Therapeutic Targeting of Anti-Cancer Drug Loaded PCL-PEG-PCL Triblock Copolymer Nanoparticles toward MCF-7 Breast Cancer Cell Line

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Abstract— Drug delivery systems based on poly (ε-caprolactone)/poly (ethylene glycol) (PCL/PEG) copolymer nanoparticles have been developed to generate new therapeutic systems with better treatment efficacy and lower side effect. In this work, poly (ε-caprolactone)-poly (ethylene glycol) - poly (ε-caprolactone) (PCL-PEG-PCL) nanoparticles with the average particle size ~ 226 ± 5 nm and spherical in shape were prepared by nanoprecipitation method using acetone as the organic solvent. Dox was encapsulated into PCL-PEG-PCL micelles with encapsulation efficiency (EE) 99.7% and drug loading (DL) 28.69 %. The PCL-PEG-PCL nanoparticles were employed to load doxorubicin by a pH-induced self-assembly method. *In vitro* release study indicated that doxorubicin release from nanoparticles at pH 5.6 was faster than that at pH 7.0. The amounts of DOX was uptake and accumulated in the MCF-7 cells from DOX-loaded micelles suggest a potential application in cancer chemotherapy.

Index Terms— Breast cancer, CARS microscopy, Doxorubicin, Drug delivery, Nanomedicine, PCL-PEG-PCL copolymer.

1 INTRODUCTION

Breast cancer is the most frequent malignancy of women worldwide. In Iraq, breast cancer ranks first among cancers diagnosed in women, the incidence of female breast cancer has risen in Iraq [1]. It is estimated that more than one million new cases of breast cancer are diagnosed all over the world annually [2]. Drugs incorporated into nanosized polymeric micelles are promising nanocarrier systems for drug delivery, because the polymeric micelles have several advantages, such as controlled drug release, enhanced tumor-penetrating ability, reduced side toxicity, increased stability, increased loading capacity and specific-tissue target ability [3]. Nanotechnology provides an important method to overcome the poor water solubility of hydrophobic drugs, hydrophobic drugs were manipulated to be entrapped into nano-scale particles, which could be well dispersed in aqueous solution to form stable and homogeneous suspension, therefore met the requirements of clinic administration [4]. Micelles prepared from synthetic biodegradable block copolymers are widely applied in drug delivery system (DDS) owing to their intrinsic core/shell architecture which demonstrates a series of attractive properties for increasing drug solubility, enhancing drug stability, and passive target effects [5]. Poly (ethylene glycol) (PEG) and poly (ε-caprolactone) (PCL) are both biocompatible and have been used in several FDA-approved products [6]. Further, drugs that are encapsulated by small-sized polymeric micelles with a hydrophilic outer shell can potentially increase the circulation time of drugs and can prevent recognition by macrophages of the reticuloendothelial system (RES) after intravenous injection [7]. Coherent anti-Stokes Raman scattering signal is greatly amplified, around five orders of magnitude over traditional Raman spectroscopy, offering new possibilities for high sensitive detection in living cells and tissue, with high chemical specificity and inherent three-dimensional (3-D) optical sectioning capability. In addition, complementary nonlinear optical (NLO) techniques, such as two-photon excitation fluorescence (TPEF) have been widely used for biological specimens imaging [8]. In combination with CARS, these modalities provide a wealth of chemical and biological information, which can help to resolve the most persistent biological questions. These techniques have the potential to revolutionize biomedical imaging offering the new insights into the biochemistry and pathways at the subcellular level [9].

2 EXPERIMENTAL METHODS

2.1 Materials

Materials used in included Acetone, Dibasic sodium phosphate anhydrous, Dipotassium phosphate (K2PO4), Disodium hydrogen phosphate (NA2HPO4), Hydrochloride acid (HCL), Isopropanol, Monobasic potassium phosphate KH2PO4, Monobasic potassium phosphate KH2PO4, Monobasic potassium phosphate KH2PO4, Phosphate Buffer Saline, Trypsin (Fisher scientific, UK), Doxorubicin, Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Phosphate-Buffered Saline (DPBS), Fetal Bovine Serum, Glutamine, Trypan blue (Sigma Aldrich , USA),

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Poly caprolacton(PCL) - Poly ethylene glycol (PEG) - Poly caprolacton (PCL) copolymer (Polysciences USA). Human breast carcinoma cell line (MCF-7) was obtained from the European Collection of Cell Cultures and cultured according to standard mammalian tissue culture protocols and sterile techniques.

