CHAPTER 2

MATERIALS AND METHODS

2.1. Materials and equipments

2.1.1. Chemicals

- Kanamycin (Carl Roth GmbH, Karlsruhe, Germany)
- DNA 1 kb ladder (Fermentas, St. Leon-Rot, Germany)
- Cholesterol (Sigma Chemical Co., St. Louis, MO, USA)
- Dicetyl phosphate (DP) (Sigma Chemical Co., St. Louis, MO, USA)
- Dimethyl dioctadecyl ammonium bromide (DDAB) (Sigma Chemical Co., St.Louis, MO, USA)
- dNTPs 10 mM (Sigma Chemical Co., St. Louis, MO, USA)
- Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA)
- Fetal calf serum (FCS) (FCS; Gibco BRL)
- Penicillin-Streptomycin for cell culture (PAA, Pasching, Austria)
- PCR forward primer and reverse primer (Biomers, Ulm, Germany)
- High-fidelity PCR enzyme mix (Fermentas, St. Leon-Rot, Germany)
- High-fidelity PCR (10X) (Fermentas, St. Leon-Rot, Germany)
- Plasmid DNA purification kit (Qiagen GmbH, Hilden, Germany)
- RNA extraction kit, Nucleospin RNA II (Macherey-nagel, Düren, Germany)
- PCR/DNA purification kit (Qiagen GmbH, Hilden, Germany)
- TAE buffer (0.04M Tris, 0.04M Acetate, 0.001M EDTA)

- *BamHI* restriction enzyme (Fermentas, St. Leon-Rot, Germany)
- *NheI* restriction enzyme (Fermentas, St. Leon-Rot, Germany)
- *HpaI* restriction enzyme (Fermentas, St. Leon-Rot, Germany)
- DNA ligase enzyme (NEB, Frankfurt, Germany)
- Pre-stained protein marker (Fermentas, St. Leon-Rot, Germany)
- Ni-NTA spin column (Qiagen GmbH, Hilden, Germany)
- PD10 column (Amersham, New Jersey, USA)
- Trypsin 0.25% (Gibco, Invitrogen Corp., Carlsbad, CA, USA)
- Tween61 (polyoxyethylene sorbitan monostearate) (Sigma Chemical Co., St. Louis, MO, USA)
- Nitrocellulose membrane (Amersham, New Jersey, USA)
- 30% Acrylamide solution (Carl Roth GmbH, Karlsruhe, Germany)
- Sodium dodecyl sulfate (SDS) (Bio-Rad, Milan, Italy)
- Anti-pentahistidine antibody horseradish peroxidase (HRP) conjugate (Qiagen GmbH, New Jersey, USA)
- 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen, UK)
- 30% hydrogen peroxide (H_2O_2) (Carl Roth GmbH, Karlsruhe, Germany)
- 4-chloronaphthol (4-CN) (Carl Roth GmbH, Karlsruhe, Germany)
- Bovine Serum Albumin (BSA) (Carl Roth GmbH, Karlsruhe, Germany)
- Imidazole (Carl Roth GmbH, Karlsruhe, Germany)
- Isopropyl-D-thiogalactopyranoside (IPTG) (Sigma, Missouri, USA)
- ProteoJet™ Mammalian cell lysis reagent (Fermentas, Ontario, Canada)
- Lysozyme (Fermentas, Ontario, Canada)
- Calcitonin (Chengdu Kaijie Biopharm, Chengdu, China)

- Tat peptide (EMC, Tübingen, Germany)
- VP peptide (Chengdu Kaijie Biopharm, Chengdu, China)
- Other solvents (analytical grade)

2.1.2. Animals

- Sprague Dawley (National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand)

2.1.3. Cell lines, bacterial cultures and plasmids

- *Escherichia coli* DH5α (Boehringer Ingelheim (BII), Germany)
- *Escherichia coli* BL21 (DE3) was kindly provided by Prof. Dr. Friedrich Götz, Department of Microbial genetics, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany
- GFPmut2 encoding plasmid (pWH105) was kindly provided by Dr. Ralph Bertham, Department of Microbial genetics, University of Tübingen, Germany
- pET28a(+) expression vector was kindly provided by Prof. Dr. Friedrich Götz, Department of Microbial genetics, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany
- Human colorectal adenocarcinoma cell (HT-29) was kindly provided by Department of Medical Microbiology, University of Tübingen, Germany
- Human mouth epidermal carcinoma cell (KB) was from American Type Culture Collection (ATCC)

2.1.4. Equipments

- CO_2 incubator (Model 2123Tc, SHEL LAB, VWR, USA)
- Transmission electron microscopy (TEM1200SJEOL, JEOL Ltd., Tokyo, Japan)

- Gel Documentation (Universal Hood, BioRad Laboratories, Milan, Italy)
- Gel Electrophoresis (SUB-CELL® GT, BioRad Laboratories, Milan, Italy)
- iCycler PCR (BioRad Laboratories, USA)
- Laminar air flow cabinet (Cytair 125, Equipments Scientifiques & Industries S.A., France)
- Lyophilizer (Christ FOC-1 Model K-40 equipment, Balzers-Pfeiffer GmbH, Asslar, Germany)
- Probe sonicator (Vibra Cell™, Sonics & Materials Inc., Newtown, CT, USA)
- Rotary evaporator (R-124 Buchi, Switzerland)
- 96 Well plates (Nunc®, USA)
- 24 Well plates (Nunc®, USA)
- Microtip probe sonicator (Banderlin Sonoplus GM70, Berlin, Germany)
- Microplate reader (Bio-Rad Model 550, München, Germany)
- Modified vertical Franz diffusion apparatus (Crown Bio Scientific, Inc., Sommerville, NJ, USA)
- pH meter (Cyberscan pH 510, Euteoh Instruments, Singapore)
- Transmitted light differential interference contrast attachment (Model CH No. 3E0245, Olympus Optical Co. Ltd., Japan)
- Ultracentrifuge (Univeral 32 R, Hettich Zentrifugen, Germany)
- Spectrophotometry (Milton Roy spectronic Genesys 5)
- Zetasizer Nano Series (Nano-S, Malvern instrument Ltd., Malvern, UK)
- Multimode Reader (Beckman, California, USA)
- Confocal laser scanning microscope (CLSM) (Zeiss CEM 902, Zeiss, Oberkochen, Germany)

- Lab-Tek® II Chamber Slide™ (Nunc, IL, USA)
- Blotting apparatus (Hölzel GmbH, Hörlkofen, Germany)
- Differential scanning calorimeter, DSC (Model 4000, Perkin Elmer, Massachusetts, USA)
- Fourier transform infrared (FT-IR) spectrophotometer (470 FT-IR, Nicolet, MA, USA)

2.2. Methods

The scope of this study was divided into 6 parts as the scheme in Figure 14.

