CHAPTER III
MATERIALS AND METHODS

3.1 Materials

The following materials were obtained from commercial suppliers.

3.1.1 Model drugs

Doxorubicin hydrochloride (obtained from Sigma-aldrich, USA)

3.1.2 Polymers and chemicals

- Chitosan with $\overline{M}_w$ of 400 kDa and a degree of deacetylation (DD) of 95% (Seafresh Chitosan (Lab) Company Limited, Thailand)
- Sodium alginate (Carlo Erba Reactifs SA)
- Poly (lactic acid) with $\overline{M}_w$ of 121,400 g/mol and consist of 4 % D-lactide, 96 % L-lactide (NatureWorks 2000D, Thailand)
- 4-carboxybenzenesulfonamide (4-CBS) 97 %, AR grade (Sigma-Aldrich,USA)
- Sodium tripolyphosphate, ($Na_5P_3O_{10}$), AR grade (Sigma-Aldrich, USA)
- Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA)
- Ethanol 95 %, commercial grade (EtOH) (Merck, Germany)
- 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC)

(Sigma-Aldrich, USA)

- Hydrochloric acid fuming (HCl) 37 %, AR grade (Merck, Germany)
- Sodium hydroxide (NaOH), AR grade (Merck, Germany)
- Lactic acid (Union chemicals, Thailand)
- Sodium hydrogen phosphate, (Na_2HPO_4), AR grade (Merck, Germany)
- Potassium dihydrogen phosphate, (KH_2PO_4) AR grade (Merck, Germany)
- Cellulose dialysis membrane with $\overline{M}_w$ cut off at 3,500 Da (Spectrum Laboratories Inc.)
- Etoposide (positive control drug for topoisomerase II study) (Sigma-Aldrich, USA)
- Plasmid PBR 322 DNA (Sigma- Aldrich, USA)
- Topoisomerase II (Amersham, UK)
- *Escherchai coli* strains HB101 (Botany faculty of science, Chulalongkorn University)
- Nutrient agar (NA) medium : 0.5% peptone, 0.5% NaCl, 0.3% beef extract, 2% agar (pH 7$\pm$ 0.2), Ampicilin trihydrate (TP drug)

