# CHAPTER III

# EXPERIMENTAL

## 3.1 Materials

The following materials were obtained from commercial suppliers; except for sodium diclofenac (DFNa) that was complimented.

### 3.1.1 Model drug

- Sodium diclofenac (compliment of Center for Chitin-Chitosan Biomaterials, Thailand)

### 3.1.2 Polymers

- Chitosan, food grade, Lot No. 496212, M.W. 50,000 – 300,000, Deacetylation 90% (Bonafides, Thailand)
- Carrageenan, food grade, type carrageenan KL-805 (Union chemical, Thailand)

### 3.1.3 Chemicals

- Glacial acetic acid 100%, AR grade (Scharlau, Spain)
- Fuming hydrochloric acid 37%, AR grade (Merck, Germany)
- Sodium chloride, AR grade (Merck, Germany)
- Sodium hydroxide, AR grade (Merck, Germany)
- Sodium hydrogen phosphate, AR grade (Merck, Germany)
- Potassium chloride, AR grade (Merck, Germany)
- Potassium dihydrogen phosphate, AR grade (Merck, Germany)
- Potassium bromide, AR grade (Merck, Germany)
- Glutaric acid, commercial grade (Aldrich, Hong Kong)
- Glutaric dialdehyde solution in water 25 %wt., commercial grade (Acros organics, USA)

## 3.2 Instruments

The instruments used in this study are listed in Table 3.1

**Table 3.1** Instruments

| Instrument | Manufacture | Model |
|---|---|---|
| UV-VIS Spectrophotometer | Milton Roy | Spectronic 601 |
| | Cary 50 | 1.00 |
| Fourier transform infrared spectrometer | Nicolet | Impect 4.1 |
| Microscope | Olympus | CH-30 |
| Scanning electron microscope | Jeol | JSM-5800 LV |
| Digital camera | Olympus | C-4040 |
| | Canon | A 80 |
| Horizontal shaking water-bath | Lab-line instruments | 3575-1 |
| pH-meter | Metrohm | 744 |
| Centrifuge | Sanyo | Centaur 2 |
| Ultrasonic bath | Ney Ultrasonik | 28 H |
| Thermogravimetric analyzer | NETZSCH | 409 C/CD |
| Differential scanning calorimeter | NETZSCH | DSC 7 |
| Freeze dryer | Labconco | Freeze 6 |
| Micropipette | Mettler Toledo | Volumate (100-1000 µl) |

## 3.3 Procedure

### 3.3.1 Preparation of the hydrogel beads

The hydrogel beads of various compositions were prepared for the study of the formulation variable effects on the properties of the beads. The procedures used for the preparations are as follow:

#### 3.3.1.1 Preliminary study

In the preliminary study, the hydrogel beads were prepared from a mixture of chitosan and carrageenan solution (weight ratio of chitosan/carrageenan is 1/1), containing 1% (w/v) DFNa. The mixture was extruded into coagulant solutions with varying in concentrations of NaOH (2.5–7.5 % w/v) and KCl (0.1-0.5M). Beads immersion time (3, 5 hours and overnight) and temperature of coagulant (10°C and room temperature) were also varied. The conditions of coagulant in the preliminary study were evaluated by the drug loading efficiency (LE). The DFNa loading of the beads was determined using an indirect method, in which the procedure is as followed.

During the hydrogel beads preparation process, the coagulant solution was collected and diluted to a suitable concentration to determine the amount of DFNa loss. The DFNa contents in the coagulant solutions were determined by UV-VIS spectrophotometer at 276 nm. The LE of DFNa in the beads was calculated from the equation indicated below [38]. All experiments were performed in triplicates.

$$\text{Loading efficiency (LE)} = \frac{(\text{The drug given} - \text{The drug loss}) \times 100\%}{\text{The drug given}}$$

### 3.3.1.2 Preparation of the hydrogel beads with various ratios of chitosan/carrageenan

The hydrogels were prepared with chitosan and/or carrageenan solution in various proportions. The compositions of these formulations (A to G) are presented in Table 3.2. The carrageenan solutions were initially prepared by dissolving carrageenan (2.5% (w/v)) in deionized water at 70±5°C. A constant 1% (w/v) of DFNa was then added to this solution. After the drug dissolved, 2.0% (w/v) chitosan solution in 2.0% (v/v) acetic acid was added to the aqueous solution of carrageenan at the specific chitosan/carrageenan ratio (w/w) of 1/0, 3/1, 2/1, 1/1, 1/2, 1/3 and 0/1. Then, the volume was adjusted to 40.0 ml for each formulation. The mixtures were stirred until homogeneous.

