# CHAPTER III

# MATERIALS AND MATHODS

## Materials

### A. Model drugs

1. Kingcol Brilliant Blue FCF (Lot No. 5-1183, Butterfield food ingredients limited, England)
2. Albumin from bovine serum, lyophilized powder (Lot No.SLBF0550V, Sigma-Aldrich, USA)
3. Albumin, Fluorescein isothiocyanate Conjugate bovine (Lot No. 080M7400, Sigma-Aldrich, USA)

### B. Polymers

1. Hydroxypropyl methylcellulose K15M; HPMC K15M, Methocel K15M (Batch No. PD300936, Colorcon Asia Pacific Pvt. Ltd, Singapore)
2. Hydroxypropyl methylcellulose E5; HPMC E5, Methocel E5LV (Batch No. UL24012404, Colorcon Asia Pacific Pvt. Ltd, Singapore)
3. Low molecular weight chitosan (MW 50,000 Dalton, 75-85% de-acetylation) (Lot No. MKBD3830, Sigma-Aldrich, USA)
4. Poly (butyl methacrylate-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate); Eudragit® E PO (Batch No. G110931200, Evonik, Germany)
5. Methacrylic Acid and Ethyl Acrylate Copolymer; Eudragit® L 100-55 (Batch No. B060204006, Evonik, Germany)
6. Polyvinyl caprolactam - polyvinyl acetate - polyethylene glycol graft copolymer; Soluplus® (Lot No. 84414368E0, BASF, Germany)

### C. Additives

1. Colloidal silicon dioxide; Aerosil® 200 (Batch No. 4161061381, Nippon Aerosil, Japan)
2. Poly (ethylene glycol); PEG (average MW 3350) (Lot No. MKBJ3254V, Sigma-Aldrich, USA)

D. Chemicals

1. Calcium chloride, dehydrate (Lot No. AF703220, Ajax Finechem, Australia)
2. D-Glucose, anhydrous (Batch No. 071125, Ajax Finechem Pty, Australia)
3. 40% Formaldehyde solution (Germany)
4. Magnesium sulphate (Lot No. 559678, Srichand United Dispensary, Thailand)
5. Mucin from porcine stomach, type II (Lot No.108K0010, Sigma-Aldrich, USA)
6. Potassium chloride (Lot No. AF501338, Ajax Finechem, Australia)
7. Potassium phosphate, monobasic (Lot No. 091128, Ajax Finechem Pty, Australia)
8. Sodium chloride (Lot No. C27348, Mallinckrodt Baker, Malaysia)
9. Sodium hydrogen carbonate (Lot No. AF701192, Ajax Finechem, Australia)
10. Sodium hydroxide (Lot No. 0B334310E, Carlo Erba Reagents, Italy)
11. Sodium phosphate, dibasic (Lot No. 480141, Carlo Erba Reagents, Italy)
12. Sodium phosphate, monobasic (Batch No. AF502342, Ajax Finechem, Australia)

E. Equipment

1. 4-digit Analytical balance (Model A200S, Sartorius, Germany)
2. 4-digit Analytical balance (Model GR-120, A&D Company Limited, Japan)
3. 5-digit Analytical balance (Model X205T, Mettler-Toledo, Switzerland)
4. 5-digit Analytical balance (Model HR-202i, A&D Company Limited, Japan)
5. Attenuated total reflectance Fourier transform infrared spectrometer; ATR FT-IR (Model Spectrum One, Perkin Elmar, USA)
6. Centrifuge (Model 5810, Eppendorf, Germany)
7. Differential scanning calorimeter; DSC (S II EXSTAR 6000, Model DSC6200, Seiko Instrument, Japan)

