# CHAPTER VI
# PREPARATION AND CHARACTERIZATION OF RHAMNOLIPID VESICLES AS POTENTIAL NANOCARRIER SYSTEMS

## 6.1 Abstract

The effect of cholesterol on the vesicle formation of a rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* SP4 was studied in the present work. The rhamnolipid vesicles were prepared in a phosphate-buffer saline (PBS) solution (pH 7.4) at a biosurfactant concentration of 0.13 wt.%, or 6.5 times the critical micelle concentration (CMC), with various amounts of cholesterol. The results showed that the rhamnolipid biosurfactant formed spherical vesicles both in the absence and presence of cholesterol, but the incorporation of the cholesterol into the bilayer membrane reduced the vesicle size as the cholesterol concentration increased. Sudan III, a water-insoluble dye, was used as a model hydrophobic compound in the encapsulation experiment. The maximum encapsulation efficiency (*E*%) (nearly 90%, or 2.2 mg of entrapped Sudan III per gram of rhamnolipid biosurfactant) was achieved at the cholesterol concentration of 100 μM and the initial Sudan III concentration of 8.8 μM.

**Keywords:** Biosurfactants; Glycolipids; Rhamnolipids; *Pseudomonas aeruginosa*; Vesicles

## 6.2 Introduction

Surfactant molecules can self-assemble to form a variety of microstructures, including micelles, vesicles, bilayers, lamellar sheets, and liquid crystalline phases, in aqueous media (Vinson *et al.* 1989). The morphology of these aggregates is known to be affected by surfactant concentration (Sánchez *et al.* 2007), pH (Dahrazma *et al.* 2008), temperature (Bakshi and Sachar 2006), counterions (Jiang *et al.* 2005), ionic strength

(Mata *et al.* 2004), and co-solutes or contaminants like alcohols and metals (Champion *et al.* 1995, Villeneuve *et al.* 1998). Among these several aggregate structures, the formation of surfactant vesicles has received great attention in recent years. The vesicles contain one or more surfactant bilayer surrounding an aqueous core (Fernandez *et al.* 2005). Therefore, they can encapsulate either water-soluble compounds in their inner aqueous phase or oil-soluble substances in their outer bilayer region, resulting in their potential use as a vehicle, or carrier, for active ingredients in cosmetics and pharmaceutical applications (Paolino *et al.* 2008, Teixeira *et al.* 2008, Worakitkanchanakul *et al.* 2008, Balakrishnan *et al.* 2009). Normally, spontaneously-formed vesicles can be obtained in various surfactant systems, such as a single surfactant solution (Champion *et al.* 1995, Kakizawa *et al.* 1996, Sánchez *et al.* 2007, Pornsunthorntawee *et al.* 2009), a surfactant mixture (Edwards and Almgren 1992, Maria *et al.* 2000, Zhai *et al.* 2001, Zhai *et al.* 2006), and with the addition of co-solutes into the surfactant solution (Devaraja *et al.* 2002, Manosroi *et al.* 2003, Muzzalupo *et al.* 2005, Bandyopadhyay and Neeta 2007).

One of the most widely-used co-solutes in the vesicle systems is cholesterol. Some surfactants cannot assemble to form vesicles without cholesterol addition due to their large head group sizes (Manosroi *et al.* 2003, Muzzalupo *et al.* 2005). The incorporation of cholesterol into the surfactant bilayer leads to a proper molecular geometry and hydrophobicity for vesicle formation (Muzzalupo *et al.* 2005). In addition, cholesterol also alters the fluidity of the hydrocarbon chains in the bilayer, thus promoting the formation of surfactant vesicles (Hao *et al.* 2004). For the vesicle formation, cholesterol can be used as a membrane stabilizer. Although some surfactants can spontaneously self-assemble to form a vesicular structure, the bilayer may be unstable and leaky (Devaraja *et al.* 2002). The inclusion of cholesterol can help stabilize the vesicle membrane and prevent the leakiness, thus further increasing the encapsulation efficiency (*E*%) of the vesicles.