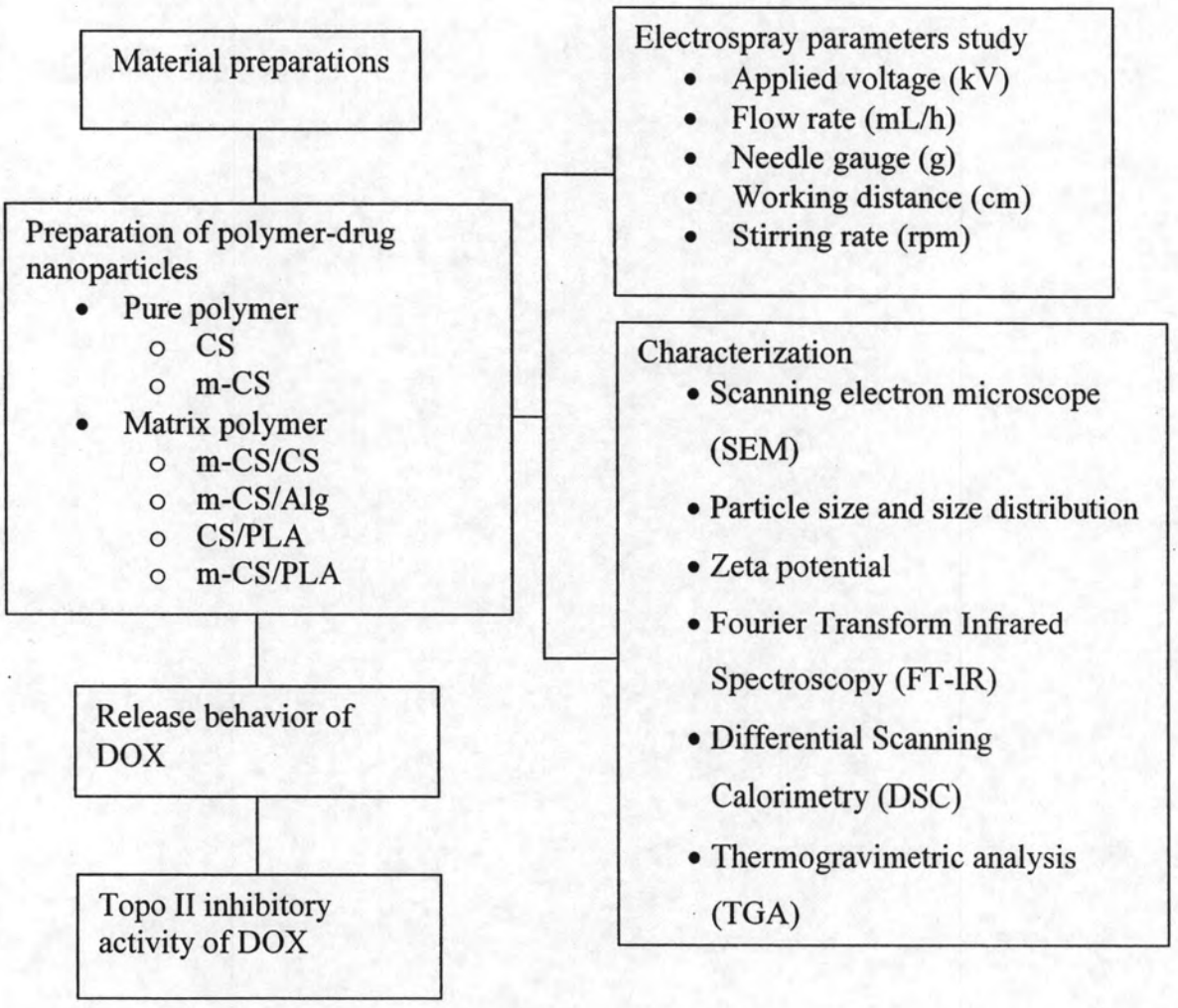
3.2 Instruments

The instruments used in this study are listed in Table 3.1

Table 3.1 Instruments

Instrument	Manufacture	Model
High voltage	Ormond beach	GAMMA
Syring pump	KDscientific	KD100
Analytical balance	Mettler	AT200
Ultracentrifuge	Refrigerated centrifuge	Sigma 30K
UV/Vis microplate reader	Sunrise TECAN	A-5082
Particle sizer	Malvern Instruments	Zetasizer nanoseries
Fourier transform infrared spectrometer	Perkin Elmer	Spectrum RX I
Scanning electron microscope	Philips	XL30CP
Differential scanning calorimeter	NETZSCH	DSC 7
	Perkin-Elmer	DSC7
pH-meter	Metrohm	744
Micropipette (100-1000 µl)	Mettler Toledo	Volumate
Magnetic stirrer	IKA®C-MAG	HS7
Freeze dryer	Labconco	Freeze 6
Ultrasonic bath	Ney ultrasomik	28 H

3.3 Method



Figuer 3.1 Flow chart of methodology

3.3.1 Synthesis of 4-carboxybenzenesulfonamide-chitosan (4-CBS-CS) conjugates

1 g of CS was dissolved in 100 ml of 1% (v/v) lactic acid at room temperature overnight to give a 1% (w/v) CS solution. 0.05 g of 4-CBS, were added into 100 ml of 1% (w/v) CS solution (CS : 4-CBS= 1 : 0.05). Next, EDAC as the coupling agent was added with the constant mole ratio of 1.2:1 of EDAC to 4-CBS and then refluxed for 6 hours. EDAC was removed by adding 1 N HCl. Then the 4-CBS-CS conjugate was precipitated by adding 1 N NaOH. Afterward, the precipitated 4-CBS-CS conjugate was filtered, washed with distilled water until neutral and air-dried. The resulted conjugate polymer was post-treatmented by dissolving in 1% (v/v) lactic acid at room temperature with shaking overnight before used.

