CHAPTER V

CAMPTOTHECIN-INCORPORATING N-PHTHALOYLCHITOSAN-g-mPEG SELF-ASSEMBLY MICELLAR SYSTEM: EFFECT OF DEGREE OF DEACETYLATION

5.1 Abstract

Amphiphilic grafted copolymers, N-phthaloylchitosan-grafted poly (ethylene glycol) methyl ether (PLC-g-mPEG), were synthesized from chitosan with different degree of deacetylation (DD = 80, 85, 90 and 95 %). Due to their amphiphilic characteristic, these copolymers could form micelle-like nanoparticles. The critical micelle concentration (CMC) of these nanoparticles with different DD in water was similar (28 μg/ml). Under transmission electron microscope (TEM), the nanoparticles exhibited a regular spherical shape with core-shell structure. The particle sizes determined by dynamic light scattering were in the range of 100-200 nm, and increased as the % DD of chitosan increased. The cytotoxicity of phthaloylchitosans (PLC) and PLC-g-mPEG in Hela cells line were evaluated. The results showed that cytotoxicity of PLC and PLC-g-mPEG increased with increasing % DD of chitosan. The cytotoxicity of PLC-g-mPEG was significantly lower than that of PLC. Camptothecin as a model drug was loaded into the inner core of the micelles by dialysis method. It was found that % DD of chitosan, corresponding to the n-phthaloyl groups in the inner core of the nanoparticle obtained, was a key factor in controlling % yield, stability of the drug-loaded micelles, and drug release behavior. As the %DD increased, the CPT-loaded micelles stability increased. Release of CPT from the micelles was dependent on the % DD and a sustained release was obtained in high % DD.

Key words: polymeric micelles, degree of deacetylation, camptothecin, N-Phthaloylchitosan

5.2 Introduction

Amphiphilic block or graft copolymers have recently emerged as a class of materials with a wide range of pharmaceutical application. It can form nanoscopic

core-shell structures above the critical micellar concentration (CMC) [1]. In the past 20 years, polymeric micelles and polymeric aggregations have attracted great interest because of their potential utilized for a drug carrier system. The hydrophobic core serve as reservoirs for hydrophobic drugs while the hydrophilic part serves as interface between the bulk aqueous phase and the hydrophobic domain. Highly hydrated outer shells of the polymeric micelles or aggregations can inhibit intermicellar aggregation of their hydrophobic inner cores. Consequently, the polymeric micelles maintain a satisfactory aqueous stability irrespective of high contents of hydrophobic drug incorporated into the inner core of micelles. Furthermore, a polymeric micelles in a size range <200 nm reduces non-selective reticuloendothelial system (RES) scavenge and shows enhanced permeability and retention effects (EPR effect) [2] at solid tumor sites for passive targeting.

Polymers used for drug delivery system (DDS) should be non-toxic, biodegradable and metabolized or excreted in the body. Chitosan is a polysaccharide derived from chitin by alkaline deacetylation and consists of 2-amino-2-deoxy-(1-4β)-D-glucopyranose residues (D-glucosamine units), and the D-glucosamine content is dependent on the degree of deacetylation (DD). Chitosan is currently receiving a great deal of attention for medical and pharmaceutical applications. The main reasons for this are undoubtedly its appealing intrinsic properties, such as biocompatibility, biodegradability, non-toxicity, adsorption properties, film-forming ability, bioadhesivity, antimicrobial activity against fungi, bacteria and viruses, in addition to its haemostatic effect [3-5]. Most of the properties of chitosan can be related to its cationic nature. At acidic pH, it is a linear polyelectrolyte with a high charge density with one positive charge per glucosamine residue and, therefore, will interact with negatively charged molecules including proteins, anionic polysaccharides and nucleic acids [6].

Camptothecin (CPT) is a potent, anticancer agent acting through the inhibition of topoisomerase I during the S-phase of the cell cycle [7]. It exists in two forms depending on the pH value, namely, an active lactone form at pH below 5 and an inactive carboxylate form at basic pH (Fig. 1) [8].

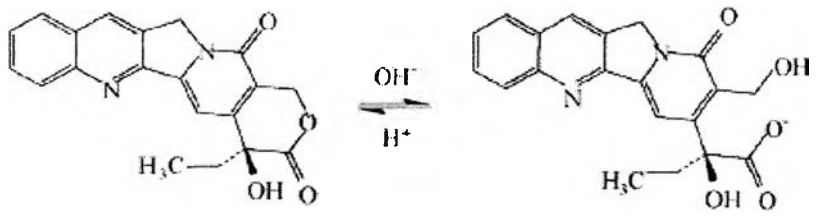


Fig. 5.1 Chemical structure of camptothecin (CPT) two forms depending on the pH value, namely, an active lactone form at pH below 5 and an inactive carboxylate form at basic pH.

