Development of Doxorubicin – Core Shell O-succinyl Chitosan Graft Pluronic®127 Copolymer Nanoparticles to Treat Human Cancer

Parichart Naruphontjirakul and Kwanchanok Viravaidya-Pasuwat

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) -carbodi imide (EDC) and N-hydroxysuccinimide (NHS) as coupling agents. 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 both pH 5.0 and pH 7.5 receiving media. The drug was rapidly and completely released from the nanoparticles at pH 5.0 nearly 100%, whereas, at pH 7.4, only 73.51 ± 2.68% to 90.26 ± 0.94% of DOX was released within 22 days. From the in vitro cytotoxicity test, DOX-NPs showed high cytotoxicity against the cancer cells. The IC50 doses determined by MTT assay showed the greater activity of DOX-NPs over free doxorubicin. Free doxorubicin was accumulated inside the MCF-7 cells as quickly as 3 hours. In contrast, DOX fluorescence from DOX-NPs in MCF-7 cells was observed after 6 hours of incubation. The results demonstrated that greater amount of free DOX and DOX-NPs were internalized in term of time dependent. Consequently, the efficacy of DOX loaded micelles was improved noticeably, owing to higher drug accumulation at the intracellular action site. O-Succinyl chitosan graft Pluronic® F127 copolymer nanoparticles have proven their potential to be used as anti-cancer drug carriers.**

Index Terms—**Core-Shell nanoparticles, Chitosan, Pluronic, and Doxorubicin.**

I. 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 in aqueous environment. 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. Moreover, 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 its 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 characterized 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, for instance, particle size and stability, encapsulation efficiency, *in vitro* drug release, cytotoxicity and drug accumulation were evaluated.

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II. Materials and Methods

A. Materials

Chitosan (medium Mw, degree of deacetylation = 85%) was purchased from Seafresh Chitosan Laboratory (Bangkok, Thailand). Pluronic® F127 was purchased from BASF Aktiengesellschaft (German). Ethanol 99.7-100% AnalaR grade was purchased from BDH (English). Doxorubicin Hydrochloride (DOX), N-Hydroxysuccinimide (NHS), and EDC (1-Ethyl-3-(3-dimethykaminopropyl) -carbodiimide), Pyridine, Phthalic anhydride, Acetone, Sodium acetate, Succinic anhydride, Ethyl ether, and 1,4-Dioxane were purchased from Sigma-Aldrich (U.S.A.). Trypsion-EDTA, Fetal Bovine Serum (FBS), Dulbecco's Modification of Eagle's Basal Medium (DMEM), and Vybant MTT cell proliferation kit were purchased from Invitrogen (U.S.A.). Dimethyl sulfoxide (DMSO) was purchased from Amresco (U.S.A.). Triethylamine (TEA), Dimethylformamide (DMF), and Hydrozine monohydrate were purchased from Carlo ERBA (France). Succinic anhydride was purchased from Fluka (U.S.A.).

B. Preparation of O-succinyl Chitosan Graft Pluronic® F127 Copolymer (CP)

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

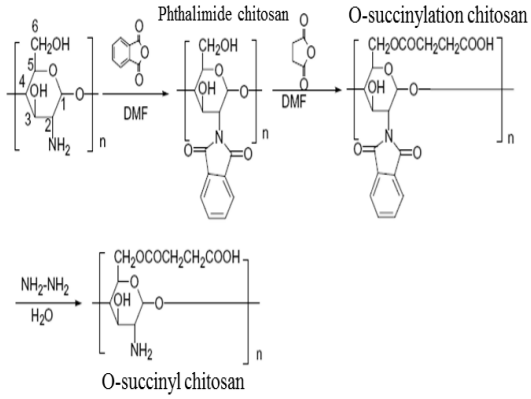

Fig.1 Synthetic route of O-succinyl chitosan

C. Preparation of Core-Shell Nanoparticles (NP) and Doxorubicin Encapsulated Nanoparticles (DOX-NPs)

The concentrations of *CP* nanoparticles used in this study were 5, 7, and 10% (w/v) in Milli Q water. To form nanoparticles, the solution was stirred at 250 rpm for 12 hours. DOX-NPs could be achieved by mixing DOX in the copolymer solution at 250 rpm for 12 hours in the dark. NPs and DOX-NPs could be separated by centrifugation at 25°C, 6,000 rpm for 2 hours. Remaining free doxorubicin in the supernatant was measured for its fluorescence intensity at excitation wavelength of 485 nm and emission wavelength of 590 nm using the Micro plate reader (TECAN Model InfiniteM200). The doxorubicin encapsulation efficiency was determined based on (1).