8. Differential scanning calorimeter (Model DSC822$^{e}$, Mettler Toledo, USA)
9. Fourier transform infrared spectrometer; FT-IR (ALPHA compact FT-IR Spectrometer, Bruker Optics, Germany)
10. Jet mill machine (Current Jet, Model CJ-10, Nisshin Engineering, Japan)
11. High speed granulator (Erweka® Model AR 400, Erweka, Germany)
12. Hot air oven (Type UL 80, Memmert, Germany)
13. Inverse microscope (Model IX51, Olympus, Japan)
14. Karl Fischer moisture titrator (MKC-500, Kyoto Electronic MFG. Co., Ltd, Japan)
15. Mastersizer 2000 (Model APA2000, Malvern, UK) equipped with Scirocco 2000 (Model ADA2000, Malvern, UK)
16. Multilabel counter (Model Victor3TM, Perkin Elmar, USA)
17. Multipoint magnetic stirrer (VARIOMAG® Multipoint 6, Thermo Scientific, Germany)
18. Orion Air Dryer (Model RAX9, Orion Machinery, Japan)
19. Optical camera (Nikon Coolpix S3300, Nikon corp., Japan)
20. pH meter (FiveEasyTM FE20-1, Mettler-Toledo, Switzerland)
21. Planetary ball mill (Model PM 100, Retch GmbH, Germany) equipped with $ZrO_2$ chamber and 10 medium-sized balls
22. Sample mill (Model T1-500ET, C M T, Japan) equipped with stainless sample chamber and 4S-sized rod
23. Scanning electron microscope; SEM (Model JSM-6510A, JEOL, Japan)
24. Scanning electron microscope; SEM (Model JSM-6400, JEOL, Japan)
25. Shaking incubator (Model LSI-3016A, LabTech, Korea)
26. Spectrophotometer (Model UV-1800, Shimadzu, Japan)
27. Circular dichroism; CD (Model J-715-150L, Jasco, Japan)
28. Water circulation bath (Polystat CC1, Huber, Germany)
29. X-ray powder diffractometer; XRPD (MiniFlex II, Desktop X-Ray diffractometer, Rigaku, Japan)
30. Zetasizer (Model Nano-ZS, Malvern, UK)

F. Laboratory supplies

1. Polytetrafluoroethylene coated sheet (Teflon® sheet)
2. Microcentrifuge tube (Corning®, UK)
3. 96 well EIA/RIA plate (Costar®, USA)
4. QuantiPro™ BCA assay kit (Sigma-Aldrich, USA)
5. Polyethersulfone membrane filter (Supor®, PALL, USA)

## Methods

### 1. Film preparation by powder casting method I

The objective of film preparation method I was to investigate appropriate type of polymer that could form film by powder casting technique. The powder casting was a technique to produce matrix film by casting powder of polymer blend on a mold and curing by low energy process. A polymer blend consisted of film-forming polymer, coalescer, and glidant, all of which were in solid powder form. Polyvinyl caprolactam- polyvinyl acetate-polyethylene glycol graft copolymer (Soluplus®; S), low molecular weight chitosan (C), HPMC K15M (HK), HPMC E5 (HE), Eudragit® L 100-55 (EL), and Eudragit® E PO (EE) were used as film-forming polymers, while polyethylene glycol (PEG or P, average molecular weight 3350) and talc were used as coalescer and glidant, respectively. All powders were sieved through 80-mesh screen before using. Each film forming polymer was varies amount of PEG and then mix with glidant as shown in **Table III-1**. The obtained polymer blend was cast in mold upon a polytetrafluoroethylene coated sheet (Teflon® sheet) mounted on a leveled glass plate, covered with 465-g of glass plate on top as weight loading, and then put in a hot air oven at 65 °C for 12 hours.



**Table III-1** Percentage amounts of coalescer and glidant in polymer blends.

| Formulation code | Amount of coalescer (% w/w) | Glidant |
|---|---|---|
| X-0 | 0 | 10% w/w of film-forming polymer + coalescer |
| X-2 | 20 | |
| X-4 | 40 | |
| X-6 | 60 | |
| X-8 | 80 | |
| X-10 | 100 | |

*Remark: X = film forming polymer; S=Soluplus®, C = low molecular weight chitosan, HK = HPMC K15M, HE = HPMC E5, EL = Eudragit® L 100-55, and EE = Eudragit® E PO.*

### 1.1 Effect of weight loading on the formation of powder casting film

The purpose of this study was to explore the influence of weight loading on the characteristics of powder casting film. A selected formulation was prepared following the aforementioned powder casting technique. The weight of covered glass loaded on top was varied ranging from 0, 105, 155, 310, 465, and 730 g, respectively.

### 1.2 Effect of glidant type

Glidant is an agent which plays an important role in improving flowability of powder mixture and enable to maintain uniformity of mixture in compression process (137). Although there are several kinds of glidants available in markets, each of them has its own advantages. Talc (T) and colloidal silicon dioxide (Aerosil® 200: A) are the popular ones, but suitable concentration for gliding properties is different. T acts as glidant at concentration of 1-10%, while concentration of A is 10 times less (138).