At physiological pH, most CPT molecules exist in the inactive carboxylate form. The stability of the lactone form of CPT is critical for its anticancer activity. In addition, the ring opening carboxylate form shows poorer diffusibility through the lipid bilayer than the lactone form. The labile lactone ring and poor aqueous solubility pose many challenges for drug development and DDS. Various types of DDS have been developed in order to reduce severe systemic toxicities and enhance antitumor effects by improving their pharmacokinetics. Previous studies showed that the lactone ring of CPT was protected upon incorporation of the drug into a lipid bilayer structure like liposomes [9-10], microspheres [11-13], upon conjugation to synthetic polymers [14-18], nanobiohybrids [19], and polymeric micelles [20-24].

Chitosan is soluble in aqueous acidic solutions, but cannot form micelles in water. Recently, the production of chitosan spheres for DDS was achieved by some specific processing techniques, such as suspension cross-linking, spray-drying coagulation, emulsification/solvent evaporation. Modifications on the chitosan structure can be carried out in order to adequate it to the intended application, such as hydrosolubility, adhesivity, haemocompatibility and others. The modification of polymeric materials by graft copolymerization has been studied deeply by several authors [25-26] because it can provide materials with desired properties through the appropriate choice of the molecular characteristics of the side chain to be grafted. Some modified chitosan e.g. N-lauryl-carboxymethyl-chitosan [27] and N-octyl-O-sulfate chitosan [28] have been reported for the preparation of polymeric micelles for paclitaxel. In our previous report, we have concentrated on the modification of

chitosan based on the balancing of polarity on the chain in order to obtain novel derivatives. We synthesized N-phthaloylchitosan-grafted poly (ethylene glycol) methyl ether (PLC-g-mPEG) which showed a self-assembly nano level sphere-like particles (80-100 nm) depending on the chain length of mPEG as observed by SEM and TEM [29]. The previous study showed that the PLC-g-mPEG with 95 % DD micellar solution had the high CPT-loaded micelles using dialysis method. We postulated that an optimal controlled release preparation would exclusively release the lactone form of CPT, moreover, CPT-loaded PLC-g-mPEG micelles showed the longer kinetic half life than CPT under the physiological conditions [30]. In this study, we aimed to determine the influence of % DD of chitosan on the physicochemical properties of PLC-g-mPEG polymers. Its cytotoxicity was evaluated in cell culture. In addition, CPT incorporation efficiency, the stability of CPT-loading micelles and in vitro release behavior were investigated.

5.3 Material and Methods

Materials

Chitosans with 80, 85, 90 and 95 % deacetylation (Mv 5.78 X 10^5 Da) were provided by Seafreach Chitosan (Lab), Co., Thailand. Phthalic anhydride and succinic anhydride were purchased from Fluka Chemika, Switzerland. Poly(ethylene glycol) methyl ethers (mPEG, MW 2,000 Da) were obtained from Aldrich Chemical Company, USA. 1-Ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) was purchased from TCI, Japan. 1-hydroxy-1H-benzotriazole, monohydrate (HOBt) was obtained from BDH Laboratory Supplies, UK. (s)-(+)-Camptothecin and pyrene were purchased from Aldrich Chem. Co., Milw., Wl, USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-chemical Co., USA. Dulbecco's modified Eagle's medium (DMEM), Trypsin-EDTA, penicillin-streptomycin antibiotics and fetal bovine serum (FBS) were from GIBCO-Invitrogen, USA. Hela cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of analytical grade.

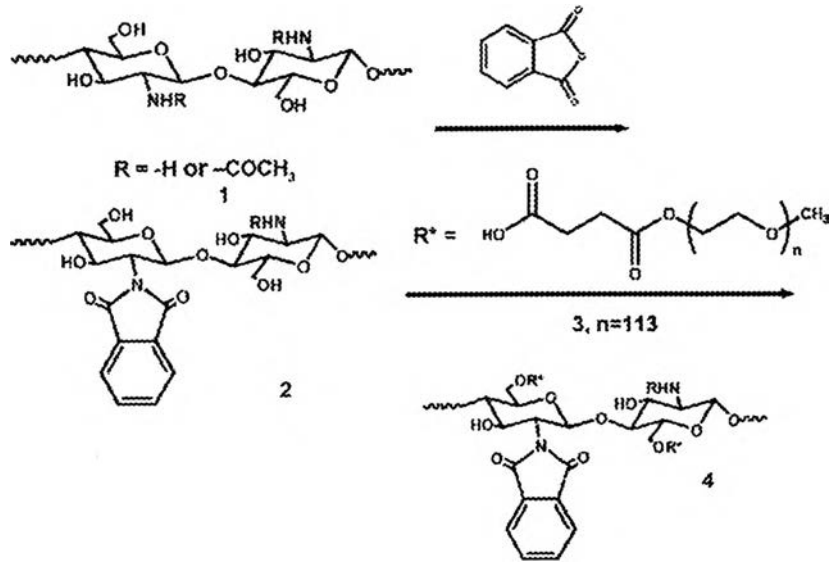
Synthesis of N-Phthaloylchitosan-grafted mPEG (PLC-g-mPEG)