3.3.2 Preparation of hydrophobic chitosan (sodium dodecyl sulfate–chitosan complex)

Preparation of the solutions: stoichiometric sodium dodecyl sulfate–chitosan complex was prepared simply by mixing acidic solutions of chitosan and SDS. Ratio of weight of chitosan : SDS = 1 : 3 were dissolved in 100 ml of 1% lactic acid solution, respectively. After completely dissolved, SDS solution was poured into chitosan solution under vigorous stirring and the mixture was gently stirred for 2 h at room temperature. The resulting precipitates were filtered off, washed three times with distilled water and finally freeze-dried to yield a white and spongy product.

In case of, sodium dodecyl sulfate-4-carboxybenzenesulfonamide-chitosan complex set was prepared similarly of sodium dodecyl sulfate–chitosan complex.

3.3.3 Particles preparation

3.3.3.1 Chitosan (CS) and 4-carboxybenzenesulfonamide-chitosan (m-CS) nanoparticles

Drug loaded polymer nanoparticles were prepared by the electrospraying technique which was developed from the principle of electrospinning technique. The polymer solution (1%w/v) was prepared by dissolving in dilute lactic acid (1%v/v) at

room temperature with stirring overnight. After stirring, the solution was then filtered through nylon cloth to dispose of impurities or undissolved chitosan. The filtrated was added into a solution reservoir using a syring pump into a syring-nozzle system. A high electric field applied to the polymer solution in the syring. Droplets fall into a coagulant bath containing 80 ml of 5 % tripolyphosphate (TPP) solution.

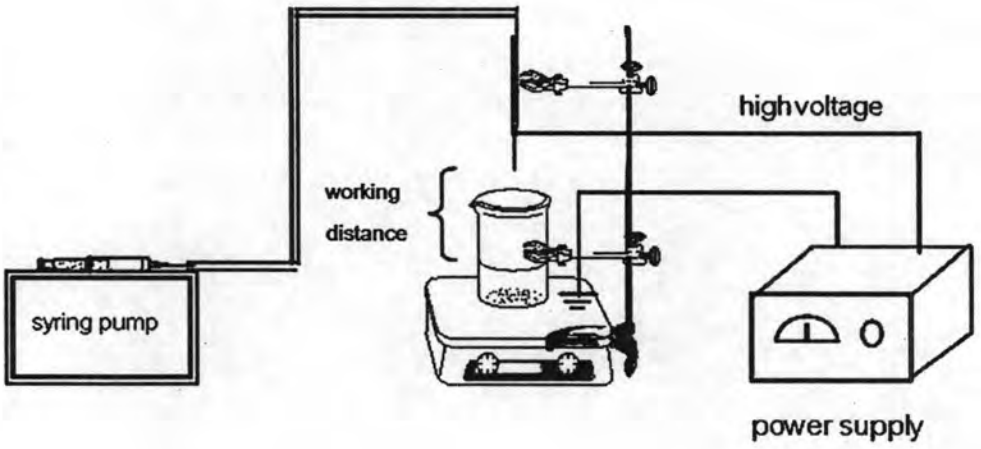


Figure 3.2 Electrospray ionization apparatus

3.3.3.2 Chitosan and 4-carboxybenzenesulfonamide-chitosan mixed poly (lactic acid) nanoparticles

The polymer solution (1%w/v) was prepared by weigh 0.5 g of hydrophobic chitosan and 0.5 g of poly (lacltic acid) dissolved in dichloromethane : toluene (v/v) = (80 : 20) at room temperature with stirring overnight. The solution was added into a solution reservoir using a syring pump into a syring-nozzle system. A high electric field applied to the polymer solution in the syring. Droplets fall into a coagulant bath containing 80 ml of 5 % tripolyphosphate (TPP) solution.

The preparation of doxorubicin-loaded various polymers nanoparticles follows the method mentioned above, but with some modifications as follows. polymer-drug solutions was prepared by adding a specified amount of doxorubicin hydrochloride into the polymer solutions under magnetic stirring at 400 rpm for 1 hour. This mixed solution was then used for particle preparation. The parameters studied for the particle preparation by using electrospay technique are shown in Table 3.2. Polymer-drug

colloidal nanoparticles were formed spontaneously under mild agitation at room temperature, after 30 mins later, polymer-drug colloidal nanoparticles were centrifuged at 4,000 rpm for 30 mins. Then, the supernatant was discarded and the deposit was re-dispersed in distilled water for further use.

