CHAPTER III

Experimental

3.1 Materials

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

3.1.1 Model drug

Sodium diclofenac (compliment from Metallergy and Material Science Research Institute (MMRI))

3.1.2 Polymers

-Chitosan, food grade, Lot No. 496212, M.W. 50,000-300,000, Deacetylation 90% (Bonafides, Thailand)

-Polyethylene glycol, M.W. 6000 (Union chemicals)

-Sodium tripolyphosphate (Union chemicals)

3.1.3 Chemicals

- Acetic acid glacial 100%, AR grade (Scharlau, Spain)
- Glutaric dialdehyde solution in water 25 %wt., commercial grade (Acros organics, USA)
- Hydrochloric acid fuming 37%, AR grade (Merck, Germany)
- Sodium chloride, AR grade (Merck, Germany)
- Sodium hydroxide, AR grade (Merck, Germany)
- Sodium hydrogen phosphate, AR grade (Merck, Germany)
- Sodium tripolyphosphate, food grade (Union chemicals)
- Potassium chloride, AR grade (Merck, Germany)
- Potassium dihydrogen phosphate, AR grade (Merck, Germany)
- Potassium bromide, AR grade (Merck, Germany)

3.2 Instruments

The instruments used in this study are listed in Table 3.1.

Table 3.1 Instruments

Instrument	**Manufacture**	**Model**
Analytical balance	Mettler	AT 200
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
Horizontal shaking water-bath	Lab-line instruments	3575-1
pH-meter	Metrohm	744
Centrifuge	Sanyo	Centaur 2
Ultrasonic bath	Ney Ultrasonik	28 H
Differential scanning calorimeter	NETZSCH	DSC 7
Freeze dryer	Labconco	Freeze 6
Micropipette (100-1000 µl)	Mettler Toledo	Volumate

3.3 Procedure

3.3.1 Preparation of the chitosan hydrogel beads

The hydrogel beads were prepared in various formulations for study of the effects on the properties of beads. The procedure of the hydrogel beads was prepared as follows:

Preparation of the chitosan/DS-loaded hydrogel beads with various DS content

The hydrogels were prepared with chitosan solution. The constant ratio of DS was firstly dissolved in 10 ml of deionized water at room temperature, then 15 ml of 2.5% (w/v) chitosan solution in 1.67%(v/v) acetic acid was added to the aqueous solution of drug at the specific chitosan/DS ratio (w/w) of 1/0, 1/0.33, 1/0.5, 1/0.67, 1/1, 1/1.5, 1/2 and 1/3. The mixtures were stirred until homogenous.

The beads were created by extruding the 20 ml of the preliminary solution, using 18-gauge needle, into 50 ml of coagulant solution, 1%(w/v) tripolyphosphate adjusted pH value to 6.0. The solutions were maintained at room temperature for 20 minutes to let the beads hardened. Then, the beads were filtered and washed with deionized water. Finally, the hydrogel beads were freeze dried at -42^{O}C for 24 hours.

Preparation of the chitosan/DS-loaded hydrogel with various coagulant conditions

From the previous study, the ratio of chitosan/DS 2/1 showed the best ratio of drug-loaded hydrogel in both encapsulation value and release behavior. Therefore, this ratio was selected to study the preparation of hydrogel beads with the various coagulant concentration and pH value.

The hydrogel beads preparations were as described previously with the exception of the concentrations of coagulant solution, Sodium tripolyphosphate (in this case 5 and 10% (w/v)) and the pH value of sodium tripolyphosphate solution (in this case 3, 6 and 8).

Preparation of the chitosan/DS-loaded hydrogel with various crosslinking time.

From the previous study, chitosan bead formulation which prepared by chitosan/DS : 2/1 weight ratio and then dropped into 10% TPP pH 6.0 was the most suitable formulation in the swelling properties and release behavior of chitosan beads.

The hydrogel beads preparation were as described previously with the exception of the gelling time (in this case 20, 30 and 60 minutes)

Preparation of the chitosan/DS-loaded hydrogel with various bead sizes

From the previous study, formulation N, 2/1 weight ratio of chitosan/DS dropped into 10% TPP pH 6.0 for 30 minute, was the suitable formulation of hydrogel preparation for studying in swelling property, encapsulation and release behavior.