N-Phthaloylchitosan **2** was prepared as previous reported [29]. Briefly, Chitosans **1** (DD = 0.80. 0.85, 0.90 and 0.95, Mv 5.78 X 10^5) were reacted with phthalic anhydride (5 mol equivalent to pyranose rings) in DMF at 100°C under vacuum for 6 h. The temperature was reduced to 60°C and left overnight. The product was reprecipitated in cold water. The precipitate was collected, washed with ethanol, and dried in vacuum to give pale yellow powder **2** (Scheme1).

Anal. Calcd. for **2** $(C_{14}H_{13}O_6N)_{0.67}$ $(C_6H_{11}O_4N)_{0.13}$ $(C_8H_{13}O_5N)_{0.05}$: (%): C, 55.54; H, 4.73; and N, 6.14. Found (%): C, 56.54; H, 5.56; and N, 3.43. FTIR (KBr, cm^{-1}): 3,474 (OH), 1,777 and 1,713 (C=O anhydride), and 721 (aromatic ring).

Compound **2** (0.40 moles equivalent to **3**) was stirred with **3** in DMF solution. 1-hydroxy-1H-benzotriazole, monohydrate (HOBt, 3 moles equivalent to **3**) was added as catalyst and stirred at room temperature until clear solution. 1-Ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide, hydrochloride (EDC, 3 moles equivalent to **3**) was reacted overnight. The mixture was dialyzed in water and washed with ethanol to obtain white particles, **4** (scheme 1).

In order to determine the substitution of mPEG and phthalimido group, 1H-NMR spectra were obtained in 7d-DMF on a Vatian Mercury-400BB spectrometer at room temperature.



Scheme 5.1 Reaction of grafting phthalic anhydride and mPEG-COOH on chitosan chain

Critical micelle concentration (CMC) determination

The CMC of PLC-g-mPEG polymer was determined using a spectrofluorophotometer (RF-1501, Shimadzu, Japan) with pyrene as a fluorescence probe. Experiments were set up with excitation and emission wavelengths of 352 and 383 nm, respectively. The concentrations of pyrene were 6.0 x 10^{-7} M. The emission and excitation spectra of pyrene fluorescence were recorded with a micelle concentration that ranged from 0.125 to 256 μg/ml. In each experiment, a 5 μl pyrene in acetone solution was added to a 4 ml polymeric micelle solution and stirred for 24 h until the acetone was completely evaporated prior to measurement. For pyrene excitation spectra, the ratio of fluorescence intensity at 334 and 337 nm (I_{334}/I_{337}) was calculated and plotted against the logarithm the concentration of micelles.

Cytotoxicity test

Cytotoxicity of tested compounds was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to Mosmann [31], with minor modifications. This assay measures the conversion of MTT to insoluble formazan by mitochondrial dehydrogenase enzymes of living cells.

The *in vitro* cytotoxicity was evaluated against human tumor cell lines (Hela cells). The concentration-dependent cytotoxic effect of PLC polymers and PLC-g-mPEG micelles was investigated. Hela cells were maintained in Dulbecco's modified Eagle's medium (DMEM) of pH 7.4, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acid solution and 0.1% penicillin- streptomycin solution in humidified atmosphere (5% CO_2, 95% air, 37 °C). The cells were grown under standard conditions for 24 hours till 60-70% confluency. The cells were then seeded at a density of 5000 cell/well in 96 well cell culture plates and preincubated for 24 h. Then, the cells were treated with PLC polymers or PLC-g-mPEG micelles at various concentrations (0.001-1 μM in medium) for 72 h under normal growing conditions. After treatment, polymers solution were removed, and fresh cell culture medium were added and incubated for 4 h to stabilize the cells. Finally, the cells were incubated with MTT containing medium (0.5 mg/ml) for 4 h. Then, the medium was removed, and the formazan crystals formed in living cells were dissolved in 100 μL dimethyl sulfoxide (DMSO). Plates were incubated for 30 min at 37 °C and absorbance was measured at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability was determined by comparing absorbance in wells containing treated cells with that of untreated cells. Eight replicates were measured for each concentration.