2.2 Method

2.2.1 Preparation of free PCL-PEG-PCL nanoparticle

The triblock copolymer PCL5000-b-PEG10000-b-PCL5000 was supplied from Polysciences Company. The number listed under each of polymer indicated the approximate molecular weight of the block segment. The PEG terminal groups are blocked as methyl ethers, but the caprolactone end group are hydroxyl and are suitable for functionalization.

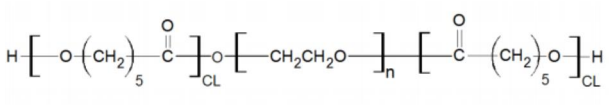

Fig. 1. Chemical structure of PCL-PEG-PCL triblock copolymer

The PCL-PEG-PCL (PCEC) nanoparticles were prepared by nanoprecipitation method by dissolved five milligrams of polymer in ten milliliter of acetone by vortexing and stirring with vortex and magnetic stirrer respectively, then the solution was added dropwise into 10 ml miliQ water under moderate stirring at 25 Cº for three hours, the solution that obtained was passed through dialysis membrane molecular weight cutoff (MWCO 3-8 KDa) for 24 hours, then the solution was filtrated throughout 0.45 μm filter membrane to remove aggregates and clumps, and then freeze-dried for further application and characterization [10].

2.2.2 Loading of doxorubicin into PCL-PEG-PCL micelles

Doxorubicin-loaded PCL-PEG-PCL nanoparticles were prepared by a pH-induced self-assembly method. 0.1 ml of PBS (10×, pH 7.4) was added into 0.35 ml of nanoparticle slurry, a volume of 0.1 ml of doxorubicin aqueous solution was added drop by drop into the nanoparticles solutions under moderate stirring. The resulting solution was placed into a dialysis bag to dialyze against 100 ml of miliQ water for 72 hours with (MWCO 10 KDa) by amicon ultra centrifugal filter unit for purification and to remove insoluble and unbounded drug. Unincorporated DOX in filtrated solution and the amount of DOX loaded in micelle was quantified by determining of fluorescence at 490 nm by using Modulus™ II Microplate Multimode Reader. The DOX solutions of various concentrations were prepared, and the fluorescence of the solutions were measured to obtain a calibration curve.

2.2.3 Characterization of PCL-PEG-PCL Nanoparticle

2.2.3.1 Particle Size

The particle size of polymeric micelles was determined by dynamic light scattering (DLS) at 25 °C using a Zetasizer with an excitation of 633 nm illuminated at a fixed angle of 90°. Aqueous micelle solutions were prepared using 1:20 (vol / vol) dilution of the NPs suspension in deionized water. The concentration of polymeric micelles was kept at 1 mg / ml. The micelle solutions were sediment for 4h then it was filtered through 0.2 μm centrifuge filter before measurements. The average values were calculated from three measurements performed on each samples. The results were expressed as the size ± SD. The size of NP's was confirmed with TEM.

2.2.3.2 Morphology

The morphology of the prepared micelles was observed under a TEM. micelles were diluted with distilled water and one drop of NP solution was placed on a carbon film-coated copper grid (400 mesh) at room temperature followed by negative staining with 2% phosphotungstic acid for 20 seconds, excess solution was absorbed with filter paper, and air drying, prior to placing it in the TEM instrument for analysis.

