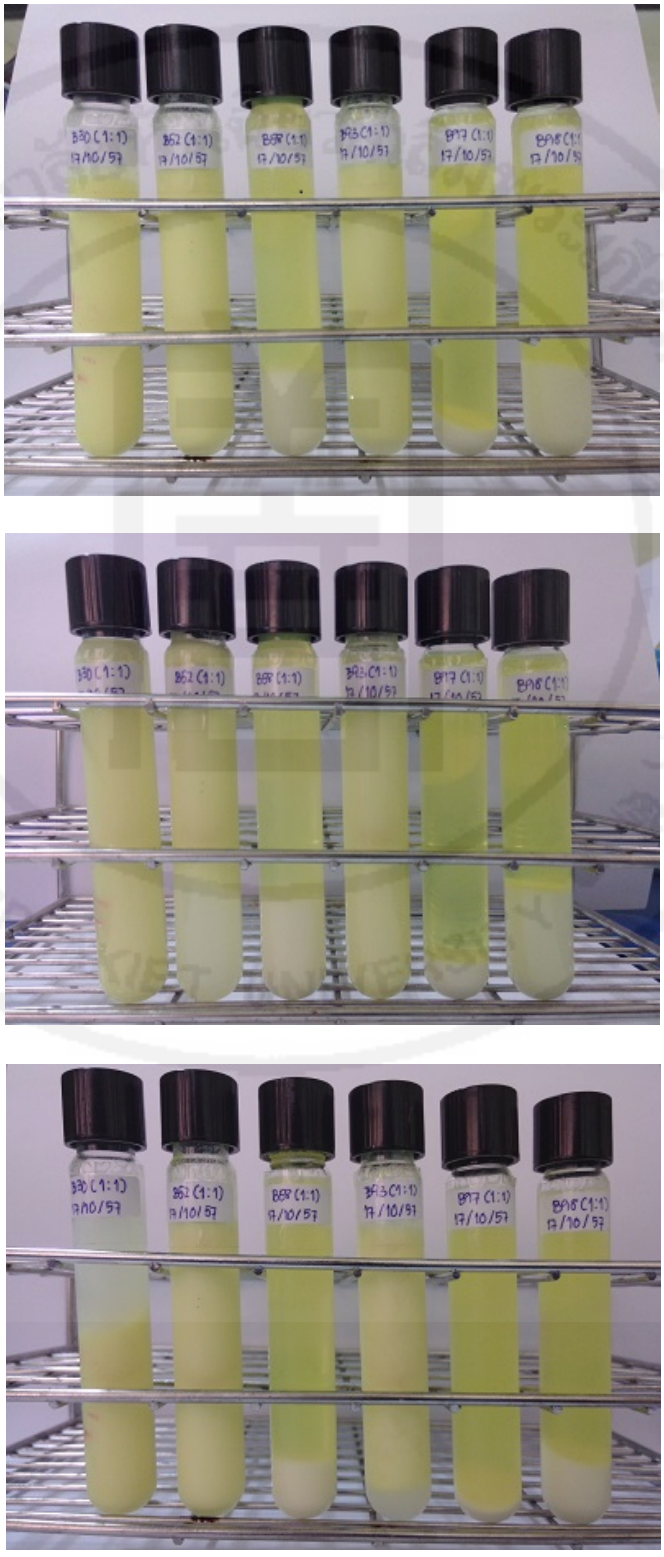


Figure 12 Photograph of quercetin niosome dispersions prepared from various non-ionic surfactants to cholesterol at 2:1 molar ratio after storage for 1 day (upper), 7 days (middle) and 30 days (lower)

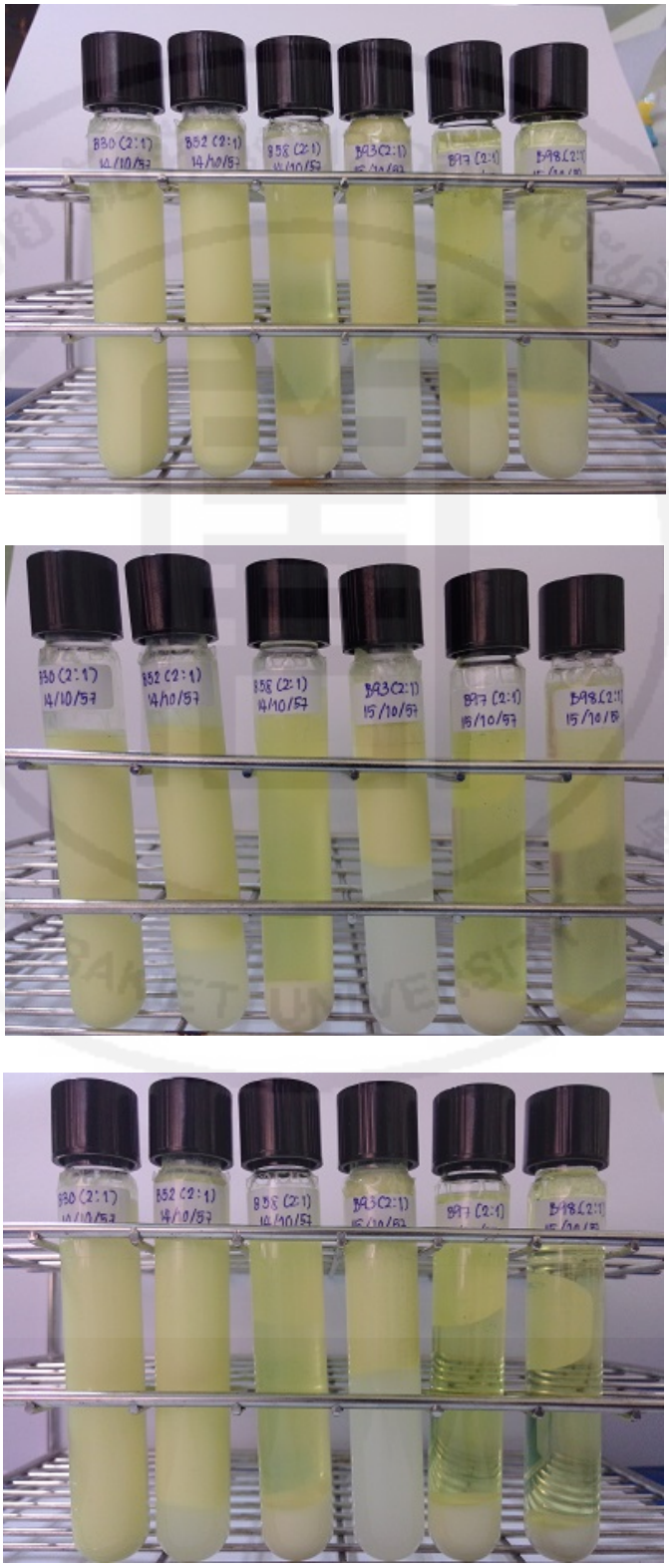


Figure 13 Photograph of quercetin niosome dispersions prepared from various non-ionic surfactant to cholesterol at 3:1 molar ratio after storage for 1 day (upper), 7 days (middle) and 30 days (lower)

