CHAPTER III

MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources and used as received. Distilled water was used throughout the experiments.

1. Lipid

1.1 Isopropyl myristate (Fluka Chemical, Switzerland, lot. No. 70120)

1.2 Soybean oil (Sigma, USA, lot, No. 98 H0172.)

2. Surfactants

2.1 Polyethylene glycol 660 (12) hydroxysterate (Solutol® HS-15) (BASF, Germany, lot. No. 52-296)

2.2 Polyoxyl 35 castol oil (Cremophor® EL) (BASF, Germany, lot. No. 423975)

2.3 Polysorbate 20 (Tween® 20) (Fluka Chemical, Switzerland, lot. No. 93773)

2.4 Polysorbate 80 (Tween® 80) (Fluka Chemical, Switzerland, lot. No. 93781)

3. Cosurfactants

3.1 Glycerylmonocaprylate (Imwitor® 308) (Sasol, Germany, lot. No. 802001)

3.2 Glycerol (BDH Laboratory Supplies, England, lot. No. K23624360708)

3.3 Glycerylmonopalmitate (Palsgaard® 0093) (Palsgaard Industry, Denmark, lot. No. ID 909305)

3.4 Propylene glycol (Sigma, USA, lot. No. 21K0011)

4. Buffer Solution

4.1 di – Sodium hydrogen orthophosphate 2- hydrate (Analar®) (BDH Laboratory Supplies, England, lot. No. K27420679010)

4.2 Potassium dihydrogen phosphate (Merck, Germany, lot. No. A262673045)

4.3 Sodium chloride (Analar®) (BDH Laboratory Supplies, England, lot. No. KK28042033031)

5. Drabkin's solution

5.1 Drabkin's reagent (Sigma, USA, lot. No. 020K6059)

5.2 Polyethylene glycol (23) laurylether (Brij® 35) (Sigma, USA, lot. No. 099H6127)

6. Dye

6.1 Methyl orange (BDH Laboratory Supplies, England, lot. No.L395018850)

6.2 Sudan III (Sigma, USA, lot. No. 41K1241)

Equipments

1. Analytical balance (Sartorius, Germany)
2. Conductivity tester (BF11a® ID 1010,Idex, USA)
3. Heating magnetic stirrer (ARE, VELP Scientifica, Italy)
4. Microcentrifuge (230 MA®, Hermle, Germany)
5. Microcomputer thermometer (HI 8424, Hanna Instruments, Hong Kong)
6. Microscope (Olympus® model CHD, Olympus Optical Co., Japan)
7. Particle size analyzer (Malvern® model 4700, Malvern Instruments Ltd., Malvern, UK)
8. pH meter (Model ф 50 pH, Beckman, USA)
9. Polarized lens (BH-POL 0183, Olympus Optical Co., Japan)

10. Refrigerated centrifuge (Himac CR 20B3, Hitachi, Japan)

11. Spectrophotometer (Milton Roy 3000, Denmark)

12. Vapor pressure osmometer (Vapor® 5520, Wescor, USA)

13. Viscometer (Model Rotovisco® RV20, Haake, Germany)

14. Water bath shaker (Heto, Denmark)

Methods

1. The preparation of micellar systems

The micelle of surfactant and cosurfactant was prepared. Each of surfactants (Tween® 20, Tween® 80, Cremophor® EL and Solutol® HS15) was matched to all cosurfactants (glycerol, propylene glycol, Palsgaard® 0093 or Imwitor® 308) at various weight ratios of surfactant to cosurfactant, 4:1, 2:1 and 1:1, and total concentrations of surfactant and cosurfactant ranging from 10 to 90 %w/w. The samples were heated up to 60-70°C with vigorous stirring for 15 minutes and then cooled down to room temperature. The optically clear solution was investigated and classified as micelle solution. Consequently, the formulations that could form micellar solution were used to formulate microemulsion.

