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Development of Doxorubicin - Core Shell Chitosan Nanoparticles to Treat Cancer

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Abstract. Biodegradable polymeric micelles encapsulating doxorubicin in the core region were prepared from a grafted copolymer composed of O-Succinyl chitosan and Pluronic® F127. This copolymer was prepared by grafting Pluronic® F127 onto chitosan using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as coupling agents. The chemical structure of the O-Succinyl chitosan graft Pluronic® F127 copolymer (*CP*) was characterized by FTIR. This polymeric micelles are self-assemblies of block copolymers of approximately 50 nm diameter in aqueous media. Anti-cancer drug (doxorubicin, DOX) can be loaded with high encapsulation efficiency (73.69 ± 0.53 to 74.65 ± 0.44%). An *in vitro* release study shows that the nanoparticle formulation exhibited a biphasic drug release with a moderate initial burst, followed by a sustained release profile in pH 7.5 receiving media. *In vitro* cytotoxicity of free doxorubicin and doxorubicin encapsulated nanoparticles (DOX-NPs) were evaluated using MCF- 7 cell line as a cancer cell model. The IC_{50} doses determined by MTT assay showed the greater activity of DOX-NPs over free doxorubicin. Consequently, the efficacy of DOX loaded micelles was improved noticeably, owing to higher drug accumulation at the intracellular action site. These results demonstrated that O-Succinyl chitosan graft Pluronic® F127 copolymer nanoparticles have the potential to be used as anti-cancer drug carriers.

Keywords: Doxorubicin, Pluronic, Chitosan, and Core-Shell nanoparticles

1. Introduction

Doxorubicin (DOX) is one of the most widely-used chemotherapeutic anticancer drugs. DOX can integrate its structure into DNA between the base pair or inhibit topoisomerase II. Unfortunately, it causes serious side effects and presents high systemic toxicity to both healthy and normal tissue [1]. Therefore, drug delivery systems (DDS) have recently emerged as an important route to unravel these obstacles. In recent years, there have been considerable interests in developing biodegradable nanoparticles as effective DDS. Amphiphilic block copolymers have been widely investigated as hydrophobic drug solubilizing agents in DDS [2]. They can spontaneously self-assemble into polymeric micelles and nanoparticles (NPs) in aqueous environments. Most polymeric micelles are composed of a hydrophobic block as the inner core and a hydrophilic block as the outer shell [3]. A hydrophobic drug can be encapsulated in the hydrophobic core of the micelles to increase the water solubility. The hydrophilic shell is able to prolong the circulation time due to a decrease in phagocytosis and renal clearance. The polymeric micelles normally have average size of approximately 50 nm diameter, allowing the particles to accumulate in tumor tissue through a mechanism called enhanced permeation and retention (EPR) effect rather than in normal tissues. This is due to the fact that tumor vessels are structurally irregular and leaky compared to normal vessels [4]. One of the most commonly used micelles in drug delivery applications is Pluronic, an amphiphilic tri-block copolymer, composed of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO). The hydrophilic (PEO) and hydrophobic (PPO) blocks form the corona and the core of the micelles, respectively. Pluronic has attracted a

lot of attention because of their low toxicity in the body and the ability to encapsulate any hydrophobic agents. However, the major problem of using polymeric micelles is their instability [5]. To overcome this limitation, grafting pluronic with chitosan to form a copolymer was suggested. Chitosan is the cationic polysaccharide derived from chitin which stimulates cell growth and protein adsorption. Chitosan has been widely used in biomedical and pharmaceutical applications because of its biocompatibility and biodegradability. Although chitosan graft Pluronic has been used in many forms such as hydrogel [5, 6], nano-aggregation [7], and nanoparticles (NPs) [8], it has never been used as a delivery vector for anti-cancer drugs. In this work, we synthesized and charaterized a novel DOX encapsulated nanoparticles delivery system using a graft copolymer composed of O-Succinyl chitosan and Pluronic® F127. The important properties of these particles such as their particle size and stability, encapsulation efficiency, *in vitro* drug release, and cytotoxicity were also evaluated.