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

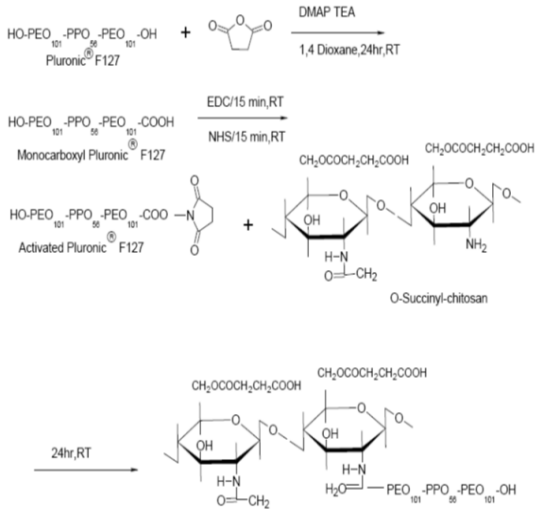

Fig.2 Synthetic route of CP using EDC/NHS.

D. Characterization of Nanoparticles

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

E. In Vitro Release Study

The DOX-NPs were dissolved in 1 ml PBS at pH 7.5 which is the pH value found in the blood stream, and at pH 5.0 which is the pH value found in the tumor tissue. An in vitro release study was conducted at 37 °C in the dark. 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 to separate the nanoparticles from the receiving media 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 test samples containing the nanoparticles. The cumulative doxorubicin release was determined by using (2).

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

F. Biocompatibility Test

The culture of L929 cell line was maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% FBS. To prepare for the cytotoxicity experiment, the cells were trypsinized and centrifuged to form a pellet of the cells. The supernatant was discarded. The cell pellet was then resuspended in its growth medium. The cell stock was diluted to the desired concentration ($5x10^3$ cells/ml). The cell suspension was transferred to 96 well plates by adding 100 µl of the cell suspension to each well. The plates were incubated in a humidified atmosphere at 37 °C and 5% CO_2 for 3 days to allow the cells to be in their exponential growth phase at the time that DOX-NPs were added.

Serial dilutions of the nanoparticles stock solution in the growth medium were prepared to give 8 final concentrations (control, 10 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml, 100 1

mg/ml, 10 mg/ml). The spent medium was discarded and replaced by fresh medium with an appropriate concentration of the nanoparticles. After 72 hours of incubation, the spent medium was discarded and replaced with the MTT assay solution. The plates were incubated for 1 hour in the absence of light at 37 °C. A number of live cells were determined by measuring the absorbance at 570 nm, and expressed as percent survival using the following (3):

$$Cell\ Viabi\ lity\ (\%) = \frac{OD570\ of DOX\ tr\ eated\ samp\ le}{OD570\ of untrea\ ted\ contro\ l\ sample} \times 100 \quad (3)$$

G. 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 103 cells were seeded on 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.

$$Cell\ Viabi\ lity\ (\%) = \frac{OD570\ of DOX\ tr\ eated\ samp\ le}{OD570\ of untrea\ ted\ contro\ l\ sample} \times 100 \quad (3)$$

H. Drug Accumulation Study

The accumulation of DOX released from different nanoparticle formulations in human breast cancer, MCF-7, was investigated. MCF-7 cells were cultured on glass cover slips for three days before the experiment. In this study, the cells were incubated in DMEM containing free DOX and doxorubicin encapsulated nanoparticles (DOX-NPs) at 37 ºC. This DMEM solution was prepared by dissolving free DOX and DOX-NPs in DMEM + 10 % of FBS and 1 % of antibiotic to obtain final equivalent DOX concentration of 0.5 µg/ml.

