CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

1. Acetone (Lot No. 10100223, LAB-SCAN, USA)
2. Calcium chloride dried granular (Code No. 328757, Carlo Erbra, Germany)
3. Chitosan 20 kDa, 87%DD (Lot No. COA050507, Seafresh Co. Ltd., Thailand)
4. Chitosan 200 kDa, 87%DD (Lot No. COA240720, Seafresh Co. Ltd., Thailand)
5. Dimethyl sulfoxide (Lot No. 2216B073, Amresco, USA)
6. Dulbecco's modification of Eagle's medium (DMEM, Lot No. 861491, Gibco™,USA)
7. Fetal Bovine Serum (Lot No. 41F6394K, Gibco, EU)
8. Glacial acetic acid (Lot No. 6M387197A, CARLO ERBA, Italy)
9. Hydrochloric acid 36.5-38% (Lot No. E15W66, J.T. Baker, USA)
10. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Lot No. D00121274, Calbiochem, Germany)
11. Potassium bromide (KBr, Lot No. B01351607 905, Merck, Germany)
12. Potassium chloride (Lot No. AF501338, Ajax Finechem, Australia)
13. Sodium acetate (Lot No. AF604190, Ajax Finechem, Australia)
14. Sodium hydroxide (Lot No. B0035298, Merck, Germany)
15. Tris (hydroxymethylaminomethane) (Lot No. Y70885, Research organic, INC, USA)
16. Phthalic anhydride (Lot No. S5335992 917, Merck, Germany)

3.2 Equipments

1. Aluminum pans (Perkins, P/N SSC0000E030 open sample pan, Japan)
2. CO_2 Incubator (Heraeus, Germany)
3. Desiccators (Biologix Reseach Company, USA)
4. Differential scanning calorimeter (DSC 6200; SII Seiko instruments Inc., Japan)
5. Disintegration testing apparatus (Sotax DT3, Switzerland)
6. Fourier transform infrared spectrophotometer (Nicolet 4700, USA)
7. Freeze dryer (Free Zone2.5, Labconco, USA)
8. Hot air oven (Heraeus, Germany)
9. Laminar air flow cabinet (Hera Safe, Heraeus, Germany)
10. Magnetic stirrer and Magnetic bar (Mettler-toledo GmbH, Germany)
11. Microplate reader (Packard BioScience AOPUS01, USA))
12. Moisture balance (Sartorius YTX01L, Germany)
13. Nuclear magnetic resonance spectroscopy (ADVANCE 300, Bruker, Germany)
14. pH meter (Mettler Toledo seveneasy, Switzerland)
15. Powder X-ray diffractometer (Miniflex II, Rigaku, Japan)
16. Schott DURAN (250, 500, 1000 mL)
17. Spectrophotometer (Lamda2, Perkin-Elmer, USA)
18. Texture analysis (TA.XT. Plus, UK)
19. Thickness meter (Minitest 600B, Typ 80-121-0306, Germany)
20. UV-VIS spectrophotometer (Lambda 2, Perkin Elmer, USA)
21. Viscometer (Brook field digital viscometer, DV-III ULTRA, USA)
22. Vortex mixer (Gibthai VX-100, Thailand)
23. Water bath (SANYO, Walk-Ins, Japan)
24. 96 well cell culture plates (Costar®, USA)

3.3 Methods

3.3.1 Preliminary study

CS (20 kDa, 87%DD), 10% w/v in 20 mL of 2.5% v/v aqueous acetic acid solution was prepared (Figure 13). The proposed reaction between chitosan and phthalic anhydride is shown in Figure 14. The very low amount of medium was used to prevent the hydrolysis of phthalic anhydride (PA). PA, 5% w/v in acetone was dropped in CS acidic solutions at 1:1 mole ratio of CS:PA under stirring for 4 and 24 h. The temperature was controlled at 25 and 40°C. The excess acid in viscous dispersion was neutralized with 0.5 N NaOH. Then, the dispersion was dropped in acetone followed by filtration. The precipitates were washed with mixture of acetone:water, 8:1 v/v and then with excess acetone. Finally, the precipitates were dried in oven at 40°C for 2 h and the obtained powder was collected. The chemical structure using FTIR spectroscopy and the solubility by measurement of % transmittance of solutions of the obtained powder was characterized.