Therefore, the purpose of this study was to evaluate efficiency of glidants used; T and A. The S formulation was chosen. T of 10% w/w or A of 1% w/w was added in S formulation with 60% w/w of PEG (**Table III-2**). The polymer blends were manually mixed for 10 minutes and cast following the same procedure in film preparation method I.

**Table III-2** Formulations with different types and concentrations of glidant.

| Ingredients | Formulation A | Formulation T |
|---|---|---|
| Film forming polymer | S | S |
| PEG (P) | 60% | 60% |
| A | 1% w/w of amount of S+P | - |
| T | - | 10% w/w of amount of S+P |

### 1.3 Mechanism of film formation by powder casting method

This study was performed in order to elucidate the possible mechanism of film formation by powder casting technique. Formulations having a good characteristic film with smooth surface were selected and prepared following the mentioned procedure. Polymer blends were cast on a polytetrafluoroethylene coated sheet mounted on leveled glass plates without any weight loading, and ovened at 65°C at different time intervals.

## 2. Film preparation by powder casting method II

The objective of film preparation II was to investigate appropriate weight ratio of selected film forming polymer to positive charge polymer which could form film by powder casting technique.

In film preparation II, two film forming polymers were combined. One of them was positively charged polymer; C or EE, because there were several literatures indicating that positive charge polymer such as C enhanced mucoadhesive properties of mucosal drug delivery, because of electrostatic interaction (91, 139-141) and transient opening tight junction (142). The other was selected from film forming polymer in film preparation I that could form a completely matrix film with 40% w/w and 60% w/w of PEG and provided film with smoothness and brittleness. Owing to concern about mucosal irritation (143, 144), 40% and 60% w/w of PEG was selected.

For film preparing method, a polymer blend consisted of two film-forming polymers, coalescer, and glidant. Weight ratios of film-forming polymers were from 10:0 to 0:10 of selected film forming polymer in film preparation I and either C or EE, and then mixed with 40% w/w or 60% w/w of PEG as well as 1% w/w of A as glidant. A polymer blend was cast on a polytetrafluoroethylene coated sheet mounted on a leveled glass plate and ovened in hot air oven at 65 °C for 3 and 4 hours for formulations containing 60% w/w and 40% w/w of PEG, respectively.

**Table III-3** Formulations of combined polymers used in film preparation: SC formulations.

| Formulation codes | Ratios of film-forming polymers | | P | A |
|---|---|---|---|---|
| | S | C | | |
| $S_4C_6$-4 | 4 | 6 | 40% w/w of S+C | 1% w/w of S+C+P |
| $S_5C_5$-4 | 5 | 5 | | |
| $S_6C_4$-4 | 6 | 4 | | |
| $S_7C_3$-4 | 7 | 3 | | |
| $S_8C_2$-4 | 8 | 2 | | |
| $S_{10}C_0$-4 | 10 | 0 | | |
| $S_4C_6$-6 | 4 | 6 | 60% w/w of S+C | 1% w/w of S+C+P |
| $S_5C_5$-6 | 5 | 5 | | |
| $S_6C_4$-6 | 6 | 4 | | |
| $S_7C_3$-6 | 7 | 3 | | |
| $S_8C_2$-6 | 8 | 2 | | |
| $S_{10}C_0$-6 | 10 | 0 | | |

*Remark: Subscripted numbers meant ratio of film forming polymers in combined polymers and for the last number, -4 and -6 meant 40% w/w and 60% w/w of PEG, respectively.*