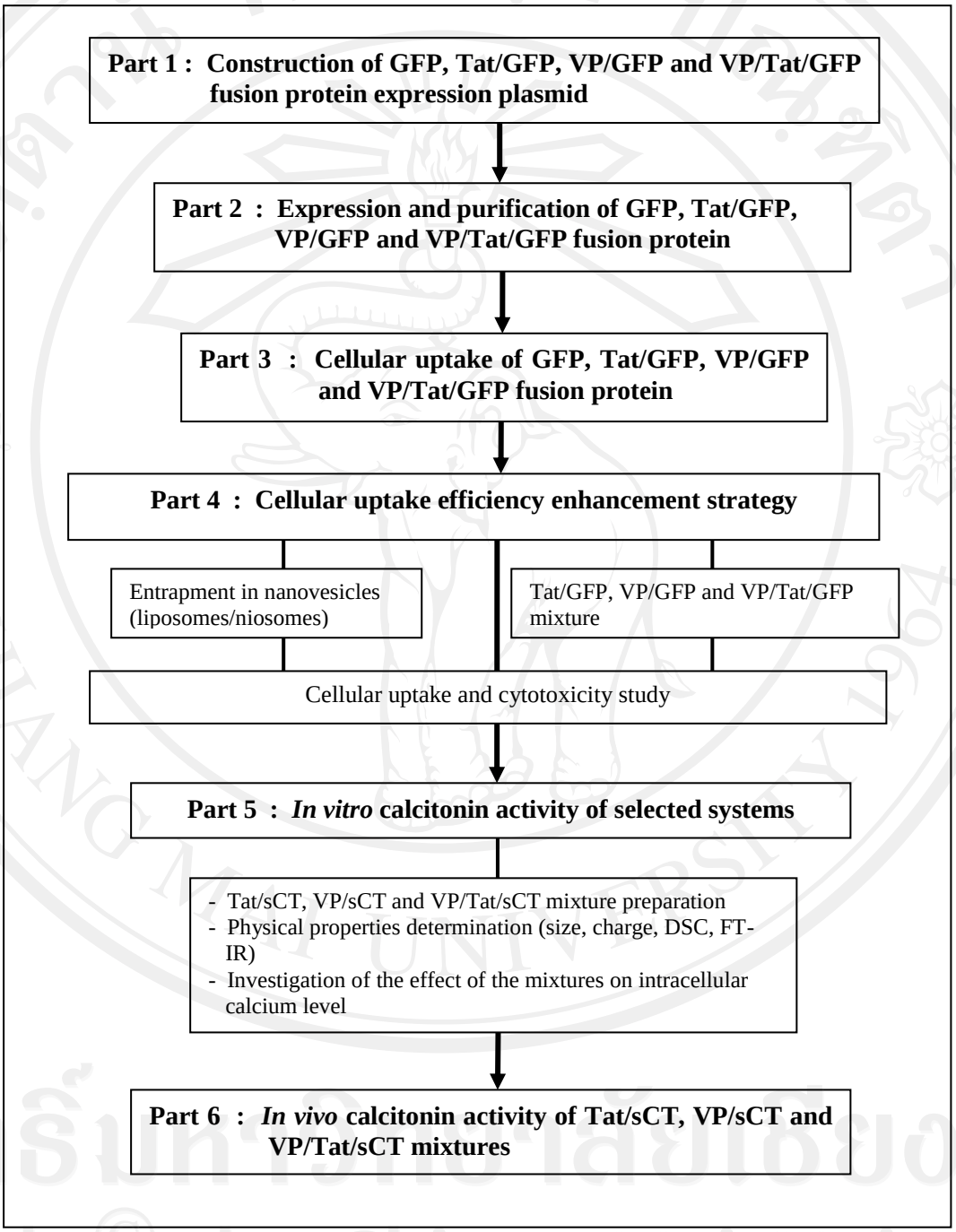

Figure 14 Scope of the study

Part 1 : Construction of GFP, Tat/GFP, VP/GFP and VP/Tat/GFP fusion protein expression plasmid

1.1. Primer design

The expression vector for GFP, N-terminal Tat fusion GFP (Tat-GFP), C-Terminal Tat fusion GFP (GFP-Tat), N-Terminal VP fusion GFP (VP-GFP) and Tat-GFP-VP were produced. The plasmid pWH105 encoding complete green fluorescent protein derivative gene (GFPmut2) with *HpaI* restriction site was used as a template for GFP, Tat-GFP, GFP-Tat and VP-GFP amplification, while pET28a(+) encoding Tat-GFP was used as a template for Tat-GFP-VP amplification. All forward primers were designed to have *Nhe I* restriction site (GCTAGC) as a flanking region at the 5' end, while the reverse primers were designed to have *BamHI* restriction site (GGATCC) as a flanking region at the 5' end. The difference between forward primer and reverse primer for the same PCR reaction should not higher than 2°C. The possibility to get the right clone was checked by Vector NTI program (Invitrogen, California, USA). Nucleotide sequences of all forward and reverse primers were as follows.

