CHAPTER III

MATERIALS AND METHODS

Materials

Alamar Blue, Lot No. 1457565A, BioSource, USA

Cetylpalmitate, Lot No. 06816KC, Sigma-Aldrich Inc., Germany

Chitosan, Flonac-C®, MW=50-100 kDa with 90.7% deacetylation, Japan

Cholesterol, Lot No. 1026620, Fluka Inc., Germany

Citric acid (monohydrate), Lot No. 788, Srichand United Dispensary Co., Ltd., Thailand

Dimethyldioctadecyl ammonium bromide, Lot No. 1072028, Fluka Inc., Germany

Dioleoyl phosphatidyl ethanolamine, Lot No., 114K5208, Sigma-Aldrich Inc., Germany

Disodium hydrogen phosphate, Lot No. F3E048, Ajax Finechem, Australia

Dulbecco's modified Eagle's medium, Lot No. 1309768, Invitrogen, USA

Ethylenediaminetetraacetic acid disodium, Lot No. 7727, Labguard, USA

Fetal bovine serum, Lot No. 41F7063K, Invitrogen, USA

Flask for cell culture, Lot No. 34904007, Costar, USA

Gelatin powder, Lot No. 424331/1, Fluka, Germany

Glacial acetic acid, Lot No. K32754317349, Analar, UK

Goat anti-mouse IgG conjugated to horseradish peroxidase, Lot No. K6902-11706, Southernbiotech, USA

HeLa cell, human epithelial cervix carcinoma cell lines, used in this study were provided from the Division of Allergy and Clinical Immunology, Faculty of Medicine, Chulalongkorn University

HIV-1 gag p24 recombinant viral protein, Lot No. 6B0027, ABI Inc., USA

Hydrochloric acid, Lot No. 315, Merck, Germany

Hydrogen peroxide, Lot No. 749938DP, Panreac, Spain

Loading Buffer, Lot No. G190A, Promega, USA

Methyl paraben, Lot No. MFB 47/947, Srichand United Dispensary Co., Ltd, Thailand

Mouse monoclonal antibody to capsid protein p24 (gag) of HIV-1, Lot No. 5L0006, ABI Inc., USA

o-Phenylenediamine, Lot No. 60706127, Zymed Laboratories, USA

pHIS-HIV-hugag, pDNA expressing a human multiple epitope gag protein used in this study were kindly provided from the Division of Allergy and Clinical Immunology, Faculty of Medicine, Chulalongkorn University

Phosphate Buffer Saline, Lot No. 1310578, Invitrogen, USA

Potassium chloride, Lot No. F1G253, APS, Australia

Potassium dihydrogen phosphate, Lot No. F2H145, Ajax Finechem, Australia

Propyl paraben, Lot No. LI 2011, Srichand United Dispensary Co., Ltd, Thailand

Propylene glycol, Lot No. PL90/925, Srichand United Dispensary Co., Ltd, Thailand

Sodium carbonate, Lot No. AF405220, Ajax Finechem, Australia

Sodium chloride, Lot No. K26811304952, Merck, Germany

Sodium hydrogen carbonate, Lot No. AF310196, Ajax Finechem, Australia

Sodium hydroxide, Lot No. D1311198 214, Merck, Germany

Span 85, Lot No. 52246, East Asiatic (Thailand) Public Co., Ltd., Thailand

Sulfuric acid, Lot No. 651, Merck, Germany

Thimerosol, Lot No. 1154686, Fluka Inc., Germany

TM-Rhodamine (Label IT® reagent), Lot No. 6449 Mirus Bio Corporation, USA

Tripolyphosphate, Lot No. 07027HI, Aldrich, Germany

Tris base, Lot No. 11200, Sigma-Aldrich, Germany

Trysin-EDTA, Lot No. 1344950, Invitrogen, USA

Tween 20, Lot No. S4232415510, VWR Internatioanl Ltd., UK

Tween 80, Lot No. 405854, Srichand United Dispensary Co., Ltd., Thailand

6-Well plate, Lot No. 31003018, Costar, USA

96-Well plate, Lot No. 07205008, Costar, USA

Animals

Dermatomed porcine skin

Balb/c Mice, National Laboratory Animal Center Service, Mahidol University, Thailand