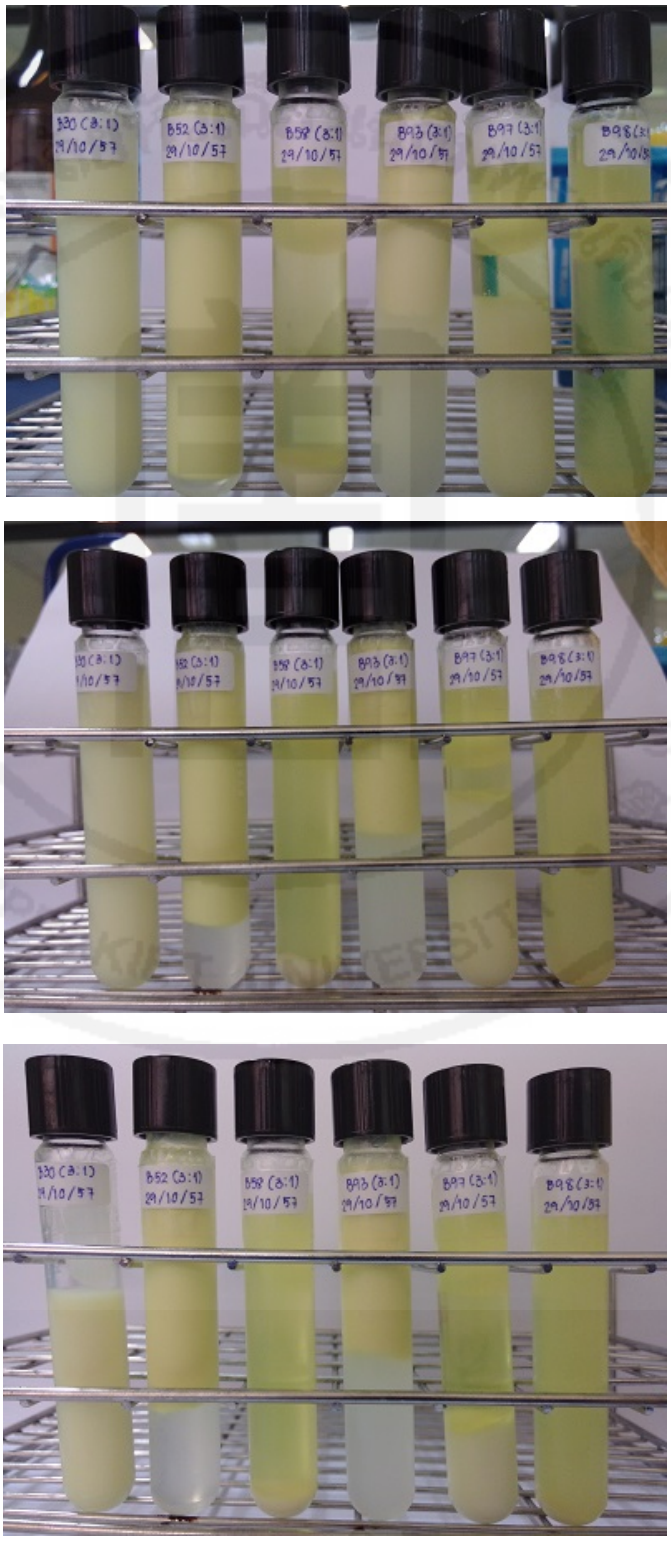


Fig 11-13 illustrate the photographs of all formulations at various cholesterol ratios. It is noticeable that the dispersion from high HLB values non-ionic surfactant (Brij 58, Brij 97 and Brij 98) mostly present the deep yellow color which indicates that quercetin is solubilized by surfactant during dilution.

Table 20 Sizes and size distribution of niosomes prepared at the 1:1 molar ratio of surfactant to cholesterol at 1 day, 7 and 30 days (n=3)

formula	Vesicle size (nm) (mean±SD)					
	1 day		7 days		30 days	
	size	PI	size	PI	Size	PI
Brij 30	4159.2 ±238.81	N/A	3008.6 ±253.44	N/A	4452.1 ±188.48	N/A
Brij 52	2670.8 ±341.03	N/A	3794.3 ±179.88	N/A	7975.6 ±390.55	N/A
Brij 58	3361.4 ±350.23	N/A	2634.4 ±361.82	N/A	1984 ±1052.83	N/A
Brij 93	4793.8 ±61.10	N/A	4927.3 ±132.35	N/A	4372.77 ±113.67	N/A
Brij 97	1873.8 ±66.44	N/A	7924.7 ±1610.01	N/A	11325.3 ±1201.95	N/A
Brij 98	7565.9 ±1693.60	N/A	5087.1 ±241.82	N/A	3808.7 ±1170.67	N/A

* N/A = Not applicable

Table 21 Sizes and size distribution of niosomes prepared at the 2:1 molar ratio to cholesterol at 1 day, 7 and 30 days (n=3)

formula	Vesicle size (nm) (mean±SD)					
	1 day		7 days		30 days	
	size	PI	size	PI	Size	PI
Brij 30	7773.8 ±246	N/A	5991.1 ±174.63	N/A	7313.9 ±545.43	N/A
Brij 52	2780.3 ±81.92	N/A	2542.83 ±121.35	N/A	2198.9 ±21.43	N/A
Brij 58	2906.6 ±1094.05	N/A	980.47 ±72.24	N/A	2135.5 ±841.82	N/A
Brij 93	3316.23 ±86.64	N/A	4554.13 ±194.13	N/A	4910.8 ±129.37	N/A
Brij 97	5366.2 ±1567.476	N/A	8091.23 ±5017.47	N/A	7049.87 ±5601.48	N/A
Brij 98	3995.9 ±490.75	N/A	4699.93 ±3564.98	N/A	2466.5 ±317.05	N/A

* N/A = Not applicable

Table 22 Sizes and size distribution of niosomes prepared at the 3:1 molar ratio to cholesterol at 1 day, 7 and 30 days. (n=3)

formula	Vesicle size (nm) (mean±SD)					
	24 hr.		7 days		1 month	
	size	PI	size	PI	Size	PI
Brij 30	6999.67 ±351.83	N/A	6305.8 ±301.48	N/A	6290.5 ±313.68	N/A
Brij 52	7079.5 ±134.28	N/A	6396.43 ±326.27	N/A	10039.53 ±617.43	N/A
Brij 58	1561 ±290.80	N/A	542.33 ±16.36	N/A	844.1 ±101.21	N/A
Brij 93	6135.6 ±220.48	N/A	5379.7 ±238.33	N/A	5793.37 ±137.23	N/A
Brij 97	8660 ±366.43	N/A	7323.43 ±1825.32	N/A	9349.5 ±493.52	N/A
Brij 98	554.43 ±26.01	N/A	389.8 ±35.16	N/A	375.6 ±5.51	N/A

* N/A = Not applicable

Tables 20-22 show the vesicle sizes and size distribution of different surfactants at various molar ratios to cholesterol at various periods of storage.

In the study, the hydrodynamic size was measured by light scatting technique. If the size of vesicles are not in nano-scale (more than 7000 nm). the PI values are very high and not applicable. After hydration 1 g of lipid mixture with 10 ml of pH 6.0 phosphate buffer under sonication 3 min for 3 rounds. Large size vesicles were formed. Size of all vesicle were found in micron size range. For the certain and fixed preparing method of the proniosome gels using different HLB values and melting points of the surfactant used might not be appropriate for all kinds and all molar ratios since cholesterol is dissolved in the lipid mixture instead of melting. The coacervation step or hydration step might not be complete. The observation

under microscope at 400x reveals the vesicle morphology and the wellness of drug entrapment. Quercetin is not soluble in water, thus the unentrapped crystal is usually observed under microscope.

Fig 14-19 are opticalmicrography at 400x of quercetin niosome dispersions prepared from Brij 30, Brij 52, Brij 58, Brij 93, Brij 97 and Brij 98, respectively.

It was found that quercetin niosomes form Brij 30 at 1: 1 and 2: 1 molar ratios reveal the unentrapped crystals and the incomplete assembling of vesicles. At 3:1 molar ratio, the vesicles are completely formed with high polydispersity index and the quercetin is well incorporated.

Brij 52 (Fig 15) shows high ability to assemble into quercetin niosomes at all ratios with large size and high polydispersity index and quite well incorporated of quercetin. While Brij 58 (Fig 4.10) which the HLB value is more higher than Brij 52 (14 and 5, respectively), did not obviously demonstrate the complete vesicles but small pieces of lipids and the agglomeration were observed. The large size and high polydispersity index might be due to the agglomeration of the particles. For very high HLB value of POAE, the polyoxyethylene head groups is more likely to dissolve in the medium, thus spheric micelles might occur and they solubilize the added quercetin.