Forward primer for GFP and GFP-Tat : 5'-ATATGCTAGCATGAGTAAAG GAGAAGAACTTTTCAC-3'

Forward primer for Tat-GFP and Tat-GFP-VP : 5'-ATATGCTAGCAGGA AGAAGCGGAGACAGCGACGAAGAATGAGTAAAGGAGAAGAACTTTTCAC -3'

Forward primer for VP-GFP : 5'-ATATGCTAGCACCGTGGATAACCCAG CTTCCACCACGAATAAGGATAAGCTATTTGCAGTGATGAGTAAAGGAGAA GAACT-3'

Reverse primer for GFP, Tat-GFP and VP-GFP : 5'-ATATGGATCCTTATTT GTATAGTTCATCCATGCC-3'

Reverse primer for GFP-Tat : 5'ATATGGATCCTTATCTTCGTCGCTGTCT CCGCTTCTTCCTTTTGTATAGTTCATCCATGC CATG-3'

Reverse primer for Tat-GFP-VP : 5'-ATATGGATCCTTACACTGCAAAT AGCTTATCCTTATTCGTGGTGGAAGCTGGGTTATCCACGGTATATGGATCC TTATTTGTATAGTTCATCCATGCC-3'

1.2. Amplification of genes encoding GFP or fusion protein

Gene fragment of GFP or other fusion proteins were amplified by polymerase chain reaction (PCR). The amount of each PCR compositions in the reaction was shown in Table 5. All PCR components were pipette into 200 µl PCR tube and placed into iCycler PCR machine. The reaction was started by heating the reaction to 94°C and hold on for 5 min. Then, the cycle of 94°C for 45s, 50°C for 30s and 72°C for 90s was repeated for 30 times. Further elongation was perform by incubated the reaction at 72°C for additional 5 min. The PCR products were purified using PCR/DNA purification kit according to the manufacturer guideline and resolved in 1% agarose gel in 1X TAE buffer. PCR products were kept in refrigerator until further experiments.

1.3. Linearization of pET28a(+) expression vector and PCR fragment digestion

The preparation of pET28a(+) and PCR fragments for the ligation reaction were performed by digested pET28a(+) and PCR fragments with *Nhe I* and *BamHI* restriction enzyme. According to the higher activity of *BamHI* restriction enzyme and the difference of appropriate buffer for *Nhe I* and *BamHI* digestion, the reaction for

each restriction enzyme digestion was performed separately. The pET28a(+) and PCR fragments were first digested with *Nhe I* restriction enzyme in Tango buffer supplied with enzyme at 37°C for 2 hr. The digested pET28a(+) and PCR fragments were purified by DNA purification kit and further digested with *BamHI* restriction enzyme in *BamHI* buffer supplied with enzyme at 37°C for 2 hr. The digested pET28a(+) and PCR fragments were purified again and analyzed by agarose gel electrophoresis.

Table 5 The amount of each component (µl) in PCR reaction

Component	Volume (µl)
Template (100 ng/µl)	2
Forward primer	1
Reverse primer	1
10X High fidelity PCR buffer	5
10 mM dNTPs	1
High fidelity PCR enzyme	1
DI water	39

1.4. Ligation reaction

The digested pET28a(+) and PCR fragments were ligated together using DNA ligase enzyme. The ligation reaction was perform overnight at 16°C. The expression vector with insertion was resolved in 1% agarose gel comparing with non-inserted vector. The insertion was confirmed by *HpaI* restriction enzyme digestion and sequencing.

Part 2 : Expression and purification of GFP, Tat/GFP, VP/GFP and VP/Tat/GFP fusion protein

2.1. Transformation

The pET28a(+) containing required gene fragment were transformed into *E. coli* DH5α and *E. coli* BL21 (DE3) for the plasmid propagation and protein expression step, respectively. The competent *E. coli* was obtained by using calcium chloride method. Briefly, the *E. coli* was cultured in 50 ml of LB broth with 150 rpm shaking overnight at 37°C. The cells were harvested by centrifugation at 6,000 g, 4°C for 10 min and resuspended in 20 ml of ice-cold 100 mM $CaCl_2$. The bacterial cell suspension was kept on ice for 15 min and centrifuged at 6,000 g for 10 min. The obtained competent *E. coli* cells were resuspended in 4 ml of ice-cold 100 mM $CaCl_2$ and kept as 200 µl aliquots at -70°C until use. The plasmids from the ligation reaction (5µl) was pipetted into the 200 µl competent *E. coli* DH5α cells, gently mixed, kept on ice for 30 min, incubated at 42°C for 2 min and immediately on ice for 2 min. Then, 800 µl of LB broth were added and the transformed cells were shaken at 37 °C, 150 rpm for 1 hr and spread on LB agar containing kanamycin at 30 µg/ml. The transformed colony was selected, cultured in 5 ml of LB broth supplemented with 30 µg/ml of kanamycin and shaken at 150 rpm, 37°C until OD600 was about 0.6. Then, the 5 ml culture was transferred into 50 ml of LB broth supplemented with 30 µg/ml of kanamycin and shaken at 150 rpm, 37°C overnight. The plasmid was purified from bacterial culture using DNA plasmid purification kit. Briefly, the 50 ml bacterial culture was transfer to a sterile 50 ml tube and the cell suspension was spin down at 6,000 g, 4^{o}C for 5 min. The supernatant was discarded and the cell pellets

were resuspend in resuspension buffer (P1) and vortex. Add lysis buffer (P2) and mix by inverting the tubes 4-6 times. Add neutralization buffer (N3) and mix by inverting the tubes, then place on ice for 15-20 min. Centrifuge for 10 min at 13,000 rpm and equilibrate Qiagen-tip column by adding QBT buffer. Apply the supernatant to the equilibrated Qiagen-tip column and allow it to enter the resin by gravity flow. Wash Qiagen-tip column by adding QC buffer and discard the flow-through. Elute plasmid DNA with Buffer QF, precipitate DNA by adding isopropanol and wash DNA pellet with 70% ethanol. Air-dry DNA pellet for 5 min and redissolve DNA in 0.5-1 ml TE buffer (pH 8.0). The concentration of the obtained plasmid was estimated from the band density of plasmid on agarose gel in comparing with DNA ladder. The amount of 10 ng plasmid with insertion was then transformed into *E. coli* BL21 (DE3) and repeated the same procedure as *E. coli* DH5α.