Rhamnolipid, a glycolipid biosurfactant produced by *Pseudomonas aeruginosa* strains, is a vesicle-forming surfactant (Champion *et al.* 1995, Helvaci *et al.* 2004,

Sánchez *et al.* 2007, Pornsunthorntawee *et al.* 2009). The rhamnolipid species are composed of a hydrophilic head formed by one or two rhamnose molecules and a hydrophobic tail containing one or two fatty acid chains (Figure 6.1). The vesicle formation of the rhamnolipid species has been reported to be affected by concentration (Sánchez *et al.* 2007, Pornsunthorntawee *et al.* 2009), pH (Champion *et al.* 1995), and additives, like salt and alcohol (Helvaci *et al.* 2004; Pornsunthorntawee *et al.* 2009). However, the rhamnolipid vesicles have rarely been characterized, and the preparation of the rhamnolipid vesicles for a specific purpose has seldom been reported. Knowledge about the characteristics of the rhamnolipid vesicles can perhaps be used to introduce this biosurfactant into high value-added utilization, such as in cosmetics and pharmaceutical applications. It was reported that a rhamnolipid biosurfactant produced by *P. aeruginosa* SP4 self-assembled to form the vesicular structure in a phosphate-buffer saline (PBS) solution (pH 7.4) at a concentration greater than its critical micelle concentration (CMC), which was 0.02 wt.% (200 mg/l) (Pornsunthorntawee *et al.* 2008a, 2009). However, the preliminary experiment showed that the *E*% of the rhamnolipid vesicles was low (Pornsunthorntawee *et al.* 2009). In the present work, the SP4 biosurfactant was used to prepare the rhamnolipid vesicles in a PBS solution (pH 7.4) at a biosurfactant concentration of 0.13 wt.% (6.5 times the CMC). Cholesterol was added to enhance the vesicle formation and to improve the *E*% of the rhamnolipid vesicles. Sudan III, a water-insoluble dye, was used as a model hydrophobic substance in the encapsulation experiment to evaluate the potential use of the rhamnolipid vesicles as carriers in either cosmetics or delivery systems.

## 6.3 Experimental

### 6.3.1 Materials

Cholesterol (≥95% purity) was supplied by Fluka. 1-[4-(phenylazo)phenylazo]-2-naphthol, or Sudan III, (90% dye content) was purchased from Sigma. The chemical structures of the cholesterol and the Sudan III are shown in Figures

6.2 and 6.3, respectively. All chemicals were used as received without further purification.

6.3.2 Production and Chemical Structures of Rhamnolipid Biosurfactants

*P. aeruginosa* strain SP4, a biosurfactant-producing microorganism, was isolated from petroleum-contaminated soil in Thailand (Pornsunthorntawee *et al.* 2008b). The isolated strain was maintained on nutrient agar slants at 37°C and was sub-cultured every two weeks.

To produce the biosurfactant, an inoculum was first prepared by transferring the bacterial colonies into a nutrient broth, and the culture was incubated at 37°C in a shaking incubator at 200 rpm for 22 h. Then a nutrient broth containing 2% inoculum and 2% palm oil was incubated at 37°C under aerobic conditions in a shaking incubator at 200 rpm for 48 h to obtain the highest microbial and biosurfactant concentrations (Pornsunthorntawee *et al.* 2008b). After that, the culture medium was centrifuged at 8,500 rpm at 4°C for 20 min to remove the bacterial cells. The supernatant was further treated by acidification to pH 2.0 using a 6 M hydrochloric solution, and the acidified supernatant was left overnight at 4°C for complete precipitation of the biosurfactant (Yakimov *et al.* 1996). After centrifugation, the precipitate was removed and was dissolved in a 0.1 M sodium bicarbonate solution, followed by the biosurfactant extraction step with a solvent, having a 2:1 chloroform-to-ethanol ratio, at room temperature (Zhang and Miller 1992). The organic phase was transferred to a round-bottom flask connected to a rotary evaporator to remove the solvent at 40°C. After solvent evaporation, about 5.2 grams of a viscous honey-colored biosurfactant product was extracted per liter of the culture medium. The chemical structure of the most abundant component in the biosurfactant product was identified as Rha-$C_{10}$-$C_{10}$ (73.5%), while the others were characterized as Rha-Rha-$C_8$-$C_{10}$ (0.7%), Rha-$C_8$-$C_{10}$ (1.5%), Rha-$C_{10}$-$C_{12:1}$ (9.5%), Rha-$C_{10}$-$C_{12}$ (13.3%), and Rha-Rha-$C_{10}$-$C_{14:1}$ (1.4%), with small contributions of their structural isomers (Pornsunthorntawee *et al.* 2008a).