**Table III-4** Formulations of combined polymers used in film preparation: SEE formulations.

| Formulation codes | Ratios of film-forming polymers | | P | A |
|---|---|---|---|---|
| | S | EE | | |
| $S_0EE_{10}$-4 | 0 | 10 | 40% w/w of S+C | 1% w/w of S+C+P |
| $S_3EE_7$-4 | 3 | 7 | | |
| $S_4EE_6$-4 | 4 | 6 | | |
| $S_5EE_5$-4 | 5 | 5 | | |
| $S_6EE_4$-4 | 6 | 4 | | |
| $S_7EE_3$-4 | 7 | 3 | | |
| $S_8EE_2$-4 | 8 | 2 | | |
| $S_9EE_1$-4 | 9 | 1 | | |
| $S_{10}EE_0$-4 | 10 | 0 | | |
| $S_0EE_{10}$-6 | 0 | 10 | 60% w/w of S+C | 1% w/w of S+C+P |
| $S_3EE_7$-6 | 3 | 7 | | |
| $S_4EE_6$-6 | 4 | 6 | | |
| $S_5EE_5$-6 | 5 | 5 | | |
| $S_6EE_4$-6 | 6 | 4 | | |
| $S_7EE_3$-6 | 7 | 3 | | |
| $S_8EE_2$-6 | 8 | 2 | | |
| $S_9EE_1$-6 | 9 | 1 | | |
| $S_{10}EE_0$-6 | 10 | 0 | | |

*Remark: Subscripted numbers meant ratio of film forming polymers in combined polymers and for the last number,-4 and -6 meant 40% w/w and 60% w/w of PEG, respectively.*

3. Pulverization processes

3.1 Film pulverization by jet mill (JM)

Film formulations obtaining from selected film-forming polymer in film preparation I with 40% and 60% w/w of PEG as well as selected combined polymers in film preparation II which could form smooth and brittle film were chosen to pulverize by JM. All selected polymeric films were coarsely ground by high speed granulator with 30-mesh sieve and then pulverized by a JM machine supplying with 6-7 bars of cool dry air for 10 minutes before collecting powders. The collected powders were kept in a desiccator until further use.

3.1.1 Effect of cycle numbers

The objective of this study was to investigate the appropriate cycle number of JM process providing appropriate particle size and size distribution. The selected formulation was milled by JM process with different milling cycles from 1 cycle to 3 cycles.



3.1.2 Effect of feeding amounts

In order to evaluate effect of feeding amounts, the selected formulation was pulverized with an optimum condition by JM process with different feeding amounts ranging from 40 to 150 g.

3.2 Film pulverization by planetary ball mill (PBM)

Effect of milling time

A film formulation was selected in order to be milled by PBM. Film formulation was coarsely ground by high speed granulator with 30-mesh sieve before pulverizing by PBM having zirconium oxide ($ZrO_2$) chamber with 10 medium-size balls and operated with 400 rpm for 10, 30, and 60 minutes.

### 3.3 Film pulverization by cryomill (CM)

The 3 g of selected polymeric films were milled by using Sample mill with stainless sample chamber and 4S rod under cryogenic condition supplied by liquid nitrogen to control temperature of chamber between (-)160 and (-)180°C. The process was operated for 90 minutes. The obtained powders were kept in a desiccator until further use.

### 3.4 Pulverization of BSA incorporated film formulations

The selected formulations were incorporated with lyophilized BSA powder in the amount of 10% w/w, prepared by powder casting and then pulverized the formulations with the different milling processes under appropriate conditions. The obtained powder samples were retained in desiccator for further studies.



## 4. Characterizations of powder blends prior to casting

### 4.1 Bulk and tapped density

The apparent bulk density is defined as a ratio of the mass of the untapped powder sample to its volume depending on both the density and spatial arrangement of powder particles in sample (145). Accurate weight ($W_0$) of a powder sample was filled in graduated cylinder and then the apparent volume ($V_0$) was read. The tapped volume ($V_t$) was determined by tapping the cylinder until the different of succeeding volume is less than 2%. The apparent bulk and tapped density can be calculated in g/ml as the following equation:

$$\rho_{bulk} = W_0/V_0 \quad [1]$$

$$\rho_{tapped} = W_0/V_t \quad [2]$$

### 4.2 Flowability

Compressibility index and Hausner ratio become one of the common reported methods for testing powder flow characteristics, because of simple, fast,

and popular method of predicting. They are evaluated by measuring and calculating using bulk density ($\rho_{bulk}$) and tapped density ($\rho_{tapped}$) as follows:

$$\text{Compressibility Index (\%)} = 100 \times [(\rho_{tapped} - \rho_{bulk})/\rho_{tapped}] \quad [3]$$

$$\text{Hausner ratio} = \rho_{tapped}/\rho_{bulk} \quad [4]$$

The measurement was conducted in triplicate (145).