2.2. Protein expression

After the transformed colony was selected, the transformed bacterial cells were cultured in LB broth supplemented with 30 µg/ml of kanamycin and shaken at 150 rpm, 30°C until OD600 = 0.8-0.9. The protein expression was induced by the addition of IPTG to the final concentration of 0.1 mM and further incubated for 3 hr. The cells were harvested, lysed by lysozyme and sonicated in the lysis buffer (NPI-10) (10 mM imidazole, 300 mM NaCl, 50 mM $NaH_2PO_4.H_2O$, pH 8.0). After the removal of cell debris by centrifugation at 6,000 g for 10 min, the supernatant was collected for protein purification by Ni-NTA spin column for hexahistidine (6xHis) tagged protein.

2.3. Purification and analysis

2.3.1. Ni-NTA purification

The Ni-NTA spin column was equilibrated with 600 µl of NPI-10 buffer and centrifuged for 2 min at 890 g. The cleared lysate was loaded onto the pre-equilibrated nickel-NTA column and centrifuged for 5 min at 270 g. Then, the nickel-NTA column was washed twice with 600 µl of wash buffer (NPI-20) (20 mM imidazole, 300mM NaCl, 50 mM $NaH_2PO_4.H_2O$, pH 8.0) and centrifuged for 2 min at 890 g. The 6xHis-tagged protein was eluted twice with 300 µl of elution buffer (NPI-500) (500 mM imidazole, 300mM NaCl, 50 mM $NaH_2PO_4.H_2O$, pH 8.0) and desalted by PD10 column.

2.3.2. SDS-PAGE and western blot analysis

Purified Protein samples were analyzed by resolved in 12% SDS-polyacrylamide gel. Briefly, the gel plates were assembled to the spacers according to the manufacturer's instructions and marked the level to which the separating gel should be poured (a few millimeters below the level where the wells will be formed by the comb). For 8×10 cm minigel, mix 2.2 ml of 2.2 ml 30% acrylamide/0.8% bis-acrylamide stock solution, 2.2 ml of 2.5X separating gel buffer, 1.1 ml distilled water and 5 µl TEMED in a beaker. Just before pouring, add 50 µl of 10% ammonium persulfate, and mix well. Pour the gel between the assembled gel plates to the level marked on the gel plate and overlay with isopropanol. After polymerization is complete (around 20 min), pour off isopropanol, rinse with water and dry. For the stacking gel, mix 0.28 ml of 30% acrylamide/0.8% bis-acrylamide stock solution, 0.33 ml of 5X stacking gel buffer, 1 ml distilled water and 2 µl TEMED in a beaker. Just before pouring, add 15 µl of 10% ammonium persulfate, and mix well. Pour on top of the separating gel. Insert comb, avoiding introduction of air bubbles. After the stacking gel polymerizes (around 10 min), the gel can be placed in the electrophoresis

chamber. Fill the chamber with electrophoresis buffer and remove the comb. Before loading, add 1 volume 5X SDS-PAGE sample buffer to 4 volumes of protein sample. Vortex briefly and heat at 95°C for 5 min. Load samples and run gel at 100 mV until the bromophenol blue dye reaches the bottom edge. After electrophoresis running, the gel was visualized by incubated the gel in Coomassie staining solution (0.1% coomassie, 50% methanol and 10% acetic acid in distilled water) for 30 min. Destained the gel with 10% acetic acid until the background become clear. After destaining the proteins appear as blue bands against a clear gel background. The molecular weight of protein can be determined by comparing with pre-stained protein marker.

For immunodetection by western blot analysis, the proteins were resolved in 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The blot was blocked with blocking reagent in blocking buffer and washed twice with TBS-Tween/Triton buffer. Anti-pentahistidine antibody chemically coupled to the horseradish peroxidase (HRP) for direct detection of 6xHis-tagged proteins was diluted to 1:2000 in TBST buffer. The nitrocellulose membrane was incubated at room temperature (25°C) for 1 hr with 20 ml of Penta-His-HRP conjugate and then washed twice with TBS buffer. The reaction of horseradish peroxidase was developed by the addition of the solution containing 4-chloronaphthol and 30% hydrogen peroxide (H_2O_2).

2.3.3. Protein concentration determination by Bradford method

The protein concentrations were determined by a Bradford protein assay kit (Bio-Rad, Milan, Italy) using BSA as a standard and measured the absorbance at 595 nm (Bradford, 1976).

2.3.4. Fluorescent intensity measurement

Fluorescent intensities were determined by Multimode Reader with the excitation and emission wavelength at 485 and 510 nm, respectively.

Part 3 : Cellular uptake of GFP, Tat/GFP, VP/GFP and VP/Tat/GFP fusion protein

3.1. Cell culture

KB (human mouth epidermal carcinoma) and HT-29 (human colon adenocarcinoma) cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin). The cells were cultured at 37 °C under 5% CO_2 humidified.

3.2. Cellular uptake study

Cellular uptake study of GFP and Tat and/or VP fusion GFP were performed to evaluate the effects of N-terminal or C-terminal fusion on GFP uptake, and also to compare the effects of Tat fusion and VP fusion on GFP uptake as well. The cells were seeded onto 24-well plates and cultured to reach 90% confluence. The effects of N-terminal or C-terminal fusion on the enhancement of GFP uptake were investigated by using Tat-GFP and GFP-Tat fusion protein. The culture medium was aspirated, washed twice with phosphate buffer saline (PBS) and incubated with various concentrations of the GFP and Tat/GFP fusion protein samples and time intervals. After incubation, the medium was again aspirated and the cells were washed twice with PBS, lysed by ProteoJet™ Mammalian cell lysis reagent. The fluorescent intensity of the supernatant was determined by a Multimode Reader. The concentration of the uptake GFP or Tat/GFP fusion proteins were calculated from the

standard curve plotted between GFP or Tat/GFP fusion protein concentration and fluorescent intensity. The percentages of cellular uptake were calculated by the following equation:

$$\text{Cellular uptake (\%)} = \frac{\text{The amount of the uptake proteins}}{\text{The amount of the proteins added in each well}} \times 100$$

The effects of Tat fusion and VP fusion on the enhancement of GFP uptake were investigated by using Tat-GFP and VP-GFP fusion protein. Tat-GFP-VP fusion protein was used for evaluated the synergistic effect of Tat and VP on the enhancement of GFP uptake. The cellular uptake experiment was performed as the same procedure as above, but the concentration of all fusion proteins were fixed at 1 μM and the incubation time was 1 hr.