6.3.3 Preparation of Rhamnolipid Vesicles

The rhamnolipid vesicles were prepared in a PBS solution (pH 7.4) at a biosurfactant concentration of 0.13 wt.% (6.5 times the CMC) with various cholesterol concentrations using the thin film hydration method. Briefly, the extracted biosurfactant and cholesterol at any desired concentrations were dissolved in chloroform. The solvent was evaporated in a vacuum oven until dry, and the obtained film was subsequently hydrated with the PBS solution to achieve a constant biosurfactant concentration of 0.13 wt.%. The sample was vortexed at room temperature to get a homogeneous solution before being filtered through a 0.45-μm pore size nylon filter at least two times to remove any solid particles. The sample was left at room temperature for a day to ensure equilibration before taken for measurements and analysis of vesicle formation.

6.3.4 Analytical Methods and Measurements

*6.3.4.1 Turbidity Measurement*

The turbidity of the biosurfactant solutions at various cholesterol concentrations was measured by using a UV/Vis spectrophotometer (Shimadzu, UV-2550) at room temperature. The reported values were corresponded to the absorbance at a wavelength (λ) of 600 nm (Sánchez *et al.* 2007).

*6.3.4.2 Zeta Potential Measurement*

The zeta potential of the vesicles formed in the biosurfactant solutions prepared at different cholesterol concentrations was measured by using a dynamic light scattering instrument (Brookhaven, ZetaPALS) at 25±1°C.

*6.3.4.3 Dynamic Light Scattering Measurement*

The dynamic light scattering (DLS) technique was used to measure the size of the rhamnolipid vesicles at various cholesterol concentrations. The DLS instrument (Brookhaven, ZetaPALS) was operated at 90° scattering angle and 25±1°C. The size of the rhamnolipid vesicles was expressed in terms of hydrodynamic diameter, which was calculated from the diffusion coefficients obtained from computer analysis by using the software provided with the instrument.

*6.3.4.4 Transmission Electron Microscopy Examination*

The morphology of the rhamnolipid vesicles was observed with transmission electron microscopy (TEM) using the negative-staining technique. A drop of the test biosurfactant solution sample was placed onto a copper grid and was stained with a 1% uranyl acetate aqueous solution. The excess sample solution was adsorbed with a piece of filter paper. The grid was dried in a vacuum desiccator for at least 6 h. The samples were imaged under a transmission electron microscope (JEOL, JEM-2100). The diameter of the rhamnolipid vesicles was then determined from TEM micrographs using SemAfore software, version 5.00 (JEOL (Skandinaviska) AB, Sweden).

6.3.5 Encapsulation Experiment

The *E*% of the rhamnolipid vesicles was evaluated by using Sudan III, a water-insoluble dye, as a model lipophilic compound. A stock solution of Sudan III was prepared in chloroform at a desired concentration. Then, 100 µl of the stock solution was added to an empty vial and the solvent was evaporated in a vacuum oven until dry. After that, 10 ml of the biosurfactant solution was added and allowed to equilibrate for one week. The residual insoluble dye was separated from the mixture by centrifugation at 10,000 rpm for 10 min. The colored supernatant was further filtered through a 0.45-µm pore size nylon filter at least two times, and the amount of the entrapped dye was then determined from the absorbance at a wavelength of 512 nm by using the UV/Vis spectrophotometer (Shimadzu, UV-2550). The concentration of the entrapped dye was calculated by using a calibration curve of Sudan III prepared in chloroform in the concentration range of 0.3 to 35 µM. The *E*% was defined as the ratio of the concentration of the entrapped Sudan III to the initial dye concentration.

6.3.6 Statistical Analysis

The experimental data are presented in terms of arithmetic averages of at least three replicates, and the standard deviations are indicated by error bars. The analyses were done using SigmaPlot software, version 8.02 (SPSS Inc., UK).

## 6.4 Results and Discussion

### 6.4.1 Vesicle Characterization

The biosurfactant extracted from the liquid culture of *P. aeruginosa* SP4 was used to prepare rhamnolipid vesicles in a PBS solution (pH 7.4) at a constant biosurfactant concentration of 0.13 wt.%, or 6.5 times the CMC (Pornsunthorntawee *et al.* 2008a, 2009). The influence of cholesterol on the vesicle formation was investigated by using turbidity, zeta potential, and DLS measurements. The morphology of the rhamnolipid vesicles was also observed with the use of TEM. The maximum cholesterol concentration used in the present work was 400 μM because a higher cholesterol concentration causes incomplete inclusion of the cholesterol in the rhamnolipid vesicles, leading to the formation of cholesterol precipitates.