Table 3.2 The parameters studied for the nanoparticles preparation by using electrospray technique

Needle gauge (g)	20, 24 and 26
Applied voltage (kV)	10, 13 and 15
Flow rate (mL/h)	0.5, 5 and 10
Working distance (cm)	8, 10 and 12

3.3.4 Characterization of polymer-drug nanoparticles

3.3.4.1 Scanning electron microscope (SEM)

The samples for the SEM analysis were prepared by sprinkling the nanoparticles on one side of a double adhesive stub. The stub was then coated by gold under vacuum. The microcapsules were then observed via scanning electron microscope (SEM, Phillips XL30CP).

3.3.4.2 Particle size and size distribution

The Particle size and size distribution of nanoparticles are tested by a Particle Size Analyzer (Zetasizer nano series, Malvern instruments). Nanoparticles are suspended in 5% Sodium tripolyphosphate. Size calculation was based on DLS method as a software protocol. The scattering light was collected at an angle of 90° through fiber optics and converted to an electrical signal by an avalanche photodiode array (APDs) [58]. All samples were sonicated and run in triplicate with the number of runs set to 5 and run duration set to 10 seconds.

3.3.4.3 Zeta potential

Zeta potential of CS-DOX nanoparticles was determined using Particle Size Analyzer (Zetasizer nano series, Malvern instruments). The analysis was performed at a scattering angle of 90°. All samples were sonicated and run in triplicate with the number of runs set to 5 and run duration set to 10 seconds.

3.3.4.4 Fourier Transform Infrared Spectroscopy (FT-IR)

The infrared spectra of polymer and polymer-drug all formulations were recorded with FT-IR (Perkin Elmer Spectrum RX-1 FT-IR system). FT-IR spectra were recored in the wavelength region 400-4000 cm^{-1} at ambient temperature.

3.3.4.5 Differential scanning calorimetry (DSC)

Approximately 10 mg of CS-DOX nanoparticles were weighed in the aluminum pan, then crimped with the sealed pan for determinations. And empty pan, sealed in the same way as the sample, was used as a reference. DSC was obtained with Perkin-Elmer DSC 7 instrument under nitrogen atmosphere. The temperature range is 25-300 C° and heating rate of 10 K/min.

3.3.4.6 Thermogravimetric analysis (TGA)

The mass of the samples was generally in the range of 7-10 mg. The platinum pan was placed in the balance system equipment. The mass of the platinum pan was continuously recorded as a function of temperature. TGA was obtained with a Perkin Elmer/Pyris Daimond TG/DTA instrument in under nitrogen atmosphere. The temperature range is 25-800 C° and heating rate of 10 K/min.

3.3.5 Evaluation of drug entrapment efficiency

3.3.5.1 Calibration curve of doxorubicin for UV spectroscopy

Doxorubicin hydrochloride stock solution 1 mg/ml was diluted to 1, 5, 10, 25, 50, 75 and 100 μg/ml with phosphate buffer (pH 7.4) in volumetric flask.

The absorbance of known doxorubicin concentration was determined by UV/Vis microplate reader (Sunrise TECAN) at a wavelength of 480 nm. The phosphate buffer (pH 7.4) was used as blank medium. The absorbances and the calibration curves of doxorubicin in phosphate buffer (pH 7.4) are presented in Table 1B and Figure 1B (Appendix B), respectively.

3.3.5.2 Determination of drug entrapment efficiency (EE)

A known weight of CS-DOX nanoparticles were suspended in phosphate buffer (pH 7.4). The suspension was centrifuged at 4000 rpm for 30 min at 25°C to separate the free drug in the supernatant from the drug incorporated in the particles. The amount of DOX was measured by UV-vis spectrophotometer at 480 nm. The calibration curves were performed using standard DOX diluted in phosphate buffer (pH 7.4). The percentage of encapsulation efficiency was calculated as follows:

$$DOX\ encapsulation\ efficiency = \frac{weight\ of\ the\ total\ DOX - weight\ of\ free\ DOX}{weight\ of\ the\ total\ DOX} \times 100\%$$

3.3.6 *In vitro* drug release

The DOX release from polymer nanoparticles were studied in phosphate buffer pH 7.4 by dialysis bag diffusion technique [59]. The accurately weighed quantities of 10 mg particles were enclosed in a dialysis bag with a molecular weight cutoff of 3500 Da and immersed into 50 ml of phosphate buffer pH 7.4 in a flask. The flask was placed in a shaken water bath at speed of 100 rounds per minutes and incubated at 37±1 °C. The incubated solution was collected at designated interval of time points and equal volume of fresh medium was compensated. The released DOX amount was determined in 30 days by UV-vis spectroscopy, detection at 480 nm.