## 5. Characterizations of matrix films

### 5.1 Physical evaluation of film formation by powder casting method

After at 65°C for 12 hours, the obtained samples were peeled off from polytetrafluoroethylene coated sheet. Smoothness, brittleness, and completeness of obtained films were physically and visually observed and optical photographs were also taken.

### 5.2 Film characteristics by scanning electron microscopy (SEM)

SEM studies on film topography of samples were carried out using JSM-6400 SEM. Before observation under the microscope, the samples were attached on a sample stub and sputter-coated with gold. SEM images were taken at various magnifications.

### 5.3 Content and weight uniformity of Brilliant Blue in film preparation

To investigate the effect of glidant type, the selected formulations incorporated with Brilliant Blue (BB) of 10% w/w as an indicator were determined for their weight and content uniformity. Three obtained film formulations were accurately weighed in weight uniformity, while the content uniformity of BB in formulations was analyzed by UV spectrophotometer. Sample was randomly selected and prepared by weighing matrix film approximate 50 mg, dissolving in 1000 ml of ultrapure water, and analyzing at 629 nm. Matrix film without BB was used as a blank. The measurement was conducted in triplicate and %labeled amount of BB in each formulations was calculated.

## 6. Characterization of pulverized formulations

### 6.1 Bulk density, tapped density and flowability determination

Selected pulverized formulations were determined for their bulk density, tapped density, and flowability as the described methods in 4.1 and 4.2.

### 6.2 Process efficiency

The process efficiency of powder production was accessed by calculating percentage of production yield. Production yield (%) was calculated from the weight of initial feed amount ($W_1$) and the weight of powder collected from collector ($W_2$) as formula;

$$\text{Production yield (\%)} = 100 \times [W_2/W_1] \quad [5]$$



### 6.3 Particle size and size distribution determination

Particle size and size distribution of the obtained powders were measured by laser light scattering technique equipped with dry dispersion unit for dry sample. Approximately 1-2 g of sample was placed on the tray, and the powder was fed through the orifice with aid of 2.5 bars of air compression. The obscuration value was kept between 0.5-6.0%. Each sample was evaluated in triplicate. The particle size was measured in the volume-weighted mode and the diameter of particles referred to 50% under size diameter (d(v,0.5)). The particle size distribution was determined by span value which was calculated from the formula;

$$\text{Span value} = [d(v,0.9) - d(v,0.1)] / d(v,0.5) \quad [6]$$

The d(v,0.1) and d(0.9) were 10% and 90% under size diameter, respectively.

### 6.4 Particle morphology

The morphology of particles after JM process with different grinding cycles was recorded by using JSM-6400 or JSM-6510 SEM. The samples were attached on a sample stub and sputter-coated with gold before observing under microscope. SEM images were taken at various magnifications.

## 7. Physicochemical characterization of matrix film and pulverized formulation

### 7.1 Moisture content by Karl Ficsher method

Water content determination of the obtained powder formulation was carried out using Karl Fischer moisture titrator after generating powder from different comminution processes. Approximately 5 mg of sample was weighed into the titration cell and water content (%) was automatically calculated by the moisture titrator. The measurement was conducted in triplicate.

### 7.2 Differential scanning calorimetry (DSC)

To characterize solid state properties of drug and excipients used in the formulations, DSC was performed. The DSC thermograms of film and powder formulations were analyzed by a DSC using either DSC6200 or DSC822$^{e}$. Samples were approximately weighed of 5 mg and hermetically sealed in aluminum pan. Samples were being scanned from 25-400°C with heating rate 10°C per minute under nitrogen purge rate of 60 mL per minute. Pure indium, a reference, was used for calibration the instrument.



### 7.3 Fourier transform infrared (FT-IR) spectroscopy

Physicochemical interaction was also investigated using FT-IR spectrometer. For matrix film samples, FT-IR spectrometer with Attenuated Total Reflectance (ATR) technique was performed. Sample was pressed into direct contact with the ATR crystal and performed from 4000-515 $cm^{-1}$ at a resolution of 4 $cm^{-1}$. As powder samples utilized an ALPHA compact FT-IR spectrometer, sample was prepared by mixing with potassium bromide (KBr) powder and compressed into a disc. The disc was scanned from 4000-400 $cm^{-1}$ at a resolution of 4 $cm^{-1}$.