2.2.4 Evaluation of Drug loading and encapsulation efficiency

The drug loading and encapsulation efficiency of Dox-PCEC were determined by a subtraction method [11]. Amount of 0.2 ml was centrifuged using filter tube with a MWCO 10 KDa. Although the free DOX could pass through, the doxorubicin-encapsulated PCEC nanoparticles could not pass through the filter. The unbounded doxorubicin was quantified by determining the fluorescence spectroscopy with excitation at 490 nm and emission wavelength of (510 – 570) nm, with a slit width of 5 nm.The drug loading (DL) and encapsulation efficiency (EE) was calculated according to the following equations:

$$DL\ (\%) = ([Total\ Drug] - [Free\ Drug]) / ([Polymer] + [Total\ Drug] - [Free\ Drug]) * 100 \quad (1)$$

$$EE\ (\%) = ([Total\ Drug] - [Free\ Drug]) / ([Total\ Drug]) * 100 \quad (2)$$

2.2.5 *In vitro* drug release

The *in vitro* release behavior of DOX from the drug loaded PCL-PEG-PCL micelles was studied as modified dialysis method. A volume of 5 ml of the DOX-loaded micelle solutions were put in dialysis membrane (MWCO 10kDa) and dialyzed against 30 ml of pH 5.6 and pH 7.4 of Sorenson's Buffer at 37 ºC. Then, 5 ml of the released solution was withdrawn for fluorescence measurements and replaced by 5 ml of fresh buffer solution. The concentration of DOX was determined by fluorescence spectroscopy with excitation at 490 nm and emission wavelength of 510 – 570 nm. Each experiment was carried out in triplicate, and average values plotted. The percent of the drug release was calculated using the equation given below:

$$\%\ Drug\ Releasing = (1 - Fluorescence\ (t)) / (Fluorescence\ (t_\circ)) * 100 \quad (3)$$

When (t) being the time at which the fluorescence is measured and ($t_\circ$) the initial time [12]. All release experiments were conducted in the dark by covering tubes with aluminum foil.

2.2.6 Cellular Uptake of DOX with 3D Multimodal Imaging Using CARS a6+nd TPEF Multimodal Microscopy

The experimental setup for imaging used in this study was described by Mouras, et al. [13]: A mode-locked Nd: YVO4 laser source produces the Stokes pulse (6 ps, 1064 nm) used in the CARS process. The source also produces a 5-ps, frequency-doubled, 532-nm beam, which was used to pump a picosecond optical parametric oscillator (OPO). The OPO delivers a signal tunable in the range 700–1000 nm, which was used as a pump in the CARS process resulting in an observable range of ~600–4000 cm−1 covering all the biologically relevant molecular vibrations. The two beams are combined by a dichroic mirror (DM) and focused onto a single-mode fiber (Thorlabs SM980-5.8–125, single modefrom ~780 to>1064 nm) for ease of alignment, which is connected to the input of a laser-scanning confocal inverted optical microscope. The pulses are synchronized in time by adjusting a micrometer-driven delay stage. These wavelengths are reflected towards a 60× oil immersion objective with 1.4 numerical aperture (NA) by a DM which removes most of the backscattered laser light. The configuration of the system enables both backward (epi-) and forward detection schemes. The backward signal is collected by the same objective and directed either to two different photomultiplier tubes (PMTs) or to an avalanche photodiode through a multimode fiber. The forward signal is collected by an air condenser (NA 0.55) and detected by a third PMT detector. An appropriate set of short-pass and band-pass filters are used to selectively transmit the non-linear (NLO) signals. Although picosecond pulse widths are far less efficient than femtosecond pulses (of the same average power) for TPEF imaging, we able to use significantly more power with picosecond sources. Photo damage occurs at far higher powers with a picosecond source, and hence imaging rates for two photon microscopy (TPEF) are quite similar for picosecond and femtosecond sources. The MCF-7 cells with confluent (70%-80%) were cultured in intracel glass bottom dishes with DMEM supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, then the cell line was maintained at 37 ◦C in a humidified 5% CO2-containing atmosphere. The cultured cells were fixed using formaldehyde and kept in solution. The fixed cells were used for imaging at different wave numbers [14].