The amount of DOX released was calculated by interpolation from a calibration curves containing increasing concentrations of DOX. The percentages of cumulative DOX release were calculated from this equation.

$$\%Cumulative\ release = \frac{Amount\ of\ DOX\ from\ release}{Amount\ of\ DOX\ before\ release} \times 100$$

3.3.7 Topoisomerase II inhibition

3.3.7.1 Preparation of pBR322 plasmid DNA

Strain of *E. coli* HB101 was routinely grown at 37 °C for 18 h in LB medium containing 100 μg/ml of ampiciline for isolation of single colony of *E. coli*. plsmid DNA isolation from bacteria cells. Cell from overnight growth in fresh LB medium at 37°C to exponential phase (A_{600} = 0.4-0.6) was taken in an Eppendorf kit and centrifuged for 2 min. Cell lysis was achieved by adding 400 μl of lysis buffer. The suspension was kept on ice for 5 min. The lysate was centrifuged at 13,000 rpm for 2 min to pellet the cell DNA and other bacterial debris. Plasmid DNA was washed with 400 μl of wash buffer and centrifuged at 13,000 rpm for 2 min, the pellet was dried briefly by centrifuging for 2 min in an Eppendorf centrifuge and finally dissolved in 10 μl of dH_2O. Plasmid DNA was visualized in 1% agarose gel electrophoresis [60].

3.3.7.2 Topoisomerase II assay

Topoisomerase II is an important nuclear enzyme controlling DNA topology through catalysis of a breakage of double-stranded DNA, allowing for the passage of double-stranded DNA followed by a resealing of the DNA. Relaxation of DNA supercoils by topoisomerase II is considered crucial to its role in DNA replication and in transcription. To further elucidate the mechanism of action of polymer-doxorubicin for the relaxation of supercoiled plasmid pBR322 DNA was evaluated. Topoisomerase II relaxation assay was conducted using human topoisomerase II

(Amersham). Etoposide is selective topoisomerase II inhibitors was used as positive control.

Polymer-doxorubicin nanoparticles were screened for the topoisomerase II inhibition function. The activity of the nanoparticles on the relaxation of DNA topoisomerase IIα was determined by measuring the conversion of supercoiled PBR 322 plasmid DNA to its relaxed form. Topoisomerase II relaxation assay was conducted by using human topoisomerase II with doxorubicin as a positive control.

Topoisomerase II reaction was performed in 20 µl of reaction mixture containing. The reaction mixture contained 10 mM Tris-HCl (pH 7.9), 175 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5 % glycerol, 1 mM ATP, 0.5 mM dithiothreitol, 30 µg/mL bovine serum albumin, 0.2 µg pBR322 plasmid DNA, 0.3 U DNA topoisomerase IIα, and test compounds in a final volume of 50 µL. The reaction mixtures were incubated at 37 °C for 30 min and terminated by the addition of 3 µL of solution containing 0.77 % sodium dodecyl sulfate, and 77 mM EDTA. Samples were mixed with 2 µL of solution containing 30 % sucrose, 0.5 % bromophenol blue and 0.5 % xylene cyanol and subjectded to electrophoresis on a 1 % agarose gel at 1.5 V/cm for 10 h with a running buffer of Tris-borate-EDTA. Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 µg/mL). DNA bands were visualized a UV transilluminator and quantities by an image analyzer and Syngene software.

3.3.8 Statistical analysis

All measurements were performed in triplicate for each experiment. Results are presented as means ± SD. Statistical analysis was performed by one-way ANOVA using Microsoft Excel (Microsoft Corporation) with $P < 0.05$ considered to indicate statistical significance.