The hydrogel beads preparations were as described previously with the exception of the needle sizes (in this case 18, 22 and 24-gauge needle sizes).

Table 3.2 The compositions of chitosan/PEG ratio, the drug content, the conditions of tripolyphosphate solution were used in each formulation.

Formulation	Ratio of the Compositions			% GD (v/v)	Tripolyphosphate		Crossinking Time (min.)	Syringe needle No.
	CS	PEG	DS		conc. (%w/v)	pH value		
A0	1	0	0	-	-	-	20	18
A1	1	0	1	-	-	-	20	18
B	1	0	0	-	1	6.0	20	18
C	1	0	0.25	-	1	6.0	20	18
D	1	0	0.5	-	1	6.0	20	18
E	1	0	0.75	-	1	6.0	20	18
F	1	0	1	-	1	6.0	20	18
G	1	0	1.5	-	1	6.0	20	18
H	1	0	2	-	1	6.0	20	18
I	1	0	3	-	1	6.0	20	18
D	1	0	0.5	-	1	6.0	20	18
J	1	0	0.5	-	5	6.0	20	18
K	1	0	0.5	-	10	6.0	20	18
K	1	0	0.5	-	10	6.0	20	18
L	1	0	0.5	-	10	3.0	20	18
M	1	0	0.5	-	10	8.0	20	18
K	1	0	0.5	-	10	6.0	20	18
N	1	0	0.5	-	10	6.0	30	18
O	1	0	0.5	-	10	6.0	60	18
N	1	0	0.5	-	10	6.0	30	18
P	1	0	0.5	-	10	6.0	30	22
Q	1	0	0.5	-	10	6.0	30	24

CS = Chitosan

PEG = Polyethylene glycol

DS = Sodium diclofenac

GD = Glutaraldehyde

Table 3.2 (continued) The compositions of chitosan/PEG ratio, the drug content, the conditions of tripolyphosphate solution were used in each formulation.

Formulation	Ratio of the Compositions			% GD (v/v)	Tripolyphosphate		Crosslinking Time (min.)	syringe needle No.
	CS	PEG	DS		conc. (%w/v)	pH value		
PEG1	1	0.25	1	-	10	6.0	30	22
PEG2	1	0.5	1	-	10	6.0	30	22
PEG3	1	1	1	-	10	6.0	30	22
PEG4	1	2	1	-	10	6.0	30	22
PEG5	1	0.5	0.25	-	10	6.0	30	22
PEG2	1	0.5	0.5	-	10	6.0	30	22
PEG6	1	0.5	1	-	10	6.0	30	22
PEG7	1	0.5	1.5	-	10	6.0	30	22
GD2.5	1	0.5	0.5	2.5	10	6.0	30	22
GD5.0	1	0.5	0.5	5.0	10	6.0	30	22
GD7.5	1	0.5	0.5	7.5	10	6.0	30	22

CS = Chitosan
PEG = Polyethylene glycol
DS = Sodium diclofenac
GD = Glutaraldehyde

3.3.2 Preparation of the chitosan/PEG hydrogel beads

The chitosan/PEG hydrogel beads were prepared for improve the properties of chitosan hydrogel beads. The procedure of the mixed polymer hydrogel beads was prepared as followed:

Preparation of the chitosan/PEG DS-loaded hydrogel beads with various DS content

The hydrogels were prepared with chitosan and polyethylene glycol mixed solution. The constant ratio of DS was firstly dissolved in 10 ml of deionized water at room temperature, PEG powder was added to the aqueous solution of drug, then 15 ml of 2.5% (w/v) chitosan solution in 1.67%(v/v) acetic acid was added to the PEG hydrogel solution at the specific polymer/DS ratio (w/w) of 1/0, 1/0.33, 1/0.5, 1/0.67, 1/1, 1/1.5 and 1/2. The mixtures were stirred until homogenous.

The beads were created by extruding the 20 ml of the preliminary solution, using 18-gauge needle, into 50 ml of coagulant solution, 10% (w/v) tripolyphosphate adjusted pH value to 6.0. The solutions were maintained at room temperature for 30 minutes to let the beads hardened. Then, the beads were filtered and washed with deionized water. Finally, the hydrogel beads were freeze dried at -42^{O}C for 24 hours.