Equipment

Analytical balance, Model PC 440, Mettler, Switzerland

Analytical balance, Model 1615 MP, Sartorius, USA

Blot module, Biorad, USA

Cell incubator, USA

Confolcal microscope, Carl Zeiss, Germany

Geldoc documentation system, Biorad, USA

High speed stirrer, Ultra Turrax, Germany

High pressure homogenizer, Emulsiflex-B3, Avestin Inc., Canada

Microplate reader, Perkin-Elmer BioAssay reader, USA

pH meter, Model SA 520, Orion Research Inc., USA

Statistical software package, Design-Expert V. 6, StatEase Inc., USA

Stirrer bed, UK

Vertical Franz diffusion Cells, UK

Zetasizer, Malvern Instrument NanoZS, UK

Methods

1. Preparation and Characterizations of Solid Lipid Nanoparticles (SLN)

1.1 SLN Production

The SLN were produced using the hot high pressure homogenization technique as described by Mehnert and Mäder (2001). Briefly, cetylpalmitate was melted at about 10 °C above its melting point. Then, cholesterol (for some formulations) and DDAB were added and stirred continuously until they melted completely. The mixture of molten lipid was dispersed into a hot aqueous solution containing mixture of surfactants, propylene glycol and paraben concentrate and was then stirred with a high speed stirrer for 1 minute to form a pre-emulsion system. The dispersion was homogenized by using Emulsiflex-B3 at a pressure of 15,000 psi for 5 cycles and allowed to cool down at room temperature. The formulations of SLN studied in this experiment are shown in Table 3.1.

Table 3.1 Formulation composition of SLN

Ingredients	%Content (w/w)
Cetylpalmitate (as lipid matrix)	3
Surfactant*	varied
DDAB (as cationic lipid)	varied
Cholesterol	varied
Propylene glycol	5
Paraben concentrate	1
Purified water to make	100

*a mixture of Tween 80 and Span 85 at a ratio of 7:3 (w/w)

1.2 Experimental Design

To depict the influence of SLN formulation on their size, polydispersity index and zeta potential, a face-centered central composite design was selected. The advantage of this method is that the addition of center points and star points to the initial factorial design allow the fitting of experimental data to a quadratic model (Vandervoort and Ludwig, 2002) and response surfaces plotting. Once the model is calculated, it can be used to predict a certain response for a known content of formulation composition. From preliminary experiments, the three factors listed; surfactant, DDAB and cholesterol were critical variables for corresponding responses, i.e. particle size, polydispersity index and zeta potential of the fixed lipid content system. The variables and their levels for the face-centered central composite experimental design are shown in Table 3.2.

Table 3.2 Concentrations and coded level of variables for experimental design

Variable	Concentration of variable (%w/w)				
	Low level (-)	Center point (0)	High level (+)	Low level of axial point (-α)	High level of axial point (+α)
a: surfactant	2.00	5.00	8.00	2.00	8.00
b: DDAB	0.08	0.64	1.20	0.08	1.20
c: cholesterol	0.00	0.34	0.68	0.00	0.68

The design containing 18 runs; i.e., 8 (2^3) factorial points, 6 star points and 4 center points as shown in Figure 3.1 was generated and analyzed by the statistical software package Design-Expert V. 6. The generated design and responses of the design are shown in Table 3.3.

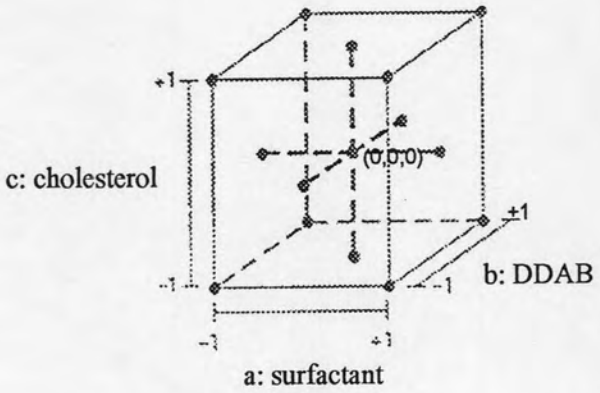

Figure 3.1 Scheme of experimental design: face-centered central composite design

Statistically significant F ratio ($p<0.05$) and adjusted determination coefficients (Adj-R^2) between 0.8-1 associated to non statistically significant lack of fit ($p>0.05$) were the criteria for validation of the chosen model, according to those previously suggested by Chacón et al. (1996). For the regression coefficients, a significance test was performed to obtain the regression equations including only the terms with statistical significance. They were also represented as three-dimension response surface plots for two variables at a time.