3.3.1.1 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of CS and N-PhCS samples were recorded with FTIR spectrophotometer (Nicolet 4700, USA) using the KBr disc method. Each sample was pulverized and blended with KBr powder and then compressed with pressure of 5 tons for 60 seconds. The KBr discs were placed in the sample holder and scanned from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}.

3.3.1.2 Solubility study

The solubility of N-PhCS in various pH media was determined by measurement of %transmission of solution using UV spectrophotometry. The sample, 50 mg, was dissolved in 5 mL of pH 1-10 buffer solutions and %transmission of the solution was measured using a UV-VIS spectrophotometer (Lambda 2, Perkin Elmer, USA) at wavelength of 600 nm.

The results from preliminary study showed that the suitable condition to prepare N-PhCS was at 25°C under stirring for 4 h. Therefore, we used these conditions for our further experiments.

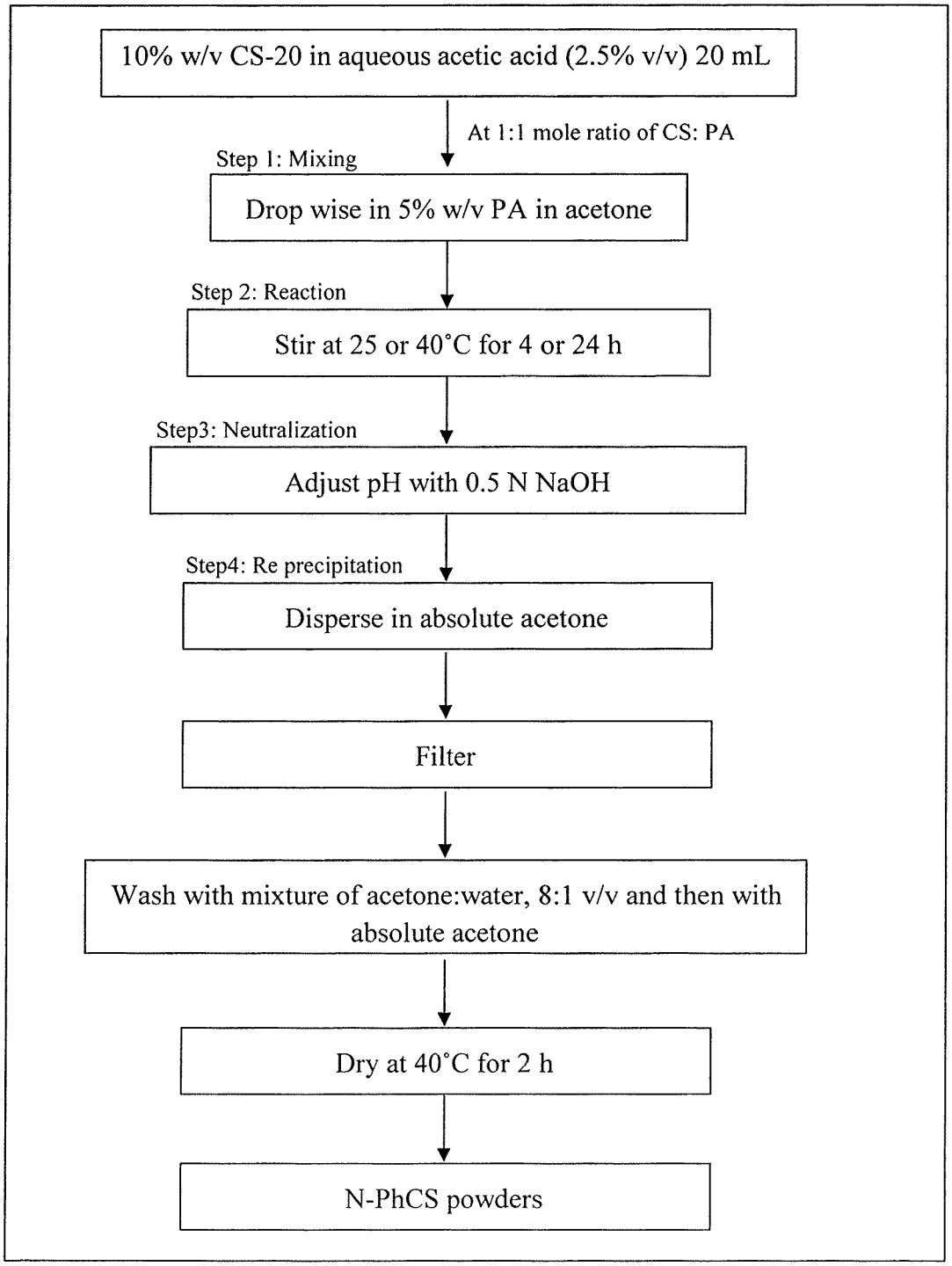

Figure 13 Preparation process of N-PhCS prepared under preliminary study.

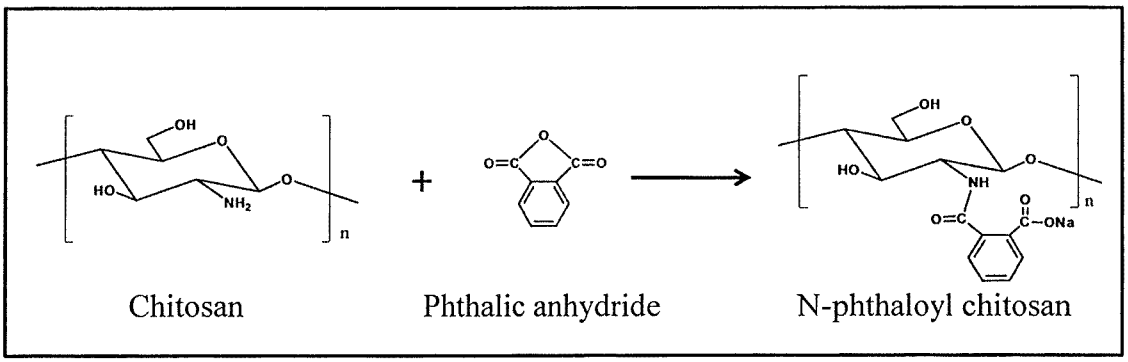

Figure 14 Proposed reaction between chitosan and phthalic anhydride.

3.3.2 Preparation of N-PhCS prepared under various conditions

N-PhCS was prepared from CS 20 kDa, 87%DD (CS-20) and 200 kDa, 87 %DD (CS-200) under varying mole ratio at 1:1, 1:3 and 1:5, respectively (Figure 15). The effect of neutralization pH was also varied at pH 4, 5 and 6, because it was found in the preliminary study that the characteristic of the precipitates depended on the amount of the added NaOH solution.

The physicochemical properties and degree of substitution of the obtained samples were characterized as well as cytotoxicity and film forming properties as follows:

