CHAPTER III

MATERIALS AND METHODS

Materials

Agarose , Lot No. AG6431, Vivantis, Malaysia

Ammonium acetate, Lot No. F3B273, Univar, Australia

Antibiotic-Antimycotic , Lot No. 1402760, Gibco, USA

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

Chitosanase (*Bacillus* sp. PI-7S), Lot No. L6040418 C7071961, US Biological, USA

D-Glucose anhydrous, Lot No. F2H113, Univar, Australia

1 kb DNA Ladder, Lot No. 6011, Vivantis, Malaysia

Dulbecco's Modified Eagle Medium High Glucose, Lot No. 287370, Gibco, USA

Ethanol, Lot No. K35754083 608, Merck, Germany

Ethidium bromide, Lot No. 1376B017, Vivantis, Malaysia

Ethylenediaminetetraacetic acid disodium dehydrate salt, Lot No. 7727,Mallinckrodt, USA

Feotal bovine serum, Lot No. 41Q6175K, Gibco, USA

Glacial acetic acid, Lot No. K32754317 349, BDH, UK

Glycolic acid, Lot No. 1300322 14306165, Fluka, USA

HeLa cell, human epithelial cervix carcinoma cell lines, were kindly provided from the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Hydrochloric acid, Lot No. A44033, J.T.Baker, USA

Kanamycin sulfate, Lot No. 0396, Meiji, Japan

Lactic acid, Lot No. 1280989 21306P21, Fluka, Spain

LB Broth, Miller (Luria-Bertani), Lot No. 6080249, Becton Dickinson, France

Lipofectamine™ Reagent, Lot No. 1371191, Invitrogen, USA

6x Loading Dye, Lot No. 4101, Vivantis, Malaysia

Lyzozyme (egg white), Lot No. NBQ A30E, Bio Basic, Canada

OPTI-MEM®I Reduced Serum Medium, Lot No. 1382451, Gibco, USA

Phenol : Chloroform : Isoamyl Alcohol 25 : 24 : 1, Lot No. 075K0720, Sigma, USA

Plasmid Enhanced Green Fluorescence Protein-C2 (pEGFP-C2), 4.7 kb, Clontech, USA

Phosphate Buffered Saline (PBS) pH 7.4, Lot No. 1380926, Gibco, USA

Potassium acetate, Lot No. 59049, Sharlau, Spain

2-Propanol, Lot No. 0380943, Fisher, UK

Ribonuclease A, Lot No. L5022550 C 5022550, US Biological, USA

RNase-free DNase was purchased together with reaction buffer and stop solution, Lot No. 19303011, Promega, USA