Table 3.3 Generated experimental design and responses of the design

Formulation	Code of (a, b, c)*	%Content (w/w)			Responses
		surfactant	DDAB	cholesterol	
SLN1	(-,-,-)	2.00	0.08	0.00	
SLN2	(+,-,-)	8.00	0.08	0.00	
SLN3	(-,+,-)	2.00	1.20	0.00	
SLN4	(+,+,-)	8.00	1.20	0.00	
SLN5	(-,-,+)	2.00	0.08	0.68	
SLN6	(+,-,+)	8.00	0.08	0.68	
SLN7	(-,+,+)	2.00	1.20	0.68	
SLN8	(+,+,+)	8.00	1.20	0.68	- partilcle size (nm)
SLN9	(-α,0,0)	2.00	0.64	0.34	- polydipersity index
SLN10	(+α ,0,0)	8.00	0.64	0.34	- zeta potential (mV)
SLN11	(0,-α ,0)	5.00	0.08	0.34	
SLN12	(0,+α ,0)	5.00	1.20	0.34	
SLN13	(0,0,-α)	5.00	0.64	0.00	
SLN14	(0,0,+α)	5.00	0.64	0.68	
SLN15	(0,0,0)	5.00	0.64	0.34	
SLN16	(0,0,0)	5.00	0.64	0.34	
SLN17	(0,0,0)	5.00	0.64	0.34	
SLN18	(0,0,0)	5.00	0.64	0.34	

*a, b, c are surfactant, DDAB and cholesterol, respectively

1.3 Differential Scanning Calorimetry Analysis of SLN

Differential scanning calorimetry (DSC) of SLN was performed by using a Mettler DSC. The SLN9, SLN10, SLN11, SLN12, SLN13, SLN14 and SLN15 were selected as representatives of all formulations because they contained extreme content of interesting factor with medium level of another factors; SLN9 and SLN10 contained low and high content of surfactant, respectively; SLN11 and SLN12 contained low and high content of DDAB, respectively, and SLN13 and SLN14 contained low and high content of cholesterol, respectively. For SLN15, it contained medium level of all factors.

Samples were accurately weighed in a range of 5-10 mg in 40 µl aluminium pan and scanned over a temperature range of 20°-180 °C at a rate of 10 K per minute under nitrogen atmosphere. However, from the preliminary experiment results, it was found that the DSC curves were interfered with a peak of water at investigated temperature range. To minimize an effect of water in the formulation, amount of water in SLN was reduced by ultracentrifugation and the sediment were collected for further study.

1.4 SLN X-Ray Diffraction Study

The X-ray Diffraction (XRD) study was performed to confirm the results obtained by DSC technique. The XRD patterns of bulk cetylpalmitate and SLN were obtained by an X-ray diffractometer (Bruker AXS Model D8 Discover). The sample were exposed with CuK_{α} radiation (40 kV, 30 mA) and scanned from 5° to 45° with step time of 1 second. The samples selected for XRD analysis were SLN10, SLN12 and SLN14, due to their high content of surfactant, DDAB and cholesterol, respectively.

2. Preparation and Characterizations of Chitosan Nanoparticles (CSN)

2.1 CSN Production

CSN were prepared by ionotropic gelation technique described by Gan et al. (2005). Briefly, 1.6 g of chitosan powder was dispersed in acetic acid solution and stirred continuously until it was transparent. Once dissolved, the 1.6 % w/v chitosan solution was divided into 12.5, 25 and 37.5 ml for the formulation CSN1, CSN2 and CSN3, respectively and diluted with purified water to make 97.4 ml of chitosan solution. The 5% w/v tripolyphosphate solution was added slowly into diluted chitosan solution at the ratio of chitosan:tripolyphosphate shown in Table 3.4. The mixture was sonicated at 10 watts for 10 minutes and stirred continuously by a magnetic stirrer at 600 rpm for 30 minutes at room temperature.