2. The preparation and determination of microemulsions

The compositions that could form clear micelles were used to prepare microemulsion. Oil (isopropyl myristate), surfactant (Tween® 20, Tween® 80,

Cremophor® EL or Solutol® HS15), cosurfactant (glycerol, propylene glycol, Palsgaard® 0093 or Imwitor® 308) and distilled water were mixed by fixing weight ratio of surfactant to cosurfactant at 4:1, 2:1 or 1:1. The concentrations of surfactant and cosurfactant mixture were varied from 10-90 % w/w and oil phase was varied from 5, 10, 15, 20-90 % w/w. Thus, there were approximately 51 mixtures in pseudo-ternary phase diagram, which were investigated for their appearance. The mixture in closed container was heated to 70-80°C in water bath for 15 minutes and cooled down at room temperature with constant stirring. The stability of mixture stored for 24 hrs, one week and one month at room temperature was observed for visual clarity and fluidity. The clear solution was determined non-birefringent property by using a polarized light microscope. The sample, which was not visible in this microscope, was classified as non-birefringence. In addition, the isotropic and clear sample, which did not show a change in meniscus after tilting to an angle of 90° was classified as gel (Kale and Allen, 1989). The mixture, which exhibited, clear and non-birefringent (isotropic) fluid, was classified as microemulsion. Only sample that was microemulsion was drawn as a dot in pseudo-ternary phase diagram. Boundaries of microemulsion formation (M) and gel formation (G) was estimated. After one month storage, these two regions in phase diagram were calculated as the percent of microemulsion and gel formation by using cut and weight method (Kale and Allen, 1989).

Then, the microemulsion in phase diagram was chosen to study the stability of microemulsion after dilution with water. The dark circle (●) and circle with a cross (⊗) represented the clear solution and turbid solution, after dilution, respectively.

To determine the effect of phosphate-buffered saline (PBS) pH 7.4 on microemulsion formation, the microemulsion systems were prepared using PBS as aqueous phase. The sample was investigated for the change in microemulsion area in phase diagram. The one liter of phosphate buffer saline pH 7.4 was prepared using 2.38 g di-sodium hydrogen orthophosphate, 0.19g potassium di-hydrogen orthophosphate and 5.0 g sodium chloride (British Pharmacopoeia, 1998).

3. Measurement of particle size and size distribution of microemulsions

Malvern® 4700, the photon correlation spectroscopy (PCS) was used to determine the size of microemulsion droplet. The PCS determines the size using dynamic techniques by which analyze the fluctuations in the intensity of scattering by droplets due to Brownian motion (Solans, Pons and Kunieda, 1997). The intensity is then calculated to obtain the Z-average dilution coefficient, which is used to determine the particle size (or the hydrodynamic radius). In the absence of interparticle interference (i.e. in diluted system), the size is directly obtained regardless of the factor

accounting for the interparticle interference. The laser wavelength, argon as light source, used in this experiment was operated at 488 nm. All measurements were made at 30°C and the intensity of the scattered light was observed at 90°. Microemulsions were freshly prepared and diluted to 1% w/w with distilled water. Then the diluted microemulsion was filtered two times using 0.22 μm microfilter in order to remove any contaminants that could interfere the measurement. The results were done in triplicate.

4. The determination of phase inversion temperature (PIT)

The temperature at which the emulsion morphology inverts is called the phase inversion temperature (Shinoda and Friberg, 1986) and is also true for microemulsion.

The microemulsion was heated up to 60-90°C with vigorous stirring. The temperature, which was the onset of turbidity of sample when heating and of clarity when cooling was noted as the PIT. The determination of PIT was the average of four determinations.

5. The type of microemulsions

In this study, the method used to determine the type of microemulsion were dye method and conductivity test.

5.1. Staining test (Dye method)

A one drop (0.2 ml) of water-soluble dye (methyl orange) or oil-soluble dye (sudan III) was dropped into 2.5 g sample at 25°C. If clear sample became orange as methyl orange could rapidly expand in external phase of sample, while sudan III was a stable dot, the sample could be o/w microemulsions. The situation converted in w/o microemulsions, that the sudan III rapidly dissolved in oil external phase.

5.2.Conductivity determination

Conductivity of microemulsion was determined using Index® ID 1010 at room temperature. An electrode was calibrated at 1410 μs/cm by using 0.01 N of potassium chloride. The electrode was dipped into the microemulsion sample and waited until a stable figure on the digital was obtained. The electrical conductivity technique determined the nature of continuous phase of microemulsions.

6. Measurement of viscosity of microemulsions

Haake viscometer®, rotational viscometer or cup and bob viscometer, was used to determine the viscosity of microemulsion at 25°C. The 9 ml sample was loaded in the viscometer and the shear rate was increased from 0 s^{-1} to 200 s^{-1} in one minute. The shear rate was maintained at 200 s^{-1} for two minutes, and the last step was one minute to decrease the shear rate back to 0 s^{-1}. The viscosity of microemulsion was an average of ten measurements at shear rate of 200 s^{-1}.