Sodium chloride, Lot No. K21636604 514, Merck, Germany

Sodium dodecyl sulfate, Lot No. 0248390, Fisher UK

Sodium hydroxide, Lot No. B281598 308, Merck, Germany

Sodium sulfate, Lot No. T4593049 930, Merck, Germany

Thiazolyl blue tetrazolium bromide (MTT), Lot No. 085K5304, Sigma, USA

Tris/Acetic acid/EDTA (TAE) buffer, Lot No. 210003220, Bio-Rad, USA

Tris base, Lot No. 034964, Fisher, USA

Trypsin-EDTA, Lot No. 300163, Gibco, Canada

Equipment

Analytical balance, Model AG204, Mettler Toledo, Switzerland

Analytical balance, Model PB3002, Mettler Toledo, Switzerland

Centrifuge, Model 2K15, Sigma, Germany

Flow cytometer, FACS Calibur CellQuest Pro Software, Becton Dickinson, USA

Fluorescence microscope, IX51, Olympus, Japan

Laminar air flow, Model HBB 2448, Holten, USA

Microplate reader, Model 3550, Bio-Rad, USA

Orbital Shaker, Model SO3, Stuart scientific, UK

pH meter, Model 210A, Thermo Orion, USA

Transmission Electron Microscope, Model JEM-1230, JEOL, Japan

UV/VIS Spectrophotometer, Model V-530, Jasco, Japan

Vortex mixer, Scientific Industries, USA

Water-jacketed Incubater, Model 3164, Forma Scientific, USA

Zetasizer, NanoZS Malvern Instrument, UK

Laboratory supplies

Centrifuge tubes, Corning, UK

Glasswares, Pyrex, USA

Microcentrifuge tubes

Pipette tips, Corning, UK

0.2 μm syringe filter, Corning, Germany

Tissue culture flasks, Nunc, Denmark

Tissue culture plates, Nunc, Denmark

Methods

1. Plasmid DNA and preparation of plasmid DNA

pEGFP-C2, encoding a green fluorescent protein, was amplified in *Escherichia coli* and prepared by alkaline lysis technique. The preparation was modified from Sambrook and Russell (2001). Briefly, a single colony of E. coli was inoculated with LB Broth containing kanamycin, the selected marker. The culture was incubated at 37°C with vigorous shaking overnight. The bacterial cells were harvested from the culture by centrifugation and supernatant was decanted. The cell pellets were resuspended and 1% sodium dodecyl sulfate (SDS) in 0.2 N sodium hydroxide solution was added. The strongly anionic detergent at high pH opened the cell wall, denatured chromosomal DNA and protein, and released plasmid into the supernatant. Then, the potassium acetate solution was added and mixed gently by swirling. A flocculent white precipitate consisting of chromosomal DNA, protein and cell wall was formed. The precipitate was eliminated by centrifugation and then was discarded. The nucleic acid was precipitated by adding ethanol and, then ribonuclease A (RNase A) was added to digest RNA.

Phenol/chloroform/isoamyl alcohol 25:24:1 was added to remove the remaining protein. And DNA was precipitated again with ethanol. Then, the pellet was dissolved in water and the plasmid DNA (pDNA) was measured for the concentration and purity.

The concentration of pDNA was determined by measuring UV absorbance at 260 nm (1 OD = 50 μg/ml). The purity of pDNA was evaluated by OD_{260}/OD_{280} ratio higher than 1.8 (Huang et al., 2005).

2. Preparation of chitosan-pDNA nanoparticles

There were three variables in this study, one was medium of chitosan (acetic acid, lactic acid and glycolic acid). Another was medium of pDNA (5 mM sodium sulfate, 5 mM sodium chloride and water). The medium of pDNA acted as a coacervating agent and pDNA in water represented the formulation without coacervating agent. The last was amino group to phosphate group ratio or N/P ratio (0.5:1, 1:1, 2:1, 3:1, 5:1 and 7:1).

The nanoparticles were prepared by complex coacervation method. This preparation was modified from Mao et al. (2001). Chitosan was dissolved in three types of medium with gentle heating and the pH of chitosan solutions were adjusted to 5.5 with sodium hydroxide. The solutions were diluted to obtain chitosan in 5 mM sodium acetate buffer, chitosan in 5 mM sodium lactate buffer and chitosan in 5 mM sodium glycolate buffer. The pH of diluted solutions was readjusted to 5.5, and sterile filtered through a 0.2 μm filter. The nanoparticles were prepared at selected N/P ratios by varying the concentration of chitosan. pDNA was dissolved in three types of medium at a fixed concentration of 100 μg/ml. Chitosan solutions and pDNA solutions were heated to 55°C separately. Then, equal volume of chitosan solutions and pDNA solutions were quickly mixed together and vortexed with maximum speed for 30 seconds. The nanoparticles were left at room temperature for 30 minutes to form complete complexes. All nanoparticles were freshly prepared before used.

3. Physical properties measurement of chitosan-pDNA nanoparticles

3.1 Measurement of particle size, PI and zeta potential

Measurement of particle size of the obtained nanoparticles was performed by photon correlation spectroscopy (PCS). One milliliter of the samples was added into a cuvette with no further dilution, and then characterized using Zetasizer. The zeta potential measurements were performed with the same instrument. Particle size and zeta potential of the samples were measured in tripicates and reported in a form of mean±standard deviation (SD).