### 7.4 X-ray powder diffractometry (XRPD)

In order to investigate the polymorphic state of all ingredients used, physical mixtures, and pulverized powder formulations with different kinds of milling techniques, XRPD studies were carried out. Diffraction patterns were recorded using

X-ray powder diffractometer. The patterns were performed under CU K-alpha radiation, 30 kV voltage, 15 mA current with a scanning rate of 3° $min^{-1}$ over a 2-theta range from 5° to 50°.

## 8. Determination of mucoadhesive properties

### 8.1 *In vitro* mucoadhesive studies

The mucoadhesive property was investigated by a modified method described by Vyas et al. (146). A fresh small intestine of porcine obtained from a local authorized slaughterhouse was kept in ice pack and used within 1 hour of killing. It was cut into 5-6 cm long pieces, then incised, and cleaned by washing with 0.9% normal saline. Sample formulations were prepared by accurate weighing of powder formulations staining with fixed amount of BB, used as indicator, while a control formulation was physical mixture of BB and lactose powder. Powders were applied onto the mucosal intestine. Next, incubation at 90% RH (147, 148), ambient temperature for 15 minutes was conducted before installing the mucosa in the funnel with an angle of 40° relative to horizontal plane and rinsing with warm simulated nasal fluids (SNF) (pH 6.0) at 32 $\pm$ 1°C (147, 148) with pump rate 5 ml/min by peristaltic pump. The washed was collected at 10, 20, 30, 60, 90, and 120 minutes, and after 120 minutes, BB were extracted from tissues by shaking tissues in SNF for 3 hours. The washed and the extracted were analyzed by UV spectrophotometer, and photographs were subsequently taken.

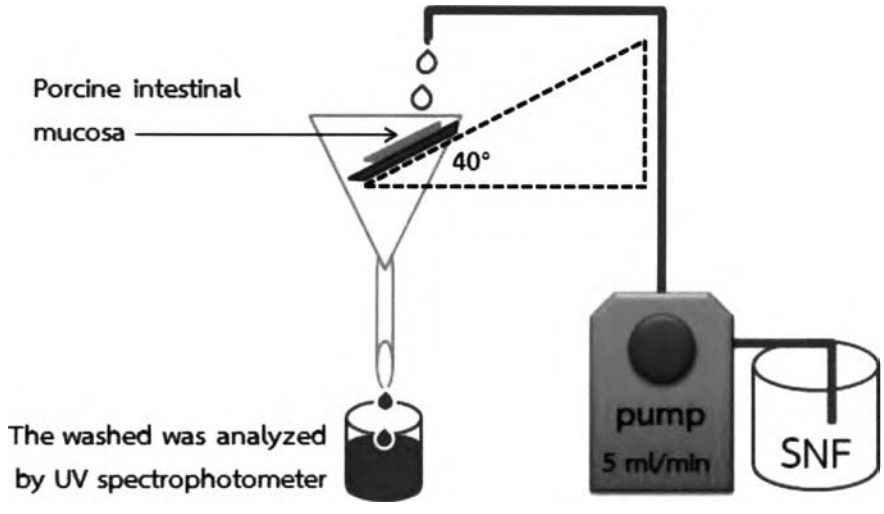


**Figure III-1** Schematic processes of mucoadhesive property evaluation using porcine intestinal mucosa.



**8.2 Viscosity evaluation**

Powder formulations were evaluated for their mucoadhesive properties as their viscosity in mucin dispersion in which the method was modified from Callens et al. (149) by using viscometers instead of oscillatory rheometer. Two types of viscometer were available; cone/plate rotational viscometer and Sine-wave Vibro viscometer. Briefly, 8% w/v mucin dispersion was prepared by dispersing porcine mucin powder in pH 6.0 SNF. Then powder formulations were dispersed with the concentration of 10% w/v in the mucin dispersion, and the obtained admixtures were incubated at 32±1°C for 15 minutes before measuring by RotoVisco-1 rotational (RV-1) viscometer. The measurement was operated by using cone/plate type (cone diameter of 60 millimeter, 1° angle C60/1) with shear rate of 3500 $sec^{-1}$. The 8% mucin dispersion in SNF was used as a control. The measurement was done in triplicate.

Since this type of viscometer may breakdown polymer-mucin network during continuous flow (149), Sine-wave Vibro viscometer, which operated by vibrating of tuning-fork and did not damage to the sample (150), was selected to confirm and support the results from rotational viscometer. Therefore, the viscosity of the

obtained admixtures was also carried out by another viscometer, SV-10 Vibro viscometer. The measurement was also done in triplicate.