Table 3.4 Ratio of chitosan:tripolyphosphate for CSN production

Formulation	Ratio of chitosan:tripolyphosphate (w/w)
CSN1	2:1.3
CSN2	4:1.3
CSN3	8:1.3

3. Physical Properties Measurement of SLN and CSN

3.1 Size, PI and Zeta potential Measurement

Particle size and PI of SLN, CSN, and also nanoparitlces-pHIS-HIV-hugag complexes were analyzed by photon correlation spectroscopy (PCS) technique, briefly, 0.1-ml of sample was diluted with purified water to make 1 ml, then they were filled into a 1-ml cuvette for measuring particle size and PI by using Zetasizer (Malvern Instrument NanoZS). Zeta potential was measured in purified water at pH 7.0 by using the same equipment. They were measured in 3 replicates and reported in a form of mean±standard deviation (SD).

3.2 Morphology Observation

The morphological characteristics of SLN were observed by

1. Cryo-scanning electron microscopy (Cryo-SEM) technique, briefly; a capillary containing the sample was frozen in liquid nitrogen and broken off to open the sample surface. Then, the surface of frozen sample was imaged using a JSM-5410LV (JEOL-Japan) scanning electron microscope.

2. Transmission electron microscopy (TEM) technique, briefly; one drop of diluted sample was placed on a copper grid coated with carbon film, then stained with 0.5% w/v uranyl acetate solution and allowed to dry under room temperature. The grids were imaged using a JEM-1220 (Japan) transmission electron microscope.

The morphological characteristics of CSN were visualized by

1. Cryo- SEM technique with gold coating, briefly, a capillary containing the sample was frozen in liquid nitrogen and broken off to open the sample surface. Then, the surface of frozen

sample was coated with gold vapor for improving picture resolution. The gold coated samples were imaged using a JSM-5410LV (JEOL-Japan) scanning electron microscope.

2. Transmission electron microscopy (TEM) technique performed by using the same procedure described in morphology observation of SLN.

The pHIS-HIV-hugag, HIV pDNA vaccine were complexed with either SLN or CSN and then visualized by using negative stain transmission electron microscopy (TEM) technique.

3.3 Physical Stability Study

Particle size, PI and zeta potential of SLN and CSN stored in air-tight colorless glass bottles at room temperature and protected from light by wrapping with aluminium foil were measured at day 1, day 7, day 14, day 30, and day 60 after they had been prepared. The measurements were performed in 3 replications and reported as mean±SD.

4. Ability of SLN and CSN to Form Complex with pHIS-HIV-hugag

The ability of SLN and CSN to form complex with polyanionic pDNA were studied by analysis of the electrophoretic mobility of pDNA within an agarose gel so-called electrophoretic mobility shift assay. In this study, the influence of formulation composition on the ability of SLN to form complex with pHIS-HIV-hugag was evaluated by comparing SLN formulations as listed; 1) effect of surfactant concentrations i.e. SLN9, SLN10 and SLN15; 2) effect of DDAB concentrations i.e. SLN11, SLN12 and SLN15; 3) effect of cholesterol concentrations i.e. SLN13, SLN14 and SLN15. Moreover, all of CSN formulations were also evaluated.

SLN and CSN were diluted with purified water and mixed with pDNA suspension at the ratio of nanoparticles:pDNA (weight by weight) 10,000:1, 5,000:1, 1,000:1, 500:1, 100:1 in microfuge tubes. The mixtures were allowed to form complex for 30 minutes at room temperature and were then loaded into 0.8% w/v agarose gels (Sigma-Aldrich Inc., Germany) immersed in tris-acetate EDTA (TAE) running buffer. The electrophoresis were run for 90 minutes at electricity of 5 volts/cm. The gels were taken to stain with 0.7%w/v ethidium bromide solution (Invitrogen, USA) and destained with purified water after electrophoresis was finished. The fraction of mobile pDNA was observed under UV light. Images were obtained using a Geldoc documentation system.

5. Cell Culture

HeLa cells, human epithelial cervix carcinoma cell lines, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were incubated at 37 °C with 5% CO_2 atmosphere and subcultured every 3 to 4 days using trypsin-EDTA solution.