Incorporation of CPT into polymeric micelles

The incorporation of CPT into polymeric micelles was carried out by a dialysis method [30]. Briefly, 5 mg of PLC-g-mPEG polymer and CPT (5-40 % of polymer) were dissolved in 2 ml of DMSO in a glass tube. The mixture was stirred at room temperature until completely dissolved. The mixture was then placed in a dialysis bag (membrane: Spectra/Por® 12,000-14,000 MWCO, Spectrum Laboratories, USA), dialyzed against distilled water overnight. The mean particle diameters were determined by dynamic laser light scattering (DLS) using Zetasizer®3000 (Malvern Instruments, Southborough, MA, USA). CPT-loaded polymeric micelles were dissolved in a mixture of DMSO:H_2O (9:1). The amount of

CPT incorporated into polymeric micelles was determined by UV-VIS absorption at 365 nm. The incorporation efficiency was calculated as the percentage share of the initial drug used in the preparation for incorporation into the micelles.

Gel permeation Chromatography (GPC)

The stability of drug-loaded micelles was determined by GPC as described previously [20,21]. High performance liquid chromatography (HPLC) was carried out using an Agilent HPLC system (Agilent 1100 series, USA) equipped with a Tosoh TSKgel G3000PW$_{XL}$ column at 40 °C. Samples (50 µl) were injected into the column and eluted with distilled water at a flow rate of 1.0 ml/min. The detection was performed by absorption at 351 nm using a UV detector and a refractive index (RI) detector.

In vitro release

Release of CPT from CPT-loaded micelles was measured using a dialysis bag (membrane: Spectra/Por® 12,000-14,000 MWCO, Spectrum Laboratories, USA) as described previously [20]. One hundred milliliters of phosphate-buffered saline (PBS) at pH 7.4 was used as a medium at 37 ± 0.1 °C under constant stirring. One milliliter CPT-loaded micelles were placed in a dialysis bag and immersed in the medium. At certain time intervals, 1 ml aliquots of the medium were withdrawn and the same volume of fresh medium was added. The sample solution was analyzed by reverse-phase HPLC. All experiments were performed in triplicate.

Reverse-phase HPLC analysis of CPT

Concentrations of CPT were determined using a reverse-phase HPLC system [20]. A lactone form and the open carboxylated form of CPT were separated within a single chromatographic run. The reverse-phase HPLC system for this determination consisted of an Agilent HPLC system (Agilent 1100 series, USA) at a flow rate of 1.0 ml/min at 40 °C. For separation a Waters µBondasphere C_{18} reverse-phase column (3.9 x 150 mm, Nihon Waters, Tokyo, Japan) was used. The mobile phase was composed of 23% acetonitrile and 77% aqueous buffer (0.1 M KH_2PO_4, 0.5 mM

tetrabutylammonium dihydrogen phosphate and 0.4 mM triethyl amine at pH 6). The detection was performed using a fluorescence detector with an excitation wavelength of 360 nm and emission wavelength of 430 nm.

Statistical analysis

All measurements were collected in triplicate experiments. Values are expressed as mean ± standard deviation (SD). Statistical significance of differences in amount of CPT was examined using one-way analysis of variance (ANOVA) followed by LSD post hoc test. The significance level was set at $p < 0.05$.

5.4 Results and Discussion

Polymeric micelles have received much attention as a material for drug delivery systems since the incorporation can be achieved without the active sites being destroyed by harsh reactions in conjugation step, and at the same time the self-assembly is eventually formed without a cross-linker. We have recently successfully synthesized the self-assembly of chitosan based on the balance of polarity on the chain, namely, N-Phthaloylchitosan-grafted poly (ethylene glycol) methyl ether (PLC-g-mPEG) [29]. The previous study showed that the PLC-g-mPEG with 95 % DD micellar solution had the high CPT-loaded micelles [30]. In the present work, we aimed to determine the influence of % DD of chitosan on the physicochemical properties of PLC-g-mPEG polymers. Its cytotoxicity was evaluated in cell culture. In addition, CPT incorporation efficiency, the stability of CPT-loading micelles and in vitro release behavior were studied.

Characterization of PLC-g-mPEG

The PLC-g-mPEG polymers with four different DD (80, 85, 90 and 95 %) of chitosan were synthesized. ^{1}H-NMR exhibited the peaks at 3.1-4.1 ppm belonging to methylene protons of mPEG, whereas the peaks at 3.1–4.2, and 7.6-7.9 refer to pyranose ring, and aromatic protons, respectively. Substitution degree of mPEG onto N-phthaloylchitosan was 16.50 % and substitution of phthalimido group was 66-69 % as calculated by ^{1}H-NMR. Calculated results were summarized in Table 1. The chemical structure of these graft copolymers was characterized by FTIR and ^{1}H-

NMR. It was found that DD increased ranging from 80 to 95 % with an increase in the phthalimido group on chitosan chain.

