CHAPTER III
EXPERIMENTAL

3.1 Materials

Porcine gelatin 180 g Bloom (PorGel) and cold-water fish gelatin (FishGel) used as a matrix, were supplied from (AR grade) Fluka and Sigma, respectively. Salicylic acid (SA) and 5-sulfosalicylic acid (SSA) used as the model drugs were purchased from (AR grade) Fluka. Glutaraldehyde (50% in water AR grade) was purchased from Fluka and used as the crosslinking agent. Sodium acetate and Glacial acetic acid were purchased form Ajax Finechem and (AR grade) Merck, respectively. Those were used to buffer solution.

3.2 Methodology

3.2.1 Preparation of Model Drugs-loaded Porcine Gelatin Film

PorGel powder was dissolved in deionized water at 60 °C for 1 h to prepare a gelatin solution at a fixed concentration of 10% w/v. After the solution was cooled down to room temperature, the model drugs (SA and SSA) were loaded into the gelatin solution at 1 wt% (based on the weight of gelatin powder) under constant stirring for 1 h. Then glutaraldehyde, the crosslinking agent, was added into the solution in order to fabricate cross-linked gelatins at various crosslinking ratios 0.25, 0.50, 0.75, 1.00, 3.00, and 7.00% (based on weight of gelatin powder). The solution was mixed very slowly to prevent the formation of air bubbles (Peppas *et al.*, 1998). Immediately after mixing the solution, the mixture was cast on the petridish (diameter 9 cm, film thickness 0.45-0.50 mm) and then cooled to room temperature.

3.2.2 Preparation of Model Drugs Loaded Fish Gelatin Film

FishGel powder was dissolved in deionized water at 60 °C for 1 h to prepare a gelatin solution at a fixed concentration of 10% w/v. After the solution was cooled down to room temperature, the model drugs (SA/SSA) were loaded into the gelatin solution at 1 wt% (based on the weight of gelatin powder) under constant

stirring for 1 h. Then the glutaraldehyde, the crosslinking agent, was added into the solution in order to fabricate cross-linked gelatins at various crosslinking ratios 1.00, 3.00, and 7.00% (based on weight of gelatin powder). The solution was mixed very slowly to prevent the formation of air bubbles (Peppas *et al.*, 1998). Immediately after mixing the solution, the mixture was cast on the petridish (diameter 9 cm, film thickness 0.45-0.50 mm) and then cooled to room temperature.