3.2 Morphological Observation

The morphology of the nanoparticles was performed by transmission electron microscopy (TEM). A drop of the freshly-made nanoparticles was placed on a copper grid. The sample was negatively stained with uranyl acetate solution and left to dry under room temperature. Observation was done using a transmission electron microscope.

4. Gel retardation assay

The nanoparticles formation was evaluated by using agarose gel electroporesis. The nanoparticles formulated with chitosan in acetic acid and pDNA in sodium sulfate (CSA/DNA S nanoparticles) at N/P ratios of 0.5:1, 1:1, 2:1, 3:1, 5:1 and 7:1 were used in this srudy. The samples were mixed with 6x loading dye to a final loading dye concentration of 1x. Then, 15 μl of the mixtures were loaded onto a 0.8% agarose gel in TAE buffer. The samples were run on the gel at a constant voltage of 80 volts for 50 minutes. Then gel was stained with 0.5 μg/ml ethidium bromide and visualized under UV light. The naked pDNA was used as a control and a 1 kb DNA ladder as a reference.

5. Protection effect of nanoparticles to pDNA

Stability of chitosan-pDNA nanoparticles from deoxyribonuclease (DNase) degradation was examined using DNase I. This assay was modified from Mao et al. (2001), Xu et al. (2004), Huang et al. (2005) and Guliyeva et al. (2006). Briefly, 1 μg of naked pDNA in 20 μl of solution or chitosan-pDNA nanoparticles 20 μl containing 1 μg of pDNA were incubated with 0.25 units of DNase I for 15 minutes at 37°C. The DNase activity was stopped by adding 6 μl of stop solution, a DNase I inhibitor, and incubated for 10 minutes at 65°C according to manufacturer's instruction (Promega, USA). Then, these nanoparticles were subjected to chitosanase and lyzozyme digestion.

Chitosan was sensitive to chitosanase and lysozyme digestion, although the site of digestion was different for these two enzymes. Lysozyme preferentially acted on N-acetyl-D-glucosamine sequences with more than three residues and could not act on D-glucosamine sequences (Aiba et al., 1992), whereas chitosanase more likely acted on D-glucosamine residues (Seino et al., 1991). Chitosanase from *Bacillus* sp. PI-7S showed the highest activity for highly deacetylated chitosan according to Seino et al. (1991). Therefore the combination of chitosanase from *Bacillus* sp. PI-7S and lysozyme was chosen to digest the chitosan-pDNA nanoparticles. The activity of chitosanase was 0.15-0.35 U/mg and that of lysozyme was >20,000 U/mg.

The chitosan digestion followed the procedure from Mao et al. (2001), Xu et al. (2004) and Huang et al. (2005). Mao et al. used chitosanase 0.08-0.1 mg/ml and lysozyme 10 μg/ml (digestion 1). Xu et al. used chitosanase 0.15U/ml and lysozyme 15U/ml (digestion 2), and Huang et al. used 5 μl of solution of 10 mg/ml chitosanase and 3 mg/ml lysozyme (digestion 3). The condition of digestion 1 and digestion 2 was 37°C for 4 hours, while that of digestion 3 was 37°C for 12 hours. The CSA/DNA S nanoparticles formulated at N/P ratio 2:1 (2:1 CSA/DNA S nanoparticles) was selected for the chitosan digestion study.

The integrity of the DNA was analyzed by 0.8% agarose gel electrophoresis as described above. Again the naked pDNA was used as a control and a 1 kb DNA ladder as a reference.

6. Cell line and cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic-antimycotic agents. The cells were maintained at 37°C in a humidified 5% CO_2 incubator and subcultured every 3 to 4 days using trypsin-EDTA.