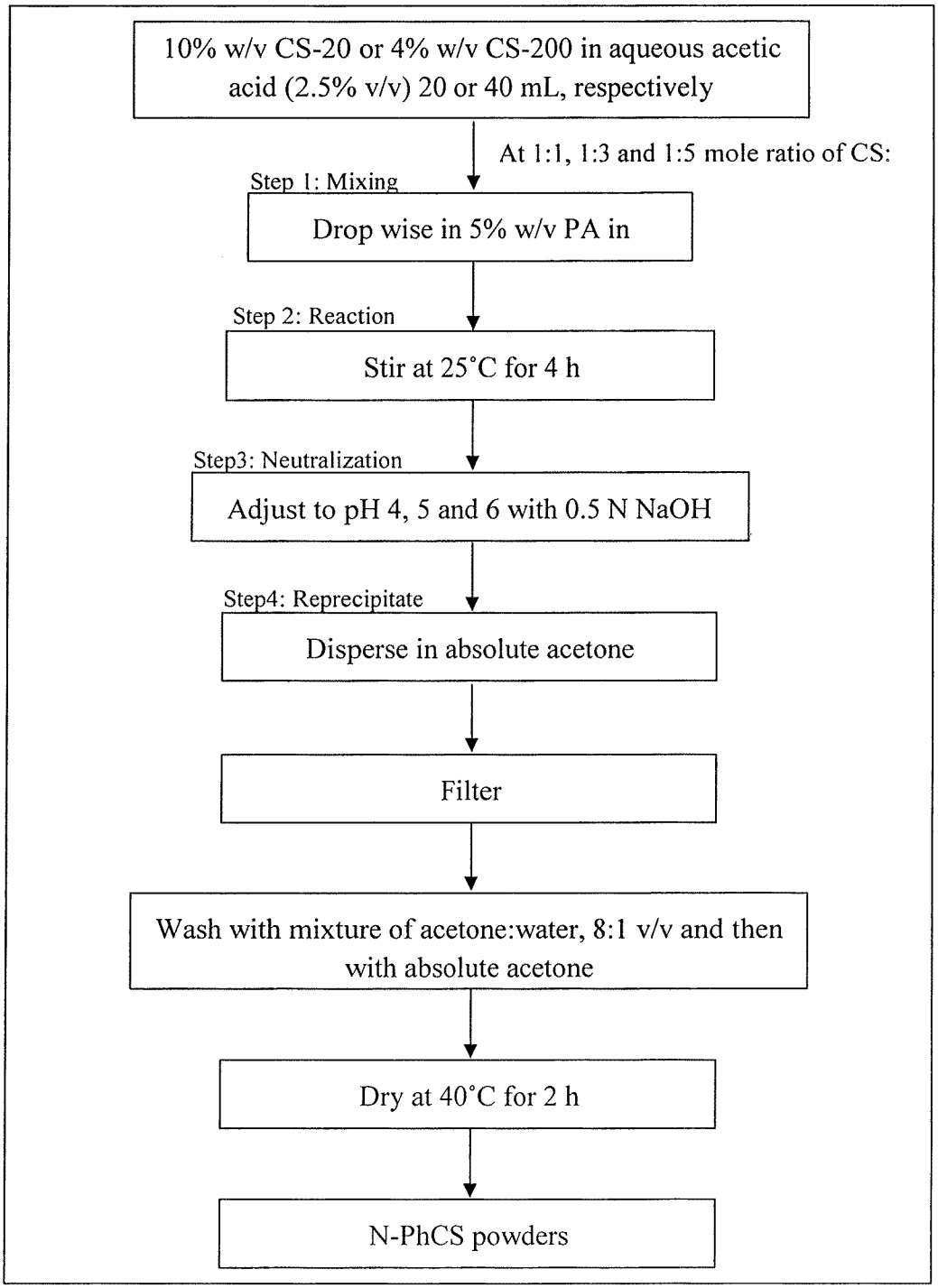

Figure 15 Preparation process of N-PhCS prepared under various conditions.

3.3.3 Characterization of N-PhCS prepared under various conditions

3.3.3.1 Fourier transformed infrared (FTIR) spectroscopy

FTIR spectra of N-PhCS were studied following the method as described in section 3.3.1.1.

3.3.3.2 Powder X-ray diffractometry (PXRD)

N-PhCS and CS powder was loaded into PXRD plate and scanned by powder X-ray diffractometer (Rigaku, Miniflex II, Japan). The PXRD data were recorded on a Rigaku Miniflex System using Ni-filtered, Cu-K (alpha) radiation, 30 kV, 15 mA with a scanning ratio: 20°= 4°/min.

3.3.3.3 Differential scanning colorimetry (DSC)

The DSC thermograms of N-PhCS and CS samples were determined by differential scanning calorimetry (DSC 6200; SII Seiko instruments Inc., Japan) using indium as a standard. About 3-4 mg of powder sample were accurately weighed and placed in a closed aluminum solid pan. The aluminum pan was then transferred into the furnace. The thermal behavior of the samples was determined at heating rate of 10°C per min from 60-350°C using an empty closed aluminum solid pan as a reference. The measurement was done under nitrogen gas at a flow rate of 10 mL per min.

