CHAPTER III

Experimental

3.1 Materials

Commercial grade L-lactic acid (88 % w/w) obtained from fermentation was used in this research. L-lactide and glycolide had purified by recrystallization from distilled ethyl acetate and dried in vacuum oven at room temperature. All of the catalysts, and other chemicals from the suppliers were used without further purification. AR grade ethyl acetate and methanol, and commercial grade solvents were distilled before use.

3.1.1 Model drug

-Nicotine : AR grade, Fluka

3.1.2 Monomer and polymer

- Glycolide monomer : AR grade, Purac
- Polyvinyl alcohol (*M*w 72 000 g mol^{-1}, 98%, hydrolyzed) : AR grade, Merck

3.1.3 Chemicals

- L-lactic acid : Commercial grade, Archer Daniels Midland Company
- Stannous(II) 2-ethylhexanoate : AR grade, Sigma
- Toluene-4-sulfonic acid monohydrate : AR grade, Fluka
- Zinc powder : AR grade, Merck
- Ethyl acetate : AR grade, Fisher scientific
- Methanol : AR grade, Merck
- Dichloromethane : AR grade, Fisher scientific
- Chloroform-d : AR grade, Aldrich

- Acetone	: AR grade, Merck
- Phosphate buffered saline (pH 7.4)	: AR grade, Fluka
- Tetrahydrofuran	: AR grade, Merck
- Glutaraldehyde	: AR grade, Fluka
- Nitrogen gas	: High purity 99.99%, Thai Industrial Gas

3.2 Instruments

The instruments used in this study are listed

Instrument	Model	Company/Country
Nuclear Magnetic Resonance Spectrometer (NMR)	: Varian mercury-400 spcetrometer	Varian, USA
Gel Permeation Chromatograph (GPC)	: Waters 150-CV Degas: ERC-3415α Column: Waters Styragel HR columns (HR 1, 3, and 4), PL-gel 10 μm Pump: Waters 600 Controller Refractive Index Detector: Waters 2414	Waters, USA
Differential Scanning Calorimeter (DSC)	: NETZSCH DSC204F1 Phoenix	Phoenix, USA
Centrifuge	: KR-20000T	Shimadzu, Japan
Vacuum Drying Oven	: DP 41	Yamato Scientific, Japan

Instrument	Model	Company/Country
Scanning electron microscope (SEM)	: JSM-5410 LV	JEOL, Japan

3.3 Procedure

3.3.1 Preparation of the L-lactide

L-lactide synthesis was divided into two steps. The first step was to produce low molecular weight PLLA and the second step was L-lactide ring formation by decomposition of low molecular weight PLLA.

Step I: Linear polymerization of L-lactic acid to low molecular weight PLLA

Under nitrogen atmosphere L-lactic acid solution (150 g) was first heated with toluene-4-sulfonic acid monohydrate 1% w/v (1.50 g) in a silicone oil bath at 140°C for 2 hours to remove any free water. The pressure of the reaction system was then reduced to gentle vacuum for the next 30 minutes, followed by a gradual temperature rise up to approximately 160°C until water ceased to distill. The resulting viscous low molecular weight PLLA was obtained.

Step II: Thermal decomposition of the low molecular weight PLLA to L-lactide

The Zinc catalyst (1.0 weight% of low molecular weight PLLA), was added into the low molecular weight PLLA. The resulting mixture was heated to 160°C and stirred for 30 minutes. The pressured was then reduced to 760 mmHg and temperature was increased to 220°C. The reaction mixture was remained at 760 mmHg, 220 °C until L-lactide crystal was formed in the air condenser. The L-lactide crystal was removed and washed with cold de-ionized water and then vacuum filtered, L-lactide crude was dissolved in warm ethyl acetate to remove insoluble materials and filtered.

The filtrate was then heated to evaporate the remaining ethyl acetate and precipitated white needle-like crystal of L-lactide was obtained. This reprecipitation was repeated three times. The lactide was further dried under reduced pressure at room temperature for 24 hours before use.

