CHAPTER 3

MATERIALS AND METHODS

All the materials used in this study are listed along with the name of the vendors as followings:

1. Materials

1.1. Solid lipids

Beeswax	Phatthanakit bee farm Ltd., Thailand
Cholesterol	Merck, Darmstadt, Germany
Mango butter	Henry lamotte oils GmbH, Bremen, Germany
Orange wax	Koster Keunen LLC, Connecticut, USA

1.2. Liquid lipids

Pomegranate seeds oil	Guangzhou herbs-ex INC, China
Rice bran oil	Surin bran oil Co.Ltd., Bangkok, Thailand
Sesame oil	Siam sesame Co.Ltd., Thailand

1.3. Active compound

Lycopene (90.25%)	Xian Guanyu Bio-technique Co.,Ltd., China

1.4. Surfactants

Dermofeel™ SL	Dr.Straetmans GmbH, Hamburg, Germany
Eumulgin™ SG	Cognis, Dusseldorf, Germany
Plantacare™ 1200	Cognis, Dusseldorf, Germany
Polysorbate 80 (Tween™ 80)	Nam siang Co.,Ltd., Thailand
Surfhope™ C-1216	Mitsubishi-Kagaku Foods Corporation, Tokyo, Japan

Surfhope™ C-1616	Mitsubishi-Kagaku Foods Corporation, Tokyo, Japan
Surfhope™ C-1815	Mitsubishi-Kagaku Foods Corporation, Tokyo, Japan
Surfhope™ C-1816	Mitsubishi-Kagaku Foods Corporation, Tokyo, Japan
Inutec™ SP 1	Beneo-Bio Based Chemicals, Belgium
Tegocare™ 450	Evonik Goldschmidt GmbH, Germany
Poloxamer™ 188	BASF Corporation, Ludwigshafen, Germany
Poloxamer™ 407	BASF Corporation, Ludwigshafen, Germany

1.5. Solvents

THF	Rankem, New delhi, India
Acetone	RCI Labscan limited, Thailand
ACN	RCI Labscan limited, Thailand
Absolute EtOH	Merck, Darmstadt, Germany
95% EtOH	Merck, Darmstadt, Germany
MeOH	Merck, Darmstadt, Germany
Chloroform	Merck, Darmstadt, Germany
Hexane	Merck, Darmstadt, Germany
Toluene	Merck, Darmstadt, Germany
DMSO	Sigma-aldrich, Switzerland

1.6. Antioxidant tests

ABTS	Sigma, Steinheim, Germany
Potassium persulphate	BDH Chemicals Ltd, England
DPPH	Sigma-aldrich, Switzerland
Trolox™	Sigma-aldrich, Switzerland

1.7. Zeta potential measurement

Sodium chloride	Merck, Darmstadt, Germany
Ultra-purified water	MilliQ Plus system, Millipore, Schwalbach, Germany

1.8. Silanized solution

Dichlorodimethysilane	Sigma-aldrich, Switzerland

1.9. Solubility test

Cetylpalmitate	Gattefosse (Weil am Rhein), Germany
Dynasan™ 118 (glycerol-tristearate)	Huls AG, Witten, Germany
Miglyol™ 812 oil	SASOL Germany GmbH, Witten, Germany
Myristol™ 318	Baff Global Co., Ltd., Switzerland
Safflower oil	Henry lamotte oils GmbH, Bremen, Germany
Cranberry oil	Henry lamotte oils GmbH, Bremen, Germany
Broccoli oil	Henry lamotte oils GmbH, Bremen, Germany
Lorbeer oil	Henry lamotte oils GmbH, Bremen, Germany
Olive oil	Kalichem, Italy
Soybean oil	Henry lamotte oils GmbH, Bremen, Germany
Labrasol™	Gattefosse (Weil am Rhein), Germany
Avocado oil	Henry lamotte oils GmbH, Bremen, Germany
Macadamia oil	Henry lamotte oils GmbH, Bremen, Germany
Almond oil bitter	Henry lamotte oils GmbH, Bremen, Germany
Triton™ X100	Sigma, Missouri, USA
Lycosol™	LiBiol, Germany
Isopropyl myristate	Chemtex USA, Inc., Montville, USA
Mineral oil	Sciencelab.com, Inc., Smith Rd., Houston, Texas