3 RESULTS

Triblock copolymers of PCL with PEG was chosen and successfully was self-assembled to form nano-sized micelles by bottom-up approach starting from amphiphilic block copolymers that self-assemble in a selective solvent such acetone to form micellar aggregates consisting of a hydrophilic outer shell and a hydrophobic inner core in aqueous media, such micelles with core-shell structure can readily incorporate lipophilic drugs doxorubicin (DOX) into their cores, while the hydrophilic shell can provide stabilization for the was used. During the preparation of PCL-PEG-PCL nanoparticles with long PEG chains, water cannot freely penetrate the inner part of the PCL core due to the strong hydrophobic and crystallized character of the PCL block. Thus, the hydrolytic degradation of ester bond first takes place at the interface between the PCL core and PEG shell, resulting in the partial cleavage of ester bonds of PCL-PCL and PCL-PEG on the surface of the PCL core, resulting PCL-PEG-PCL with core-shell micelles formation. The poly caprolactone-poly ethylene glycol copolymer displays a desirable properties for drug delivery applications [15].

3.1 Loading of Doxorubicin (DOX) into Polymeric PCL-PEG-PCL Micelles

The triblock copolymer of PCL-PEG-PCL contains the hydrophobic segment, enabling the encapsulation of the hydrophobic drug in the core of micelle. In an attempt to overcome the disadvantages of toxicity and drug-resistance and increase selectivity towards cancer cells, the hydrophobic DOX was physically entrapped in the core of PCL-PEG-PCL micelle by a self-assembly method. This procedure for preparing Dox / PCL-PEG-PCL micelles was very simple and easy to scale up, also no surfactants and organic solvents were applied in this procedure. After DOX was entrapped into the core of the amphiphilic polymeric micelles, drug-loaded micelles could be well and stably dispersed in water solution to meet the requirement of intravenous injection. The ability of the core to encapsulate drug is largely dependent upon the compatibility between the hydrophobic core and the drug molecule. In their study, Yan et al., found that there is compatibility between DOX and PCL [16]. The amount of DOX that loaded in PCL-PEG-PCL micelles was determined by calculation the Drug Loading (DL) and the Encapsulation Efficiency (EE) with various initial concentration ratios of copolymer to DOX (table 1).

Table 1
The concentration, drug loading and encapsulation efficiency of dox / pcl-peg-pcl nanoparticles

Drug/ Nanoparticle	Concentration of nanoparticle (mg/ml)	Concentration of Drug (mg/ml)	Drug loading (%)	Encapsulation Efficiency (%)	Ratio of PCL-PEG-PCL micelles:DOX (%)
DOX /PCL –PEG-PCL solution	12.4	5	28.69	99.7	70:30

Recently, biodegradable polymeric micelles are highlighted as advanced drug delivery systems for cancer therapy due to their unique core-shell structure, which could solubilize hydrophobic anticancer drug into the hydrophobic inner core and release the drug in a controlled manner at a later stage [17].

3.2 Characterization of PCL-PEG-PCL Nanoparticles

TEM image revealed that the micelles that prepared by nano-precipitation were spherical shape in solution shown in Fig. 2. The micelles was shown by light spherical entities surrounded by dark staining, it apparent that the hydrophobic PCL segments were assembled in the micelle core and the hydrophilic PEG backbone was exposed to the shell.

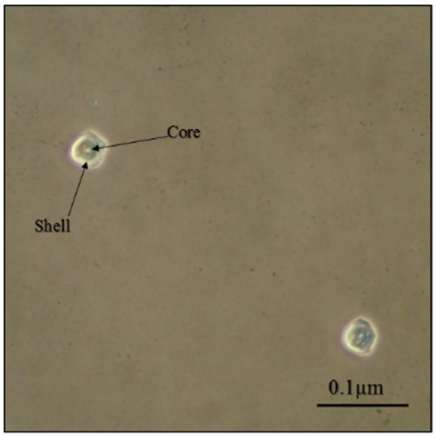

Fig. 2. TEM image of PCL-PEG-PCL micelles
Note: magnification: 8000x; scale bars correspond to 0.1 µm.

Dynamic Light Scattering (DLS) was employed to evaluate the size and polydispersity index (PDI) of the obtained nanoparticles. The particle size and PDI was illustrated in Fig. 3. DLS studies indicated that the average particle size of obtained micelle was 226±5nm and polydispersity index (PDI) was 0.26± 0.034 with a narrow monodispersed unimodal size distribution pattern.

