CHAPTER III

EXPERIMENTAL

Materials

1. Model protein

- Albumin from bovine serum (Lot No. 017K0726, Sigma-Aldrich, USA)

2. Polymers

- Pectic polysaccharide was isolated from durian rinds *Durio ziberthinus* as described earlier (Pongsamart et al.,1998; Illum,1999)
- High methoxy pectin (GENU®) (Lot No.GR81626, CP Kelco Germany GmbH, Germany)
- Low methoxy pectin (Amid CM 020®) (Herbstreith & Fox KG, New Zealand)

3. Additives

- Maltodextrin DE10 (Lot.No. DU301067, Nutrition Ltd., Thailand)
- Mannitol (Lot.No. H100509006, Forebest Chemical Co.,Ltd., China)
- Lactose monohydrate (Morkerel Meggle Wasserberg GmbH, Germany)
- Colloidal Silicon dioxide (Aerosil®200) (Lot.No. ZB56966, Wacker Chemie GMBH, Germany)
- Propylene glycol (Lot. No. 7879040502, Singapore)

4. Biomaterials

- RPMI 2650, human nasal epithelial carcinoma cell line (CCL-30™, American Type Culture Collection (ATCC), VA, USA)

5. Chemicals

- Mucin from porcine stomach, type II (Lot.No. 108K0010, Sigma-Aldrich, USA)

- Potassium dihydrogen orthophosphate (Lot. No. AF401428, Ajax Finechem, Australia)
- Sodium hydroxide (Lot. No.B131198214, Merck KGaA, Germany)
- Copper (II) sulfate solution (Lot.No. 048K5302, Sigma-Aldrich, USA)
- Bicinchoninic acid solution (Lot.No. 118K5300, Sigma-Aldrich, USA)
- Trypan blue cell culture tested (Lot.No. 25496TH, Sigma-Aldrich, USA)
- Minimum Essential Medium (MEM)-α medium (Cat No.32561037 GIBCO®, Invitrogen, USA)
- Fetal bovine serum (GIBCO®, Invitrogen, USA)
- Antibiotic-Antimycotic (100X) (GIBCO®, Invitrogen, USA)
- Hanks' balance salt solution (HBSS) (GIBCO®, Invitrogen, USA)
- 0.025% Trypsin in EDTA (GIBCO®, Invitrogen, USA)
- Phosphate buffer saline solution (PBS) pH 7.4 (GIBCO®, Invitrogen, USA)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA)
- Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, USA)
- FITC-phalloidin (fluorescein isothiocyanate conjugated phalloid) (Sigma-Aldrich, USA)
- Fluorescein isothiocyanate conjugate dextran molecular weight4000 (FD-4) (Sigma-Aldrich, USA)
- MOPS SDS Running Buffer (NuPAGE®, Invitrogen, USA)
- Tris-Glycerine SDS sample buffer (Novex® Invitrogen, Calsbad CA, USA)
- 12% Bis-Tris polyacrylamide gel (NuPAGE®Novex® 12%Bis-Tris Gel 1.0 mm, 10 well, Invitrogen, USA)
- SimplyBlue™ SafeStain (Invitrogen, Calsbad CA, USA)

6. Equipments

- Fourier Transform Infrared Spectroscopy (FT-IR) (Model 1760X, Perkin Elmer, UK)
- Analytical Balance (Model A200 S, Sartorius, Germany)
- Spray-dryer (Büchi model B290, Büchi Labortechnik, Flamil, Switzerland)
- Gel permeation chromatography (Water® Model 600E, Milford, USA)
- Sonicator bath (Elma®, Germany)