2. Materials and methods

2.1. Materials

Chitosan (medium Mw, degree of deacetylation = 85%) was purchased from Seafresh Chitosan Laboratory (Bangkok, Thailand). Pluronic® F127 was purchased from BASF Aktiengesellschaft (German). Doxorubicin Hydrochloride (DOX), N-Hydroxysuccinimide (NHS), and EDC (1-Ethyl-3-(3-dimethykaminopropyl)-carbodiimide) were purchased from Sigma-Aldrich (U.S.A.). Trypsion-EDTA, Fetal Bovine Serum (FBS), Dulbecco's Modification of Eagle's Basal Medium (DMEM), Vybant MTT cell proliferation kit, and Anti-HER2, were purchased from Invitrogen (U.S.A.). Micro BCA™ Protein Assay Kit was purchased from Thermo Scientific (U.S.A.).

2.2. Methods

2.2.1 Preparation of O-succinyl chitosan graft Pluronic® F127 copolymer (*CP*)

O-succinylation of chitosan [9] 5% and 10% (w/w of MP) was added to Activated Pluronic® F-127 [6]. This mixture was then incubated at 20 °C in an incubator shaker. After 24 hours of reaction, copolymers were separated from the solvent by a vacuum dryer overnight and followed by solvent evaporation in a desiccator for 7 days. The functional groups of the *CP* were characterized using Fourier Transform Infrared Spectroscopy (FT-IR).

2.2.2 Preparation of Core-Shell Nanoparticles (NPs, and DOX-NPs)

The concentrations of *CP* used in study were 5, 7, and 10% (w/v) in Milli Q water and stir solution at 250 rpm for 12 hours. DOX-NPs can be achieved by mixing the drug in the copolymer solution at 250 rpm for 12 hours in the dark. NPs and DOX-NPs can be separated by centrifugation at 25°C, 6,000 rpm for 2 hours. Remaining free doxorubicin in the supernatant was measured for its absorbance at λ = 485 nm by using UV-Vis Spectrophotometer (Hitachi Model U-1000). The doxorubicin encapsulation efficiency was determined based on Equation 1.

$$Encapsulation\ Effciency = \frac{Amount\ of\ DOX\ in\ micelles}{Amount\ of\ DOX\ initially\ added\ to\ the\ formulation} x100 \quad (1)$$

2.2.3 Characterization of Nanoparticles

Particle sizes were examined by photon correlation spectroscopy (Nanosizer), while their morphologies were visualized using Transmission Electron microscopy (TEM). The overall surface charges of the nanoparticles were measured as zeta potential using Zetasizer.

2.2.4 *In vitro* release study

The nanoparticles were dissolved in 1 ml PBS (pH 7.5) and kept at 37 °C. The amount of DOX released can be determined by centrifuging nanoparticles containing DOX. At predetermined time (2, 4, 6, 12, 24, 48, 72, 96, 168, 264, 456, and 528 hours), the solution containing DOX-encapsulated nanoparticles was centrifuged and 0.1 ml of the supernatant was withdrawn. The drug concentration was determined by a

fluorescence spectrophotometer at excitation wavelength of 485 nm and emission wavelength of 590 nm. The receiving medium was replenished by adding 0.1 ml of PBS into the samples of nanoparticles. The cumulative doxorubicin release was determined by Equation 2.

$$DOX\ released\ (\%) = \frac{Amount\ of\ DOX\ released}{Initial\ amount\ DOX} \times 100 \quad (2)$$

2.2.5 *In vitro* Cytotoxicity

The cytotoxicity of DOX-NPs and free DOX was performed on the human breast cancer cell line, MCF-7, using the MTT method. Briefly, 5.0 x 10^3 cells were seeded in 96-well plates and incubated for 24 hours to allow the cells to attach. Then the cells were exposed to various concentrations of free DOX or DOX-NPs at 37 °C for 72 hours. The cell viability was expressed as percent survival comparing with control where the cells were not exposed to any chemicals.