The uptake of GFP and Tat fusion proteins into the cells was also analyzed with a confocal microscope. HT-29 cells grown on chamber slide to 50 to 70% confluence were treated with 1 μM GFP or Tat fusion proteins for 1 hr. The transduced cells were washed twice with PBS and fixed in 3.7% (v/v) formaldehyde in PBS for 5 min at room temperature and staining for 30 min with 300 nM DAPI solution in PBS to visualize nuclei. The excess dye was removed by rinsed sample several times with PBS before observed with CLSM. Wavelength at 358 nm and 395 nm were used to excite DAPI and GFP, respectively and emission spectra were collected with 461 nm and 535 nm bandpass filters.

Part 4 : Cellular uptake efficiency enhancement strategy

4.1. Entrapment in nanovesicles

4.1.1. Preparation of the empty nanovesicles (liposomes/niosomes)

Blank liposomes and niosomes were prepared by the freeze dried empty liposome (FDEL) method (Kikuchi et al., 1999). The neutral, cationic and anionic liposomes were composed of DPPC/cholesterol (CHL), DPPC/CHL/DDAB and DPPC/CHL/DP at the molar ratios of 7:3, 7:2:1 and 7:2:1, respectively. The neutral, cationic and anionic niosomes were composed of Tween 61/ CHL, Tween 61/CHL/DDAB and Tween 61/CHL/DP at the molar ratios of 1:1, 1:1:0.05 and 1:1:0.05, respectively (Manosroi et al., 2008). Lipids or surfactants together with cholesterol and/or the cationic lipid (DDAB) and anionic lipid (DP) were dissolved in chloroform. The solvent was removed by a rotary evaporator to get a thin film. The film was dried by evacuation in a desiccator under reduced pressure for over 12 hrs. Phosphate buffer (100 mM, pH 7.0) was added to the film, swirled in a water bath at 45°C for 30 min to obtain a swelled dispersion. The resulting dispersion was sonicated by a microtip probe sonicator at pulse on 3.0, pulse off 2.0, 25% amplitude for 10 min and centrifuged at 2,190 g for 1 min to remove the contaminated titanium particles. The dispersion was lyophilized by a freeze-dryer and kept at 4°C until use. The Tat-GFP fusion protein was loaded in non-elastic nanovesicles by reconstitution the lyophilized blank nanovesicles with the Tat-GFP (at 1 µM) in phosphate buffer (pH 7.0), while that loaded in the elastic nanovesicles was from the reconstitution of the dried blank nanovesicles with the Tat-GFP in phosphate buffer containing 25% v/v ethanol. The resulting mixture was mixed for 10 min at room temperature (30±2°C).

4.1.2. Physical properties determination of nanovesicular formulations

A. Mean particle sizes and zeta potential

The vesicular sizes of the vesicles were measured at 25°C by a dynamic light scattering equipment using the non-negative constrained least squares (NNLS) algorithm mode particle size distribution (PSD) analysis. The measurements were performed in triplicate with 3 cycles in each measurement. The measurement conditions were set at 30s with 10s pulse between cycles. The ζ (zeta) potential values of all samples were obtained by the phase analysis light scattering (PALS) software. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90^{o}. The measurements were performed in triplicate with 5 cycles in each measurement.

B. Entrapment efficiency of Tat-GFP loaded in nanovesicles

The entrapment efficiency of Tat-GFP fusion protein was determined by gel electrophoresis and gel documentation as previously described (Manosroi et al., 2010). The gel electrophoresis was carried on a 12% SDS-PAGE in tris-glycine system buffer at a constant voltage (100 mV) for 150 min and stained by coomassie brilliant blue R-250. The bands of the free fusion protein were observed, while the loaded fusion protein in the vesicles remained at the site of application. The band densities were determined by the Quantity One Program Analysis and the entrapment efficiency was calculated as the following:

$$\text{Entrapment efficiency (\%)} = \frac{[\, I_{\text{total}} - I_d \,]}{I_{\text{total}}} \times 100$$

While, I_{total} = band densities of the total fusion protein initially loaded in the nanovesicular dispersion and I_d = band densities of the free fusion protein.

C. Deformability index (DI) determination

The elasticity value in the term of DI was determined by the extrusion method (Cevc et al., 1995). Ten ml of the nanovesicular dispersion were extruded through a polycarbonate membrane filter (50 nm pore size; Millipore, Massachusetts, USA) at a constant pressure (2.5 bars) for 10 min. The DI was calculated by the following equation (Van der Bergh et al., 2001):

$$Deformability\ Index\ (DI) = j \times (r_v/r_p)^2$$

where j is the weight (g) of the sample extruded through a polycarbonate membrane filter in 10 min, r_v is the size (nm) of the vesicles after extrusion, and r_p is the pore size (nm) of the membrane filter.

4.1.3. Cellular uptake study of Tat-GFP loaded nanovesicles

Cellular uptake experiment of Tat-GFP loaded nanovesicles was performed as described in section 3.2. The total concentration of Tat-GFP fusion protein in each well was 1 μM and the incubation time was 1 hr.

4.1.4. Chemical stability determination of Tat-GFP loaded nanovesicles

Tat-GFP loaded in non-elastic and elastic anionic niosomes were selected for the assessment of the protective effect of the Tat-GFP fluorescent signal by loading in nanovesicles. Tat-GFP in phosphate buffer, Tat-GFP loaded in non-elastic and elastic anionic niosomes were prepared. The concentration of Tat-GFP in all samples was 1 μM. All samples were kept in dark to avoid the loss of fluorescent from UV light exposure at room temperature (30±2°C) for 3 months. An aliquot of the samples was collected at 1, 2, and 3 months for fluorescent measurement. The

data were calculated as the percentages of the fluorescent signal remaining at various time intervals in comparing to at initial.