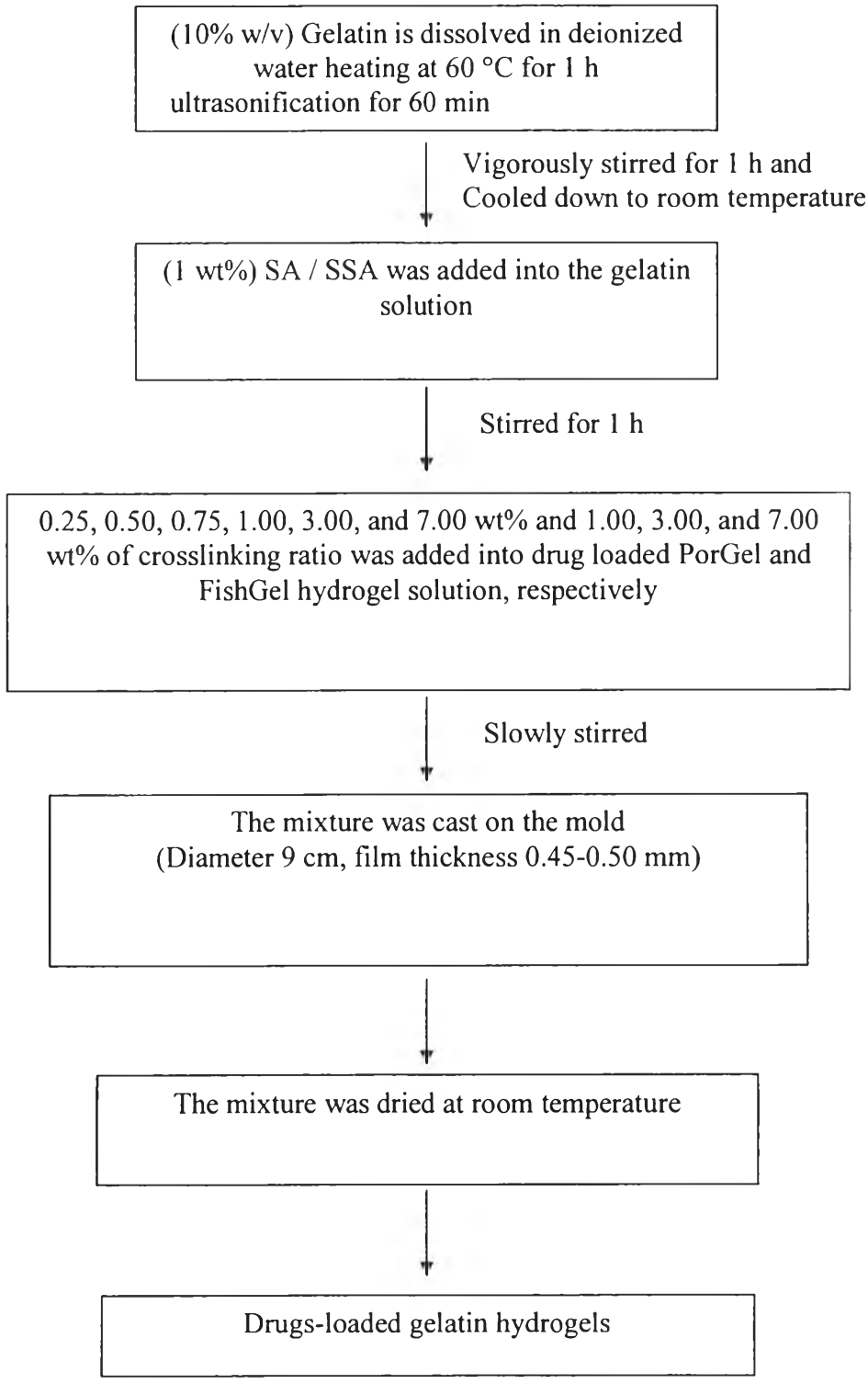

Figure 3.1 Preparation of drugs-loaded gelatin hydrogels.

3.2.3 Characterization and Testing

3.2.3.1 Fourier Transforms Infrared Spectrometer (FT-IR)

The FTIR spectrometer (Bruker, Equinox 55/FRA 1065) was used to identify the functional group of salicylic acid (SA) and5-sulfosalicylic acid. The ATR-FTIR spectroscopy (Thermo Nicolet) was used to investigate the polymer/model drug interaction in the drug-loaded gelatin hydrogels and also to investigate the polymer/cross-linked in the reaction between the gelatins with glutaraldyhyde. The sample was placed on the crystal and spectra were taken to determine any interactions between the model drug and the gelatin polymer. The observed spectra were in the range of 400-4000 cm^{-1} for FTIR and 650-4000 cm^{-1} for ATR-FTIR mode in the absorption mode with 32 scans at a resolution of 4 cm^{-1}.

3.2.3.2 Thermogravimetric Analyzer (TG-DTA)

Thermal gravimetric analyzer (TG-DTA, Perkin Elmer) were used to investigate weight loss of volatile molecule, the amount of residual water, and the degradation temperature of cross-linked gelatin with glutaraldehyde and the drug-loaded gelatin hydrogels with the temperature scan from 25 to 800 °C and with a heating rate of 10 °C/min under nitrogen atmosphere. The samples were weighed in the range of 4-6 mg and loaded into a platinum pan.

3.2.3.3 Scanning Electron Microscope (SEM)

A scanning electron microscope or SEM (JEOL, model JSM-5200) was used to investigate morphology of each crosslinked PorGel and FishGel hydrogel. The hydrogels was immersed in distilled water at 37 °C, before it was rapidly frozen in liquid nitrogen then dried it in the vacuum chamber at -50 °C. After a freeze-dry process, a piece of sample was placed on the holder with an adhesive tape and coated with a layer of gold by using a JFC-1100E ion-sputtering device for 4 min. The scanning electron micrographs of crosslinked PorGel and FishGel hydrogels were obtained by using an acceleration voltage of 15 kV at magnifications of 3000x.

3.2.4 Ubbelohde Viscometer

Ubbelohde viscometer was used to measure the intrinsic viscosity of the gelatin solution to be determined the molecular weight, M_w of porcine gelatin. The intrinsic viscosity, $[\eta]$, was determined by the measurements of solution viscosity. The measurements were made for the elution times (t = elution time of polymer solution, t_0 = elution time of solvent) from the viscometer. The times were proportional to the viscosity of the polymer solution, η, and solvent, η_o, respectively. The specific viscosity and relative viscosity were calculated by the following equations (3.1) and (3.2):

$$\eta_{rel} = \frac{\eta}{\eta_o} \tag{3.1}$$

and

$$\eta_{sp} = \frac{\eta - \eta_o}{\eta_o} = \frac{t - t_o}{t_o} = \eta_{rel} - 1 \tag{3.2}$$

The intrinsic viscosity, $[\eta]$, was calculated by the extrapolation to infinite dilution of the equation of Huggins (3.3) and Kramer (3.4) (Derosa 2008):

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \tag{3.3}$$

and

$$\frac{\ln \eta_{rel}}{c} = [\eta] + k''[\eta]^2 c \tag{3.4}$$

where η_{sp} is the specific viscosity, η_{rel} is the relative viscosity, c is the concentration of polymer in grams per deciliter (g/dL) , η_{sp}/c is defined as the reduced viscosity, η_{red}, $\ln\eta_{rel}/c$ is defined as the inherent viscosity, η_{inh}, and k' and k'' are the constants of Huggins and Kramer, respectively.