$$Cell\ Viability\ (\%) = \frac{OD570\ of\ DOX\ treated\ sample}{OD570\ of\ untreated\ control\ sample} \times 100 \quad (3)$$

3. Results and discussions

3.1 Characterization of O-succinyl chitosan graft Pluronic® F127 copolymer (*CP*)

FT-IR spectroscopy measurements were carried out to substantiate the chemical structure of *CP*. The FT-IR spectrum of *CP* shows that peaks appearing at 1648 cm^{-1} and 1561 cm^{-1}could be assigned to amide group (C=O and N–H), as a result of the bond between an amine group of chitosan and an carboxylic group of monocarboxy Pluronic, as shown in Fig. 1. The FT-IR spectrum is similar to that of the previous research [9]. This result indicates that O-succinyl chitosan graft pluronic® F127 copolymer was successfully synthesized.

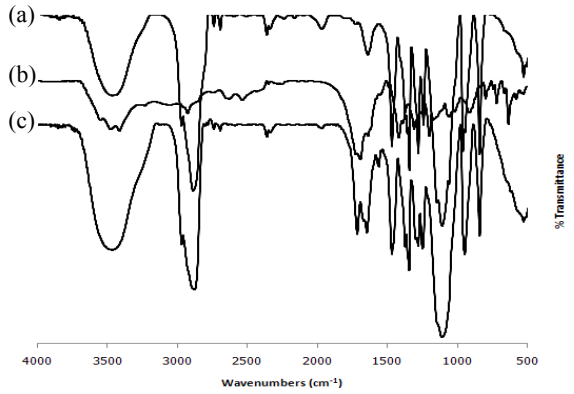

Fig. 1: FT-IR spectra of (a) Pluronic® F127, (b) Monocarboxy Pluronic and (c) *CP*

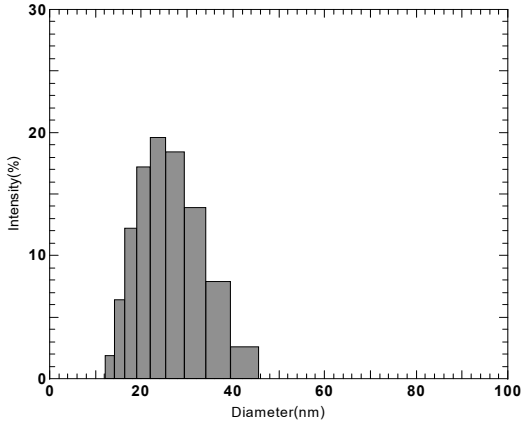

Fig. 2: Particle size distribution

3.2 Characterization and Encapsulation Efficiency of DOX encapsulated core-shell nanoparticles (DOX-NPs)

When the copolymer concentration gets closer to its critical gel concentration (CGC), the solution becomes sticky, making it difficult for the preparation. Therefore, the concentrations of *CP* used in this study were 5, 7, and 10% (w/v) which were between the critical micelle concentration (CMC) and the CGC. As shown in Table 1, the differences in average size between *CP* micelles prepared from various concentrations were not significant. The particle sizes were within 34 – 40 nm. In addition, the nanoparticle size distributions among various copolymer concentrations were quite uniform (Fig. 2). The average size of 5% *CP* nanoparticles was somewhat smaller than that of 10% *CP* nanoparticles, possibly because higher O-succinyl chitosan content led to a larger outer shell. These result from the fact that O-succinyl chitosan is a hydrophilic part. The zeta potentials of 5% and 10% *CP* nanoparticles were 34 - 39 mV and 40 - 50 mV, respectively (Table 1). The positive values were the result of positive charges on the surface of the particles due to functional group of O-succinyl-chitosan. In addition, the high zeta potentials indicated stable particles which could be suitable for drug delivery applications. DOX, hydrophobic anti-cancer drug, was encapsulated at the core of the polymeric micelles in the hydrophobic block owing to its hydrophobicity. The shape of DOX encapsulated *CP* nanoparticle (DOX-NPs) was spherical as shown in Fig. 3. In this study,