At the predetermined time, the glass cover slips were rinsed briefly with cold PBS. The cells were fixed with 10% paraformaldehyde in PBS for 45 minutes. Afterwards, the cells were rinsed three times with water and finally air dried. Fluorescence images were made using the auto-fluorescence of doxorubicin. DOX becomes auto-fluorescent when excited at 488 nm. Its fluorescence emission (between 565 and 630 nm) could be visualized using a fluorescence microscope (Olympus Fluorescence Microscope System) [10].

III. Results and Discussions

A. Characterization of O-succinyl Chitosan Graft Pluronic® F127 Copolymer (CP)

FT-IR spectroscopy measurement was carried out to substantiate the chemical structure of CP. The FT-IR spectrum of CP shows that the peaks appearing at 1648 cm^{-1} and 1561 cm^{-1}could be assigned to an amide group (C=O and N–H), as a result of the bond between an amine group of chitosan and a carboxylic group of monocarboxy Pluronic, as shown in Fig. 3. 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.

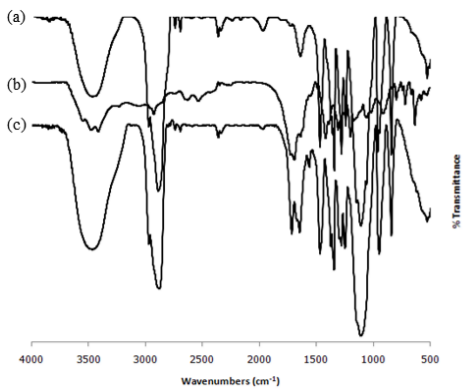

Fig. 3: FT-IR spectra of (a) Pluronic® F127,

(b) Monocarboxy Pluronic and (c) CP

TABLE I. Particle Size, Zeta Potentials of Various Nanoparticle Formulations

CP NPs	*CP* NPs concentration (%w/v)	Size (nm)	Zeta potential (mV)
5%	5%	35.12 ± 2.0	+34.182
	7%	34.75 ± 1.7	+35.115
	10%	35.97 ± 2.3	+38.504
10%	5%	40.12 ± 2.2	+40.317
	7%	39.67 ± 3.0	+43.220
	10%	39.47 ± 6.2	+49.340

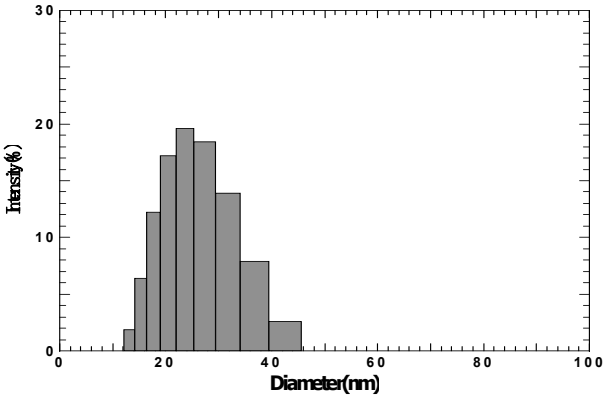

Fig. 4: Particle size distribution

B. 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 I, 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 distribution among various copolymer concentrations was moderately uniform (Fig. 4). 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.

The zeta potentials of 5% and 10% *CP* nanoparticles were

34 - 39 mV and 40 - 50 mV, respectively (Table I). The positive values were the result of positive charges on the surface of the particles due to the functional group of O-succinyl-chitosan. The zeta potential result suggests that the copolymer nanoparticles self-assembled in such a way that Pluronic® was located inside, while O-succinyl chitosan was layered on the outside. A schematic representation of O-succinyl chitosan-graft-Pluronic® copolymer nanoparticles is shown in Fig. 5. In addition, the high zeta potentials indicated stable particle which is suitable for drug delivery applications.