7. *In vitro* transfection in HeLa cells

The *in vitro* transfection was modified from Jiang et al. (2007). HeLa cells were seeded 24 hours prior to transfection into a 24-well plate at a density of $5x10^4$ cells per well in 1 ml of complete medium. At the time of transfection, the complete medium was removed and the cells were rinsed with PBS.

7.1 Transfection with naked pDNA and chitosan-pDNA nanoparticles

Naked pDNA was used as a negative control. Naked pDNA 1 μg in 20 μl of medium of pDNA or the 20 μl of chitosan-DNA nanoparticles containing 1 μg of pDNA were mixed with 480 μl of non-serum DMEM and added to each well. The cells were incubated with the nanoparticles for 4 hours at 37°C in a humidified 5% CO_2 incubator. Then they were washed once with PBS and allowed to grow for further 24 hours in 1 ml of DMEM containing 10% FBS at 37 °C under 5% CO_2.

7.2 Transfection with Lipofectamine™-pDNA complexes

Lipofectamine™ Reagent, a commercial non-viral transfection vector, was selected as positive control. Lipofectamine™-pDNA complexes were prepared with a 2:1 ratio of Lipofectamine (μl): pDNA (μg). This preparation was followed the manufacturer's protocol and modified from Lavertu et al. (2006). Cells were incubated with Lipofectamine™-pDNA

complexes in 0.5 ml of OPTI-MEM®I Reduced Serum Medium for 4 hours at 37°C under 5% CO_2. They were washed once with PBS and allowed to grow for further 24 h in 1 ml of DMEM containing 10% FBS at 37 °C under 5% CO_2.

8. Analysis of *in vitro* transfection efficiency

8.1 Fluorescence microscope

Cells were directly viewed under a fluorescence microscope to obtain an estimation of the *in vitro* transfection efficiency.

8.2 Flow cytometry

In vitro transfection efficiency in HeLa cells was quantitatively assessed by flow cytometry. After transfection, cells were washed once with PBS and detached with trypsin/EDTA. Once detached, complete medium was added to inhibit trypsin and the cells were resuspended in the complete medium. Cell suspension was transfered into flow cytometry tubes and the transfection efficiency was analyzed using a flow cytometer. Transfection efficiency was evaluated by scoring the percentage of cells expressing green fluorescence protein (GFP).

9. Cell viability tests

Cell viability tests were evaluated by thiazolyl blue tetrazolium bromide (MTT) assay. This assay was modified from Jiang et al. (2007) and Corsi et al. (2003). HeLa cells were seeded in 96-well plate at a density of $1x10^4$ cells per well in 100 µl of complete medium. After incubation of the cells in a humidified 5% CO_2 incubator at 37°C for 24 hours, 100 µl of non-serum DMEM containing chitosan-pDNA nanopaticles or Lipofectamine™-pDNA complexes or various concentration of chitosan were added to each well. The incubation was continued for 72 hours. Cell viability was determined by the ability of the cells to cleave the tetrazolium salt MTT by the succinate dehydrogenase, the mitochondrial enzyme, to give a blue formazan crystal.

MTT was dissolved in PBS at a concentration of 5 mg/ml and sterile filtered through 0.2 μm filter. Then, 25 μl of the MTT solution was added to each well and incubated at 37°C under 5% CO_2. After further incubation for 4 h, 100 μl of acid-isopropanol was added to each well and mixed thoroughly using orbital shaker at 150 rpm to dissolve the blue formazan crystals. The plates were left at room temperature for 20 minutes to ensure that all crystals were dissolved. Then, they were read on microplate reader, using a test wavelength of 595 nm.

Cell viability (%) was calculated according to the following equation:

$$\text{Cell viability (\%)} = (OD_{595(sample)} / OD_{595(control)}) \times 100 \qquad \text{Equation (1)}$$

Where $OD_{595(sample)}$ represents a measurement from a well treated with nanoparticles or chitosan solutions and $OD_{595(control)}$ from a well treated with only DMEM