3.3.2 Polymerization of the PLLGA

Bulk ROP of PLLGA was conducted. The monomer molar ratio of L-lactide (11.79 mmol) and glycolide (1.29 mmol) was 90:10, using catalyst at 0.1-0.5 mol% (0.013 to 0.065 mmol) of monomers. Under nitrogen atmosphere and with stirring, the mixture was heated at 120°C at a certain time. When using $Sn(Oct_2)$ as a catalyst, monomers were heated to 120°C until the monomers melted, and then $Sn(Oct_2)$ solution was injected into the reaction mixture. The reaction was quenched to room temperature to stop polymerization. The polymerization product was dissolved in dichloromethane and precipitated with cold methanol. This process was repeated twice to purify the copolymer. White solid was filtered out and dried in vacuum oven at room temperature for 24 hours.

Three parameters were studied in this work including type of catalysts ($Sn(Oct)_2$ and Zn dust), reaction times (4, 12, 24, and 48 hours), and catalyst concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mol% of monomers).

The different molar ratio of L-lactide and glycolide in the preparation of copolymer was investigated. The PLLGA copolymers were further characterized by IR, ^{1}H-NMR, ^{13}C-NMR, and GPC.

3.3.3 Polymer characterization

Nuclear magnetic resonance spectrometer (NMR)

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance analysis were used to characterize L-lactide, PLLA, and PLLGA products. The sample was dissolved in chloroform-d ($CDCl_3$) and vortexed until clear solution was obtained. The NMR experiment was carried out by using Varian mercury-400 spectrometer ^{1}H NMR

operating at 400 MHz and ^{13}C NMR at 100 MHz. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) using the residual protonated solvent signal as a reference.

Gel permeation chromatograph (GPC)

Gel permeation chromatography (GPC) was used to determine the molecular weight of PLLGA products. The PLLGA sample (15 mg) was dissolved in tetrahydrofuran (THF) (3 ml) and filtered by syringe filter (diameter 13 mm. 0.45 μm. nylon). GPC chromatogram of PLLGA was obtained from Waters 150-CV chromatography equipped with PL-gel 10 μm mixed B 2 columns (MW resolving range = 500-10,000,000) at 35°C. Tetrahydrofuran (THF) was used as an eluent with the flow rate of 1.0 mL/min. Degassed THF mobile phase was passed through the column for 20 minutes before sample was injected. The sample volume 100 μl was injected and run for 40 minutes. Polystyrenes (MW = 5,460-1,290,000) were used as standards for calibration. The molecular weight was determined by a reflection index detector.

Differential scanning calorimeter (DSC)

Differential scanning calorimeter was determined by NETZSCH DSC204F1 Phoenix. Technique in which the difference in energy input into a substance and a reference material is measured as a function of temperature, while the substance and reference material are subjected to a controlled temperature program.

This technique is used to determine the physical property of semi-crystalline products. In this research, DSC technique was used to study the glass transition temperature (T_g) of PLLGA products. Approximately 10-13 mg PLLGA was weighed in a sample pan. The temperature was raised from 20°C to 160°C (heating rate 20°C/min) and maintained at 160°C for 3 minutes. In this step PLLGA would be completely molten. Liquid nitrogen was used to reduce temperature to -50°C (cooling rate 20°C/min). The temperature was kept constant at -50°C for 3 minutes. Some semi-crystalline PLLGA was precipitated quickly but the amorphous could not be

precipitated in short time. The sample was heated to 200°C (heating rate 20°C/min) until completely molten again. The empty pan was used as standard calibration. The amorphous could be changed from glass-like to rubber-like at glass transition temperature.

3.3.4 Preparation of controlled release PLLGA containing nicotine

PLLGA was dissolved in a mixture of 19 ml acetone (miscible with water) and 1 ml dichloromethane (immiscible with water). The resulting organic solution was added to 20 ml of PVA solution (2%, w/v), with continuous stirring. After the end of liquid addition, stirring of the dispersion under reduced pressure was continued for a further 2 hours in order to remove the organic solvents. During evaporation of the organic solvents from the dispersed droplets of the organic PLLGA solution, the droplets were solidified in the aqueous solution, these solidified PLLGA droplets are designated nanospheres. After completion of evaporation, the dispersion was filtered to remove aggregated PLLGA particles. The dispersed PLLGA nanospheres were sedimentated by centrifugation. To clean the resulting PLLGA nanospheres, the aqueous supernatant was removed. The sedimented nanospheres were redispersed in distilled water under stirring and then sedimented as above by centrifugation twice. The supernatant was removed and the sedimented PLLGA nanospheres were redispersed in a small volume of distilled water by stirring. The final aqueous colloidal PLLGA dispersion was diluted to the desired concentration and was ready to be used.