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- Laser light-scattering analyzer (Mastersizer2000, Malvern Instrument, Malvern, UK)
- Scanning electron microscope (Jeol model JSM5410 LV, Tokyo, Japan)
- Thermogravimetric analyzer (TGA, Mettler-Toledo, USA)
- Texture analyzer (Model TA.XT plus, Stable micro systems, UK)
- Spectrophotometer (Microplate reader, VICTOR^3, Perkin-Elmer, USA)
- Electrophoresis (XCell SureLock™ Mini-Cell, Invitrogen, USA)
- Spectropolarimetry (model J-715, Jasco, Japan)
- Spectrophotometer (Microplate reader Bio-RAD® Model 3550, USA)
- Hymocytometer (Boeco®, Germany)
- Millicell® ERS meter (Millipore, Bedford, MA, USA)
- Pipette Aid (Drummond®, USA)
- Centrifuge (Model 2K15, Sigma Germany)
- Laminar air flow (Model HBB 2448, Holten, USA)
- Vortex mixer (Scientific Industries, USA)
- Water-Jacketed Incubator (Model 3164, Forma Scientific, USA)
- Micropipette (Gilson® 57329H, France)
- Micropipette (Socorex® ACURA 825, Swizerland)
- Hot Air Oven (Heraeus Model 5090E)

7. Laboratory supplies

- Centrifuge tubes (Corning®, UK)
- Microcentrifuge tube (Corning®, UK)
- 6 Well Plate Transwell® permeable support 0.4 µm polyester membrane (Costar® USA)
- Cellulose acetate membrane 0.45µm (Whatman®, UK)
- Tissue culture flask (Corning®, UK)

Methods

1. Molecular characterization of pectin powder

Molecular chromatograms of two types of commercially available pectin and pectic polysaccharide was carried out using a Water 600E gel permeation chromatography (GPC). The 0.2% w/v of sample solution was prepared using 0.05 M sodium bicarbonate buffer (pH11) as dissolution medium and volume of 20 μl was injected into the GPC. The pectin was separated using the same aforementioned medium at a flow rate of 0.6 ml/min. Approximate molecular weight of each pectin was estimated using a standard curve obtained with different molecular weight Pullulans (MW : 5,900 – 708,000).

2. Preparation of spray dried pectic polysaccharide microparticles

Pectic polysaccharide [from durian rinds (*Durio ziberthinus*), isolated by hot water extraction followed by ethanol precipitation (Hokputsa et al.,2004)], was dissolved in ultrapurified water at various concentrations of 0.2 - 4.0 %w/v and then spray dried in a bench-top spray dryer (Büchi model B290, Büchi Labortechnik, Flamil, Switzerland) at the inlet temperature, aspirator rate and feed rate of 120°C, 80% (equivalent to 32 m^3/hr) and 3 ml/min, respectively, to obtain optimal pectic polysaccharide concentration. The liquid feed was pumped peristatically and fed through a two-fluid nozzle (0.7 mm internal diameter) where it was atomized into fine droplets.

The selected concentration of pectic polysaccharide was then spray dried with the formulations including maltodextrin (MT) as filler, colloidal silicon dioxide [Aerosil®(A)] as a glidant and propylene glycol (PG) as shaper. The suitable spray drying condition was achieved by varying the inlet air temperature (90, 100, 110, 120 °C), drying air flow rate (80 and 90 % of maximum aspirator rate) and liquid feed rate (3 and 5 ml/min).

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The selected spray drying condition was used to find the suitable formulation by varying the filler [lactose (L), mannitol (M)] and then the selected filler was spray dried with various amount of Aerosil® (A). The formulation was shown in Table 1.

Each excipient was separately dissolved in ultrapurified water and then mixed with pectic polysaccharide solution of optimal concentration before adjusting to final volume. The solution of each batch was spray dried. After obtaining the suitable condition and formulation, two types of commercial pectin (high methoxylate pectin, HM-pectin and low methoxylate pectin, LM-pectin) were similarly spray dried to compare with pectic polysaccharide.**Table 1**. Formulations (%w/v in water) used to spray dry at the optimal condition.

Formulation	Mannitol (M)	Lactose (L)	Aerosil (A)	Maltodextrin (MT)	Propylene glycol (PG)
P+MT+A+PG*	-	-	0.036	0.32	0.3
P+MT+A	-	-	0.036	0.32	-
PM	0.2	-	-	-	-
PL	-	0.2	-	-	-
PLA0.1	-	0.2	0.1	-	-
PLA0.12	-	0.2	0.12	-	-
PLA0.15	-	0.2	0.15	-	-
PLA0.2	-	0.2	0.20	-	-

* This formulation from AAPS PharmSciTech (Harikarnpakdee et al. 2006).
P : pectic polysaccharide

3. Preparation of pectic polysaccharide BSA-loaded microparticles

Each excipient in the formulation, selected by considering the characterized properties such as particle size, flowability, and mucoadhesion of obtained spray dried powders, and BSA were separately dissolved in ultrapurified water and spray dried in a bench-top spray dryer with the spray drying conditionThe quantity of BSA : polymer ratio by weight was studied at 1:3, 1:5 and 1:10.