It is known that a change in the turbidity of a surfactant solution can relate to the change in the amount and/or size of the surfactant aggregates. Therefore, the turbidity of the biosurfactant solution was first measured. As shown in Figure 6.4a, the turbidity of the biosurfactant solution remained almost steady at a low cholesterol concentration (less than 50 μM) before beginning to decrease at a cholesterol concentration greater than 50 μM. The reduction of the solution turbidity implies an abrupt change in the size of the rhamnolipid vesicles, perhaps due to the incorporation of the cholesterol into the bilayer membrane.

To investigate the inclusion of the added cholesterol in the vesicle membrane, a zeta potential measurement was carried out. Figure 6.4b shows the relationship between the zeta potential and the cholesterol concentration. It was found that the absolute value of the zeta potential first reduced with increasing cholesterol concentration before turning flat at a cholesterol concentration greater than 200 μM. Rhamnolipids are composed of carboxylic groups in their chemical structures (Figure 6.1). The majority of these carboxylic groups are dissociated to form carboxylate groups when the pH is above 4.0, and the rhamnolipids begin to behave as anions (Nitschke *et al.* 2005). In this present study, the rhamnolipid vesicles were prepared at pH 7.4, so the

negative value of the zeta potential was measured. However, the incorporation of the added cholesterol into the bilayer membrane made the rhamnolipid vesicles less negatively charged, because cholesterol is non-ionic. The flat zeta potential curve at a cholesterol concentration greater than 200 μM also implies the limitation of the inclusion of added cholesterol in the vesicle membrane.

The DLS measurement was used to determine the size of the rhamnolipid vesicles in terms of hydrodynamic diameter. As shown in Figure 6.4c, an increase in the cholesterol concentration from 0 to 400 μM significantly reduced the vesicle size from 300 to 200 nm. According to the work of Champion *et al.* (1995), rhamnolipid vesicles in the 50–250 nm range are considered to be medium vesicles, while those larger than 250 nm are classified as large vesicles. From the DLS results, large-sized vesicles could be observed at a cholesterol concentration lower than 100 μM, while medium-sized rhamnolipid vesicles formed spontaneously when the cholesterol concentration was increased beyond 100 μM. A change in the vesicle size from large to medium at the cholesterol concentration of 100 μM should correspond to an abrupt decrease in the turbidity of the biosurfactant solution (see Figure 6.4a).

To verify the presence of the above-mentioned rhamnolipid vesicles, TEM was used to examine the morphology of the rhamnolipid microstructure. Figure 6.5 shows the representative negative-staining electron micrographs of the rhamnolipid vesicles prepared in the PBS solution at various amounts of cholesterol. Normally, the negative-staining technique may affect the size of the surfactant vesicles due to the solubilized staining dye and to the drying process in the sample preparation. Thus, the surfactant microstructure determined from TEM micrographs is usually slightly bigger than that obtained from the DLS measurement (Fan *et al.* 2006, Roy *et al.* 2006), and the size of the surfactant microstructure obtained from the DLS technique is much more reliable than those obtained from the TEM. In the present study, the TEM micrographs revealed that the rhamnolipids self-assembled in the PBS solution at a biosurfactant concentration of 0.13 wt.% (6.5 times the CMC) to form spherical vesicles both in the absence and presence of cholesterol. Although the rhamnolipid vesicles in the TEM

micrographs were larger than those measured by using the DLS technique, the TEM micrographs also showed that the vesicle size was decreased upon increasing the cholesterol concentration (see Figure 6.4c).

The influence of the added cholesterol on the size of the rhamnolipid vesicles was expected to be governed by the interactions in the outer bilayer region. Cholesterol is composed of a small hydrophilic hydroxyl head group and a hydrophobic chain containing a steroid ring (Figure 6.2). Hence, the cholesterol is able to interact with the rhamnolipid molecules via the hydrogen bond formation between the hydroxyl group of the cholesterol and those in the hydrophilic head group (rhamnose moiety) of the rhamnolipid molecules. The hydrophobic portion of the cholesterol can also orient itself parallel to the hydrophobic chains of the rhamnolipids via their hydrophobic interaction, leading to the inclusion of the added cholesterol in the vesicle membrane (Nasseri 2005). At pH 7.4, the majority of the rhamnolipid molecules are anions due to the dissociation of the carboxyl groups. The incorporation of cholesterol into the vesicle membrane can increase the surface area where the charge is localized, so the surface charge density is reduced and the dissociation of the rhamnolipid molecules increases. The stronger the electrostatic repulsion between the polar head groups of the rhamnolipids, the smaller the obtained vesicles.