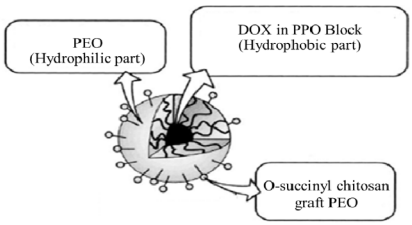

Fig. 5: A schematic representation of doxorubicin encapsulated O-succinyl chitosan–graft– Pluronic® F127 copolymer nanoparticles

DOX, hydrophobic anti-cancer drug, was encapsulated at the core of the polymeric micelles in the hydrophobic block owing to its hydrophobicity [13], [15]. DOX was encapsulated into the micelles by the self-assembly method.

The shape of DOX encapsulated CP nanoparticle (DOX-NPs) was spherical as shown in Fig. 6. In this study, DOX was successfully loaded into the hydrophobic core of the micelles via physical entrapment.

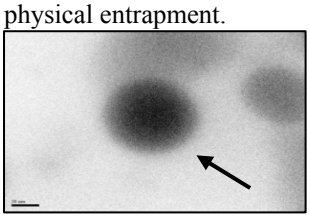

Fig. 6: TEM images of CP NPs

Table II shows the encapsulation efficiency (%) of CP nanoparticles. It can be illustrated that higher concentration of copolymer resulted in higher encapsulation efficiency. The encapsulation efficiencies were ranging from 73.69 ± 0.53% to 74.65 ± 0.44% for 5% and 10% *CP* nanoparticles, respectively. It is possibly due to the fact that DOX was limitedly loaded to the hydrophobic part of the nanoparticles. Therefore, excess DOX could not be encapsulated into the nanoparticles. The highest encapsulation efficiency was approximately 74.65 ± 0.44 % when 5 % O-succinyl chitosan in copolymer at 10% (w/v) copolymer concentration was used to encapsulate 5 μg of initial DOX.

TABLE II. ENCAPSULATION EFFICIENCIES OF VARIOUS NANOPARTICLE FORMULATIONS

CP NPs	*CP* NPs concentration (%w/v)	Encapsulation Efficiency (%)
5%	5%	74.30 ± 1.84
	7%	74.40 ± 1.60
	10%	74.65 ± 0.44
10%	5%	73.95 ± 0.85
	7%	73.69 ± 0.53
	10%	74.00 ± 1.07

In addition, the DOX encapsulation efficiencies of 5 % and 10 % O-succinyl chitosan copolymer nanoparticles was not significantly different ($P > 0.05$), indicating that the amount of O-succinyl chitosan copolymer in nanoparticle did not affect the encapsulation efficiency. 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, the nanoparticles' ability to encapsulate a hydrophobic drug was not affected. As a result, the encapsulation efficiencies of 5% and 10% *CP* nanoparticles were rather similar. Moreover, the encapsulation efficiency among 5, 7, and 10% (w/v) particle concentration were not significantly different ($P > 0.05$). This result possibly comes from comparable amount of nanoparticles in the solution.

C. *In vitro release study*

In vitro-release profiles of DOX encapsulated *CP* nanoparticle (DOX-NPs) in PBS pH 7.5 and 5.0 are shown in Fig. 7 and Fig. 8. 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% DOX-NPs exhibited similar release profiles with an initial burst release up to 39% to 42% and 29% to 39%, respectively, in the first 24 hours, followed by sustained release of the encapsulated drug of 85% to 90% and 73% to 86% after 22 days, respectively, at pH 7.5.

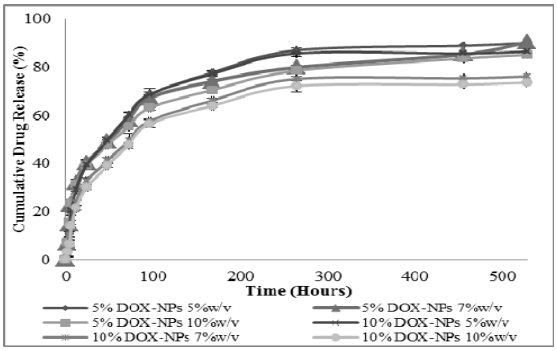

Fig. 7: Doxorubicin Release Profiles in PBS, pH 7.5

At pH 5.0, 5% and 10% DOX-NPs represented different release trend with an initial burst-release profile of 29.84% to 40.31% and 50.61% to 64.07% in the first 24 hours, respectively. After 1 day, the nanoparticles demonstrated a sustained release of the encapsulated drug, with approximately 99% cumulative drug release in 22 days. More drug was released from 5% and 10% DOX encapsulated *CP* nanoparticle (DOX-NPs) at pH 5.0 at earlier time. It was possibly because O-succinyl chitosan in the copolymer nanoparticle could dissolve better in an acidic condition [11], [12]. As a result, more O-succinyl chitosan degraded at pH 5.0 than at pH 7.5, leading to more DOX released at lower pH.