DOX was successfully loaded into the hydrophobic core of the micelles via physical entrapment. Table 1 shows the encapsulation efficiency (%) of *CP* nanoparticles. The encapsulation efficiencies were ranging from 73.69 ± 0.53 to 74.65 ± 0.44% for 5% and 10% *CP* nanoparticles, respectively. The difference between 5% and 10% *CP* nanoparticles was the hydrophilic portion of O-succinyl chitosan. Since the hydrophobic parts of both formulations remained unchanged for both formulations, the nanoparticles' ability to encapsulate a hydrophobic drug was not affected. As a result, the encapsulation efficiencies of 5% and 10% *CP* nanoparticles were quite similar.

3.3 *In vitro* release study

In vitro-release profiles of DOX-NPs in PBS at different pH 7.5 were shown in Fig. 4. The amount of DOX released was presented as cumulative percentage release at 37 °C over a period of 22 days. It was found that 5% and 10% *CP* nanoparticles exhibited similar release profiles with an initial burst release up to 39 - 42% and 29 - 39%, respectively, in the first 24 hours, followed by sustained release of the encapsulated drug of 85 - 90% and 73 - 86% after 22 days, respectively, at pH 7.5. This result suggests that there are two phases of DOX release profile. First, the initial burst release of DOX from the nanoparticles in the first 24 hours. Burst-release is the phenomenon of drug which a greater amount of initial bulky drug is immediately released prior to arriving at the steady level of the release profile. This directly affects the effective exposure time of nano-carriers [10]. In the next phase, a sustained release of the encapsulated DOX was shown after 24 hours.

Table 1. Particle size, Zeta potentials and Encapsulation efficiencies of various nanoparticle formulations

CP NPs	*CP* NPs concentration (%w/v)	Size (nm)	Zeta potential (mV)	Encapsulation Efficiency (%)
5%	5%	35.12 ± 2.0	+34.182	74.30±1.84
	7%	34.75 ± 1.7	+35.115	74.40±1.60
	10%	35.97 ± 2.3	+38.504	74.65±0.44
10%	5%	40.12 ± 2.2	+40.317	73.95±0.85
	7%	39.67 ± 3.0	+43.220	73.69±0.53
	10%	39.47 ± 6.2	+49.340	74.00±1.07