### 6.4.2 Encapsulation Results

From the cosmetics and pharmaceuticals point of view, *E*% is one of the most important parameters in vesicle formulations (Balakrishnan *et al.* 2009). To evaluate the *E*% of the prepared rhamnolipid vesicles, Sudan III, a water-insoluble dye, was used as a model lipophilic compound. The initial Sudan III concentration was varied at 8.8, 17.5, and 35 μM. Figure 6.6 shows the *E*% of the rhamnolipid vesicles prepared at different cholesterol concentrations and at various initial dye concentrations.

At all three studied initial Sudan III concentrations, the *E*% of the rhamnolipid vesicles increased with increasing cholesterol concentration before beginning to decrease at a cholesterol concentration greater than 100 μM. According to

previous works (Kirby *et al.* 1980, Bernsdoff *et al.* 1997, Manosroi *et al.* 2003, Muzzalupo *et al.* 2005, Balakrishnan *et al.* 2009), improvement of the *E*% of the rhamnolipid vesicles at a cholesterol concentration less than 100 μM is caused by an increase in the bilayer hydrophobicity and stability, and a decrease in the membrane permeability. These cholesterol effects help in the solubilization of the lipophilic compound in the outer bilayer region. The inclusion of cholesterol in the vesicle membrane also leads to a compact and well-organized bilayer. Hence, it is difficult for the entrapped compound to leak from the vesicles. However, a further increase in the cholesterol concentration beyond 100 μM reduced the *E*%, perhaps because the added cholesterol may compete with the Sudan III for inclusion in the bilayer, thus finally excluding the dye from the rhamnolipid vesicles (Balakrishnan *et al.* 2009). In addition, smaller rhamnolipid vesicles at high cholesterol concentration might not be suitable for entrapping Sudan III molecules because the size of the surfactant vesicle is another key factor affecting the *E*% (Manosroi *et al.* 2003, Pornsunthorntawee *et al.* 2009). It should be noted that, at the highest initial Sudan III concentration, the vesicle size obtained from the DLS technique was not quite different from that in the absence of Sudan III (data not shown).

It was previously reported that the *E*% of the rhamnolipid vesicles prepared from the SP4 biosurfactant in the PBS solution at a biosurfactant concentration of about 0.13 wt.%, with an initial Sudan III concentration of 35 μM, was improved by the addition of ethanol (Pornsunthorntawee *et al.* 2009). The *E*% of the rhamnolipid vesicles increased from nearly 10% (1.0 mg of entrapped Sudan III per gram of biosurfactant) in the absence of ethanol to about 14% (1.4 mg/g), 16% (1.5 mg/g), and 30% (3.3 mg/g) at ethanol concentrations of 0.1, 0.2, and 0.4 M, respectively. Compared to the present study, only 25 μM of the added cholesterol was required to achieve an *E*% of 16% at the same initial dye concentration. The influence of ethanol on the *E*% of the rhamnolipid vesicles is also governed by the interactions in the outer bilayer region, so the results suggest that cholesterol could be used more effectively as a membrane stabilizer for the rhamnolipid vesicle system, compared to ethanol. However, the

incorporation of the added cholesterol into the rhamnolipid vesicles increased the *E*% to a certain limit at a cholesterol concentration of 100 μM, and a highest *E*% of only 23% (2.2 mg/g) was obtained at an initial Sudan III concentration of 35 μM.

As shown in Figure 6.6, the initial Sudan III concentration significantly affects the *E*% of the rhamnolipid vesicles at all investigated cholesterol concentrations. The *E%* was found to be reduced upon increasing the initial dye concentration from 8.8 to 35 μM. The dye precipitate was also observed at high Sudan III content, indicating saturation of the outer bilayer region of the rhamnolipid vesicles. Our finding is also supported by previous studies (Mokhtar *et al.* 2008, Balakrishnan *et al.* 2009), which reported that the vesicle formulations enhanced the solubility of a poorly soluble compound to a certain maximum limit before any increase in the amount of the entrapped substance results in precipitation. From the results, a maximum *E*% of the rhamnolipid vesicles of about 90% (2.2 mg/g) was obtained at a cholesterol concentration of 100 μM and an initial Sudan III concentration of 8.8 μM. One advantage of vesicle formulations with a high *E*% is that less time and effort are required to remove unentrapped material (Balakrishnan *et al.* 2009).