4. Characterization of pectic polysaccharide microparticles

4.1 Production yield

The percentage of production yield was calculated from the weight of dried powders (w_1) collected from the collector and the sum of the initial dry weight of starting materials (w_2) as the following equation.

$$\text{Percentage of production yield} = \left(\frac{W_1}{W_2}\right) \times 100 \quad (1)$$

4.2 Particle size and size distribution

Particle size and size distribution of all obtained microparticles were measured by laser light-scattering method (Mastersizer2000, Malvern Instrument, Malvern, UK). Small amount of microparticles was dispersed in ten milliliters of 95%ethanol and sonicated in sonicator bath (Elma®, Germany) for about 30 seconds, in order to deaggregate the microparticles. The dispersion was loaded into a stirred sample cell, containing 95% ethanol as a measuring medium. The particle size was presented in the volume weighted mode and the 50% undersize diameter $d\ (v, 0.5)$was referred to as the particle diameter. The size distribution was determined by the span value. Triplicate measurement was conducted.

4.3 Particle morphology

Spray dried powders were mounted onto double-faced adhesive tape, which was attached on a sample stub. The samples were sputtered with gold and viewed under a scanning electron microscope (Jeol model JSM5410 LV, Tokyo, Japan)

4.4 Determination of bulk density and flowability

The apparent powder bulk density (defined as the ratio of the powder weight over the bulk volume) of the spray-dried products was determined in the cylindrical glass tube filled with accurate weight of powders. The percentage compressibility can

be used as indirect method of measuring powder flow from bulk density because it is dependent on particle packaging and changes as the powder consolidates. A consolidated powder is likely to have a greater arch strength than a less consolidated one and may therefore be more resistant to powder flow (Staniforth 2002). It was determined by measuring the unsettled apparent volume, V_0 and the final tapped volume, V_f, of the powder after tapping the spray-dried powder until no further volume change occur.(n=3) The compressibility index are calculated as the following equation:

$$\%\text{Compressibility Index} = 100 \times \left[\frac{V_0 - V_f}{V_0}\right] \quad (2)$$

4.5 Moisture content

Moisture content of the spray-dried powder was measured using a thermogravimetric analyzer (TGA, Mettler-Toledo, USA) linked to a data station (STARe software). Samples (~5 mg) were loaded in aluminium oxide 70 μl pans and heated at 10 °C/ml under 20 ml.min nitrogen gas purge. The moisture content was based on weight loss between 30 °C and 150 °C

4.6 Swelling index

The study of the swelling of spray dried powders with respect to time modified from Ph. Eur. Method 2.8.4 was performed by placing a fixed weight of spray-dried powder in a graduated cylinder before adding ethanol and purified water, then recorded the volume at time zero (V_0). The cylinder was vigorously shaken every 10 minutes for 1 hour and then allowed to stand for 3 hours. At 1.5 hours after beginning of the test, liquid retained in the layer of powders was discarded. The volume occupied by the pectic polysaccharide powders at 4 hours was recorded (V_t). The swelling index (SI) was calculated as following:

$$\text{Swelling Index} = \left[\frac{V_t - V_0}{V_0}\right] \quad (3)$$

4.7 Moisture adsorption

Spray-dried powders were precisely weighed for each formulation (m_1) and added to glass weighing vials. All vials were then stored in the simulated climate cabinet which was working fluently at the room temperature and at relative humidity (RH) of 75%. After 48 hours running of the unit, the vials were taken out and weighed again (m_2). The percentage weight gain of each formulation after storing at 75% RH was then calculated from the following equation (n=4).