Characterization of PLC-g-mPEG polymeric micelles

Initially, the suitable solvents for dissolving PLC-g-mPEG were studied. Appearance of PLC-g-mPEG dispersed or dissolved in various solvents was shown in previous reports [29]. We found that the turbidity level largely depended on the types of solvents. PLC-g-mPEG was completely dissolved in DMSO and DMF. However, it formed colloid (as seen from turbidity) in water, methanol, ethanol, 2-propanol and chloroform, and it was insoluble in toluene and n-hexane. The ability of DMSO and DMF to dissolve PLC-g-mPEG was probably due to a high dielectric constant and a high dipole moment of the solvents.

The rate of PLC-g-mPEG dissolved in DMSO was ranked as PLC (95)-g-PEG > PLC (90)-g-PEG > PLC (85)-g-PEG > PLC (80)-g-PEG after vigorous stirring using a magnetic stirrer. This might be the results of the decrease in chitin unit and the increase in chitosan and phthaloyl chitosan unit (Table 1). In this study we chose DMSO as the solvent to dissolve PLC-g-mPEG and form nano-size (50-100 nm) micellar when dialyzed against water. The tendency of these polymers to form self-assembly micellar might be based on the hydrophobic phthalimido groups on the chitosan chain and hydrophilic mPEG chain. The TEM photograph also confirmed that all the polymers could be formed spherical micelles with a nano-size (data not shown).

In order to determine the critical micelles concentration (CMC) of PLC-g-mPEG micelles, fluorescence measurements were carried out using pyrene as a fluorescent probe [32]. Pyrene was used as a fluorescence probe because its fluorescence spectrum was sensitive to the polarity of the environment. With an increase in PLC-g-mPEG polymer concentrations, the total fluorescent intensity (I) increased, and fluorescence spectrum shifted. The ratio I_{337}/I_{334} of the pyrene excitation spectra was used to determine CMC of block copolymers in water. The plot of the intensity ratio I_{337}/I_{334} of the pyrene excitation spectra against the logarithm of the polymer concentration is shown in Fig. 2.

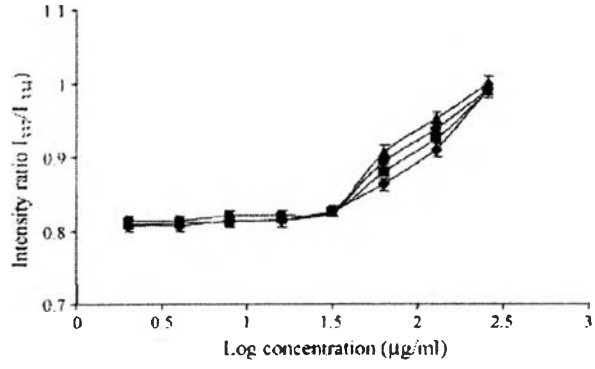


Fig. 5.2 Effect of percent degree of deacetylation of chitosan: (•) 80%, (♦) 85%, (▲) 90% and (■) 95% measured as the I_{337}/I_{334} band intensity ratio of pyrene as a function of the logarithm of the concentration of micelles forming from PLC-g-mPEG. Data are plotted in the mean ± S.D. of three measurements.

The CMC value can be determined at a polymer concentration of onset of the I_{337}/I_{334} ratio increase. The CMC value of 4 PLC-g-mPEGs (DD= 80, 85, 90 and 95 %) were equal value of 28 μg/ml. This result indicated that the increased N-phthaloylchitosan unit was not affected the CMC value.

In vitro Cytotoxicity of Phthaloylchitosan (PLC) and PLC-g-mPEGs micelles

One of the major requirements for polymers used for micelle forming should be non-toxic. Although chitosan is generally considered as a biodegradable and safe polymer, Schipper et al [33] observed some toxic effect of certain chitosans in Caco-2 cells in MTT assay. Therefore, the cytotoxicity of PLC and PLC-g-mPEG was performed by quantitative evaluation of cell viability using Hela cells. According to the MTT assay results, PLC did not remarkably affect the viability of Hela cells. All PLC polymers incubated for 72 h showed concentration dependent cytotoxicity in Hela cells. PLC (95 % DD) was the most toxic having an IC50 of 0.25 ± 0.06 μM. PLC (80, 85 and 90 % DD) have the IC50 more than 1μM. The ranking of cytotoxicity was: PLC 95 > PLC 90 > PLC 85 > PLC 80. Fig. 3 showed the effect of % DD of PLC and PLC-g-mPEG micelles on the cytotoxicity.