3.3.3.4 Thermogravimetric analysis (TGA)

The TGA thermograms of N-PhCS and CS samples were measured using a thermogravimetric analyzer (TG/DTA 6200; SII Seiko instruments Inc., Japan). About 3-4 mg of powder samples were accurately weighed into an aluminium pan. The measurements were conducted over 60-350°C at a heating rate of 10 °C/min under nitrogen purge.

3.3.3.5 Solubility study

Solubility study of N-PhCS was studied following the method as described in section 3.3.1.2.

3.3.3.6 Determination of degree of substitution (DS)

3.3.3.6.1 FTIR method

The degree of substitution of all samples was screened by calculation of the ratio of absorbance at 1645 cm^{-1} (amide I band) and the hydroxyl band at 3450 cm^{-1} of FTIR spectra (as described in section 3.3.3.1) of all N-PhCS following the Equation (1) proposed by Moore and Roberts [82]. The FTIR measurement of three discs (n=3) was used to obtain a statistical evaluation.

$$DS(\%) = \left(\frac{A_{1645}}{A_{3450}}\right)_{N-PhCS} - \left(\frac{A_{1645}}{A_{3450}}\right)_{CS} \times 100 \quad \text{.....(1)}$$

Where

- A_{1645} is the absorbance of the amide-I band as a measure of the N-acyl group content
- A_{3450} the absorbance of hydroxyl band as an internal standard to correct for film thickness or for differences in chitosan

3.3.3.6.2 ^{1}H-NMR method

According to the high accuracy of the DS investigation [28], ^{1}H NMR was used to confirm the DS of N-PhCS prepared under the suitable pH neutralization with mole ratio of CS:PA at 1:1, 1:3 and 1:5 mole ratio. N-PhCS was placed into dialysis bag (MWCO: 6000) for dialysis against excess distilled water to remove the impurities. The solution in dialysis bag was dried using freeze dryer (FreeZone 2.5, Labconco, USA). The ^{1}H NMR spectra of the obtained samples was recorded using nuclear magnetic resonance spectroscopy. The DS was determined from the ratio of area between the protons of substituted group and methyl proton of monosaccharide residue (H_3, H_4, H_5 and H_6). The chemical shifts were reported in δ (ppm). The degree of substitution was calculated using the following Equation (2):

$$DS\ (\%) = \left[\left(\frac{I_{substitution\ group}}{number\ of\ proton}\right) / \left(\frac{I_{H3+\,H4+\,H5+\,H6}}{\text{number of proton}}\right)\right] \times 100 \quad \text{.....(2)}$$

where I was the integral of the hydrogen atom.

3.3.4 Stability study

N-PhCS samples from section 3.3.3.6 with highest %DS were kept in the hot air oven at 60, 80 and 120 ± 2°C for 6, 12 and 24 hours, respectively. Then remove the sample from the oven, cool down to room temperature and determine by FTIR spectra.

3.3.5 Cytotoxicity test

Cytotoxicity of N-PhCS samples from section 3.3.3.6 with highest %DS was studied by the MTT cytotoxicity assay. Caco-2 cells were harvested and seeded in 96-well plates at a seeding density of 1 x 10^4 cells per well and pre-incubated for 24 h. Then, the cells were treated with N-PhCS sample at various concentrations ranging from 0.01-10 mg/mL in medium (pH 7.4) and incubated for 24 h. After treatment, the solutions were removed. Finally, the cells were incubated with MTT containing medium (0.05 mg/mL) for 3 h. The medium was removed and the formazan crystal that formed in the living cells was dissolved in 100 μL DMSO/well. The relative viability (%) was calculated based on absorbance at 550 nm using a microplate reader (Packard BioScience AOPUS01, USA). Viability was determined by comparing absorbance in wells containing treated cells with that of untreated cells. Eight replicates were measured for each concentration. The relative cell viability was calculated according to the following Equation (3).

$$\text{Relative cell viability} = \frac{[OD_{550,sample} - OD_{550,blank}]}{[OD_{550,control} - OD_{550,blank}]} \times 100 \qquad \text{.....(3)}$$

where $OD_{550,\ sample}$, $OD_{550,\ control}$ and $OD_{550,\ blank}$ are optical density at 550 mM of N-PhCS sample, control and blank solution, respectively.