## 6.5 Conclusions

The biosurfactant produced by *P. aeruginosa* SP4 was used to prepare rhamnolipid vesicles in a PBS solution (pH 7.4) at a biosurfactant concentration of 0.13 wt.% (6.5 times the CMC) with various amounts of cholesterol. It was found that the biosurfactant self-assembled to form spherical vesicles both in the absence and in the presence of cholesterol but the added cholesterol significantly influenced the size of the biosurfactant vesicles. The higher the cholesterol concentration, the smaller the vesicle size. The inclusion of cholesterol in the bilayer membrane improved the *E*% of the rhamnolipid vesicles to a certain limit. Regardless of the cholesterol content, the *E*% was also directly affected by the initial concentration of Sudan III, a model oil-soluble substance. The entrapment ability of the rhamnolipid vesicles suggests their potential

use as a vehicle for active ingredients in either cosmetics or pharmaceuticals applications, perhaps further enlarging the high value-added utilization of the rhamnolipid compounds.

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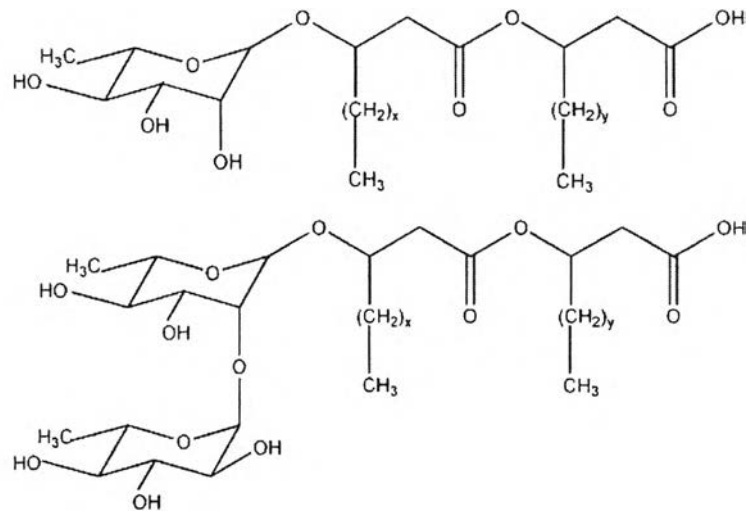