Transcutol^{TM} P	Gattefosse (Weil am Rhein, Germany)

1.10. Cream base

Cetyl alcohol	Sciencelab.com, Inc., Smith Rd., Houston, Texas
Stearyl alcohol	JEEN International Corp., Madison Road, Fairfield, New Jersey, USA
BHT	LANXESS Corporation Product Safety & Regulatory Affairs, Pittsburgh, USA
PG	Lyondell Chemical Company One Houston Center, McKinney St., Houston, Texas
Sodium metabisulfite	Sciencelab.com, Inc., Smith Rd., Houston, Texas
Glycerin	Sciencelab.com, Inc., Smith Rd., Houston, Texas
Disodium EDTA	J.T. Baker, Phillipsburg, USA
Salicylic acid	Sciencelab.com, Inc., Smith Rd., Houston, Texas
Methyl paraben	Sigma, Poole, UK
Propyl paraben	Sigma, Poole, UK

2. Method

<u>Part I</u> Preformulation study of lycopene and carriers

1. Preliminary physicochemical properties of lycopene

1.1. Lycopene identification

1.1.1. UV-visible (UV/Vis) analysis

Lycopene powder was dissolved in a THF solvent before analyzing. The UV/Vis spectrum of lycopene was obtained by scanning a wavelength from 300 to 600 nm using an UV 2450 double-beam spectrophotometer (Shimadzu, U.S.A). The recorded data represent the mean values ± SD of triplicate measurements.

1.1.2. HPLC analysis

HPLC analyses were performed using an auto-sample HPLC system model 360 with a pump system model 420 and a UV visible detector model 430 (Kontron Instruments, Groß-Zimmern, Germany) linked to a KromaSystem 2000 v. 1.83 computerized data acquisition and process system. A portion of 20 µl of lycopene solution was injected into a Eurosphere-100 C18 (5 µm) endcapped 250x 4.6 mm column with a matching pre-column (Knauer, Berlin, Germany). The column was kept at 25°C during the measurement. The mobile phase, composed of MeOH/THF/water (60:33:7) was run with a flow rate of 1.5 mL/min. The peak of lycopene solution detected at a wavelength of 475 nm was recorded.

1.2. Solubility

Pharmaceutical solvents including melted solid lipids, liquid lipids, surfactants and organic solvents were used as tested solvents. The exact amount of lycopene powder was weighted and recorded. The exact amount of tested solvents were added slowly little by little by using an adjustable speed stirrer at 80°C for solid lipids,

liquid lipids, surfactants and at room temperature for organic solvents until lycopene is completely dissolved. The total volume of tested solvents used was recorded. The value was expressed in mg/g unit which meant the maximal amount of lycopene (mg) soluble in total weight of solutions (g).

1.3. Thermal analysis

Thermal analysis was performed by using DSC (Mettler DSC 821e, Mettler Toledo GmbH, Gießen, Germany). Pure lycopene powder of an approximately 1-2 mg was weighed in 40 μL aluminium pans. The DSC was performed during 0-200°C at the rate of 10K/min. A reference is an empty aluminium pan. In a measurement, nitrogen gas was purged at a flow rate of 80 mL/min. Indium was used as a calibration standard for the instrument. Melting, crystallization, onset, integral, and enthalpy were calculated using Star^e Software 8.12 program.

1.4. Crystalline characterization

WAXS investigations were performed by using a Philips PW1830 X-ray generator (Philips, Amedo, the Netherlands) with a copper anode (Cu-Ka radiation, 40 kV, 25 mA, k = 0.15418 nm), using a Goniometer PW18120 as a detector. The measurements were analyzed at 2 theta from 0.6º to 40.0º. Bragg's equation was used to transform the data from scattering angle to the spacings of lipid chains. The data used were typically collected with a step width of 0.04º and a count time of 60 s.