6. Cytotoxicity Assay

The cytotoxicity of SLN and CSN were determined using the alamar blue bioassay. The oxidized indigo blue form of this chromogenic indicator dye is reduced by cellular dehydrogenases, specifically targeting the mitochondrial electron transport chain, to a reduced pink form (Davoren et al., 2005). The bioassay was performed according to the manufacturer's instruction. Briefly, HeLa cells were seeded on 96-well plates with a density of 10,000-20,000 cells per well and allowed to adhere overnight. SLN samples, and CSN possessing proper particle size and zeta potential that were able to immobilize pDNA at the lower ratio of nanoparticles:pDNA were diluted in DMEM without FBS at the final concentrations of 10,000, 5,000, 1,000, 500, 400, 300, 200, 100 µg/ml.

The HeLa cell monolayers, 60%-70% confluent, were washed once with phosphate buffer saline (PBS) and incubated with 200 µl of tested suspensions for 24 hours at 37 °C. The cells were washed again with PBS and incubated with 200 µl of alamar blue solution (5% v/v in DMEM without FBS) for 24 hours at 37 °C. Fluorescence was read after 24 hours using a Perkin-Elmer BioAssay reader set at wavelengths of 544 nm for excitation and 620 nm for emission. Wells containing medium and alamar blue without HeLa cells were used as negative control. The 100% cell viability was calculated from the results of wells containing HeLa cells without any addition of nanoparticles. The assay was performed in 6-replicate and reported as mean % cell viability±SD.

7. *In vitro* Transfection Study

HeLa cells were seeded on 6-well plate at a density of $2x10^5$-$3x10^5$ cells per well and allowed to adhere overnight. The medium was removed from the 70% confluent monolayer, and then cells were rinsed with PBS. The 30-µl of nanoparticles-pDNA complex was mixed with 1970 µl of DMEM (no FBS supplement) and added to cells. The cells were incubated with the

complex for 24 hours at 37 °C under 5% CO_2. They were then washed once with PBS and allowed to grow for further 24 hours in DMEM containing 10% FBS at 37 °C under 5% CO_2 atmosphere.

Fugene 6, a commercial non-viral transfection vector, was chosen as positive control to compare with SLN and CSN. The pHIS-HIV-hugag transfection in HeLA cells using Fugene 6 was performed at a ratio of Fugene 6: pDNA (volume by weight) 3:1 according to manufacturer's instruction (Roche Applied Science, USA).

Cells were washed twice with cold PBS and harvested using a cell scraper. They were collected by centrifugation at 10,000 rpm, 4 °C for 5 minutes. Lysis buffer was added to cells and allowed to be lysed for 45 minutes on ice. Cell debris was spun down at 13,000 rpm, 4 °C for 5 minutes to collect supernatant into a new tube. NuPage LDS sample buffer (Invitrogen, USA) and NuPage reducing agent (Invitrogen, USA) were added to the supernatant at a volume of 5 µl and 2 µl, respectively. The mixture was heated at 70 °C for 10 minutes and then loaded into NuPage Novex Bis-Tris gel (Invitrogen, USA) immersing in SDS running buffer (Invitrogen, USA) to run electrophoresis using 200 volts for 50 minutes.

Gag protein expressed in HeLa cells was detected by western blot technique as described in the following. The gel used in electrophoresis experiment was taken to immerse in NuPage transfer buffer (Invitrogen, USA) for 10 minutes to equilibrate. Two filter papers (Biorad, USA) and a transfer membrane (Biorad, USA) were also soaked in NuPage transfer buffer for 1 minute. Then, a soaked filter paper was placed on bottom platinum anode of a Biorad blot module followed by the soaked transfer membrane, the equilibrated gel and remaining soaked filter paper, respectively. A stainless steel cathode and safety cover were placed on. The experiment was performed using 10 volts for 30 minutes. After that, the transfer membrane was taken to wash with PBS and blocked with 10% v/v non fat milk (Skim milk, USA) in PBS for 1 hour. It was then washed with PBS three times and incubated in 183-H12-5C mouse monoclonal antibody (NIH AIDS research and reference reagent, USA) overnight. The membrane was washed, blocked and washed using the same reagents. Then, it was incubated in Anti-Mouse Immunoglobulins (DakoCytomation, Denmark) for 4 hours. The membrane was washed, blocked, washed, and then incubated in substrate solution containing BCIP (x-phosphate/5-bromo-4-chloro-3-indolyl-phosphate) and NBT (4-nitro blue tetrazolium-chloride) (Boehringer Mannheim, USA). If a lane containing gag protein, the dark blue band normally appear.