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 a 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 [13], [14]. In the next phase, a sustained release of the encapsulated DOX was shown after 24 hours, allowing for prolonged treatment.

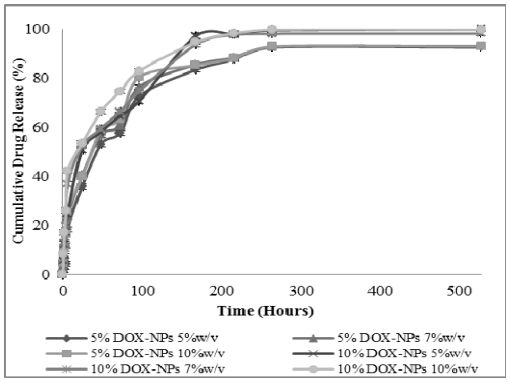

Fig. 8: Doxorubicin Release Profiles in PBS, pH 5.0

D. Biocompatibility test

The proliferation of L929 cells in the presence of the nanoparticles was studied at day 3 to determine the toxicity of the particles themselves. The cells were cultured with fresh DMEM for 1 day. Then, *CP* nanoparticles in DMEM + 10 % of FBS, supplemented with 1 % of antibiotic were added to the culture of L929. These nanoparticles were the particles without doxorubicin. The number of living cells cultured in DMEM for 3 days is shown in Fig 9. The nanoparticles without DOX or blank nanoparticles (NPs) were shown to have minimal toxic effects, indicating that these particles themselves would not cause any cellular damage. From Fig 9, significant reduction of the cell viability relative to the control was observed when 1 mg/ml of nanoparticles was used. The results show that 10 ng to 0.1 mg/ml of NPs are considered safe for use as anti-cancer carriers.

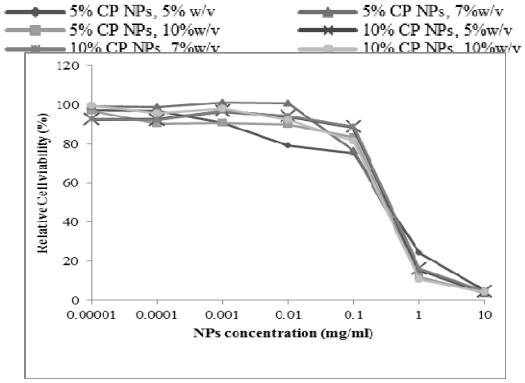

Fig. 9: In Vitro Cytotoxicity in L929 Cells

E. In vitro Cytotoxicity

The nanoparticles were tested for their cytotoxicity against MCF-7 cell line. After 72 hours of incubation, blank nanoparticles (NPs) were also tested to eliminate the possibility that they would affect the cytotoxicity. The cell viability data from the blank NPs are shown in Fig. 10. The blank 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). This result proves that the nanoparticles are biocompatible as nano-carriers when used at a concentration lower than 1 mg/ml.

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 that of 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 the encapsulated DOX concentration. This result shows that DOX could prevent cell proliferation and induce apoptosis (Fig. 11). The lower IC_{50} of DOX-NPs indicated that DOX-NPs showed a high cytotoxic activity against the cancer cells.