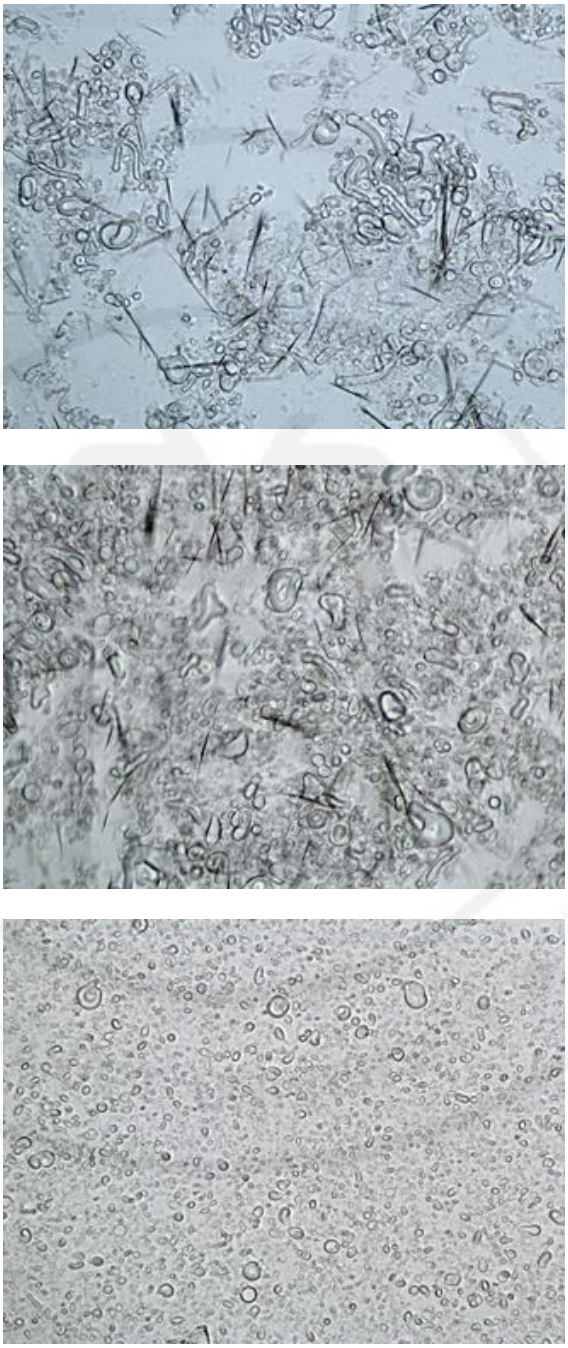
In case of Brij 93 (Fig 17) from which HLB value is 4, at all molar ratios, vesicles were formed in spheric shape and rather homogeneous. The population of small size is dominant but this does not go along with the values measured by instrument. However, this kind of surfactant was not selected because of the incomplete incorporation of quercetin.

In case of both Brij 97 and Brij 98 at 1: 1 and 2: 1 molar ratios of surfactant to cholesterol, the vesicles were not formed. The complete spheric vesicles were observed only at 3: 1 molar ratio from Brij 98 (Fig 19). this surfactant was previously used in the optimization study in 4.2.

Many other factors might affect the assembling ability of the lipid such as dilution temperature, ionic strength of the medium and sonication time were not included. Those factors should be separately studied for each certain surfactant and might not be impliable to other surfactant.

Although Brij 98 had been studied in 4.2 and showed high ability to use as drug delivery, but when compared to Brij 30 which lastly involved into the investigation study, Brij 98 had less robustness to prepare the same product upon hydration. This might be due to the physical property such as the HLB, melting point, solubility of surfactant in buffer and/or isopropanol. The processing time and the temperature might not be accurately controlled. Brij 30 shows more robustness of such proniosome gel method of preparation. Thus Brij 30 was selected to develop quercetin niosome which is tentative to used as delivery system for skin either as antioxidant or anticancer.

Figure 14 Optical micrographs (400x) of quercetin niosome dispersions prepared from Brij30:CHO at the molar ratio of 1:1 (upper), 2:1 (middle) and 3:1 (lower).

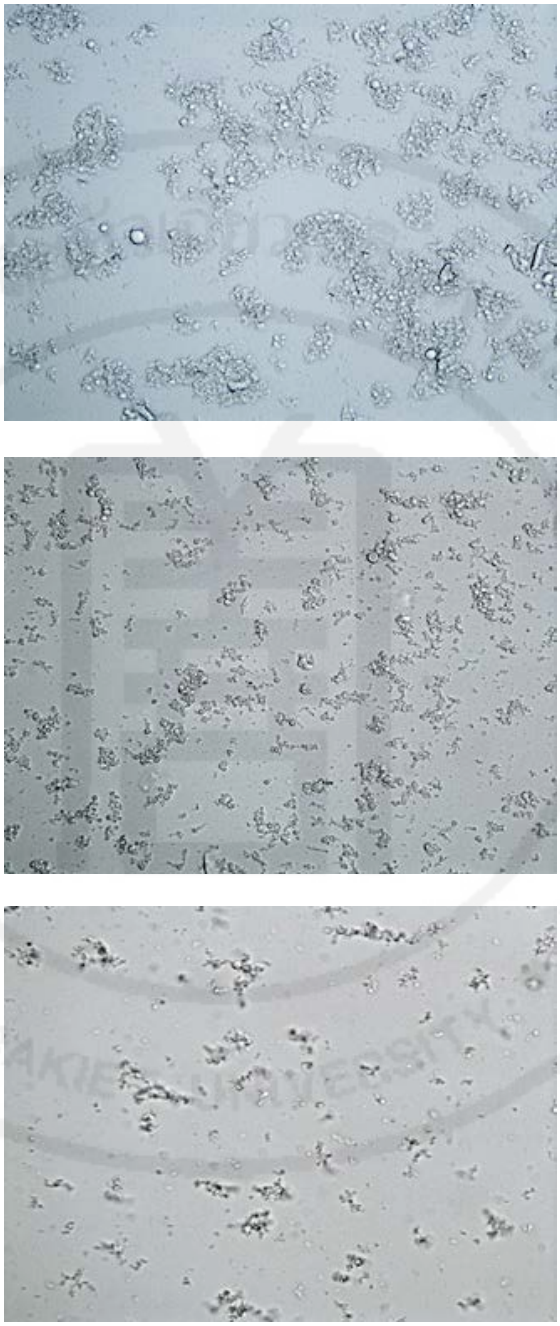


Under the microscope for molar ratio of 1:1 and 2:1, the crystals were obviously found indicating the uncompleted incorporation of quercetin into the vesicle at the used condition. For the molars ratio of 3:1 the homogeneous dispersion and completed vesicles were observed.

Figure 15 Optical micrographs (400x) of quercetin noisome dispersions prepared from Brij52:CHO at the molar ratio of 1:1 (upper), 2:1 (middle) and 3:1 (lower).

As shown in Fig 15, all the molar ratios of the lipid mixture, the vesicles formed but in incompleted manner. The quercetin crystals were found in all molar ratios. Within 1 day the vesicles and other particles settled out of the fluid.

Figure 16 Optical micrographs (400x) of quercetin noisome dispersions prepared from Brij58:CHO at the molar ratio of 1:1 (upper), 2:1 (middle) and 3:1 (lower).