8. Mice Immunization

Four- to seven-week-old female Balb/c mice were immunized with nanoparticles-pHIS-HIV-hugag complex via either intradermal or transdermal route. The nanoparticles used in this study were selected from their ability in *in vitro* transfection study. In addition, SLN containing DOPE, an accepted neutral helper lipid used in commercial non-viral transfection vectors and the successful SLN for *in vitro* and *in vivo* transfection (Babiuk et al., 2000; Cui and Mumper, 2002; Tabatt, Sametti et al., 2004; Watabe et al., 2001) (DOPE-SLN) were prepared for this study to compare with the tested nanoparticles. DOPE-SLN were prepared by using the same procedure as SLN production described previously. The formulation composition of DOPE-SLN is shown in Table 3.5. The mice immunized with naked pDNA were compared to the mice immunized with nanoparticles-pDNA. In addition, five naïve mice were also evaluated as negative control. Therefore, mice were separated into 9 groups as shown in Table 3.6. The procedure for the use and care of mice were approved by The Ethical Committee of Laboratory Animal Use of Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Table 3.5 Formulation composition of DOPE-SLN

Ingredients	%Content (w/w)
Cetylpalmitate	3
Tween 80+Span 85 (7:3 w/w)	5
DDAB	0.64
DOPE	0.68
Propylene glycol	5
Paraben concentrate	1
Purified water to make	100

Table 3.6 Groups of mice used in immunization study

Treatment	Number of Mice	
	Intradermal injection	Topical application
DOPE-SLN-pDNA complex	5	5
SLN-pDNA complex	5	5
CSN-pDNA complex	5	5
Naked pDNA	5	5
Naïve mice	5	

8.1 Intradermal Immunization

Mice were anesthetized with isofurane (Rhodia, UK), an inhaled anesthetic, and shaved on the lower back area about quarter way up starting at the base of the tail for using as an injection area.

The injection area was swabbed thoroughly with 70% ethyl alcohol and allowed to air dry. Mice were injected intradermally by using 0.5 ml disposable syringe with 25 gauge needle. The total injection volume for each mouse was 100 µl divided into 50 µl for 2 sites of injection. They were injected with either nanoparticles-pHIS-HIV-hugag complex consisting of 50 µg of pHIS-HIV-hugag and 50,000 µg of nanoparticles or naked pHIS-HIV-hugag consisting of 50 µg of pHIS-HIV-hugag and sterile water added up to 100 µl. The mice were immunized 3 times; at day 0, day 7 and day 14 and collected blood at day 13 and day 20 by tail cutting and cardiac puncture, respectively. For naïve mice, no administration was used. All of mice were sacrificed by cervical dislocation technique after the last blood collection. They were anesthetized prior to each immunization, blood collection and euthanasia.

8.2 Transdermal Immunization

Mice were anesthetized with isofurane and shaved on the back covering area of 2 cm^2 for using as a topical immunization area. Mouse skin was wiped with 70% ethyl alcohol, allowed to air dry, and 100 µl of each formulation was dripped and subsequently spread thoroughly with the syringe puncture on skin. They were topically immunized with either nanoparticles-pHIS-HIV-hugag complex consisting of 50 µg of pHIS-HIV-hugag and 50,000 µg of nanoparticles or naked pHIS-HIV-hugag consisting of 50 µg of pHIS-HIV-hugag and sterile water added up to 100 µl.

The mice were immunized 3 times at day 0, day 7 and day 14 and bled at day 13 and day 20 by tail cutting and cardiac puncture, respectively. For naïve mice, they were not administered anything. All of mice were sacrificed by cervical dislocation technique after the last blood collection. In addition, they were anesthetized prior to each immunization, blood collection and euthanasia.