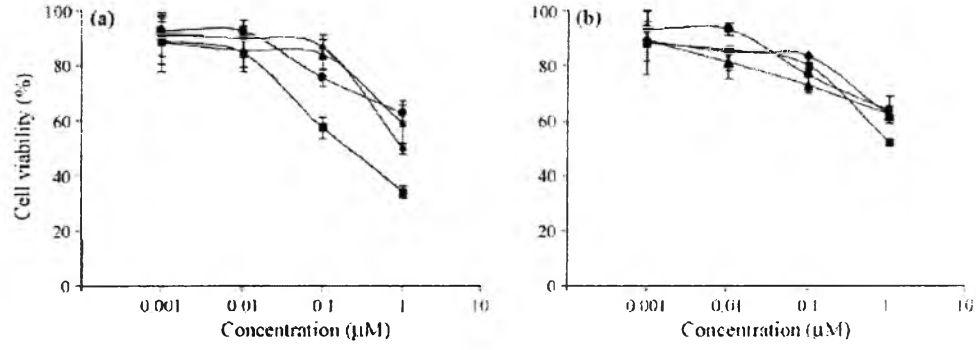


Fig. 5.3 Effect of percent degree of deacetylation of chitosan: (□) 80%, (♦) 85%, (▲) 90% and (■) 95% on cytotoxicity of (a) N-phthaloylchitosan (PLC); (b) N-phthaloylchitosan-grafted-mPEG (PLC-g-mPEG) polymeric micelles incubated with Hela cells for 72 h. Data are plotted in the mean ± S.D. of eight measurements.

All PLC-g-mPEG (80, 85, 90, 95% DD) have the IC50 more than 1μM. None of the PLC-g-mPEG micelles was cytotoxic in vitro against the Hela cells thereby indicating their potential for drug carriers. These results indicate that the introduction of some PEG content in phthaloychitosan is important to decrease their cytotoxicity.

Table 5.1 Chemical composition of N-phthaloychitosan-grafted mPEG

polymers	% DD	Chitin		Chitosan		N-Phthaloy chitosan[a]	
		Unit	%	Unit	%	Unit	%
PLC (80)-g-PEG	80	682.4	20	900.8	26	1828.9	54
PLC (85)-g-PEG	85	518.2	15	969.1	28	1967.6	57
PLC (90)-g-PEG	90	349.9	10	1039.1	30	2109.8	60
PLC (95)-g-PEG	95	177.2	5	1111.0	31	2255.7	64

[a] Calculated from ^{1}H NMR

Incorporation of CPT into PLC-g-mPEG micelles

Chitosan is hydrophilic, therefore, cannot interact with CPT molecules by hydrophobic interactions. Chitosan-mPEG can not incorporate with CPT and form micelles (data not shown). As shown in previous studies, the evaporation, dialysis

and emulsion method was found to be used for physical incorporation of CPT into the polymeric micelles [21,30]. In this study, CPT was incorporated by dialysis method, since PLC-g-mPEG and CPT was dissolved in DMSO. These solvents could not evaporate therefore evaporation and emulsion method may not be done. Fig. 4 shows the effect of %DD and initial drug on the CPT incorporation efficiency in PLC-g-mPEG micelles.

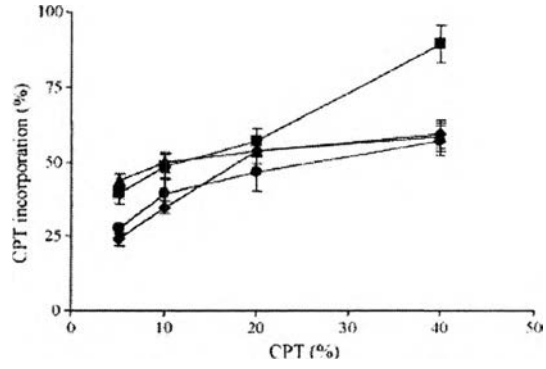


Fig. 5.4 Effect of percent degree of deacetylation of chitosan: (□) 80%, (♦) 85%, (▲) 90% and (■) 95% on the CPT incorporation efficiency in polymeric micelles. Data are plotted in the mean ± S.D. of three measurements.