$$\% \text{ Moisture adsorption } = 100\% \times \left[\frac{M_2 - M_1}{M_1}\right] \quad (4)$$

4.8 Mucoadhesive property

The mucoadhesive properties of microparticles were evaluated using a texture analyzer with a 5 kg load cell. A hydrophilic membrane (cellulose acetate membrane, 0.45 μm pore size) was first soaked for 15 min in aqueous mucin solution (10% mucin from porcine stomach, Type II), and then horizontally attached to the upper end of the Texture Profile Analysis (TPA) probe by using double-sided adhesive tape. Microparticles were placed on the lower probe, dropped pH 6.0 phosphate buffer solution and left for 1 minute. The analytical probe containing the membrane was moved down to attach the surface of microparticles for 90 seconds to ensure intimate contact between the membrane and the sample. The probe was then moved up in a vertical direction at a constant speed of 0.5 mm/s and the force required to detach the mucin membrane from the surface of micropaticles was determined from the resultant force-time plot. All measurements were performed in triplicate.

4.9 Infared absorption study

The FT-IR spectra of raw material BSA, three types of pectin (pectic polysaccharide and two types of commercial pectin) and three kinds of spray dried microparticles, including BSA and pectin in the ratio of 1:1, were examined by using the potassium bromide disc with an infared spectrophotometer (Fourier-Transform Infared Spectrophotometer model 1760X, Perkin Elmer, USA) in the range of 4000 –

450 cm^{-1}

4.10 Protein content determination

Microparticles were dissolved in distilled water. BSA content was determined by using bicinchoninic acid assay (MicroBCA, Sigma-Aldrich, USA). The reaction was run at 37°C for 2 hours. Optical density of the sample solution was read at 560 nm on a spectrophotometer (Microplate reader, VICTOR^3, Perkin-Elmer, USA) and then quantified from the calibration curve. Actual protein content was then calculated as following:

$$\%\text{protein content} = \frac{\text{actual amount of protein} \times 100}{\text{amount of microparticles}} \tag{5}$$

4.11 *In vitro* protein release

The in vitro protein release test of the microparticles was performed on modified Franz diffusion cell with cellulose acetate filter; pore size 0.45 μm. The receptor compartment contained 0.2% sodium lauryl sulfate in water and maintained 37 ± 1°C. The filter was equilibrated before carefully dispersing the microparticles of 10 mg onto the donor side. Samples were periodically withdrawn from the receptor compartment 0.5 ml, replaced with the same amount of sodium lauryl sulfate solution and assayed by MicroBCA assay.

5. Protein properties

5.1 Protein integrity

The integrity of BSA was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). BSA was discovered from microparticles by dissolving in distilled water. One part of the samples was mixed with one part of reducing sample buffer (5% β-mercaptoethanol in Novex® Tris-Glycerine SDS sample buffer, Invitrogen, Calsbad CA, USA) and heated at 95°C for five minutes. The mixture equivalent to 10 μg of BSA was loaded onto a pH 8.8 12% Bis-Tris

polyacrylamide gel (NuPAGE®Novex® 12%Bis-Tris Gel 1.0 mm, 10 well, Invitrogen, USA) and subjected to electrophoresis (XCell SureLock™ Mini-Cell, Invitrogen, USA) in NuPAGE® MOPS SDS running buffer (Invitrogen, Calsbad CA, USA) at 150 V for about two hours. The gel was stained with SimplyBlue™ SafeStain (Invitrogen, Calsbad CA, USA) for one hour and destained several times with distilled water until the protein bands were visualized.

5.2 Protein secondary structure

Circular dichroism was used to observe the secondary structure of BSA from the preparations compared with raw material. Elipicity (θ, mdeg) of the solutions was recorded between 190-250 nm on a spectropolarimetry (model J-715, Jasco, Japan). Molar ellipticity ([θ], deg cm^2/decimal) was then calculated by the following equation:

$$[\theta]=\theta\ Mp/10\ 000\ n\ C'\ l \qquad (6)$$

where Mp was the molecular weight of BSA (66 430 Da), n was the number of amino acid residues of BSA (583 residues), C′ was the concentration of BSA in sample solution (g/ml) and l was the path length of cell (0.1 cm). CD spectra was obtained by plotting molar elipticity against wavelength. The solution of corresponding blank pectic polysaccharide microparticles of each sample was prepared as afore mentioned and run as background.