The 40 ml of mixture was extruded in the form of droplets, using a 18-gauge needle, into 100 ml of 0.3M KCl/5.0% (w/v) NaOH coagulant solution except for the chitosan solution and the carrageenan solution that were extruded into 5% NaOH solution and 0.3M KCl solution, respectively. The solutions were maintained at 10°C for 5 hours to allow the beads become hardened. Then, the beads were filtered and washed with cold deionized water to remove excess NaOH and potassium ion. Finally, the hydrogel beads were freeze dried at -42°C for 24 hours.

### 3.3.1.3 Preparation of the DFNa-loaded beads with various DFNa content

It was shown, from the previous study, that the ratio of chitosan/carrageenan 2/1 (w/w) yielded the best release behavior. Therefore this ratio was selected to study the preparation of hydrogel beads with various DFNa contents.

The hydrogel beads were preparaed as described previously with the exception for the varying amounts of DFNa used (in this case 1-5% (w/v)). The formulations (C and I to L) used in the study of drug content effects are given in Table 3.2.

**Table 3.2** The compositions of chitosan/carrageenan ratio, the drug content, the type and amount of cross-linking agent used in each formulation

| Formulation | CS/CR ratio | DFNa content (%w/v) | Crosslinking | |
|---|---|---|---|---|
| | | | GA | GD |
| **Non-crosslinked beads** | | | | |
| A | 1/0 | 1 | - | - |
| B | 3/1 | 1 | - | - |
| C | 2/1 | 1 | - | - |
| D | 1/1 | 1 | - | - |
| E | 1/2 | 1 | - | - |
| F | 1/3 | 1 | - | - |
| G | 0/1 | 1 | - | - |
| H | 2/1 | - | - | - |
| I | 2/1 | 2 | - | - |
| J | 2/1 | 3 | - | - |
| K | 2/1 | 4 | - | - |
| L | 2/1 | 5 | - | - |
| **Crosslinked hydrogel solution before dropping beads (The first method)** | | | | |
| M | 2/1 | 5 | 1.00 | - |
| N | 2/1 | 5 | - | 1.00 |
| **Crosslinked beads in coagulant (The second method)** | | | | |
| O | 2/1 | 5 | 0.25 | - |
| P | 2/1 | 5 | 0.50 | - |
| Q | 2/1 | 5 | 0.75 | - |
| R | 2/1 | 5 | 1.00 | - |
| S | 2/1 | 5 | - | 5.00 |

CS = Chitosan
CR = Carrageenan
GA = Glutaric acid
GD = Glutaraldehyde
DFNa = Sodium diclofenac

### 3.3.1.4 Preparation of the crosslinked hydrogel beads

The effect of types and amounts of crosslinking agent were studied in the formulation M to S (Table 3.2). The chitosan/carrageenan (CS/CR : 2/1) hydrogel solution containing 5% (w/v) DFNa were initially prepared. Two crosslinking methods, as illustrated in Figure 3.1, were employed to prepare the eventual cross-linked hydrogel beads.