The X-axis represents the initial drug used in preparation (percentage of CPT), ranging from 5 % to 40 % and Y-axis represents the percentage of CPT incorporated into the micelles (% yield). Of all test polymers, the incorporation efficiency increased ranging from 30 to 90 % with an increase in the initial CPT loading from 5 % to 40 %. The important factor to control this incorporation process was mainly due to the hydrophobic interactions, among hydrophobic N-phthaloylchitosan chain, CPT, and solvent. Micelle formation and drug incorporation into micelle were expected to occur simultaneously. If CPT interacts more favorably with the hydrophobic polymer chain than with solvent, incorporation efficiency should be high. If CPT molecules interact with each other more strongly than with the hydrophobic polymer chain, CPT will precipitate rather than being incorporated into micelles. Increasing the initial drug loading results in the increase of hydrophobicity of the inner core, therefore, incorporation efficiency was increased. It was found that

the increase %DD slightly increased the CPT incorporated into the micelles. PLC(95)-g-mPEG Micelle with 40% initial CPT showed the highest incorporation efficiency (90% yield). As showed in Table 1, %DD was correlated to the hydrophobic N-phthaloylchitosan (PLC) units, % DD ranked from 80 to 95 with the PLC unit of 1829 and 2256, respectively. This suggests a large contribution of π-π interaction between aromatic groups of CPT molecules and the hydrophobic inner core (phthalimido group) of the PLC-g-mPEG polymers. These results showed advantages of this novel polymer since it is preferable to design drug carrier systems without using drug molecules as a chemically conjugated species. These results are likely to be consistent with previous results in which benzyl and methylnaphtyl PEG-poly(aspartate) block copolymer (phenyl/naphtyl group) at high esterification degree provided high CPT incorporation stability. In contrast, n-butyl and lauryl PEG-poly(aspartate) block copolymer showed lower stability than benzyl and methylnaphtyl PEG-poly(aspartate) block copolymer [20-21]. The diameter and distribution of the polymeric micelles were measured by dynamic laser light scattering (DLS) and data were shown in Fig. 5.

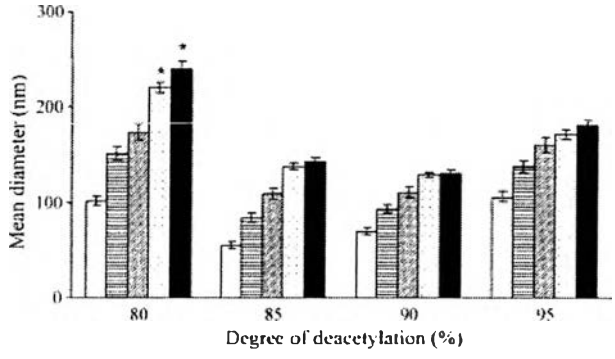


Fig. 5.5 The particle size of bare-polymeric micelles and CPT-loaded polymeric micelles prepared from PLC-g-mPEG with 80%, 85%, 90% and 95% DD chitosan. ( ) bare-polymeric micelles; (▤) 5%; (▩) 10%; (⊡) 20%; (■) 40% CTP to polymer loaded polymeric micelles. *$p < 0.05$.

The mean particle sizes of the CPT-loaded PLC-g-mPEG micelles ranged from 100 to 250 nm in diameter, which were larger than that of the bare micelles (50-

100 nm). For both bare and CPT-loaded PCL-g-mPEG micelles, the size distribution was narrow. The diameter increased with an increase in the weight ratio of CPT to (5%, 10%, 20% and 40% CPT to polymer) It was found that the increasing in %DD slightly increased the particle sizes (85-95 % DD), these might be due to higher incorporation of CPT inside the micelles. However, some aggregations were observed in 80% DD, therefore the particles sized were significant larger than that of 85-95% DD.

Stability of CPT-loaded polymeric micelles

The stability of CPT-loaded micelles was characterized by GPC. It was observed that all samples formed polymeric micelle structures with micelle peaks near the gel-exclusion volume. Micelle peaks detected by the RI detector (for polymers) showed the same retention time (4.2 min) as detected by UV absorption at 351 nm (for CPT). GPC with UV detection allowed us to evaluate the nature of the polymeric micelles obtained and the degree of drug incorporation. Therefore, the stability of CPT-loaded micelles was characterized by the peak area of the peak detected by UV absorption at 351 nm. This peak area represents the amount of CPT loaded into the micelles. The ratio of this peak area/CPT concentration, [CPT], was larger, thus CPT was more stably incorporated into the micelles. The small values of the peak area/[CPT] means that most of the CPT was adsorbed to the GPC column by hydrophobic interactions due to unstable packaging of CPT in the micelles. The values of peak area/[CPT] of this CPT-loaded micelles were decreased with an increase in the weight ratio of CPT to polymer (5 - 40 % CPT to polymer), as shown in Fig. 6