### 8.3 Zeta potential evaluation

In order to substantiate charged effect to mucoadhesive properties, zeta potential of powder formulations was determined by a Zetasizer. Samples were prepared by dispersing powder formulations in ultrapure water and 8% mucin dispersion to 1% w/v, and the experiment was conducted in quintuplicate.

## 9. Protein Integrity study

### 9.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) study



The primary structure of the BSA incorporated in the formulations was determined by SDS-PAGE. In addition to determine the primary structure of protein, SDS-PAGE technique also provided information related to MW, charge of proteins, and purity. Powder formulations were dissolved in PBS (pH 6.0) to the BSA concentration of 200 µg/ml. The samples, native BSA, and a MW reference marker (SeeBlue® Plus2 Prestained Standard) were mixed with sample buffer (NuPAGE® LDS Sample Buffer), reducing agent (NuPAGE® reducing agent), and deionized water, and then were boiled for 10 minutes. The electrophoresis was run in vertical electrophoresis cell. The bands were observed by Coomassie blue (SimplyBlue® SafeStain) staining.

### 9.2 BSA secondary structure determinations

Circular dichroism (CD) spectroscopy is a technique to evaluate structural information of protein; secondary and tertiary structure. In the far-UV region (240-180 nm), CD spectrum is used to determine the protein secondary structure, while in the near-UV region (320-260 nm) the tertiary structure was observed (151, 152).

The secondary structures of the BSA incorporated formulations were measured on a CD compared with native BSA solution. Each 11-mg powder

formulation containing 1-mg BSA was dissolved with 10-ml of PBS pH 6.0, as 0.1 mg/ml of native BSA solution was prepared as a control. CD spectra were recorded in ellipticity ($\theta$, mDeg) as a function of wavelength ranging from 250 to 190 nm. PBS (pH 6.0) was run as background.

For CD operating condition, samples were measured in 0.1-cm pathlength of rectangular quartz cell at room temperature. Scanning wavelength was from 250 to 190 nm with 1.0 nm of resolution, 1.0 nm of bandwidth, 100 mDeg of sensitivity, and 100 nm/min of scan rate. The number of scan was 5.

**10. BSA content determination**

Selected film and powder formulations obtaining from JM, CM, and PBM were dissolved in PBS (pH 6.0) and content of BSA was determined by using bicinchoninic acid protein assay kit (QuantiPro™ BCA assay kit). Each protein solution was mixed with BCA working reagent at ratio 1:1 in 96-well plate and then incubated at 37 °C for 2 hours. The absorbance of mixtures was measured at 560 nm on a spectrophotometer (Microplate reader, VICTOR$^3$, Perkin-Elmar, USA). The calibration curve of BSA was used to quantify amount of BSA in formulations. Actual amount of BSA content was then calculated as followed:

% BSA content = 100 x (actual amount of BSA/theoretical amount of BSA) [7]

**11. *In vitro* BSA release studies**

The *in vitro* BSA release test of the powder formulations was determined by vertical Franz diffusion cells and equipment. Hydrophilic polyethersulfone filter (PES, Supor® membrane filter) with pore size 0.45 micron was selected as membrane for *in vitro* release because it has extremely low protein binding properties (153). Such a membrane was treated by pre-hydrating with SNF (pH 6.0) at least 2 hours prior used. The pre-hydrated filters were mounted on receptor compartments which were filled with pH 6.0 SNF as well as magnetically stirred at 100 rpm, and then the diffusion cells were allowed to equilibrate at 32±1°C for 30 minutes. The 11-mg powder formulations were dispersed on the membrane surface in the donor compartments.

One-ml sampling volumes were periodically extracted for BCA assay, and fresh replacement medium of same volumes was reintroduced into receptor compartments via sampling arms. The accumulative amount of BSA release of each formulation versus time was plotted. Additionally, the data were analyzed by DDSolver program (154), an add-in program for drug release modeling, in order to calculate the fitted release kinetic model.