3.3.3 Characterization and physical properties of the hydrogel beads

Morphology and particle size

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

Fourier transform infrared spectroscopy (FT-IR)

Infrared spectroscopy was used to confirm the functional groups of substances and samples by observing the positions and intensities of IR peaks.

The infrared spectra of all beads were recorded 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}.

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).

- *The different scanning calorimetry (DSC)*

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

Approximately 3-6 mg of the dried beads was weighed into the aluminum pan, were crimped with the sealed pan for 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.

3.3.4 Determination of encapsulation efficiency (EE) and drug loading efficiency (LE)

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

The drug content in the DS-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 [33]. 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 DS content was calculated from the calibration curve of DS in phosphate buffer saline pH 7.4. All experiments were performed in triplicates.

The drug loading efficiency (LE) study was done as follows:

The drug content in the DS-loaded hydrogel beads was quantitatively determined by immersing the 100 mg. of ground beads in 250 ml of phosphate buffer saline pH 7.4 to dissolve the drug dispersed inside the beads [34]. After sonication for 60 minutes, the solution was collected and the drug content entrapped inside the beads were determined by UV-VIS spectrophotometer at 276 nm.

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

The amount of DS loading of the beads, Drug loading efficiency (LE), was determined by using an indirect method, in which procedure is as follows.

During the beads preparation process, the coagulant solution was collected to determine the amount of DS loss. The DS contents in the coagulant solutions were determined by UV-VIS spectrophotometer at 276 nm. The LE of DS in the beads was calculated from the equation indicated below [35]. All experiments were preformed in triplicates.

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

3.3.5 Swelling study

The swelling behaviors of the beads were studied by observing the change of diameter of the beads. The beads were studied at the ambient temperature in two dissolution systems as follows; simulated gastric fluid (pH 1.2), simulated intestinal fluids both in pH 7.4.

The diameter of swollen beads was determined at specific time intervals for 1 hour after beads immersed in the solutions.

The swelling behavior was determined by measuring the change of the diameters of the bead using a micrometer scale with standard light microscope. The swelling percent for each sample determined at time t was calculated using the following equations [34].

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

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

3.3.6 Calibration curve of sodium diclofenac

In 0.1N HCl (pH 1.2)

DS 50 mg was accurately weighed and dissolved with deionized water into a 500 ml volumetric flask and adjusted to volume (100 ppm). The solution was used as stock solution.

The 2, 4, 6, 8 and 10 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 absorbance of standard solutions was 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.

In phosphate buffer saline pH 6.6

DS 62.5 mg was accurately weighed and dissolved with phosphate buffer saline pH 6.6 into a 250 ml volumetric flask and adjusted to volume (250 ppm). The solution was used as stock solution.

The 1, 2, 5, 10 and 20 ml of stock solution was individually pipetted into a 50 ml volumetric flask and then diluted to volume with phosphate buffer saline pH 6.6. The final concentration of each solution was 5, 10, 25, 50 and 100 ppm, respectively.

The absorbance of standard solutions was 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

In phosphate buffer saline pH 7.4

DS 62.5 mg was accurately weighed and dissolved with phosphate buffer saline pH 7.4 into a 250 ml volumetric flask and adjusted to volume (250 ppm). The solution was used as stock solution.

The 1, 2, 3, 4, 5, 6, 7 and 8 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 5, 10, 15, 20, 25, 30, 35 and 40 ppm, respectively.

The absorbance of standard solutions was 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.

3.3.7 In vitro release studies

The DS 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-change method [2]. The beads (100 mg) were enclosed in a teabag and placed into a flask that contained 250 ml of the dissolution medium. The flask was placed on a horizontal shaking water bath; shaking rate was 50 rounds per minute and incubated at $37\pm1^{\circ}c$.

In the dissolution model with pH-change, the pH of the dissolution medium was 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 different time intervals, 3 ml of the dissolution medium were withdrawn and the same volume of fresh buffer replaced to maintain of incubation volume. 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 and fresh buffer was replaced.

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