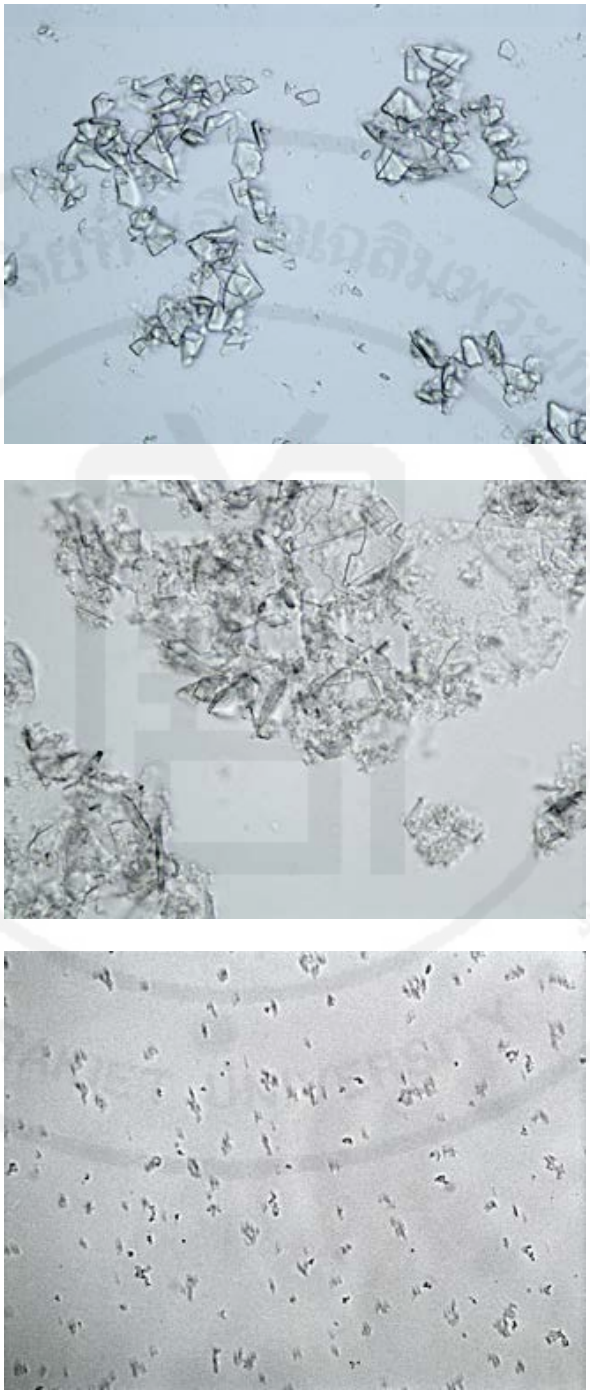


At all cholesterol ratios, vesicles were not completely formed and became agglomeration. No quercetin crystal was observed. Since the micelle might form and solubilization occurred. After storing for a month, the dispersion separated by settling down.

Figure 17 Optical micrographs (400x) of quercetin noisome dispersions prepared from Brij93:CHO at the molar ratio of 1:1 (upper), 2:1 (middle) and 3:1 (lower).

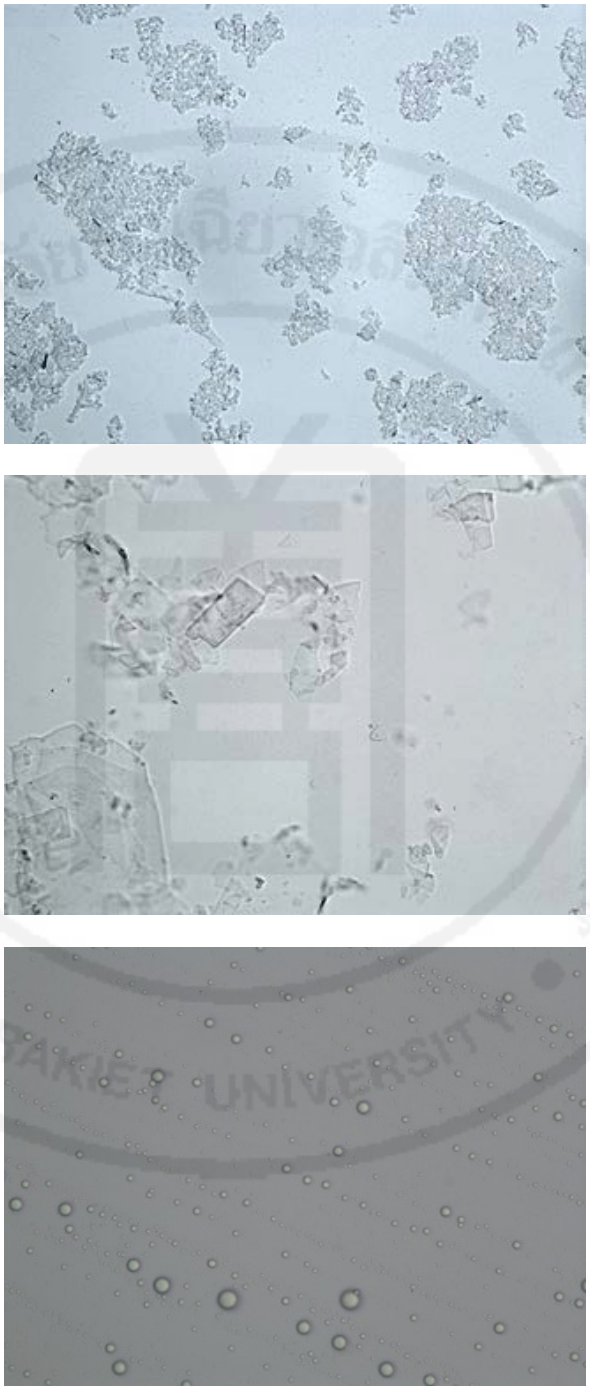
For all surfactant to CHO ratios, the vesicles were incompletely assembled. At 2:1 ratio, the small and homogeneous vesicles were observed with unentrapped quercetin in needle shape. The higher in cholesterol ratio, the lesser of quercetin crystal was found. This might be due to the higher solubility of the quercetin in the lipid bilayer.

Figure 18 Optical micrographs (400x) of quercetin noisome dispersions prepared from Brij97:CHO at the molar ratio of 1:1 (upper), 2:1 (middle) and 3:1 (lower).



For all surfactant to CHO ratios, the vesicles were not formed. At higher cholesterol ratio, the lipid mixture did not spontaneously form into vesicles but the quercetin completely dissolve. The solubilization of quercetin may occur.

Figure 19 Optical micrographs (400x) of quercetin noisome dispersions prepared from Brij98:CHO at the molar ratio of 1:1 (upper), 2:1 (middle) and 3:1 (lower).



At 1:1 and 2:1 molar ratios, the vesicle was not observed. At the 3:1 molar ratio, the homogenously rounded shape of niosome vesicles were observed.

4.3.2 Morphology of quercetin niosome from Brij 30

Proniosome gel containing quercetin (0.02 g/g lipids) appeared in yellowish transparent and viscous gel. The gel was confirmed to be lamella liquid crystalline by observing under cross polarizer and found to be a dark background. Upon hydration with excess aqueous phase, lipid lamella swells and randomly forms multivesicular structures.

Figure 20 Photomicrograph of quercetin niosome from Brij:CHO at 3:1 molar ratio. The niosome dispersion contains small and large unilamellar and multilamellar vesicles.

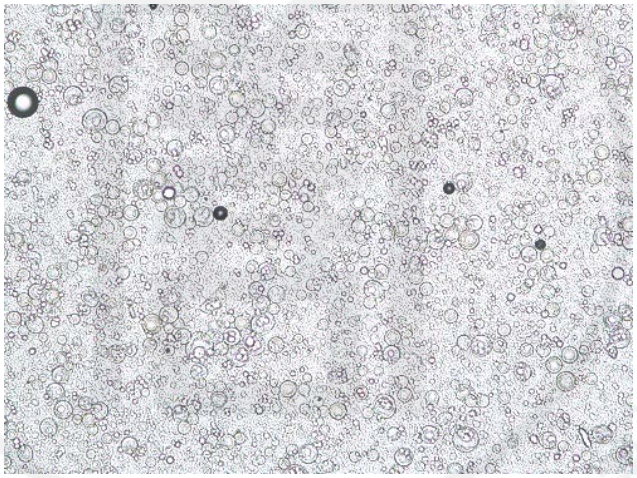


Figure 21 Photomicrograph of the same preparation captured under cross polarizer. The x-cross of the vesicles indicates the formation of vesicle.