PLLGA recovery was calculated based on the ratio of the weight of the nanospheres (after vacuum drying) to that of the starting PLLGA polymer.

$$PLLGA\ recovery = \frac{Weight\ of\ PLLGA\ nanospheres\ (after\ vacuum\ drying) \times 100\%}{Weight\ of\ starting\ PLLGA}$$

PLLGA film was prepared from 20 ml colloidal PLLGA dispersion. 3 %, 5 %, and 7 % v/v glutaraldehyde (GD) was used as a crosslinking agent. 0.159 g nicotine was added to the mixture. The mixture was stirred at ambient temperature. After that the film solution was poured onto the petri dish (9 cm diameter). The solvent was allowed to evaporate slowly in air at room temperature for 5 days. The resulting film was cut into 1×1 cm.

Table 3.1 The compositions of PLLGA/PVA ratio were used in each formulation.

Formulation	Ratio of the composition		PLLGA		PVA (2%, w/v)	
	PLLGA	PVA	Weight (g)	Volume[a] (ml)	Weight (g)	Volume[b] (ml)
A	0.5	2	0.1	20	0.4	20
B	1	2	0.2	20	0.4	20
C	1.5	2	0.3	20	0.4	20
D	2	2	0.4	20	0.4	20

[a] = The volume of acetone (19 ml) and dichloromethane (1 ml)

[b] = The volume of aqueous solution

3.3.5 Film Characterization

Morphology of film

The surface morphology of the films was further studied using a scanning electron microscope (SEM), JSM-5410 LV model. 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.

3.3.6 Swelling study

The swelling behaviors of the films were studied from the observation of the change of weight of the films. The films were studied at the ambient temperature in Phosphate buffered saline (PBS) pH 7.4 at room temperature.

The percentage of weight ratio changes was determined at various time intervals after films immersed in the solutions. The swelling percent for each sample determined at time *t* was calculated using the following equations [Soppimatha, *et al.*, 2001]. Where S_w is swelling degree, W_t is the weight of the films at time *(t)* and W_o is the initial weight of the dried films.

$$S_w = \frac{(W_t - W_o) \times 100\ \%}{W_o}$$

3.3.7 In vitro release studies

3.3.7.1 Calibration curve of nicotine

Nicotine 10 mg was accurately weighed and dissolved with PBS pH 7.4 into a 10 ml volumetric flask and adjusted to volume (1,000 µg/ml). The solution was used as stock solution.

The 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ml of stock solution was individually pipetted into a 10 ml volumetric flask and then diluted to volume with PBS pH 7.4. The final concentration of each solution was 10, 20, 30, 40, 50, 60 and 70 µg/ml, respectively.

The absorbance of standard solutions was determined by UV-VIS spectrophotometer at 260 nm. Each concentration was determined in triplicates. The absorbance and calibration curve of nicotine are shown in Table C1 and Figure C1 (Appendix C), respectively.

absorbance and calibration curve of nicotine are shown in Table C1 and Figure C1 (Appendix C), respectively.

3.3.7.2 Determination of controlled release in the PLLGA containing nicotine

The nicotine release study of the film from each formulation was performed in PBS solution pH 7.4. The films (1 x 1 cm) were placed into a flask that contained 100 ml of the phosphate buffer saline. The flask was placed at room temperature. In this solution, at various time intervals, was stirred for 2 minutes and samples were periodically withdrawn from the flask using a 2 mL pipette.

Each sample solution was diluted to a suitable concentration if necessary. The release rate of nicotine was assayed by UV-VIS spectrophotometer at 260 nm. All experiments were performed in triplicates. The amount of nicotine released was calculated by interpolation from a calibration curves containing increasing concentrations of nicotine. A cumulative correction was made for the previously removed sample to determine the total amount of drug release.