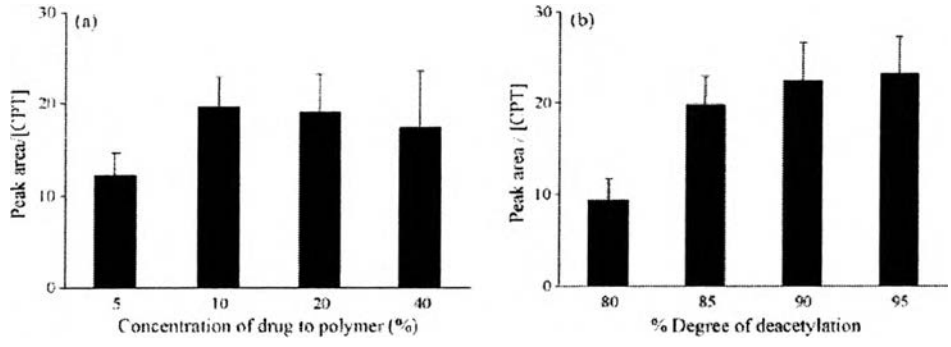


Fig. 5.6 (a) effect of CPT concentration (5–40%) forming from PLC(85)-g-mPEG (b) effect of percent degree of deacetylation of chitosan using CPT 10% on the CPT-loaded micelles stability by using the gel permeation chromatography (GPC). Data are plotted in the mean±S.D. of three measurements.

At 10% CPT initial drug loaded, we found that PLC(95)-g-mPEG showed the highest values of peak area/[CPT].These results indicated that not only the drug content but also a certain quantity of hydrophobic component (phthalimido group) was essential to form stable CPT-loaded micelles. Particularly, greater than 85 % DD was enough for stable micelle formation with high CPT incorporation. These results in agreement with the previous study showed that benzyl content of PEG-poly(benzyl L-aspartate) block copolymer greater that 57% was enough for stable micelle formation [20].

Micelle drug release

Polymeric micelle drug delivery systems are advantageous for their wide applicability in delivering hydrophobic drugs. Micelle stability, long-circulation properties, and sustained drug release are critical factors for achieving highly selective delivery to tumor target sites. As previously reported, the CPT release rate was the slowest when it showed the highest GPC stability [20]. The weak interaction between drug and inner core of micelles partially contribute to the fastest release of drug. Therefore, in this studied we investigated the release behavior of CPT-loaded

PLC-g-mPEG micelles. The CPT release from PLC-g-mPEG micelles with 80, 85, 90 and 95 % DD and have the 10 % CPT initially loaded is shown in Fig. 7.

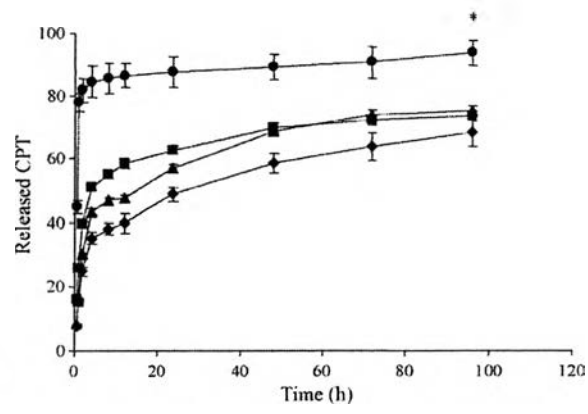


Fig. 5.7 Release profile of CPT from the micelles forming from N-phthaloylchitosan-grafted mPEG. (•) 80%, (♦) 85%, (▲) 90% and (■) 95% DD. Data are plotted in the mean ± S.D. of three measurements. $^{*}p < 0.05$.

The resulted showed that CPT release from PLC-g-mPEG micelles was sustained over 96 h and depend on the % DD. As the %DD increased up to 85%, the release rate was retarded. A greatest retarded release was exhibited by 85% DD following by 90, 95 % DD. This implied that PLC-g-mPEG with % DD higher than 85% might be a good carrier for CPT incorporation and sustained release for passive tumor targeting.

5.5 Conclusion

A water-insoluble anticancer agent, CPT, was successfully incorporated into polymeric micelles formed from N-Phthaloylchitosan-grafted mPEG polymer by a dyalysis method. Phthaloylchitosan unit of the hydrophobic chain of the polymers and CPT content were the important factors for the incorporation efficiency and stability of drug loaded micelles. PLC-g-mPEG ($\geq$ 85 % DD) has an appropriate amount of phthalimido group on chitosan chain that showed high stability and retarded release. Therefore, this novel PLC-g-mPEG polymer presents considerable

potential interest in the development of CPT carrier for long circulation of passive tumor targeting.

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