1.5. Morphology

The appearance of lycopene powder was observed. Then, visualization was recorded by using a digital camera. Morphology of lycopene was characterized by light microscopy and the crystalline characteristic was performed by polarized light microscopy as following;

1.5.1. Light microscopy and polarized light microscopy

An optical microscope (Leitz, Wetzlar, Germany) equipped with a CMEX-1 digital camera (Euromex microscopes, Arnheim, The Netherlands) connected to Image Focus software version 1.3.1.4. (Euromex microscopes, Arnheim, The Netherlands) was used. Magnifications of the analyzed material by 16x10, 40x10, 63x10 and 100x10-fold were possible with this equipment. Lycopene was dispersed in rice bran oil before investigation.

1.6. Biological action (antioxidant activities)

1.6.1. ABTS assay

The method introduced by Tachakittirungrod *et al*. (2007b) was performed with some modifications. ABTS radical monocation ($ABTS^{\bullet+}$) was generated during the reaction of 7 mM aqueous ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) aqueous solution. The ABTS solution was allowed to stand for 12-16 h and diluted with absolute EtOH to an absorbance of 0.7±0.2 at 750 nm before used. Lycopene powder was dissolved in acetone. The reaction was allowed to stand for suitable time approximately 5 min and measured spectrophotometrically at 750 nm by using a microplate reader spectrophotometer (Product of Bio rad, Model 680). All measurements were carried out in triplicate. The free radical scavenging activity of samples was calculated as % inhibition and compared to standard Trolox™, then expressed as TEAC value.

1.6.2. DPPH assay

The method introduced by Okonogi *et al.* (2007) was performed with some modifications. DPPH radical solution was prepared at the concentration of 10^{-4} M by using absolute EtOH as solvent. Lycopene powder was dissolved in acetone. To start

the reaction, 20 μL of sample solution was added to 180 μL of 10^{-4} M DPPH solution. The lycopene solution was stand for 3 min in the dark at room temperature. The absorbance of the mixture was measured at 540 nm by microplate reader spectrophotometer (Product of Bio rad, Model 680).

1.7. Stability of lycopene

Lycopene was exposed to light and oxygen for 7 days. The color change of lycopene was recorded by digital camera and by visualization.

1.7.1. Crystalline characterization

Crystalline characterization was performed using the same method as 1.4.

2. Preliminary physicochemical properties of carriers (solid lipids and liquid lipids)

2.1. Physicochemical properties of solid lipids

The property data of some interesting solid lipids were collected and investigated. Solid lipids, which have suitable properties, were used for further experiments. The properties of solid lipid include its benefits to skin and its multi-functional properties which make it suitable for cosmetic applications, the proper melting property, the re-crystallization of lipid and the lycopene solubility. Melting point and the re-crystallization properties of solid lipids were investigated as following;

2.1.1. Morphology

The appearance of lipid was performed using the same method as 1.5.

2.1.2. Thermal analysis

Thermal analysis by DSC was performed using the same method as 1.3 except for the temperature run program (20-80°C).

2.1.3. Crystalline characterization

Crystalline characterization was performed using the same method as 1.4.

2.2. Physicochemical properties of liquid lipids

The property data of some interesting liquid lipids were collected and investigated. Liquid lipids, which have suitable properties, were used for further experiments. The properties of liquid lipid include its benefits to skin and its multi-functional properties which make it suitable for cosmetic applications, the proper melting property and the lycopene solubility. The selected liquid lipids were mixed with only promising selected solid lipid; orange wax, to predict the melting properties of lipid matrices. Thermal behavior of lipid matrices was investigated by thermal behavior analysis. The only promising selected liquid lipids were investigated for antioxidant activities as following;

2.2.1. Thermal analysis

Thermal analysis was performed using the same method as 1.3 except for the temperature run program (0-60°C).

2.2.2. Biological action (antioxidant activities)

Antioxidant activities were performed using the same method as 1.6.