6. *In vitro* study in cell culture

6.1 Cell cultures

Nasal cell line (ATCC RPMI2650) isolated from a human nasal septum carcinoma were grown in horizontal plastic cultural flask. Cells were maintained in minimum essential medium (MEM) alpha-medium (GIBCO Cat. No.32571063) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution and incubated in humidified atmosphere of 5%CO_2 at 37°C until 70-80% confluence.

These cells were then trypsinized and seeded in 96-well plates for cytoxicity study and seeded in 6-well Transwell® plates for permeation study

6.2 Cytotoxicity study

Nasal cells were cultured in 96-well plate at a density of 3 x 10^5cells/ml for 100 μl/well. In the next 2 days, 100-μl samples of particle loading concentration of 1.0, 2.0, 3.0 mg/ml (6 wells per concentration-group plus one control group) were incubated in culture medium as described above for 48 hours. Control group consisted of cells in media was processed identically and incubated simultaneously as treated groups. The 50-μl MTT solution (1 mg/ml in MEM) was added to each well after removing sample in media solution and had been incubated for 3 hours. The medium was replaced with 100 μl dimethyl sulfoxide (DMSO), agitated for 15 – 20 minutes and the absorbance was read at 595 nm on Bio-RAD® Model 3550 microplate reader. Cell viability was expressed as a percentage of the control group.

6.3 Permeation study

Nasal cells were seeded on polyester filters, 0.4 μm pore size, of 6-well Transwell® at a density of 4 x 10^5 cells/ cm^2. The apical and basolateral compartments received 1.5 and 2.6 ml of culture medium, respectively. These were cultured until monolayer was reached and the medium was changed every second day. The monolayer integrity was assessed firstly by the inverted microscope, and confirmed again before permeation study by the measurement of trans-epithelial electrical resistance (TEER).

Permeation of FITC-labeled dextran (FD-4) model substance (MW 4,000) through nasal epithelial monolayer in comparison to blank filter was to quantify the barrier function of the cultivated human cell monolayer. The FD-4 in Hanks' balance salt solution (HBSS) at the concentration of 200 μg/ml was added to the apical compartment of Transwell. The 2-ml samples were withdrawn from the basolateral chamber and replaced by fresh HBSS at time intervals. The amounts of permeated FD-4 were determined by a fluorometry at the excitation of 490 nm and emission of

515 nm (Microplate reader, VICTOR^3, Perkin-Elmer, USA).

For drug permeation study, the confluent cell monolayers were rinsed and filled with HBSS at both compartments and allowed to equilibrate for 1 hour. Microparticles were scattered over the polyester filters. The 2-ml samples were withdrawn from the basolateral chamber and replaced by fresh HBSS at time intervals. The amounts of permeated BSA were determined by MicroBCA assay. Results were expressed as cumulative transport as a function of time.

6.4 Tight junction integrity study

TEER measurement was performed during the drug permeation at 60 minute intervals until 5 hours and 24 and 48 hours after permeation experiment. For negative control, no microsphere was applied to the monolayers. After permeation study, the microspheres were carefully removed. The monolayers of cell culture were fixed with 3.7% formaldehyde in PBS, washed 3 times with PBS, permeabilized cell with 0.5%tritonX-100 in PBS, washed 3 times with PBS, and stained with a 50µg/ml FITC-labeled phalloidin solution in PBS (Sigma-Aldrich, USA) after that observed under a fluorescence microscope (E200, Nikon, Japan)

6.5 Viability of cell monolayer

Plasma membrane integrity was analyzed by performing trypan blue dye exclusion. Both the apical and basolateral sides of the cell monolayers were washed several times with PBS after finishing permeation experiment. The cell monolayer was typsinized and diluted with the same amount of 0.4% trypan blue solution (Sigma Aldrich, USA). Viable and non viable cells presented as absence and presence of intracellular trypan blue were counted separately by a heamocytometer slide, calculated as percentage of viable cells.