## 12. *In vitro* permeability studies

In order to evaluate the potential of a specific anatomical site such as nasal as a route of drug delivery, permeation study was performed by using Franz diffusion cell and porcine nasal mucosa which is comparable with human mucosa with large available area of mucosa (155) and is also easily accessible and available in Thailand. In addition, intranasal drug delivery is a promising route for brain targeting, and two main pathways from nose to brain are relevant to olfactory and respiratory regions (52). Thus, permeation study had to perform on both olfactory mucosa (OLF) and respiratory mucosa (RES).



### 12.1 Olfactory and respiratory tissue preparation

Porcine OLF and RES were isolated from domesticated pigs after slaughtered in the local authorized slaughterhouse. The heads were longitudinally incised along the lateral walls of the nasal cavity and transported in ice box to the laboratory. Two anatomical types of mucosae were obtained. OLF, pale yellow-colored mucosae, were located on the ethmoturbinate bone and the last portion of dorsal nasal conchae near the roof of the nasal cavity, while RES, reddish pink mucosae, were obtained from the ventral nasal concha at the anterior part of the nasal cavity (156, 157). The excised mucosae of each type were directly cleaned as well as separately stored in pH 7.4 PBS on ice for further use. The experiments were performed within 1 hour of procurement of the mucosae.

### 12.2 Tissue integrity by trans-epithelial electrical resistance (TEER) measurement

The integrity of tissues used were determined before, during, and after the experiment by observing the TEER value (156, 158, 159). Briefly, epithelial Volt-Ohm meter were modified by immersing one electrode on donor compartment above tissue about 1 millimeter, as the other in receptor compartment at the determined time (0, 30, 60, and 120 minutes). Tissues with TEER value less than 40 $\Omega.cm^2$ or decreasing greater than 20% of the initial value were excluded (155, 156).

### 12.3 *In vitro* permeation

For *in vitro* permeation study, selected powder formulation was incorporated with FITC-BSA as an indicator compared to solution formulation. For sample preparation, 11-mg powder formulation consisted of 500 µg FITC-BSA and 500 µg BSA without conjugating with FITC, while solution formulation was prepared by dissolving 500 µg FITC-BSA and 500 µg non-FITC conjugated BSA in 200 µl of pH 6.0 PBS.



The permeability of FITC-BSA through OLF and RES was investigated by vertical Franz diffusion cells. The diffusion chambers were filled with freshly prepared PBS (pH 7.4) and controlled temperature at 37±1°C by warm recirculating water bath with stirring rate of 500 rpm. The excised mucosa was mounted between the donor and receptor compartment which had 0.45 micron of Supor® filter as a supportive membrane. The epithelial and ventral sides of mucosa were contacted with the upper donor compartment and the supportive membrane, respectively. The system was equilibrated for 30 minutes prior to conducting an experiment. Eleven-mg of powder formulation or 200 µl of solution formulation was introduced on the epithelial side on the donor compartment. At the determined time (before applying sample, 10, 20, 30, 45, 60, 90, and 120 minutes), 0.5-ml sampling volume was periodically withdrawn, and fresh replacement medium of same volume was reintroduced into a receptor compartment via the sampling arm. The amount of FITC-BSA permeated through both types of nasal mucosae was analyzed by a fluorescence spectrophotometer. Samples were made in triplicate and in

quintuplicate for permeation through OLF and RES, respectively. The accumulative percentage of FITC-BSA release of each formulation was plotted versus time.

After finishing permeation study, amount of FITC-BSA which was not permeated through the mucosae on the donor compartment was investigated by a fluorescence spectrophotometer.

### 12.4 Histomicroscopic examinations

The porcine OLF and RES which were treated and untreated after the 2 hours of the permeation study were performed by histomicroscopic study in order to evaluate any epithelial damages caused by the formulations. The mucosae were immediately fixed in 10% neutral buffered formaldehyde solution, embedded in paraffin wax, and prepared according to standard procedures. Each 5-μm thick section was stained with hematoxylin and eosin (H&E) and analyzed under an inverse microscope with phase contrast and fluorescence.



## 13. Data analysis and statistical methods

Data were presented as mean±SD for the results performed up to three measurements. The independent paired t-test or simple analysis of variance (One way-ANOVA) were utilized. For multiple comparisons among group, Tukey's and Dunnett's T3 pairwise comparisons were performed, if there was or was not homogeneity of variance (Levene's test), respectively. All analysis were statistically determined using IBM® SPSS® statistics version 21 and significant differences were considered at a level of $p\text{-value}<0.05$.