Part II Optimization of formulations

1. Effect of surfactant

1.1. Preparation of lipid nanoparticles

The promising selected lipid matrix; the blend of orange wax and rice bran oil was used. The lower HLB surfactants give a w/o emulsion while the higher HLB molecules provide an o/w emulsion. Because NLC is derived from an o/w emulsion

system, the selected surfactants used in this study were of a higher HLB value group which is preferably dissolved in an aqueous external phase of the emulsion. Moreover, as the aim to develop the lycopene-loaded NLC for dermal application, the non-ionic surfactants particularly with a basic chemical structure of sugar ester were used in this study because they had been reviewed earlier and were claimed to be skin friendly. Besides their good compatibility properties with the skin, these surfactants also showed a drug enhancement effect in percutaneous absorption.

Five different surfactants with its own specific property were selected; C-1816, C-1216, C-1616, C-1815, and Plantacare™ 1200.

Table 3-1 The properties of selected surfactants.

Trade name	Structure	C atom	HLB value
Plantacare™ 1200	lauryl glycoside	12	16
C-1216	sucrose laurate	12	16
C-1616	sucrose palmitate	16	16
C-1816	sucrose stearate	18	16
C-1815	sucrose stearate	18	15

In this study, formulations containing 5% of selected lipid matrices have been prepared by the hot temperature HPH technique described in detailed by Müller and Lucks (1996) using a Micron LAB 40 homogenizer (APV Systems, Unna, Germany) or another homogenizer (Avestin, model C3, Germany). The lipid phase was melted to above lipid melting point 10-15°C. The aqueous phase was separately heated to the same temperature of lipid phase. The melted lipid phase was dispersed in a hot surfactant solution by using an Ultra-Turrax T25 mixer (Janke and Kunkel GmbH,

Staufen, Germany) for high speed stirring at 12,000 rpm for 30 seconds to obtain a pre-emulsion. This hot pre-emulsion was further processed by HPH applying for suitable amount of cycle, pressure, and temperature. After the HPH was applied, the lipid dispersion was suddenly quenched and abruptly cooled down to room temperature and solidified to obtain the aqueous SLN/NLC dispersions. The lipid particles were obtained in nanometer size range.

1.2. Effect of surfactant on contact angle

Several surfactants which showed different contact angle to the major lipid of NLC were firstly investigated. The hypothesis is that the contact angle might be a useful tool for selection of the suitable surfactant for small size NLC within a short time. The effect of surfactant on contact angle was performed as following;

1.2.1. Goniometric measurement

In this study 0.1% (w/v) solutions of surfactants in purified water were freshly prepared. The solid surface was prepared on a microscopic glass slide using an appropriate amount of lipid. Goniometric measurement of the contact angle, is assessed directly by measuring the angle formed between the solid and the tangent to the drop, was performed 15 sec after application of a drop on the solid using a contact angle meter G1 (Kruss, Hamburg, Germany) at room temperature. Determination was repeated 3 times.

1.3. Effect of surfactant on the particle size

The effects of surfactant include surfactant's molecular size and HLB values of surfactant were investigated. To study the effect of surfactant's molecular size on the particle size, they were compared in 2 states. To study the effect of hydrophilic head group, the particle size from formulation using Plantacare^{TM} 1200 was compared

with C-1216. To study the effect of lipophilic tail, the particle size from formulations using C-1216, C-1616, C-1816 were compared. To study the effect of HLB, the particle size from formulation using C-1815 was compared with C-1816. Particle size analysis was performed as following;

1.3.1. Particle size analysis

Analysis of the particle size was performed by photon correlation spectroscopy (PCS) with a Malvern Zetasizer IV (Malvern Instruments, UK). PCS yields the mean particle size (z-ave) and the PdI which is a measure of the width of the size distribution (Junyaprasert and others, 2009). The z-ave and PdI values were obtained by averaging of ten measurements at an angle of 273° in 10 mm diameter cells at 25°C. Prior to the measurement, all samples were diluted with bidistilled water to an appropriate count rate in order to eliminate multiple scattering and to have a suitable scattering intensity. The average diameter was calculated according to Stokes–Einstein equation after a curve fitting of the correlation function was done.