4.1.5. Cytotoxicity of nanovesicular formulations by the SRB assay

The cytotoxicity as determined by cell viability in both HT-29 and KB cells was performed in a 96-well plate using sulforhodamine B (SRB) assay (Skehan et al., 1990). The cells were cultured in 96-well-plate ($1x10^4$ cells/well) overnight in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The adherent cells were incubated with test samples for 1 hr. Then, the culture medium was removed, the adherent cells were fixed in situ by the addition of 50 µl of cold 50% (w/v) trichloroacetic acid (Merck, Darmstadt, Germany) and kept at 4°C for 60 min. The supernatant was discarded, washed for three times with distilled water and air-dried. SRB solution was added and the cells were allowed to stain for 30 min at room temperature (27±2°C). The unbound SRB was removed by washing for three times with 1% acetic acid solution. The plate was air-dried and the bound stain was dissolved with the unbuffered Tris base (Sigma Chemical Co., Missouri, USA). The optical density was measured at 540 nm using a Microplate Reader. Cell viability (%) was calculated as the following :

$$\text{Cell viability (\%)} = \frac{\text{Optical density at 540 nm of the treated cells}}{\text{Optical density at 540 nm of the untreated cells}} \times 100$$

4.1.6. Development of low toxic elastic anionic niosomes

Low toxic elastic anionic niosomes were prepared by the reduction of ethanol concentration in the formulation or using the edge activators, sodium cholate (NaC) and sodium deoxycholate (NaDC), instead of ethanol. For the preparation of

NaC or NaDC containing elastic anionic niosomes, NaC or NaDC at various concentrations (1-25 mol%) was added in the step of film forming to the mixture of Tween 61, CHL and DP, dissolved in 1:1 volume ratio of chloroform and methanol. The solvent was removed by a rotary evaporator to get a thin film. The film was then rehydrated with phosphate buffer to obtain elastic niosomes containing edge activators. The Tat-GFP fusion protein was loaded into the vesicles as described in section 4.4.1. The cellular uptake and cytotoxicity in HT-29 and KB cells of Tat-GFP loaded in the developed nanovesicular formulation was also performed**.** The formulation which gave the highest cellular uptake enhancement was selected for visualized by TEM using negative staining technique employing 2% (w/v) of ammonium molybdate solution. A drop of the dispersion was applied on a 300-mesh formvar copper grid on paraffin and allowed the sample to adhere on the formvar for 10 min. The remaining dispersion was removed and a drop of 2% aqueous solution of ammonium molybdate was applied for 5 min. The remaining solution was then removed. The sample was air dried and examined with a TEM.

4.1.7. Transdermal absorption study

The nanovesicular formulation which gave high cellular uptake with lowest cytotoxicity was selected for this study. The samples for transdermal study were GFP, Tat-GFP fusion protein, Tat-GFP fusion protein loaded in non-elastic anionic niosomes and Tat-GFP fusion protein loaded in elastic anionic niosomes (1% NaC).

A. Preparation of rat skin

Full-thickness abdominal skin was obtained from the male Sprague Dawley rats, weighing between 150 and 200 g. Abdominal hair was shaved off and

left overnight. The rats were sacrificed and the abdominal skin was removed, trimmed off the subcutaneous fat and freshly used. The protocol for all procedures has followed to the "Principles of Laboratory Animal Care" (Górska, 2000).

B. Transdermal absorption experiment

Transdermal absorption study was determined by the Franz diffusion cell with some modifications (Birchall et al., 2000; Foldvari et al., 2006). All samples were prepared in 100 mM phosphate buffer (pH 7.0) at the concentration equivalent to 5 µM of GFP. Briefly, on the Franz diffusion cell, the rat skins were mounted on the receptor compartment with the stratum corneum (SC) side facing upwards to the donor compartment (Figure 15). The donor chamber was filled with 0.5 ml of the samples. The available diffusion area (rat skin) was 2.46 cm^2. An amount of 14 ml of phosphate buffer saline (PBS), pH 7.4 was put in the receiver chamber. The receiving chamber was maintained at 37±2ºC and stirred by a magnetic bar. The donor port was occluded with parafilm. The experiments were performed in triplicate. At 1, 3 and 6 hr, the diffusion cells were stopped. The skin was removed and swung twice in 100 ml of distilled water. For SC, the amount of the fluorescent protein in SC was collected by stripping the skin with a 1 cm × 1 cm 3M Scotch Magic™ tape (Plessis et al., 1992). Nine pieces of the tape were used for each skin sample. The tapes were cut into small pieces, pooled in a 5 ml vial and 3 ml of 100 mM phosphate buffer were added. The vial was vortexed for 5 min, allowed to stand for 10 min and vortexed again for 5 min. For viable epidermis and dermis (VED), the stripped skin was cut into small pieces and pooled in 1.5 ml microcentrifuge tube containing 1 ml of 100 mM phosphate buffer, vortexed for 5 min, allowed to stand for 10 min and vortexed again for 5 min. All samples including the receiving solution

were centrifuged at 16,350 g for 10 min and determined the fluorescent intensity of the supernatants. The amounts of GFP or Tat-GFP in each sample at various time intervals were calculated from the standard curve between the fluorescent intensity and the GFP or Tat-GFP concentrations. Cumulative amounts (μg/cm^2) and fluxes (μg/cm^{2}h) of each compartment were presented.