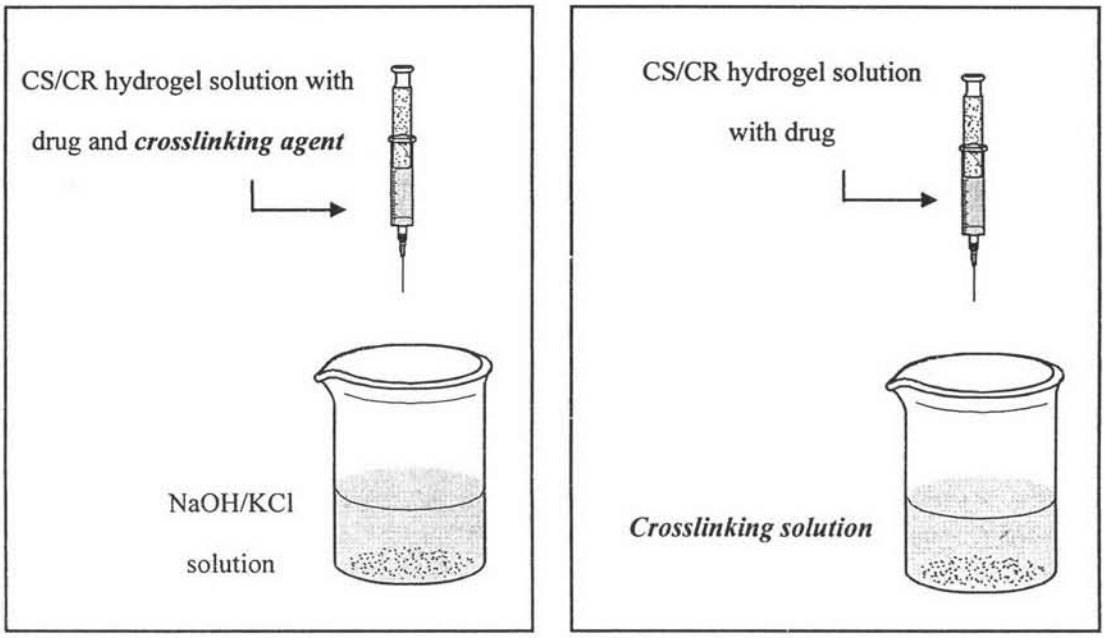


**Figure 3.1** Preparation of the crosslinked hydrogel beads by the two crosslinking methods

In the first method (Formulation M and N), the cross-linking agent (glutaric acid or gluaraldehyde) at 1.00% (w/v) was added into the hydrogel solution containing 5 % (w/v) of drug. The solution was mixed until it was homogeneous. Next, the hydrogel solution was extruded into 0.3M KCl/5.0% (w/v) NaOH coagulant solution. The beads were hardened in the coagulant solution for 5 hours at 10°C. Then, the obtained beads were filtered and washed with cold deionized water. The hydrogel beads were freeze dried at -42°C for 24 hours afterward.

In the second method (Formulation O to S), the hydrogel solution was extruded into two different types of the cross-linking coagulation either glutaric acid or glutaraldehyde. The glutaric acid cross-linking solution contained 0.3M KCl/5.0% (w/v) NaOH and glutaric acid at different concentration (0.25-1.00% (w/v), Formulation O to R). The glutaraldehyde cross-linking solution was prepared using 5.00% (w/v) glutaraldehyde solution in deionized water containing 0.3M KCl (Formulation S). The crosslink time was 5 hours at 10°C for both formulations. Then, the beads were filtered and washed with cold deionized water. The hydrogel beads were freeze dried at -42°C for 24 hours thereafter.

### 3.3.2 Characterization and physical properties of the hydrogel beads

#### 3.3.2.1 Morphology and particle size

The shape, size, surface and cross-section morphology of the beads were observed using a microscope and a scanning electron microscope (SEM). For the SEM examination, the samples were mounted on metal grids and coated by gold under vacuum prior to the observation. The photographs were taken at different magnifications.

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

Infrared spectroscopy was used to confirm the functional groups of substances and samples.

The infrared spectra of all beads were acquired using a KBr disc method. The dried sample was mixed with potassium bromide in agate mortar and pestle by geometric dilution technique. The mixture was then transferred to a hydraulic pressing machine and pressed into a thin disc. The KBr disc was then measured within the wave numbers of 4000-400 $cm^{-1}$.

### 3.3.2.3 Thermal analysis

Thermal analysis is the most common approach to study physicochemical interaction of two or more component systems. Thermal analysis used in this study was the differential scanning calorimetry (DSC) and the thermogravimetric analysis (TGA).

(1) The differential scanning calorimetry (DSC)

The differential scanning calorimetry analysis was used to characterize the thermal behavior of the bead components. This analytical method was carried out on isolated substances and their physical mixture.

Approximately 3-6 mg of the dried beads were weighed into an aluminum pan, were crimped, sealed and immediately made a few holes for the determinations. An empty pan, sealed in the same way as the sample, was used as a reference. All samples were run at a heating rate of 10°C per minute and in the range of 25°C to 350°C.

(2) Thermogravimetric analysis (TGA)

The compositions of the beads were determined by thermogravimetric analysis. This technique measures the weight (%) of the sample as a function of temperature (°C).

The weight of the dried beads for the TGA experiment was about 13 –16 mg. The experiments were conducted using closed aluminum pans with a cover hole. The sample was examined under the nitrogen flow rate of 20 ml/min at a scan rate of 10°C /min starting from 25°C to 550°C.

### 3.3.3 Determination of encapsulation efficiency

The encapsulation efficiency (EE) study was carried out as follows:

The drug content in the DFNa-loaded hydrogel beads was quantitatively determined by immersing the dried beads (100 mg) in 250 ml of phosphate buffer saline (pH 7.4) to dissolve the drug dispersed inside the beads [39]. After sonication, the solution was collected and the total drug content entrapped inside the beads were determined by UV-VIS spectrophotometer at 276 nm.

The DFNa content was calculated from the calibration curve of DFNa in phosphate buffer saline pH 7.4. All experiments were performed in triplicates.

### 3.3.4 Swelling study

The swelling behavior of the chitosan/carrageenan beads were studied by observing changes in the diameter of the beads. The beads were studied in three dissolution systems, i.e. 0.1N HCl (pH 1.2), phosphate buffer saline pH 7.4 and the pH-alternating system.