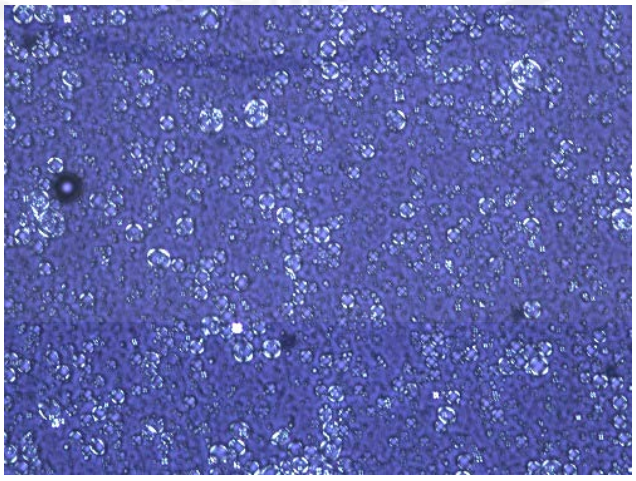
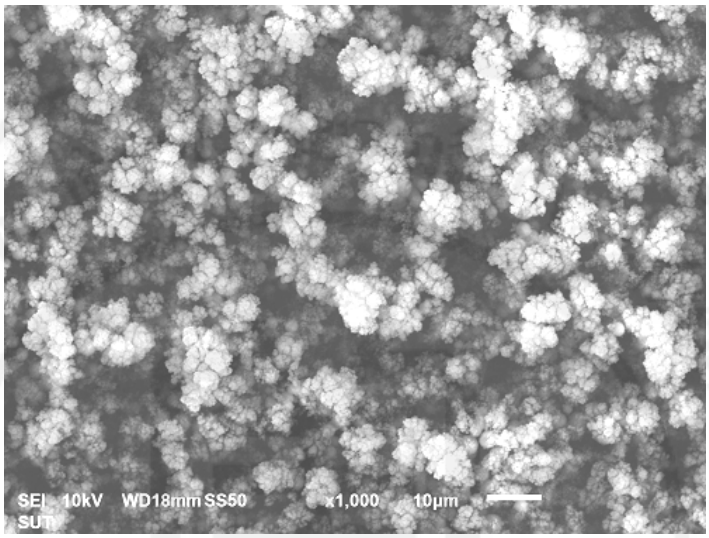


Figure 22 Cryo-scanning electron micrograph of the same quercetin niosome from Brij 30, which reveals the agglomeration of small spherical vesicles that causes the very large size measured by dynamic light scattering technique.



The large size and wide size distribution of all the niosomes in the study come from the inadequate of sonication time of the hydration process and the volume of dilution medium. To improve preparation parameters, sonication time was increased totally upto 30 minutes and the dilution volume was varied by diluting an aliquot of 100 mg of the same batch of proniosome gel (Brij 30: CHO = 3: 1 molar ratio, DCP = 0.005 g, quercetin 0.02 g) with 1, 3 and 5 ml. The resulting niosomes were characterized for mean size and polydipersity index, zeta potential and %EE as shown in Table 23.

Table 23 Size, zetapotential and % EE of the niosomes upon the increase of dilution volume from 10 to 50 ml per 1 g of proniosome gel.

Dilution volume (ml)	Mean size (nm) (±SD)	PI	Zeta potential	%EE
10	356.1 ± 116.79	0.234 ± 0.0005	-2.02 ± 2.23	97.10 ± 1.33
30	114.33 ±40.33	0.268 ± 0.0221	-1.67 ± 1.16	94.26 ± 1.25
50	170.33 ± 95.64	0.245 ± 0.0127	-1.45 ± 1.64	89.15 ± 2.79

Table 23 shows the decrease of size from 356.1 ± 116.79 to 170.33 ± 95.64 nm and almost identical polydispersity index as the dilution volume increase. However, both zeta potential and % EE were a little bit lower. The % EE (89.15 ± 2.73 – 94.26 ± 1.25%) are considered high which might be attributed to the lipophilic nature of the quercetin that it is readily dissolved in the lipid bilayer.

4.3.3 Stability study

DPPH method was used to estimate the stability of quercetin in the niosome formulations kept at various conditions. DPPH method is simple and popular since DPPH is a stable free radical that provides the use in interpretation of antioxidant activity.

DPPH radical solution presents the violet color, when it is mixed with substance that can donate a hydrogen atom like quercetin, it will change into a reduced form which losses of the violet color. The wavelength of maximum absorbance to be used to measure may vary from 515 – 520 nm and the reaction time is around 30 – 45 min. (41). In this study, the selected wavelength to measure the residual of free radicals is 520 nm, the reaction is often 45 min of the incubation time in dark. The antioxidant activity of the niosome is interpreted as % inhibition which is defined by

$$\% \text{ inhibition} = 100\ (A_O - A_C)/A_O$$

Where A_O is the initial absorbance of DPPH radical solution

A_C is the absorbance after adding the sample of quercetin

The linearity of the reaction was controlled by concomitantly study using standard solutions of quercetin ranging from 0 – 100 µg/ml. The % inhibitions are ploted vs concentrations as shown in Table 4.14 and Fig 4.17. It is notable that the DPPH reacts with quercetin under Beer-Lambert relation which can be used to estimate the activity of the samples in the stability study.

The stability of the samples that kept in various conditions at various time intervals was measured as the % inhibition of those compared to the initial time as shown in Table 25.

Table 24 Absorbance of DPPH radical solution after reacting with quercetin standard solutions

Concentration of quercetin standard solution (µg/ml)	Absorbance
0	0.8118
20	0.7063
40	0.6114
60	0.5061
80	0.3828
100	0.2513

*A 200 µl of standard solution was mixed with 7 ml of DPPH radical solution, the absorbance = A_c

**An absorbance of DPPH radical of 0 µg/ml (0.8118) was used as initial absorbance value = A_o

Diagram 1 % inhibition of standard quercetin solution ranging from 0-100 µg/ml

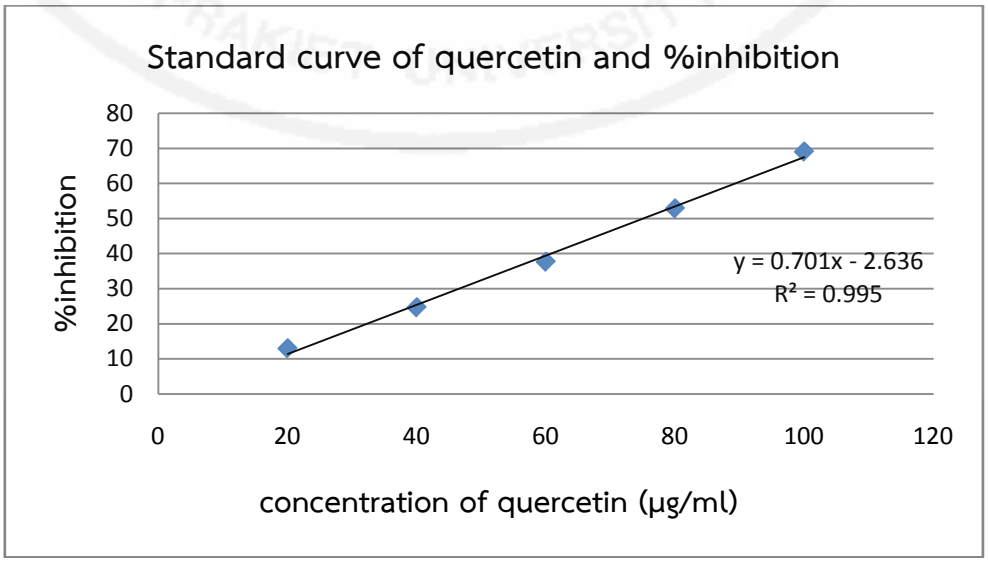


Table 25 % inhibition of quercetin niosome from Brij 30:CHO (3:1 molar ratio) kept in various conditions

Temp.	%Inhibition (mean±SD) (n=3)			
	1 day	15 days	30 days	90 days
4°C	64.14 ± 0.71	61.37 ± 1.91	61.50 ± 1.24	62.64 ± 2.21
25°C	63.68 ± 0.13	60.74 ± 1.57	62.44 ±7.82	62.99 ± 0.37
25°C in dark	66.65 ± 4.93	63.49 ± 9.35	65.36 ± 2.05	64.21 ± 0.26
45°C	63.07 ± 0.53	62.65 ± 1.09	N/A	N/A

* N/A = the niosome vesicle aggregated and was not detected

As shown in Table 25, the % inhibition of quercetin are not significantly affected after storage at 4°C and 25°C either in the dark or not. It presented a strong antioxidant activity (62.64 ± 2.21 to 64.21 ± 0.26) upto 90 days of storage.