**Figure 6.1** Chemical structures of rhamnolipid biosurfactants.

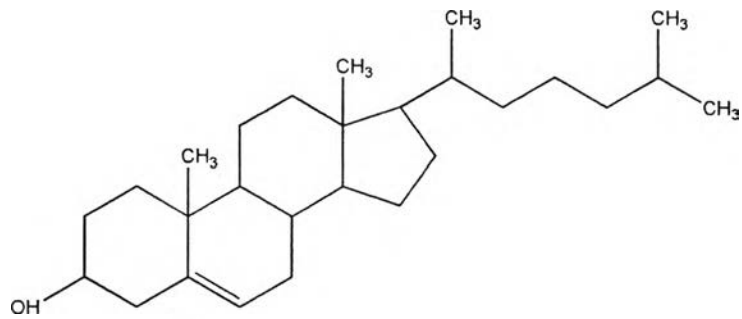


**Figure 6.2** Chemical structure of cholesterol.

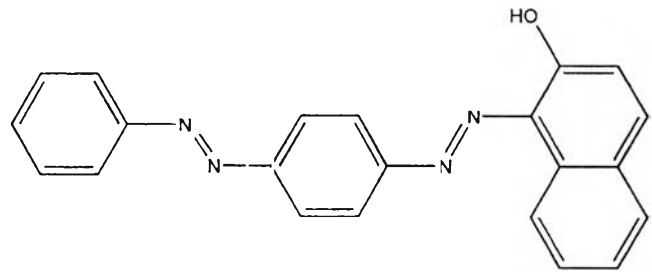


**Figure 6.3** Chemical structure of Sudan III.

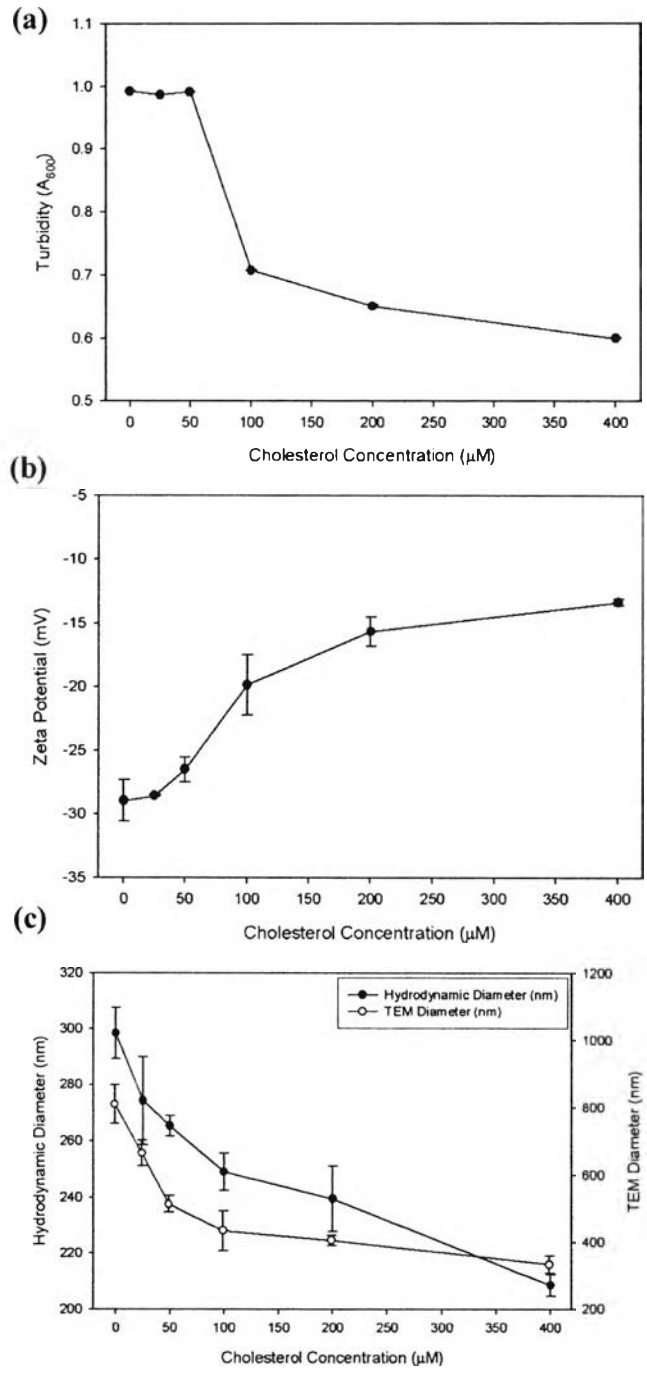


**Figure 6.4** Characteristics of the rhamnolipid solution prepared in a PBS solution (pH 7.4) at a biosurfactant concentration of 0.13 wt.% at different cholesterol concentrations: (a) Solution turbidity (absorbance at 600 nm), (b) Zeta potential, and (c) Vesicle size.