The beads were immersed in either 0.1N HCl (pH 1.2) or phosphate buffer saline pH 7.4. The diameters of swollen beads were determined at specific time intervals for 5 hours.

In the pH-alternating system, the beads were firstly immersed in 0.1N HCl (pH 1.2) for 2 hours. Then, the dissolution medium was changed to phosphate buffer saline pH 7.4. In this solution, the diameters of the beads were observed for 5 hours.

The swelling behavior was determined by measuring the change of the diameter of the bead using a microscope with a micrometer. The swelling ratio for each sample determined at time $t$ was calculated using the following equation[40].

$$S_w = \frac{D_t}{D_o}$$

Where $D_t$ is the diameter of the beads at time *(t)* and $D_o$ is the initial diameter of the dried beads.

### 3.3.5 Calibration curve of sodium diclofenac

#### 3.3.5.1 In 0.1N HCl (pH 1.2)

DFNa 50 mg was accurately weighed and dissolved with deionized water into a 250 ml volumetric flask and adjusted to volume (200 ppm). The solution was used as stock solution.

The 1, 2, 3, 4 and 5 ml of stock solution was individually pipetted into a 100 ml volumetric flask and then diluted to volume with 0.1N HCl. The final concentration of each solution was 2, 4, 6, 8 and 10 ppm, respectively.

The absorbances of standard solutions were determined by UV-VIS spectrophotometer at 276 nm. The 0.1N HCl was used as a reference solution. Each concentration was determined in triplicates. The absorbance and calibration curve of sodium diclofenac in 0.1N HCl are shown in Table A1 (Appendix A) and Figure A10, respectively.

#### 3.3.5.2 In phosphate buffer saline pH 6.6

DFNa 50 mg was accurately weighed and dissolved with phosphate buffer saline pH 6.6 into a 250 ml volumetric flask and adjusted to volume (200 ppm). The solution was used as stock solution.

The 1, 2, 3, 4 and 5 ml of stock solution was individually pipetted into a 100 ml volumetric flask and then diluted to volume with phosphate buffer saline pH 6.6. The final concentration of each solution was 2, 4, 6, 8 and 10 ppm, respectively.

The absorbances of standard solutions were determined by UV-VIS spectrophotometer at 276 nm. The phosphate buffer saline pH 6.6 was used as a reference solution. Each concentration was determined in triplicates. The absorbance and calibration curve of sodium diclofenac in phosphate buffer saline pH 6.6 are shown in Table A2 (Appendix A) and Figure A11, respectively

#### 3.3.5.3 In phosphate buffer saline pH 7.4

DFNa 50 mg was accurately weighed and dissolved with phosphate buffer saline pH 7.4 into a 200 ml volumetric flask and adjusted to volume (250 ppm). The solution was used as stock solution.

The 0.5, 1, 2, 3, 4, 5, 6 and 7 ml of stock solution was individually pipetted into a 50 ml volumetric flask and then diluted to volume with phosphate buffer saline pH 7.4. The final concentration of each solution was 2.5, 5, 10, 15, 20, 25, 30 and 35 ppm, respectively.

The absorbance of standard solutions were determined by UV-VIS spectrophotometer at 276 nm. The phosphate buffer saline pH 7.4 was used as a reference solution. Each concentration was determined in triplicates. The absorbance and calibration curve of sodium diclofenac in phosphate buffer saline pH 7.4 are shown in Table A3 (Appendix A) and Figure A12, respectively.

### 3.3.6 In vitro release study

The DFNa release study of the beads from each formulation was performed in 0.1N HCl (pH 1.2), phosphate buffer saline pH 6.6 and pH 7.4 by pH-alternating method [2]. The beads (100 mg) were enclosed in a teabag and placed into a beaker that contained 250 ml of the dissolution medium. The beaker was placed on a horizontal shaking water bath; shaking rate was 50 rounds per minute and incubated at 37±2°C.

In the dissolution model with alternating pH, the formulated beads were initially kept in 0.1N HCl (pH 1.2) for the first 2 hours. Then, the dissolution medium was changed to phosphate buffer saline pH 6.6 for 1 hour. At each of the specific time intervals, 5 ml of the dissolution medium were withdrawn. Finally, the release dissolution medium was changed to pH 7.4 and maintained up to 24 hours. In this solution, at various time intervals, 2 ml of the dissolution medium were withdrawn.

Each withdrawal of the sample was centrifuged and diluted to a suitable concentration if necessary. The release rate of DFNa was assayed by UV-VIS spectrophotometer at 276 nm. All experiments were performed in triplicates. The amount of DFNa released was calculated by interpolation from a calibration curve containing increasing concentrations of DFNa. A cumulative correction was made for the previously removed sample to determine the total amount of drug release.