Molecular weight, M_w, of the porcine and fish gelatin were determined by the capillary viscometer. The relation between $[\eta]$ and M_w can be

described in terms of the Mark–Houwink–Kuhn–Sakurada (MHKS) equation (3.5) (Enrione *et al.*, 2011):

$$[\eta] = KM_w{}^a \tag{3.5}$$

where K and a are the constant values of gelatins.

3.2.5 Swelling Behavior

The degree of swelling and the weight loss of the gelatin hydrogels were measured in an acetate buffer solution at 37 °C for 48 h (Taepaiboon *et al.*, 2006) using the following equations (3.6) and (3.7):

$$\text{Degree of swelling (\%)} = \frac{M_s - M_d}{M_d} \times 100 \tag{3.6}$$

and

$$\text{Weight loss} = \frac{M_i - M_d}{M_i} \times 100 \tag{3.7}$$

where M_s is the weight of the sample after submersed in the buffer solution, M_d is the weight of sample after submersed in the buffer solution as dry state, M_i is the initial weight of the sample without submersed in the buffer solution as dry state.

3.2.6 Determination of the Molecular Weight between Crosslinks, the Mesh Size, and the Crosslinking Density

To determine the molecular weight between crosslinks, $\bar{M}_c$, the mesh size, ξ, and the crosslinking density, ρ_x, the sample of gelatin film was cut immediately after crosslinking (1 cm^2). This sample was weighted in air and heptane. The sample was then placed in distilled water at 37 °C for 5 days that allow it swelling to equilibrium, then weighted in air and heptane again. Finally, the sample was dried at 25 °C in vacuum oven for 5 days. Once again, it was weighted in air and heptane. The volumes of the polymer sample in the dry, relaxed, and swollen states were calculated by using equations (3.8) - (3.10), respectively.

$$V_d = \frac{W_{a,d} - W_{h,d}}{\rho_h} \tag{3.8}$$

$$V_r = \frac{W_{a,r} - W_{h,r}}{\rho_h} \tag{3.9}$$

$$V_s = \frac{W_{a,s} - W_{h,s}}{\rho_h} \tag{3.10}$$

where, $W_{a,d}$ is the weight of the dry polymer in air, $W_{h,d}$ is the weight of the dry polymer in heptane, $W_{a,r}$ is the weight of the relaxed polymer in air, $W_{h,r}$ is the weight of the relaxed polymer in heptane, $W_{a,s}$ is the weights of the swollen polymer in air and $W_{h,s}$ heptane, ρ_h is the density of heptanes, V_d is the volume of the polymer sample in the dry states, V_r is the volume of the polymer sample in the relaxed states, and V_s is the volume of the polymer sample in the swollen states.

The calculattion of the polymer volume fraction in the relaxed, $\upsilon_{2,r}$, and swollen states, $\upsilon_{2,s}$, and by using equations (3.11) and (3.12), respectively (Peppas *et al.*, 1998):

$$\upsilon_{2,r} = \frac{V_d}{V_r} \tag{3.11}$$

and

$$\upsilon_{2,s} = \frac{V_d}{V_s} \tag{3.12}$$

The molecular weight between crosslinks, $\bar{M}_c$, was calculated from the swelling data by using equation (3.13) (Peppas *et al.*, 1998):

$$\frac{1}{\bar{M}_c} = \frac{2}{\bar{M}_n} - \frac{\frac{\bar{\upsilon}}{\bar{V}_1}[\ln(1-\upsilon_{2,s}) + \upsilon_{2,s} + \chi\upsilon_{2,s}^2]}{\upsilon_2[\left(\frac{\upsilon_{2,s}}{\upsilon_{2,r}}\right)^{1/3} - \frac{1}{2}\left(\frac{\upsilon_{2,s}}{\upsilon_{2,r}}\right)]} \tag{3.13}$$

where $\bar{M}_n$ is the number averaged molecular weight of the polymer before cross linking, $\bar{\upsilon}$ is the specific volume of gelatin ($\bar{\upsilon}$ = 0.69 cm^3/g of gelatin) (Sutter *et al.*, 2007), $\bar{V}_1$ is the molar volume of water ($\bar{V}_1$ = 18.1 mol/cm^3), χ is the Flory interaction parameter of gelatin (χ = 0.49) (Bohidar 1998), and the dissociation constant p*K*a is 4.7.