Table 26 % EE of quercetin niosome from Brij 30:CHO (3:1 molar ratio) kept in various conditions

Temp.	%EE (mean±SD)			
	1 day	15 days	30 days	90 days
4°C	95.13 ± 0.74	96.86 ± 0.61	94.11 ± 3.72	96.8 ± 0.59
25°C	90.65 ± 6.44	96.59 ± 1.77	92.94 ± 1.88	95.59 ± 0.77
25°C in dark	96.69 ± 0.54	96.53 ± 1.00	93.09 ± 0.91	95.09 ± 2.00
45°C	90.10 ± 0.35	87.56 ± 3.11	N/A	N/A

* N/A = the niosome vesicle aggregated and was not detected

Table 26 shows the % EE for each aliquot of quercetin niosome to be stored at each condition. At the initial times, the % EE are ranging from 90.10 ± 0.35 - 96.69 ± 0.54. At the end of 90 days storage period, all the conditions except at 45°C, % EE are found to be identical to the initial time. Storing at high temperature, 45°C, the niosome went on high degree of aggregation thus % EE was not detected.

To confirm the physical stability of the dispersions. Optical micrographs of them were taken and show in Fig 23 to 24 It was found that the storage at 4°C causes the crystallization of quercetin. Thus, the %EE might be over estimate since the quercetin crystal outside the vesicles is involed in the detection.

However, the optical micrographs of niosomes at both 25°C and 45°C have no remarked effect on vesicle morphology.

Figure 23 Photomicrograph of quercetin niosome from Brij 30:CHO at 3:1 molar ratio after storing for 90 days. The captured under cross polarizer.

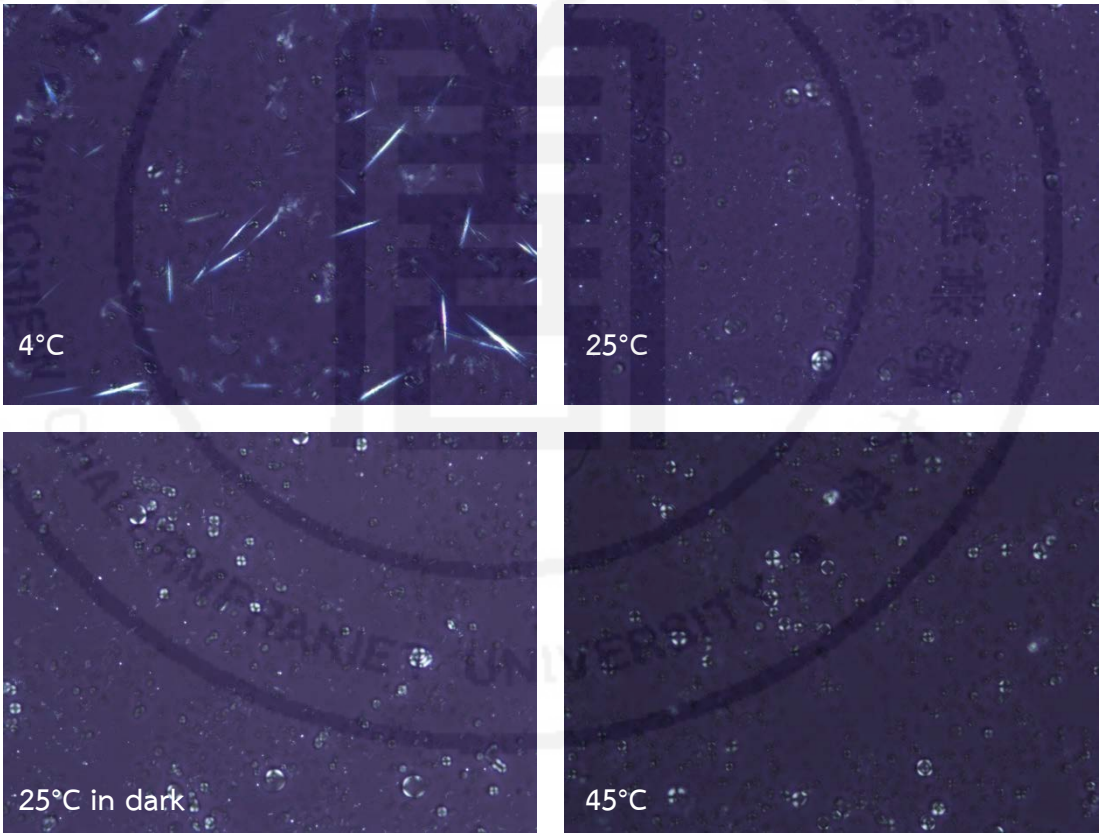
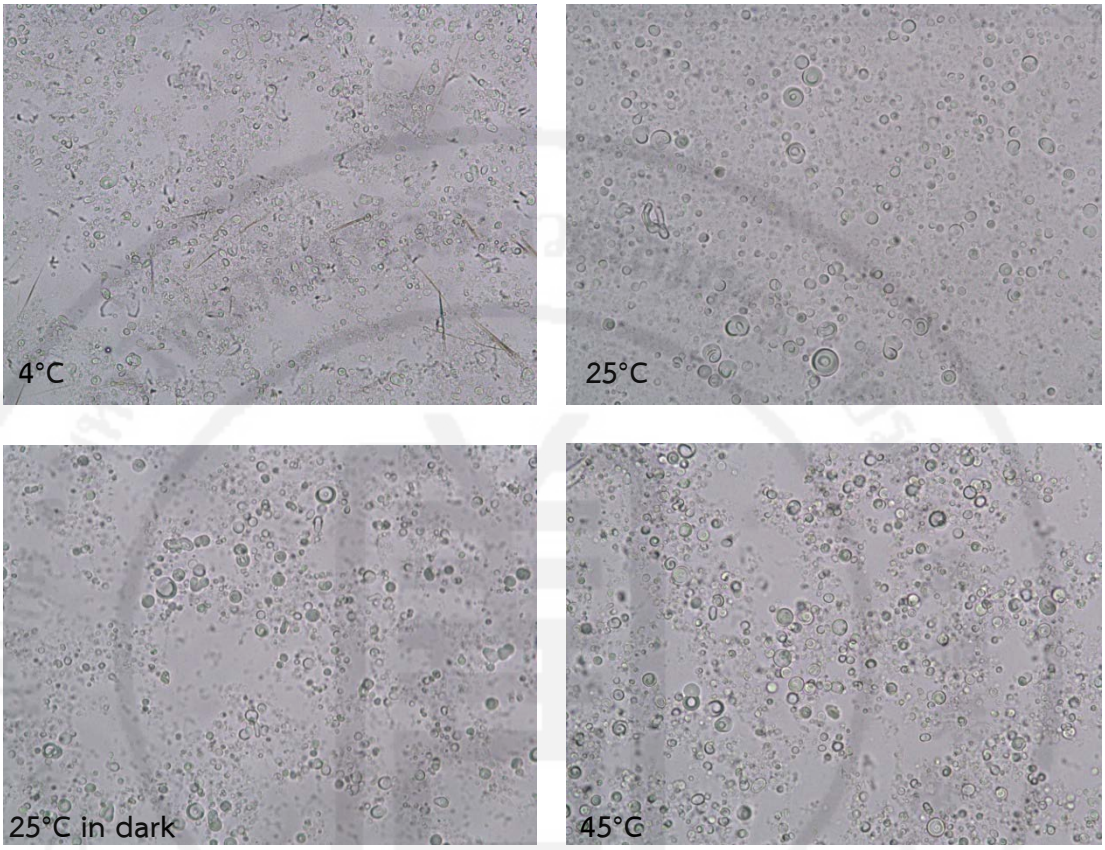


Figure 24 Photomicrograph of quercetin niosome from Brij 30: CHO at 3: 1 molar ratio after storing for 90 days.