1.4. Effect of surfactant on the physical stability of the NLC

The physical NLC stability was performed as following;

1.4.1. Particle size analysis

Particle size analysis was performed using the same method as 1.3.1 (part 2).

1.4.2. Zeta potentiometry

The ZP was measured by determining the electrophoretic mobility using the Malvern Zetasizer IV (Malvern Instruments, UK). The measurements were performed in bidistilled water adjusted to a conductivity 50 μS/cm with sodium chloride solution (0.9% w/v). The pH was in the range 5.5–6.0 and the field strength was 20 V/cm.

1.5. Chemical stability of the lycopene

The chemical stability profile of lycopene was performed as following;

1.5.1. UV/Vis spectrophotometry

The specific absorption peak at 475 nm was recorded using an UV 2450 double-beam spectrophotometer (Shimadzu, U.S.A). The recorded data represent the mean values ± SD of triplicate measurements (n=3). Sample containing lycopene was dissolved in THF prior to the experiment.

2. Effect of different solid lipids

Three promising selected solid lipids; orange wax, mango butter and beeswax were prepared. From the results of the effect of surfactants, Plantacare^{TM} 1200 was selected as a stabilizer.

2.1. Preparation of lipid nanoparticles

Preparation of lipid nanoparticles was performed using the same method as 1.1. (part 2). The particle size analysis was performed by PCS and LD. The ZP was measured by determining the electrophoretic mobility of nanoparticle.

2.2. Particle size analysis

2.2.1. Photon correlation spectrophotometry

Particle size analysis was performed using the same method as 1.3.1 (part 2).

2.2.2. Low angle static light scattering

The laser diffractometer LS 230 from Beckman-Coulter (Krefeld, Germany) was used. The experiments were performed by pure LD, the PIDS technology was excluded in all measurements. The analysis used Mie approximation. Concerning the optical parameters for lipid nanoparticles, the real part was considered to be 1.456 and the imaginary part was 0.01 (Souto and others, 2005a). LD data were evaluated using

the volume distribution diameters of LD (0.50), LD (0.90) and LD (0.99). The LD (0.50) is often referred to be a measure for the mean particle size, whereas the LD (0.90) and LD (0.99) are utilized for the presence of larger particles. Each sample was measured five times consecutively.

2.3. Zeta potentiometry

The ZP was measured by determining the electrophoretic mobility using the Malvern Zetasizer IV (Malvern Instruments, UK). The measurements were performed in bidistilled water adjusted to a conductivity 50 μS/cm with sodium chloride solution (0.9% w/v). The pH was in the range 5.5–6.0 and the field strength was 20 V/cm.

3. Effect of the amount of cycle and pressure

Orange wax was selected for further study. Plantacare™ 1200 was selected as a stabilizer. Maintaining all other variables constant, two formulations have been homogenized at two different homogenization pressures (800 bar and 500 bar) and the number of homogenization cycles was 1, 2, 3, 4 or 5 cycles.

3.1. Particle size analysis

Particle size analysis by PCS and LD were performed using the same method as 2.2 (part 2).

4. Effect of different liquid lipids

To meet the criterions mentioned above, rice bran oil, sesame oil, and pomegranate seeds oil were selected in this study to blend with orange wax. Plantacare™ 1200 surfactant was fail in providing long-term stability of the formulation; therefore, it was excluded from this study. Three pairs of surfactant combination were selected to stabilize both lipid and aqueous phase of NLC. The 1st surfactant combination is between Tegocare™ 450 and Tween™ 80 at the same

weight ratio (1:1). The 2nd surfactant combination is between Tegocare™ 450 and Plantacare™ 1200 at the same weight ratio (1:1). The 3rd surfactant combination is between Tegocare™ 450 and Inutec™ SP 1 at the same weight ratio (1:1).

4.1. Particle size analysis

Particle size analysis by PCS and LD were performed using the same method as 2.2 (part 2).