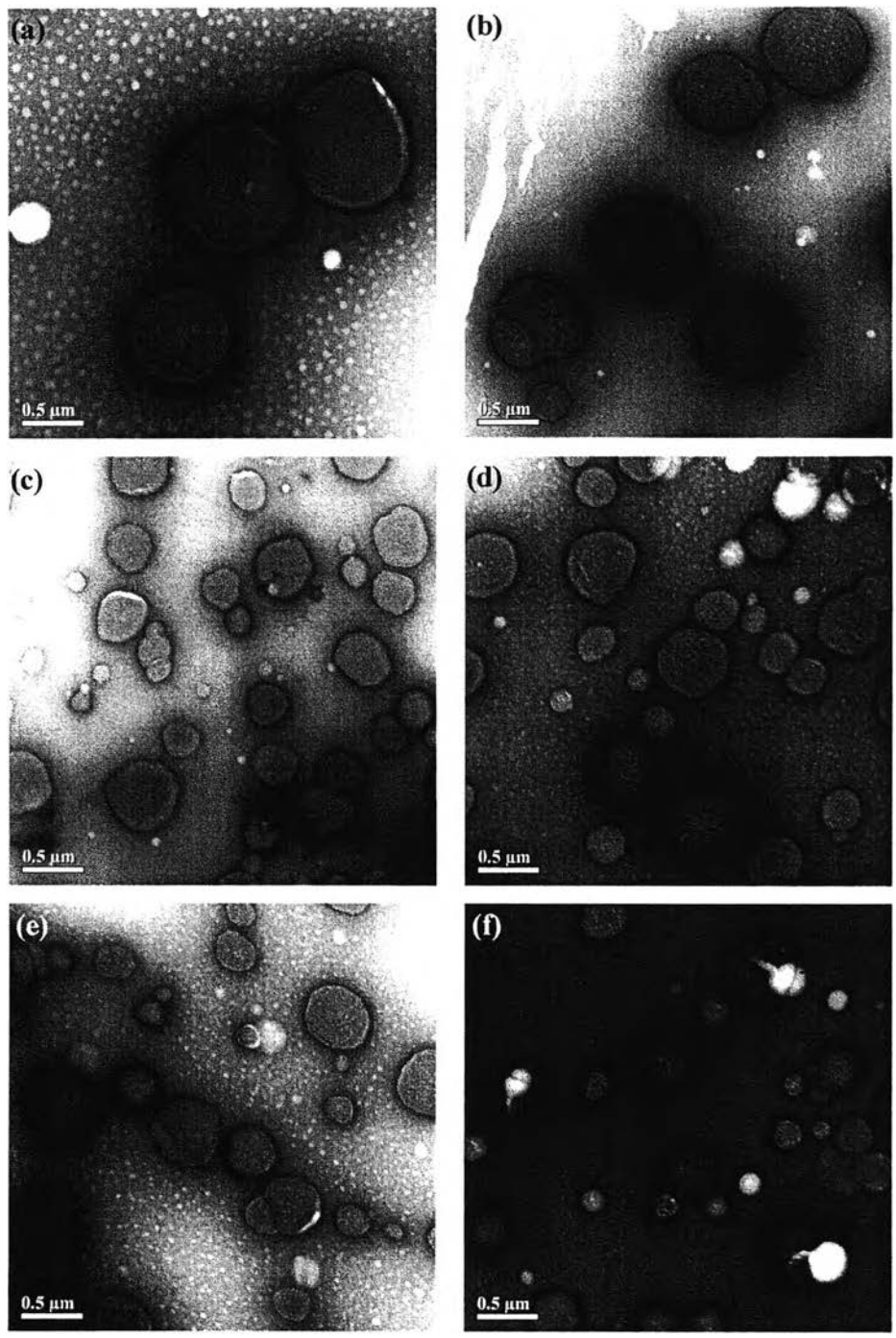


**Figure 6.5** TEM micrographs of the rhamnolipid vesicles formed in a PBS solution (pH 7.4) at a biosurfactant concentration of 0.13 wt.% and a cholesterol concentration of (a) 0 μM, (b) 25 μM, (c) 50 μM, (d) 100 μM, (e) 200 μM, and (f) 400 μM.

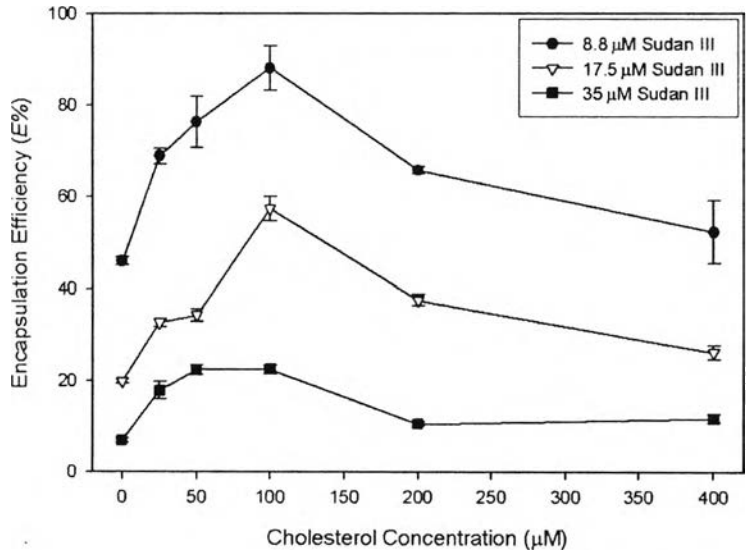


**Figure 6.6** Encapsulation efficiency ($E$%) of the rhamnolipid vesicles formed in a PBS solution (pH 7.4) at a biosurfactant concentration of 0.13 wt.% at various cholesterol concentrations and initial Sudan III concentrations.