This study demonstrated that niosome stored at 4°C forced the quercetin recrystallization as seen in needle shape under microscope. These needle are the same characteristic as the crystal found in the investigation study of six Brij:CHO with 1:1, 2:1 molar ratios. The found crystals may be the unentraped quercetin which does not dissolve well in the lipid mixture. The niosome size is very small compared to those other storage conditions that might be due to the coagulation among the vesicles. There is no crystal observed and no significant change in vesicle shape although storing at 45°C for 90 days. It is noteworthy that niosome from Brij 30 is stable at 25°C and 45°C. The cool place like 4°C is not appropriate to store due to the crystallization of quercetin. The system can maintain the DPPH-scavenging activity of quercetin. Niosome form Brij 30 may be considered as a promising delivery system for quercetin.

CHAPTER 5
CONCLUSION AND RECOMMENDATION

In this study, quercetin was successfully entrapped within niosome from Brij 30: cholesterol at 3: 1 molar ratio using a simple process named coacervation-phase separation method. This method is socalled proniosome gel method. The lipid mixture, (non-ionic surfactant, cholesterol, DCP and quercetin) were mixed with a limit amount of isopropanol and buffer at 60°C to form proniosome gel, a liquid crystalline stage which is subsequently hydrated with access amount of buffer to form niosome dispersion. This method is simple and free from hazardous inorganic solvent and can apply to various non-ionic surfactants. The initial condition of proniosome gel preparation used in the screening study is chosen from the previous report by Fang et al. (14) Four series of non-ionic surfactant which are Tween, Span, Brij and Myrj were screened. Among those, Brij series showed the highest ability to form vesicles under the specified proniosome gel method and Brij 98 was selected into optimization study to investigate the effect of DCP, quercetin loading amount and the pH of buffer on the size and stability of the vesicles.

Minitab 14 was used in data analysis and it was found that DCP always need to be incorporated into the lipid bilayer since it stabilizes the niosome from aggromeration by the repulsion of negative charge. From the optimization study, 0.02 g of quercetin can be completely incorporated into the lipid bilayer with smaller size compared to the adding of 0.01 g per a gram of lipid mixture. This may be due to the higher amount of quercetin affects the transition temperature of the lipid mixture in higher degree nevertheless this presumption does not find out. However, the quercetin of 0.02 g was used for all experiments. Brij 98 was found to provide good size and size distribution and showed promising good stability compared to others. But it possesses the inconstancy in construction into vesicles. The non-ionic surfactant to cholesterol ratio at 1:1 and 2:1 cannot form vesicle at all. At 3:1 molar ratio, is good but it lacks of reproducibility if the conditions of preparation are not well controlled enough. One reason might be from the HLB value of about 15 is too high since it has been reported that HLB value of a non-ionic surfactant plays a key

role in controlling the drug entrapment. High HLB values in the ranges of 14-17 are not suitable to produce niosome, since the hydrophilic polar head groups dissolve in the aqueous medium and the micelle will likely form instead of niosome. (6) For this reason, the non-ionic surfactant: cholesterol molar ratios (1:1, 2:1, 3:1) at the total weight of 1 g, 0.005 g of DCP and 0.02 g of quercetin were used to investigate the formation of niosomes from various Brij which are Brij 30, Brij 52, Brij 58, Brij 93, Brij 97 and Brij 98. They were characterized and compared for their size, polydispersity index and physical stability after storing upto 1 month. It was found that niosome from Brij 30 at 3:1 molar ratio and Brij 52 at 2:1 molar ratio were found to be the most complete assembled vesicles. Although the vesicle size and size distribution from the measurement are large and indicate the wide range dispersibility due to the too short sonication time after hydration. However, the morphology of quercetin niosome from Brij 30 are sphere and agglomerated. To find out, the quercetin niosome from Brij 30 (3:1 molar ratio) was further investigated for improving the hydration method by increase the hydration volume from 10 ml/g to 50 ml/g. The longer sonication time from 9 minutes to 30 minutes was applied. The size, % EE and zetapotential were measured. Size and size distribution reduced upon dilution from 356.1±116.79 to 170.33±95.64, the PI values were indentical (between 0.234±0.0005 to 0.245±0.0127). The stability was tested at 4°C, 25°C, 25°C in dark and 45° and the samples were monitored for physical appearance, % EE and antioxidant activity. It was found that at 45°C upto 30 days, the dispersion became more viscous and the color changed thus it did not be monitored after 30 days. Drug crystals were observed after 90 days upon storage at 4°C. The samples both at light exposure and light protection were stable at 25°C. The DPPH-scavenging activity of quercetin was maintained at 4°C and both at 25°C. The % EE did not significantly change.

The present study shows that proniosome gel method is simple, reproducible and does not need the expensive instrument and hazardous inorganic solvent to prepare. Quercetin niosome from Brij 30 is successfully prepared and provides a promising delivery system for tentative used as skin application.

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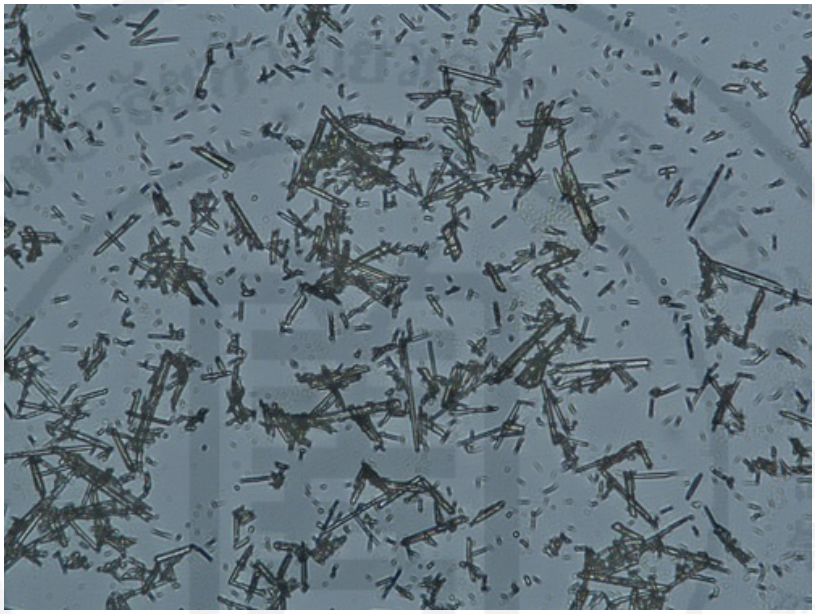
APPENDIXS

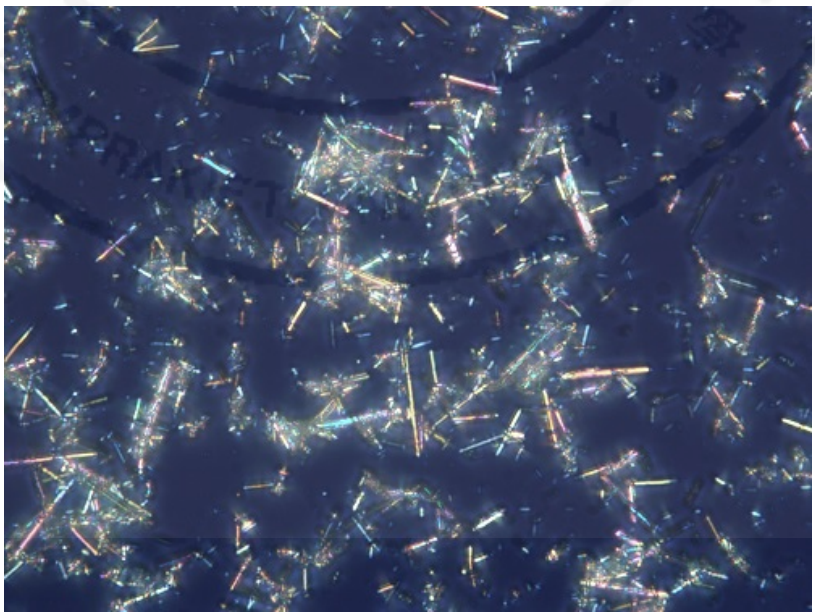
APPENDIX

Photomicrography of the active ingredients used in the preparation of quercetin niosome by proniosome gel method.