5. Comparison of NLC system *versus* NE system

5.1. The effect of lipid nanoparticle formulations on the particle size

Lycopene loaded NLC and o/w NE and lycopene-free NLC and o/w NE were produced by hot HPH (3 cycles, 500 bar, at 75°C). The compositions of these 4 formulations are shown in Table 3-2. In the o/w NE the orange wax was replaced by rice bran oil. The particle size analysis was performed as following:

Table 3-2 The percentage of compositions used in the NLC and NE formulations.

Components	NLC		NE	
	Free	Load	Free	Load
Orange wax	4.5	4.5	0.0	0.0
Rice bran oil	0.5	0.5	5	5
Lycopene	0.0	0.005	0.0	0.005
Tegocare™ 450	0.5	0.5	0.5	0.5
Tween™ 80	0.5	0.5	0.5	0.5
MilliQ	qs 100	qs 100	qs 100	qs 100

5.1.1. Particle size analysis

Particle size analysis by PCS was performed using the same method as 1.3.1 (part 2).

5.2. Entrapment efficiency

Formulations were centrifugation at 50,000 rpm for 30 min by an ultra-centrifugation (Optima L-90K model, Beckman Coulter, Germany) to separate the particle and the aqueous phase. The drug quantity in the aqueous phase was determined by PLM. The percentage of incorporated lycopene (entrapment efficiency) was determined by spectrophotometric determination at 475 nm using an UV 2450 (Shimadzu, U.S.A). UV visible spectrophotometry was performed using the same method as 1.5.1 (part 2). The amount of free drug was detected in the supernatant and the amount of incorporated drug was determined as a result of the initial drug load minus the free drug.

5.3. Biological action (antioxidant activities)

Antioxidant activity was performed using the same method as 1.6. (part1)

5.4. Stability test

The stability on the particle size as well as the PdI was monitored over the period of 30 days as following;

5.4.1. Particle size analysis

Particle size analysis was performed using the same method as 1.2.1 (part 2)

5.4.2. Zeta potentiometry

ZP measured on day 0 was performed using the same method as 1.4.2 (part 2)

6. Effect of surfactant on NLC stability

To provide long term NLC stability, the steric effect and electrostatic effect of surfactant were investigated. The particle size analysis was performed as following;

6.1. Steric effect

In this study, Poloxamers™ 188 and Poloxamer™ 407 were used due to theirs steric stabilization (Heurtault and others, 2003).

6.1.1. Particle size analysis

Particle size analysis by PCS and LD were performed using the same method as 2.2 (part 2).

6.2. Electrostatic effect

Dermofeel™ SL and Eumulgin™ SG were selected as represent small anionic surfactants which are available.

6.2.1. Particle size analysis

Particle size analysis by PCS and LD were performed using the same method as 2.2 (part 2).

<u>Part III</u> Preparation and physicochemical study of lycopene-loaded NLC

1. Development of skin friendly lycopene-loaded NLC

1.1. Morphology and re-crystallize of lipid matrix

Morphology of lipid matrix was performed using the same method as 1.5. (part 1). Lipid matrix was melted and dropped on the glass slide. Then, cover the lipid matrix immediately with the cover slide.

1.2. Effect of lipid combination

1.2.1. Thermal analysis

Thermal analysis was performed using the same method as 1.3.

1.3. Preparation and characterization of the investigated formulations

1.3.1. Preparation of lipid nanoparticle

1.3.1.1. Preparation of lipid nanoparticle

Preparation of lipid nanoparticle was performed using the same method as 1.1. (part 2).

1.3.2. Particle size analysis

Particle size analysis by PCS was performed using the same method as 1.3.1 (part 2).

1.3.3. Electric conductivity measurements

The electrical conductivity of the investigated formula was measured by using Cyberscan CON 11: hand-held conductivity meter (Eutech Instruments, Singapore) connected with conductivity/TDS electrode cell. The experiment was performed at 25±1.0°C by dipping the electrode into the test sample until equilibrium was reached and reading became stable. The measurements were performed in triplicate.