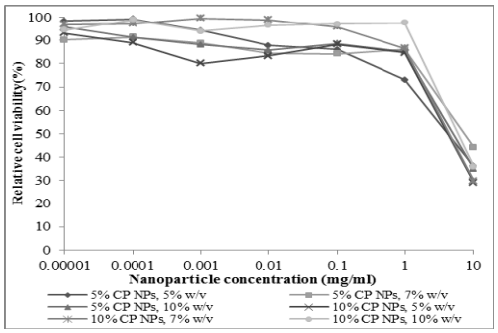

Fig. 10: Cytotoxicity of NPs

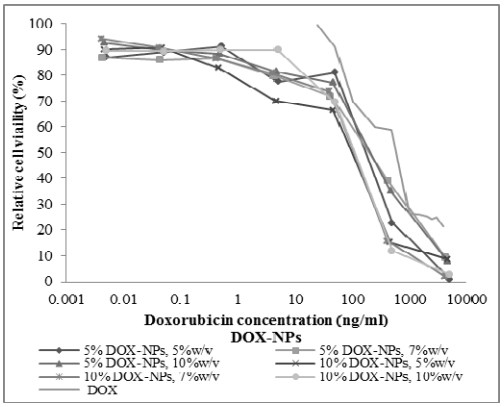

Fig. 11: Cytotoxicity of DOX-NPs

F. Drug Accumulation Study

Intracellular uptake of DOX-NPs was compared with that of free DOX using fluorescence microscopy. The cellular uptake studies showed that both 10% w/v particle concentration of 5% *CP* nanoparticles and 10% *CP* nanoparticles were initially adsorbed onto the cell surface, while free DOX could easily diffuse into the cytoplasm as shown in Fig.11. Because the nanoparticles were bigger than the free drug, they could only enter the cells via endocytosis which would take longer.

The results from Fig. 12 – 13 illustrated that a greater amount of free DOX and DOX-NPs were internalized in terms of time dependent. After 3 hours of free DOX incubation, DOX fluorescence was detected in the cytoplasm and the nuclei of the cells (Fig.12). In contrast, when incubated with DOX-NPs for 3 hours, most of DOX fluorescence was detected only in the cytoplasm. DOX fluorescence intensity of 6-hour incubation was higher than of 3-hours. DOX-NPs were detected in the cytoplasm and in the cell nucleus (Fig.13).

IV. Conclusions

O-Succinyl chitosan graft Pluronic® F127 copolymer (*CP*) was successfully synthesized by grafting Pluronic® F127 onto chitosan via EDC using NHS coupling agents. The graft copolymer could form self-aggregated micelles of about 50 nm in size in an aqueous medium. DOX could be successfully loaded into the synthesized *CP* nanoparticles with high

encapsulation efficiency. The release profiles of DOX at both pH 5.0 and pH 7.5 showed a sustained release profile within 22 days. The release rate of DOX from DOX-NPs was faster at pH 5.0 than at pH 7.5 due to the faster degradation rate of O-succinyl chitosan at the outer shell of the nanoparticles in an acidic solution. The IC_{50} of DOX-NPs was approximately 3.6 times lower than that of free DOX, suggesting higher therapeutic efficacy of the drug encapsulated nanoparticles. Thus, O-Succinyl chitosan graft Pluronic® F127 copolymer nanoparticles are promising as drug carriers which can lead to effective cancer treatment.

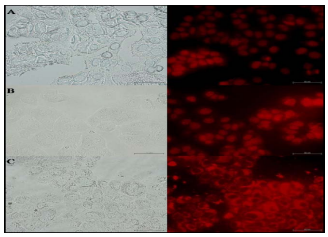

Fig. 12: Intracellular distribution of DOX and DOX-NPs after 3 hours of drug exposure in MCF-7 cell line at 37 °C A. Free DOX, B. 5% CP NPs -10%w/v, C. 10% CP NPs -10%w/v (The left images are Phase contrast, while the right images are red fluorescent of DOX in the same area.) Bar represents 50 µm.

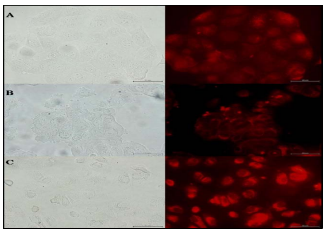

Fig. 13: Intracellular distribution of DOX and DOX-NPs after 6 hours of drug exposure in MCF-7 cell line at 37 °C A. Free DOX, B. 5% CP NPs -10%w/v, C. 10% CP NPs -10%w/v (The left images are Phase contrast, while the right images are red fluorescent of DOX in the same area.) Bar represents 50 µm.

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