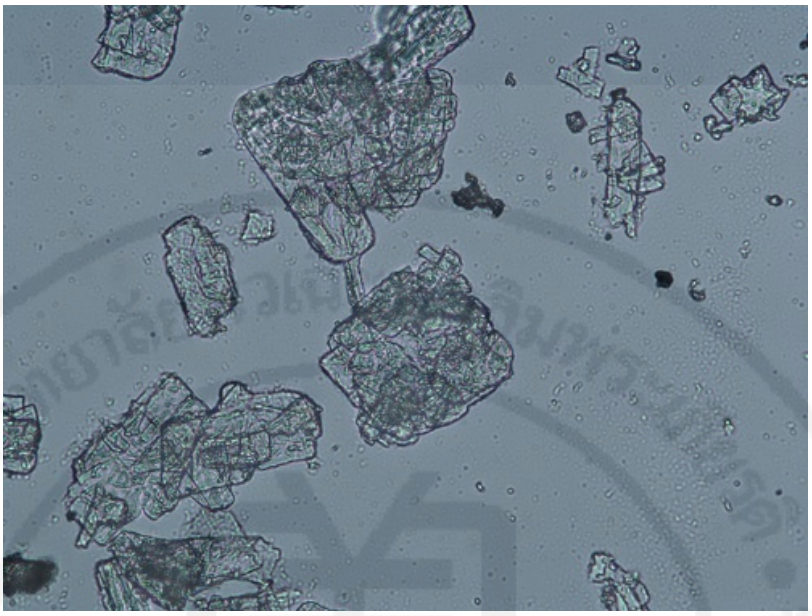
1A. Photomicrograph of quercetin.



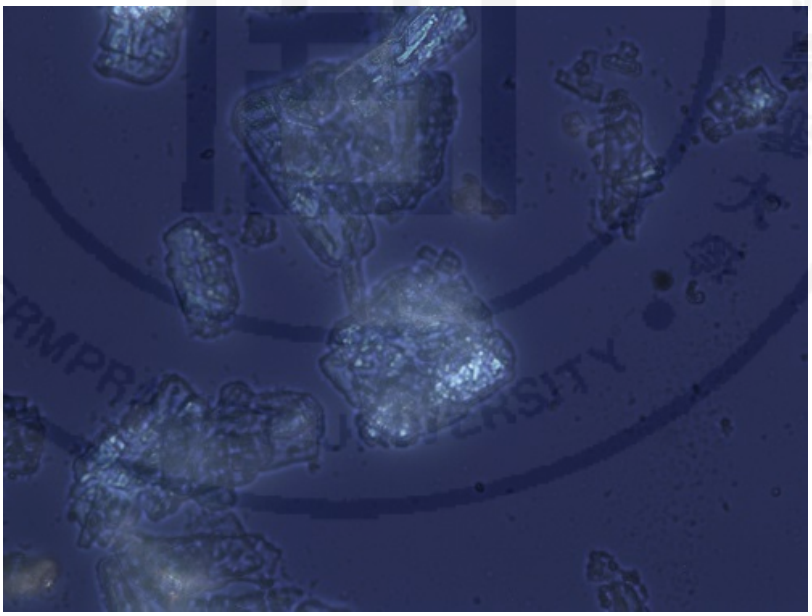
2A. Photomicrograph of quercetin under cross polarizer.



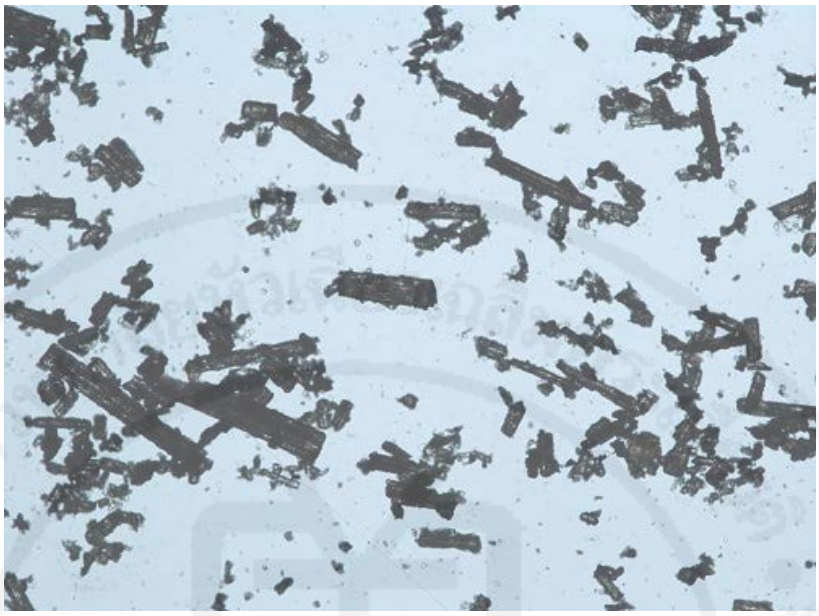
1B. Photomicrograph of dihexadecyl phosphate.



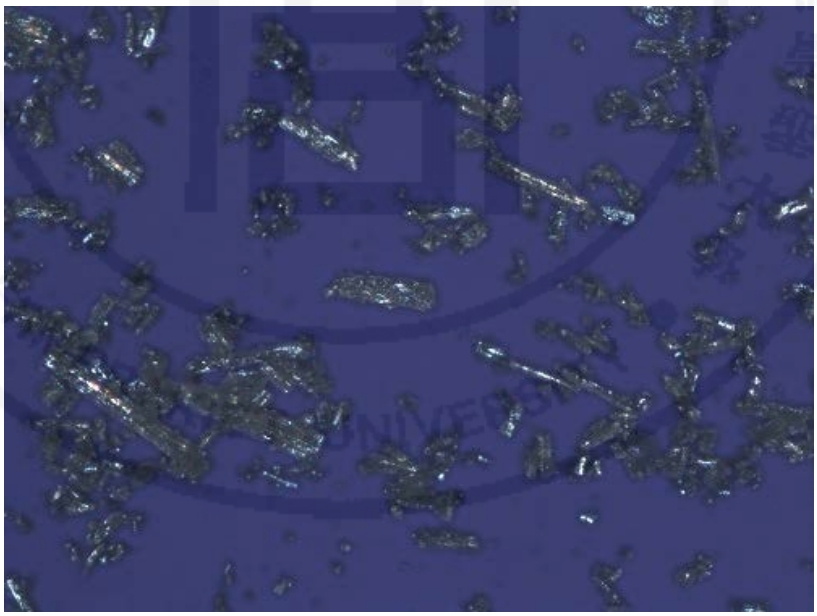
2B. Photomicrograph of dihexadecyl phosphate under cross polarizer.



1C. Photomicrograph of cholesterol.



2C. Photomicrograph of dihexadecyl phosphate under cross polarizer.



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