1.4. Effect of lipid on the state of the internal phase

1.4.1. Thermal analysis

Thermal analysis was elucidated by using the same method as 1.3 except for the temperature run program was 20-80°C. (part 1)

1.5. Effect of rice bran oil and cholesterol on the state of the internal phase

1.5.1. Thermal analysis

Thermal analysis was performed using the same method as 1.3 except for the

temperature run program was 20-80°C. (part 1)

1.6. Effect of rice bran oil and cholesterol on particle size of the internal phase

1.6.1. Particle size analysis

Particle size analysis was performed using the same method as 1.2. (part 2)

1.7. Effect of rice bran oil and cholesterol on zeta potential of the internal phase

1.7.1. Zeta potentiometry

ZP was performed using the same method as 1.4.2 (part 2).

1.8. Stability

1.8.1. Particle size analysis

Particle size analysis by PCS and LD were performed using the same method as 2.2 (part 2).

1.8.2. Zeta potentiometry

ZP was performed using the same method as 1.4.2 (part 2)

1.8.3. Stability profile of lycopene

Stability profile of lycopene was performed by UV visible spectrophotometry using the same method as 1.5.1 (part 2)

1.9. Occlusion property

The *in vitro* occlusion test was adapted by de Vringer (1992; 1999). Beakers (100 mL) with a diameter of 3.9 cm were filled with 50 mL of water, covered with Whatman™ no. 1 filter paper (cellulose filter, 70 mm, Buckinghamshire, UK) and sealed. 400 µL of aqueous NLC dispersions or 400 mg of topical dosage forms were spread evenly with a spatula on the filter surface (11.94 cm^2), leading to an applied

amount of 33.5 μL/cm^2. The samples were stored at 32 ± 1°C (skin temperature) and 60% ± 5% relative humidity for 48 h. The samples were weighed after 6, 24 and 48 h, giving the water loss due to evaporation at each time (water flux through the filter paper). Beakers covered with filter paper but applied Milli Q water served as reference values. Every experiment was performed in triplicate (n = 3).

2. The investigation on crystallization behavior of lycopene-loaded NLC

2.1. Thermal analysis

Thermal analysis was performed using the same method as 1.3 except for the temperature run program was 20-80°C. (part 1)

2.2. Crystalline characterization

2.2.1. WAXS

Crystalline characterization was performed using the same method as 1.4. (part 1)

2.2.2. Electron diffraction mode of TEM analysis

TEM was performed using JEM-1200 (JEOL Co. Ltd., Tokyo, Japan). The sample was diluted 100-fold with double-distilled water. NE, SLN, or NLC dispersion was placed on a carbon-coated copper grid to form a thin-film specimen and then a drop of 1% w/w uranyl acetate in EtOH covered on the dispersion. The grid was dried at room temperature and then observed by diffraction mode to obtain the ED pattern.

2.3. The bright field cryo-TEM analysis

The sample for cryo-TEM was prepared at an ambient temperature of 30°C and saturated humidity in a custom-made CEVS apparatus (controlled environment vitrification system) according to (Bellare and others, 1988). Under these temperature and humidity controlled conditions a droplet (6μL) of the solution was placed on

hydrophilized (60 s plasma treatment at 8W using a BALTECMED 020 device), perforated carbon filmed Quantifoil grids (R1/4 batch of Quantifoil Micro Tools GmbH, Jena, Germany). The excess fluid was blotted off to create an ultra-thin layer (typical thickness of 100–200 nm) of the solution spanning the holes of the carbon film. The grid was immediately vitrified by propelling the grid through a shutter of the CEVS into a vessel placed right below the shutter containing liquid ethane at its freezing point (−184°C). Ultra-fast cooling is necessary for an artifact-free thermal fixation (vitrification) of the aqueous solution avoiding crystallization of the solvent or rearrangement of the assemblies. The vitrified samples were transferred under liquid nitrogen into a Philips Tecnai F20 transmission electron microscope (FEI company, Oregon, USA) using the Gatan (Gatan Inc., California, USA) cryoholder and stage (Model 626). Microscopy was carried out at −175°C sample temperature using the microscope's low-dose mode at a calibrated primary magnification of 62,000X. Images were recorded using a 2 k-Eagle CCD camera at full resolution (2048×2048 pixel). The accelerating voltage was 160 kV and the defocus was chosen to be 2 μm.