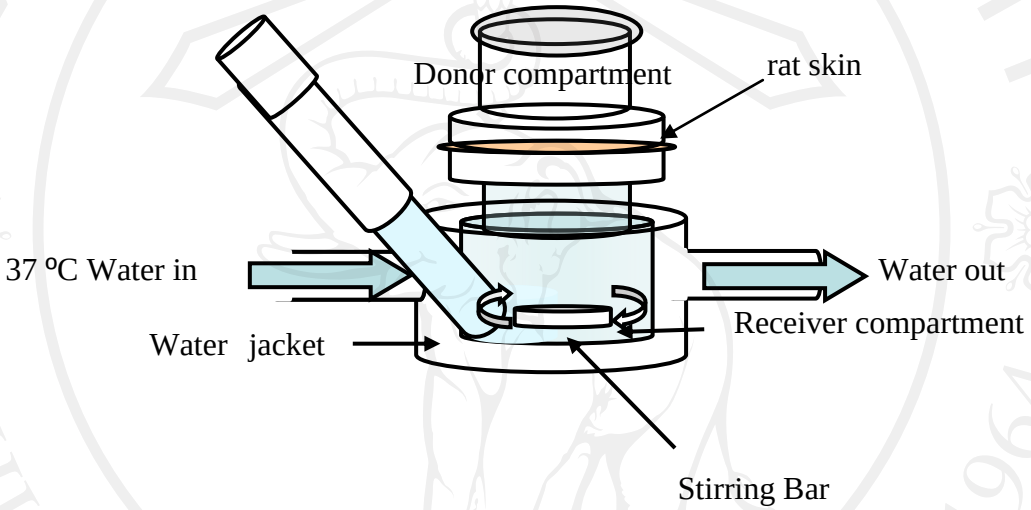

Figure 15 Experimental set-up of Franz diffusion cell apparatus

4.2. Tat/GFP, VP/GFP and VP/Tat/GFP mixture

4.2.1. Evaluation of simple mixing method as cargoes transport strategy

Tat/GFP mixture at 1:1 molar ratio was used in this study. Cellular uptake, transepithelial study and trandermal transport of the mixture was compared with Tat-GFP fusion protein.

A. Cellular uptake study

The procedure of cellular uptake study was the same as described in section 3.2.

B. Transepithelial study

The ability to cross epithelium barrier of GFP, Tat-GFP and Tat/GFP was performed in HT-29 cells. An amount of 1×10^5 HT-29 cells was seeded in each cup of a 6-well Transwell™ (0.4 µm pore size; Corning Costar, NY, USA) and cultured for at least 10 days. The quality of the cell monolayer was determined by the 100% confluence growth using phase contrast microscope. The medium was changed both in the lower (2.6 ml) and the upper (1.5 ml) chamber every 2–3 days. Sample at a concentration equivalent to 2 µM/well of GFP in PBS, was added to the upper Transwell™ chamber and the cells incubated with PBS was served as a control. A 500 µl sample was collected from the lower chamber and replaced by fresh medium at various time intervals. The fluorescence intensity was measured at 485/510 nm by a Multimode Reader. The fluorescent intensity of aliquot sample from control was substracted from the value recorded from aliquot sample of the treated group before calculated the percentage of samples permeated through the HT-29 cells as follows:

$$\text{Percentage of permeation (\%)} = \frac{\text{fluorescence intensity of the collected samples}}{\text{fluorescence intensity of the tested samples}} \times 100$$

C. Transdermal delivery through rat skin

The permeability through the excised rat skin was compared among GFP, Tat/GFP and Tat-GFP. The experiment was performed as described in section 4.1.7.

4.2.2. Tat/GFP, VP/GFP and VP/Tat/GFP mixture preparation

GFP at fixed amount of 1 µM was mixed with Tat or VP in MilliQ (MQ) water at different molar ratios of Tat/GFP or VP/GFP of 6:1, 3:1, 1:1 and 1/3:1. The mixture of VP/Tat/GFP was prepared at different molar ratios of 1:6:1, 6:1:1,

1:3:1, 3:1:1, 1:1:1, 1/3:1:1 and 1:1/3:1. The mixtures were incubated at room temperature (25±2°C) for 1 hr.

For the effect of order of mixing on the uptake efficiency, the VP/Tat/GFP mixture at the molar ratio which gave the highest uptake efficiency in each cell line was used. Three types of mixing order: VP, Tat and GFP; VP, GFP and Tat; Tat, GFP and VP were evaluated. The first two peptides were mixed and allowed to form complexes at room temperature (25±2°C) for 1 hr. Then, the third peptide was added and further incubated for 1 hr.

4.2.3. Cellular uptake study of Tat/GFP, VP/GFP and VP/Tat/GFP mixture

Tat/GFP, VP/GFP and VP/Tat/GFP mixture at all molar ratio as indicated in section 4.2.1 were used for cellular uptake study. The procedure of cellular uptake study was the same as described in section 3.2. The results were compared between fusion protein and protein mixture system and the system which gave better result was selected for further experiment with calcitonin.

Part 5 : *In vitro* calcitonin activity of Tat/sCT, VP/sCT and VP/Tat/sCT mixtures

5.1. Mixture preparation

sCT aqueous solution at 100 pg/ml was mixed with Tat or VP in MilliQ (MQ) water at different molar ratios of Tat/sCT or VP/sCT of 6:1, 3:1, 1:1 and 1/3:1. The mixture of VP/Tat/sCT were prepared at different molar ratios of 1:6:1, 6:1:1, 1:3:1, 3:1:1, 1:1:1, 1/3:1:1 and 1:1/3:1. The mixtures were incubated at room temperature (25±2°C) for 1 hr.

5.2. Physical properties of the mixture

5.2.1. Sizes and zeta potential determination

The mean particle size of sCT, Tat, VP, Tat/sCT mixture, VP/sCT mixture and VP/Tat/sCT mixture were determined with the same procedure described in section 4.1.2. The mixture solution was lyophilized to get the dry powder for DSC and FT-IR analysis.

5.2.2. Differential scanning calorimetry (DSC)

Thermograms of sCT, Tat, VP, Tat/sCT mixture, VP/sCT mixture and VP/Tat/sCT mixture were obtained with a DSC. Briefly, accurately weighed 3 mg of each sample was placed onto standard aluminum pans and sealed. An empty pan was used as a reference. DSC scans were performed at a heating rate of 10°C/min in a nitrogen atmosphere. To calibrate temperature and energy scale of the DSC instrument, aluminum oxide was used as a standard reference material.