8.3 Determination of Antigen-Specific IgG Titer

The p24-specific IgG titer was quantified by ELISA as previously described by Bråve et al. (2005). Briefly, high binding assay plates were coated with 100 µl HIV-1 gag p24 recombinant viral protein (10 µg/ml) overnight at 4 °C. The plates were washed three times with PBS/Tween 20 and then blocked for 1 hour at 37 °C with 3% w/v gelatin in PBS/Tween 20 solution (100 µl/well). The plates were washed three times with PBS/Tween 20. Mouse serum diluted at ratio 1:10, 1:100, 1:500, 1:1,000, 1:2,500, 1:10,000 and 1:12,500 in 1% w/v gelatin in PBS/Tween 20 were added to wells and incubated for 1 hour at 37 °C. After washing three times with PBS/Tween 20, goat anti-mouse IgG conjugated to horseradish peroxidase diluted at 1:4,000 in 1% w/v gelatin in PBS/Tween 20 was added (100 µl/well) and incubated for 1 hour at 37 °C. Plates were washed three times again with PBS/Tween 20. Finally, the samples were developed with 100 µl o-phenylenediamine in substrate buffer and incubated for 30 minutes at room temperature and then the reaction was stopped with 50 µl of 4 N H_2SO_4. The OD of each well was measured using a microplate reader (Perkin-Elmer BioAssay reader, USA) at 490 nm. In this study, mouse monoclonal antibody to capsid protein p24 of HIV-1 at ratio 1:100,000 was used as a positive control. Antibody titer (units/ml) was defined as a reciprocal of the highest dilution that gave OD>2 standard deviations above the mean of negative control wells (Baliga et al., 2006)

9. Study of Skin Penetration Pathway of Nanoparticles-pHIS-HIV-hugag Complex

9.1 pHIS-HIV-hugag Labeling

The pHIS-HIV-hugag was labeled with TM-Rhodamine. The procedure for labeling was followed the manufacturer's instruction. Briefly, the labeling reaction was prepared by mixing the ingredients shown in Table 3.7 in a microfuge tube and incubated at 37 °C for 1 hour. A quick spin was performed during the incubation after 30 minutes of beginning. The labeled sample was purified by using a G50 microspin purification column. The column was prepared before

purification process to obtained high efficiency of purification. The resin in the column was resuspended by vortex mixer. Then, the column cap was loosened and the bottom closure was pulled out. The column was placed in 1.5 ml screw-cap microfuge tube for support and spun for 1 minute at 735xg continuously. The buffer collected during spin was discarded. The purification of labeled pDNA was performed by applying the sample prepared in labeling reaction to the top center of the resin. The column was supported by microfuge tube and spun at 735xg for 2 minutes. Finally, the purified sample was collected at the bottom of the support tube.

Table 3.7 Formulation for labeling reaction

Ingredients	Content (µl)
Purified water	35
10x labeling buffer A	5
1 mg/ml pHIS-HIV-hugag	5
Label IT® reagent	5

9.2 Preparation of Nanoparticles-pHIS-HIV-hugag Complex

SLN and CSN were incubated with 2.5 µg of labeled pHIS-HIV-hugag at the ratio of nanoparticles:pDNA equaled to 1,000:1 with the total volume 80 µl. The complexes were allowed to form at room temperature for 30 minutes before the skin penetration experiment was performed.

9.3 Skin Penetration Experiment

The dermatomed porcine skin (750 µm thickness) was thawed and placed between the donor and receptor compartment of vertical diffusion cells (area = 0.79 cm^2). The receptor compartment was filled with phosphate buffer saline pH 7.4 and stirred continuously throughout the experiment. The system was equilibrated at room temperature for 30 minutes before the experiment was performed, then, 80 µl of nanoparticles-labeled pHIS-HIV-hugag complexes was applied on the skin in donor compartment and let to penetrate for 24 hours.

9.4 Confocal Laser Scanning Microscopy

After the penetration experiment was finished, the excess nanoparticles-labeled pHIS-HIV-hugag complex was removed from the skin surface by washing with phosphate buffer saline pH 7.4. The skin was mounted on a glass slide and covered with cover glass. The samples were observed under LSM 510 invert Laser Scan Microscope (Carl Zeiss, Germany) without additional tissue processing. Laser excitation wavelength of 543 nm was selected to scan nanoparticles-labeled pHIS-HIV-hugag complex. The images were obtained using a Plan-neofluar 10x/0.3. To visualize the distribution of nanoparticles-labeled pHIS-HIV-hugag complexes, confocal images were first obtained in the xy-plane. The skin surface ($z = 0$ μm) was defined as the imaging plane of bright fluorescence with a morphology characteristic of stratum corneum surface. To generate an xz-section, a horizontal line was drawn across a region of interest in the $z = 0$ μm-xy-plane and then optically sliced through the digitized image data of the successive xy-sections to generate xz-planar optical cross-sections. All images were obtained with the same optical aperture and lens.