7. Measurement of osmolality

The 10 μl microemulsion using PBS as the continuous phase was filled into a solute-free paper disc in the sample holder of Vapor® 5520 osmometer. The osmometer was an electronic adaptation of the hygrometric method of vapor pressure determination. The equipment was calibrated to vary osmolality of 100, 290 and 1000 mOsm/kg by using 100, 290 and 1000 Opti-mole® standard solutions. A fine-wire thermocouple hygrometer was the sensing element suspending in a metal mount. While vapor pressure was equilibrated in the chamber airspace, the thermocouple sensed the ambient temperature of the air, establishing the reference point for the measurement.

Under electronic control, the thermocouple then seeked the dew point temperature within the enclosed space, giving an output proportional to the different temperature. The difference between the ambient temperature and the dew point temperature was the dew point temperature depression that was an explicit function of solution vapor pressure.

8. Measurement of pH

The pH of microemulsion using phosphate-buffered saline (pH 7.4) as aqueous phase was measured at room temperature. The pH meter was calibrated at pH 4, 7 and 10 using Fisher's standard buffer.

9. Hemolysis study

9.1 Sample preparation for hemolysis studies

Microemulsions and micelle in PBS were used for hemolysis studies. The samples were diluted with the buffer to obtain the total concentrations of surfactant and cosurfactant of 0.005, 0.01, 0.05. 0.075, 0.1, 0.25, 0.5 and 1% w/v

9.2 Erythrocyte hemolysis (Gould et al., 2000)

9.2.1 Removing of plasma and buffy coat

Human blood was a donated blood with normal blood chemistry from Pranangklao Hospital. The plasma and buffy coat were removed by centrifuging at 2,200 rpm for 10 minutes and the erythrocytes were washed three times in buffer for at least five times of their volumes. The erythrocytes were adjusted to have approximately 12% hematocrit. The erythrocyte suspension was stored at 4 ^{0}C and used within 48 hours of collection.

9.2.2 Drabkin's solution preparation

The one liter of Drabkin's solution was prepared by adding 1.25 g of Drabkin's reagent and 0.5 ml of Brij 35 solution into distilled water.

9.2.3 Hemolysis test of microemulsions and micelle

The 0.2 ml of test sample was mixed with 0.2 ml of uncentrifuged mixtures of erythrocyte suspension then the mixture was incubated for 15 minutes in shaking water bath at 37 ^{0}C. After incubation, the mixture was spun in a microcentrifuge for 15 seconds, and then 0.2 ml of the resulting supernatant was added with 3 ml of Drabkin's solution and waiting for 15 minutes (time of the reaction) to assay for the amount of hemoglobin released. The mixture was added in quartz cell to determine spectrophotometrically at 540 nm and values were expressed as a percentage of the maximum hemolysis.

A positive control was prepared by mixing 0.2 ml uncentrifuged mixtures of erythrocyte suspension and 0.2 ml buffer to intend of test sample. This mixture was measured the absorption at 540 nm after added with 3 ml of Drabkin's solution. The absorbance of positive control was assumed to 100 % hemolysis. A negative control, composed of 0.2 ml buffer mixed with 0.2 ml supernatant of centrifuged erythrocyte suspension and 3 ml Drabkin's solution, was used to assess the levels of spontaneous hemolysis. The absorbance for each sample was used to calculate the percentage hemolysis by the following relationship:

$$\% \text{ Hemolysis} = \frac{A_f - A_0}{A_{100}} \times 100 \qquad (6)$$

where;

A_f = Absorbance of hemolysis in the supernatant of test sample

A_o = Absorbance of hemolysis in the supernatant of negative control

A_{100} = Absorbance of hemolysis in the supernatant of positive control

The percent hemolysis of test sample in each concentration was an average from nine measurements.

The hemolytic activity is measured of the amount of hemoglobin released. In fact, the erythrocyte lyses with adding Brij 35 in erythrocyte solution. The Drabkin's

reagent contains potassium ferricyanide, potassium cyanide and sodium bicarbonate. In nature, the ferrous ions of the hemoglobins are oxidized to the ferric state by potassium ferric cyanide to form methemoglobin. The methemoglobin then reacts with cyanide to form cyanmethemoglobin, which can detect the absorbance at 540 nm.

The relationship between percent hemolysis and concentration in logarithmic scale of test sample was plotted as y axis and x axis, respectively. Generally, the relationship between respond and concentration was presented as sigmoidal (S-shape) curve. The S-shape curve could be converted into linear regression line in order to further calculate the inhibitory concentration (IC_{50}) of the test sample. The y-axis was transformed into the probit scale (Bowman and Rand, 1980). The method for calculation is shown in Appendix F.