5.2.3. Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectroscopy sCT, Tat, VP, Tat/sCT mixture, VP/sCT mixture and VP/Tat/sCT mixture were performed to study the interaction between sC and V. Samples were mixed with KBr powder and the KBr disks were prepared by using pressing force of 7.54 kgf/cm2. The IR absorbance were scanned at the range from 400 to 4000 cm^{-1} using FT-IR spectrophotometer.

5.3. *In vitro* calcitonin activity experiment

In vitro calcitonin bioactivity was determined in HT-29 and KB cells. The amount of 1×10^5 cells were seeded into each well of a 24-well plate and grown to 90% confluence. Then, the culture medium was removed and replaced with 1 ml of 100 pg/ml sCT, Tat, VP or various molar ratio of Tat/sCT mixture, VP/sCT mixture and VP/Tat/sCT mixture (equivalent to 100 pg/ml of sCT) dissolved in medium

containing 0.2 mg/ml calcium chloride. DMEM was used as control. After 1 hr incubation at 37°C, samples were removed and cells were washed twice with PBS. DI water (500 µl) was added into each well and cells were lysed by sonication using ultrasonic bath (Model 690D, Crest, NJ, USA) for 1 hr. After removal of the cell debris by centrifugation, the supernatants were collected for intracellular calcium measurement using calcium OCPC kit and detected the purple coloured complex at 570 nm by microplate reader. The intracellular calcium level was calculated as relative to control.

5.4. Determination of poliovirus receptor (PVR) expression

The overexpression of PVR (*CD155*) on HT-29 and KB cell surface was determined. Total cellular RNA was extracted from cultured cells with Nucleospin RNA II (Macherey-nagel, Düren, Germany) according to the manufacturer's instructions. The reverse transcription reaction and PCR amplification were performed in iCycler thermal reactor. Primers were designed from the sequence of the human *CD155* gene (Koike et al., 1990). They were designed to amplify cDNA but not contaminating genomic DNA. The primers used to locate the expression of the *CD155* gene in a region corresponding to the extracellular domain of the protein, which is conserved in all splice variants (Masson et al., 2001). The sequences of these primers were 5'-TATCTGGCTCCGAGTGCTT GCC-3' and 5'-ATCATAGCCA GAGATGGATACC-3' for forward primer and reverse primer, respectively. Amplified fragments were identified by electrophoresis on a 1% agarose gel stained with ethidium bromide. For quantification, the bands densities were determined by the Quantity One Program Analysis using Gel documentation.

Part 6 : *In vivo* calcitonin activity of Tat/sCT, VP/sCT and VP/Tat/sCT mixtures

6.1. Animals

In vivo calcitonin bioactivity assessment was performed in Sprague Dawley rat weighing between 200-250 g. After 3 days of acclimatization, the rats were divided randomly into groups of three. The animal experiments in this study were conformed to the guidelines of the Animal Welfare Commission of Chiang Mai University.

6.2. Subcutaneous administration of sCT

For the positive control studies, sCT in 0.9% normal saline solution (NSS) at the dose of 10, 50, 100, 250 and 500 µg/kg was subcutaneously injected to each group of rats at the dorsal skin using a 27-gauge needle. At 0, 1, 2, 4, 8, 12 and 24 hr after injection, blood samples (100 µl) were collected from tail vein, centrifuged at 5,000 g at 15°C for 10 min. The supernatant was collected and assayed the serum calcium level by calcium OCPC kit. The relative serum calcium was calculated comparing with control.

6.3. Oral administration of Tat/sCT, VP/sCT and VP/Tat/sCT mixtures

Tat/sCT mixture, VP/sCT mixture and VP/Tat/sCT mixture at molar ratio which showed the highest *in vitro* activity in KB and HT-29 cells were selected for oral administration. sCT (12.5 µg/ml), Tat/sCT mixture, VP/sCT mixture and VP/Tat/sCT mixture (equivalent to sCT of 12.5 µg/ml) in MilliQ water were prepared. The amount of sCT Tat/sCT mixture, VP/sCT mixture and VP/Tat/sCT mixture equivalent to 50 µg/kg of sCT was orally administered to each group of rats using 4 inches feeding tube. At 0, 1, 2, 4, 8, 12 and 24 hr after administration, blood

samples (100 µl) were collected, assayed the serum calcium as described in section 7.2 and calculated as the relative serum calcium comparing with the control. Further transdermal absorption and chemical stability study of the mixture which demonstrated highest in vivo hypocalcemic activity was performed in comparing with sCT solution.

6.4. Transdermal absorption of sCT and Tat/sCT at 1:1 molar ratio

The permeation through the excised rat skin was compared between sCT aqueous solution and Tat-sCT mixture at 1:1 molar ratio. The concentration of sCT in each sample was fixed at 2.5 mg/ml. The experiment was performed as described in section 4.1.7. sCT contents in viable epidermis and dermis (VED) and the receiver chamber were analyzed by HPLC. A phenomenex C 18 (250x4.6 mm, 10µm) HPLC column was used. The mobile phase was a mixture of 0.05% TFA in acetonitrile (ACN) as solvent A and 0.1% trifluoroacetic acid (TFA) in water as solvent B with UV detection at 214 nm. HPLC was performed at ambient temperature with the gradient of solvent A: solvent B at 7:93. The gradient of mobile phase was altered gradually to 100:0 of solvent A: solvent B. The mobile phase was filtered through 0.45 µm membrane filter before use. The flow rate was set at 1.0 ml/min. The injection volume was 20 µl. The peak areas were calculated and the concentrations of sCT were determined from the standard curve.

6.5. Chemical stability of sCT and Tat/sCT mixture

An aliquot of the freshly prepared sCT aqueous solution and Tat/sCT mixture (1:1 molar ratio) with the concentration of sCT at 1 mg/ml were filled in transparent vials. The vials were kept at 4±2 ºC, room temperature (27±2 ºC) and 45±2 ºC for 1 month. At 1, 2 and 4 weeks, the samples were withdrawn and the sCT

contents in the samples were analyzed by HPLC according to the procedure previously described in section 7.4.