<u>Part IV</u> Effect of lycopene concentration on lycopene-loaded NLC

1. Effect on the particle size and ZP

1.1. Preparation of lipid nanoparticle

Preparation of lipid nanoparticle was performed using the same method as 1.1. (part 2).

1.1.1. Particle size analysis

Particle size analysis by PCS was performed using the same method as 1.3.1 (part 2).

1.1.2. Zeta potentiometry

ZP was performed using the same method as 1.4.2 (part 2)

2. Entrapment efficiency

Entrapment efficiency was performed using the same method as 5.2 (part 2).

3. The *in vitro* release study

3.1. Membrane free release

A release test model was developed to allow the relative (not absolute) comparison of lycopene releases from the different concentrations e.g. 0.005%, 0.025%, 0.050% w/w. The test model consists of an aqueous phase NLC dispersion and an acceptor medium. This release test model allows to determine the release profile of lycopene from their carrier systems independently of the membranes. The diffusion of the lycopene from the NLC formulations to the acceptor medium (mineral oil) was observed. Six millilitres of mineral oil were placed in vials without the membrane and maintained at 37°C in order to ensure the surface skin temperature (32°C). One millilitre of aqueous lycopene loaded NLCs was filled beneath the acceptor medium. Saturation of the lycopene in the acceptor medium was not allowed to be reached. The concentrations of the lycopene have been assessed at defined time intervals by withdrawing 2 mL from the acceptor medium and analyzing it using a double-beam spectrophotometer, UV-2450 (Shimadzu, USA), at 475 nm. Two millilitres of mineral oil was added to the acceptor medium to substitute the withdrawn volume.

3.2. Release from membrane

To elucidate the mechanism of active release through membrane, *in vitro* release studies using dialysis membrane has been performed. Cellulose acetate membranes (Sartorius, Germany) with MW cutoff 12,000 Da were selected. The acceptor chamber was maintained at 37°C, in order to ensure the surface skin temperature at the membrane. Nine millilitres of propylene glycol was used as an acceptor medium due to lycopene soluble in it. One millilitre of aqueous lycopene loaded NLCs was filled inside the dialysis membrane. Three millilitres of samples were collected over 24 h and analyzed by spectrophotometric determination at 475 nm. After each sample taking, acceptor chamber was filled up with acceptor medium, in order to ensure the sink conditions during the experiment. For each formulation, the release studies were performed in triplicate. UV/Vis spectrophotometric quantifications of lycopene were carried out using an UV 2450 (Shimadzu, USA)

4. Occlusive property

Occlusion test was performed using the same method as 1.9. (part3)

5. Biological action (antioxidant activities)

Antioxidant activity was performed using the same method as 1.6. (part1)

6. Stability test

6.1. Particle size analysis

Particle size analysis was performed using the same method as 1.2.1 (part 2)

6.2. Zeta potentiometry

ZP was performed using the same method as 1.3. (part 2)

6.3. Stability profile of lycopene

Stability profile of lycopene was performed by UV/Vis spectrophotometry

using the same method as 1.5.1 (part 2)

Part V Preparation and evaluation of topical dosage forms

1. Preparation of topical dosage form

The lipid and aqueous phases of the cosmetic o/w cream were heated separately to 80°C. The hot aqueous phase was added to the lipid phase under constant stirring. The suitable NLC dispersion (10%) was added to the emulsion which cooled down to 40°C. Stirring was continued until the o/w cream reached ambient temperature.

2. Occlusion property

Occlusion test was performed using the same method as 1.9. (part3)

3. Stability test

3.1. Stability profile of lycopene

Stability profile of lycopene was performed by UV visible spectrophotometry using the same method as 1.5.1 (part 2)

4. Antioxidant activities

Antioxidant activity was performed using the same method as 1.6. (part1)

5. Statistical analysis

The significance of difference was evaluated using one-way ANOVA at the probability level of 0.05.