The hydrogel mesh size, ξ, defines the linear distance between consecutive crosslinks. It indicates the diffusional space available for solute transport and was calculated by using equation (3.14) (Peppas *et al.*, 1996);

$$\xi = \upsilon_{2,s}{}^{-1/3}\left[C_n\left(\frac{2\bar{M}_c}{\bar{M}_r}\right)\right]^{1/2} \cdot l \qquad (3.14)$$

where C_n is the Flory characteristic ratio for gelatin (C_n = 8.8) (Deiber *et al.*, 2009), $\bar{M}_r$ is the average molecular weight of one amino acid of the gelatin chain ($\bar{M}_r$ = 100 g/mol) (Sutter *et al.*, 2007), and l is the carbon–carbon bond length (l = 1.54 Å).

The crosslinking density of the hydrogel, ρ_x, was calculated by using equation (3.15) (Peppas *et al.*, 1996).

$$\rho_x = \frac{1}{\bar{\upsilon}\bar{M}_c} \qquad (3.15)$$

3.2.7 Drug Release Experiments

3.2.5.1 Preparation of Acetate Buffer

Acetate buffer was chosen to simulate human skin at the pH value of 5.5. To prepare 1000 ml of the acetate buffer solution, 150 g of sodium acetate was dissolved in distilled water. Then 15 ml of glacial acetic acid was added very slowly into the aqueous sodium acetate solution. The solution was poured into the receptor chamber of a modified Franz-Diffusion cell.

3.2.5.2 Spectrophotometric Analysis of Model Drug

A UV-Visible spectrophotometer (TECAN, Infinite M200) was used to determine the spectra of model drugs. Each model drug, in aqueous solution was prepared for scanning the maximum absorption wavelength and the characteristic peak was observed. The absorbance value at the maximum wavelength of the model drug was read that can be related with the model drug, so the calibration curves with various model drugs were generated.

3.2.5.3 Actual Drug Content

The actual amount of drug in the drug-loaded gelatin hydrogels (circular disc about 2.5 cm in diameter, thickness 0.45-0.50 mm) was quantified by dissolving the sample in 4 ml of dimethyl sulfoxide (DMSO) and then 0.5 ml of the solution was added into 8 ml of the acetate buffer solution. The amounts drugs in the solution were measured by used the UV-Visible spectrophotometer at a wavelength of 298 nm for salicylic acid and 5-sulfosalicylic acid.

3.2.5.4 Transdermal Transport Studies

To studied diffusion the custom built modified Franz-Diffusion cells were used. A diffusion cell consisted of two compartments. First one is a water jacket compartment that was used to expose to an ambient condition. Other one was a receptor chamber that was used to contain an acetate buffer solution pH 5.5 and maintained at 37 °C by a circulating water bath. In the study of the effect of crosslinking ratio, a unit of drug-loaded gelatin hydrogels with various crosslinking ratios (0.25, 0.50, 0.75, 1.00, 3.00, and 7.00) of PorGel and (1.00, 3.00, and 7.00) of FishGel, were placed on the nylon net (mesh size = 2.25 mm^2) over the receptor chamber which contained the acetate buffer solution. The nylon net was allowed to come into contact with the buffer solution. The buffer was magnetically stirred during the experiment period (48 h) at temperature 37±2 °C. The drugs diffused through the polymer matrix and the net towards the buffer solution. 0.1 ml of the sample was withdrawn at various time intervals and simultaneously replaced with an equal volume of the fresh buffer solution. The amount of drug in solution was determined by the UV-visible spectrophotometer.

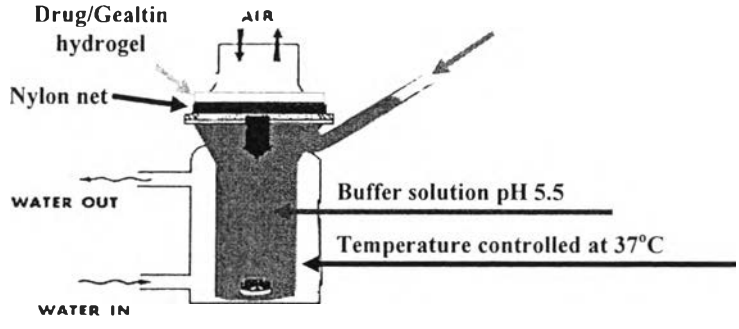

Figure 3.2 Schematic diagram of experimental set up of transdermal transport studies.