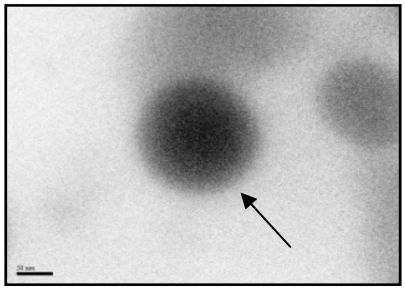

Fig. 3: TEM images of *CP* NPs

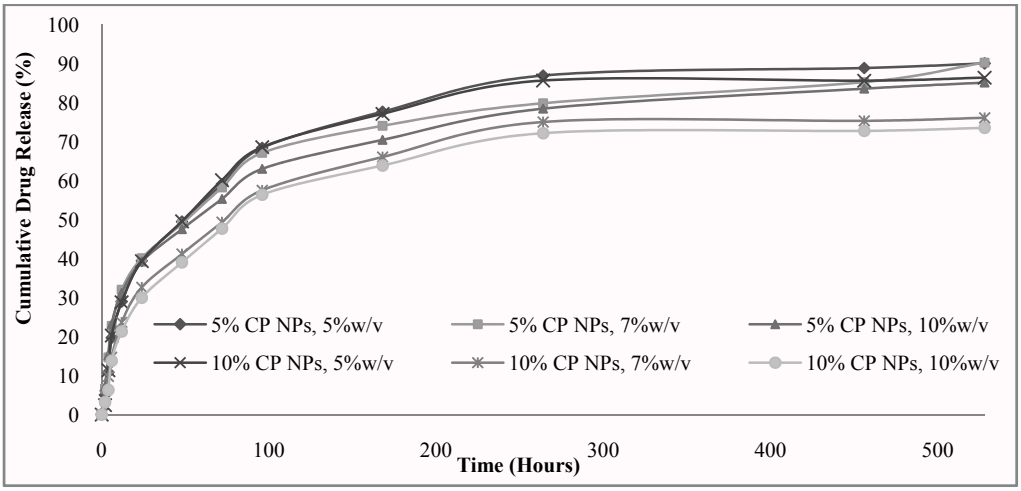

Fig. 4: Doxorubicin Release Profiles in PBS, pH 7.5

3.4 *In vitro* Cytotoxicity

The nanoparticles without DOX (NPs) were shown to have minimal toxic effects and these particles themselves would not cause cellular damage (Range of IC_{50}: 4.32 to 7.60 mg/ml NPs) as shown in Fig. 5. The results of the *in vitro* cytotoxicity test showed that the nanoparticles are biocompatible, if they were used as nano-carriers. The IC_{50} of free DOX against MCF-7 cell was 0.67 µg/ml which was about 1.58 to 3.60 times higher than DOX-NPs (range from 0.19 to 0.42 µg/ml). DOX-NPs represented a decrease in cell survival as nanoparticle concentration increased due to an increase in DOX concentration. DOX has a

property that prevents cell proliferation and induces apoptosis (Fig. 6). These results indicated that DOX-NPs showed high cytotoxicity against the cancer cells.

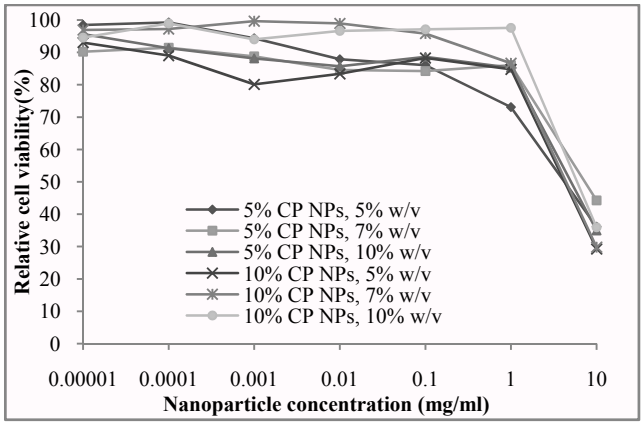

Fig. 5: Cytotoxicity of NPs

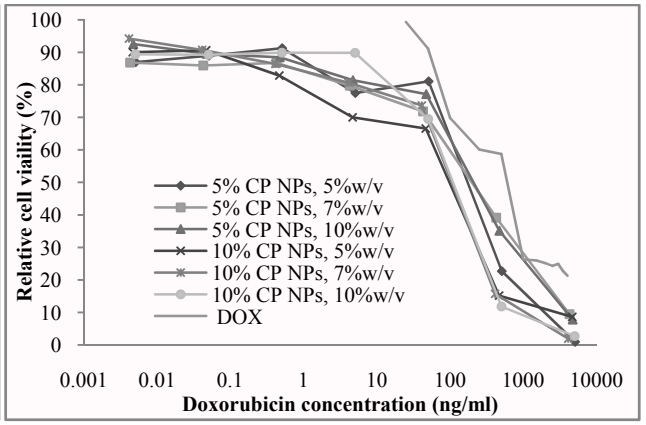

Fig. 6: Cytotoxicity of DOX-NPs

4. Conclusions

DOX can be successfully loaded into the synthesized O-Succinyl chitosan graft Pluronic® F127 copolymer with high encapsulation efficiency. The release profile of DOX at pH 7.5 showed a sustained release profile within 22 days. DOX-NPs were more cytotoxic against MCF-7 cells than was Free DOX. Thus, O-Succinyl chitosan graft Pluronic® F127 copolymer nanoparticles are promising as drug carriers that can lead to effective cancer treatment.

5. Acknowledgements

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