3.3.6 Preparation and characterization of N-PhCS and chitosan acetate films

The films of N-PhCS from section 3.3.3.6 with highest %DS were prepared by casting method. N-PhCS was dissolved in distilled water at concentration of 4% w/v. The solution, 100 mL, was then poured on a silicone coated

glass plate (15 x 15 cm^2) and dried at 50°C for 4-5 h. The films were peeled off and kept in a desiccators containing dried silica gel to control the moisture for all films prior to test. Chitosan acetate (CSA) films were also prepared in the same manner by dissolving CS-20 at concentration of 2.5% w/v in 0.5 N acetic acid solution.

3.3.6.1 Films thickness

The film thickness was determined at ten points by using a thickness gauge Mini-Test 600 (Elektro Physik Dr. Steingroever GmbH & Co. KG, Germany).

3.3.6.2 Mechanical properties of films

Mechanical properties of films were measured by texture analyzer; model TA.XT plus (Stable Micro Systems Ltd., United Kingdom). A maximum load of 50 N was used. The prepared films were cut in a dumbbell shape with length of 25 mm and width of 6.25 mm. The thickness of the films was measured using a micrometer. The test speed was 1 mm/sec. The tensile strength, the elongation at break and gradient stress-strain of 10 samples were reported [83].

3.3.6.3 Water vapor permeability of films

Water vapor permeability (WVP) was conducted using modifications of the standard procedure of the ASTM standard method E96-95 (ASTM, 1995) described by Zavareze et al. [84]. Each film sample was sealed over the circular opening of a permeation cell containing with 30 g dried-granular calcium chloride. These cells were then placed on desiccators with a saturated sodium chloride solution (75% RH) at 25°C. After the samples reached steady-state conditions, the cell weight was recorded every 24 h for 10 days. The WVP of at least six cells for all films was calculated using the following Equation (4):

$$\text{WVP coefficient} = \frac{(W \times t)}{(A \times \Delta P)} \qquad \text{.....(4)}$$

where WVP coefficient is water vapor permeability coefficient (g h^{-1} m^{-1} Pa^{-1}),

W is the amount of water permeated through the film (g/h),

t is the thickness of film (m),

A is exposed area of film (m^2),

ΔP is the vapor pressure difference (ΔP = 5386.21 Pa)

3.3.6.4 Moisture content

The film of N-PhCS was accurately weighed (W_0) and dried by loss on drying measurement (W_1). The weight of film before and after drying was calculated using the following Equation (5). All samples were performed in triplicate.

$$\text{Moisture content (\%)} = 100 \times \frac{(W_0 - W_1)}{W_0} \quad \text{.....(5)}$$

where W_0 and W_1 were the constant weight before and after drying, respectively.

3.3.6.5 Percentage dissolved of the films

The film was cut in a square of 1.5 cm × 1.5 cm, weighed and placed in each tube of the basket of USP disintegration apparatus. The simulated gastric fluid (SGF, pH 1.2) was used as immersion fluid for the first 2 h. After immersion in SGF, the film was transferred to simulated intestinal fluid (SIF, pH 6.8 Tris buffer) for the next 3 h. The temperature was controlled at 37 ± 2 °C. The rest film was dried at 70°C for 3 h, and reweighed. The percentage of dissolved film was calculated from the percent weight loss of the film. In case of the film was completely dissolved within 3 h in SIF solutions, the dissolving time was recorded.

3.3.7 Statistic analyses

Data of research were expressed as mean ± standard deviation (SD). The statistical analysis was carried out using analysis of variance at the 0.05 significance level.