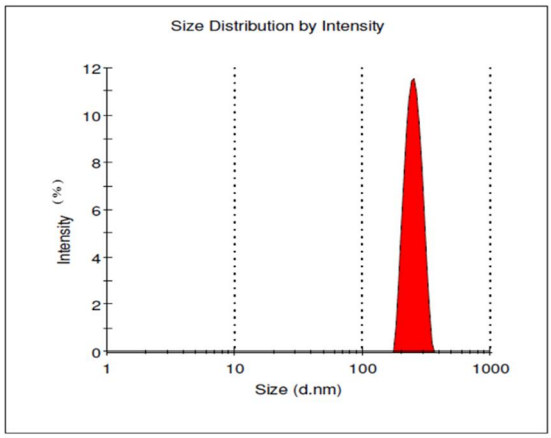

Fig. 3. Particle size distribution of PCL-PEG-PCL micelles determined by Dynamic Light Scattering (DLS). Mean ± standers deviation.

3.3 *In vitro* Release of Doxorubicin from Nanoparticle

The release profile of DOX from the PCL-PEG-PCL nanoparticles was studied using a dialysis method. In this study, pH 5.6 was selected to mimic pH of cancer cells' microenvironment, while pH 7.4 was selected to mimic pH of the healthy cells. As shown in Fig. 4., it could be observed that DOX release from Dox-PCEC faster at pH 5.6 than at pH 7.4.

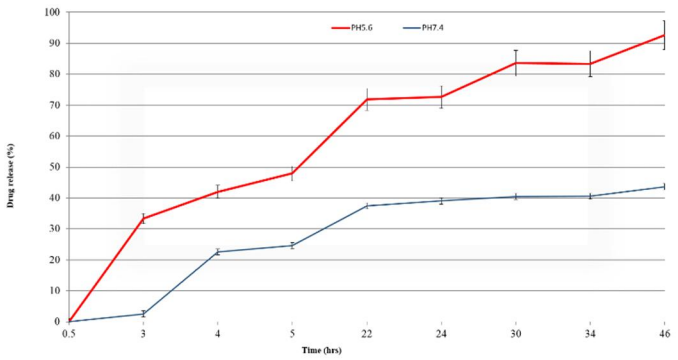

Fig. 4. *In vitro* release profile of DOX from PCL-PEG-PCL micelles during time of incubation with Sorenson's Buffer solutions at different pH.

3.4 Cellular uptake of DOX loaded with PCL-PEG-PCL by MCF-7 breast cancer cell line using CARS and TPEF spectroscopy

Multimodal spectroscopy was employed to visualize the cellular uptake of DOX and the internalization of DOX-loaded micelles and free DOX into the MCF-7 cells illustrated in Fig. 5. It can be used DOX directly to track its cell uptake without introducing additional fluorescent probes because its fluorescence property. The visualization of the 3D distribution of DOX by CARS and TPEF showed that nuclei of MCF-7 accumulate a high concentration of free DOX after 10h35m, while for DOX loaded PCL-PEG-PCL after 2 hr 20 min, strong DOX fluorescence was observed only in the cytoplasm, after 5h50m, DOX appeared to be exclusively located within the cytoplasm and concentrated in a perinuclear area with negligible levels accumulation in nuclei. After 10h 35 min, the observation of fluorescence in cytoplasm indicated that the DOX loaded PCL-PEG-PCL micelle was internalized by the cells through endocytosis and DOX was distributed in the cytoplasm after escaping from the endosome and/or the lysosome then diffused from the cytoplasm to the nucleus where it will intercalate in the DNA in the cells and MCF7 show the early signs of their reaction to the drugs by shrinking and start to undergo apoptosis suggesting that DOX was may delivered into nuclei and successfully inhibited the proliferation of MCF-7 cells. However the diffusion of Dox from the cytoplasm depends on the time taken during the incubation [18]. Micellar doxorubicin is released in a controlled manner from the micelle particles and eventually enters the nucleus where DOX is known to exert its cytotoxicity during DNA synthesis.

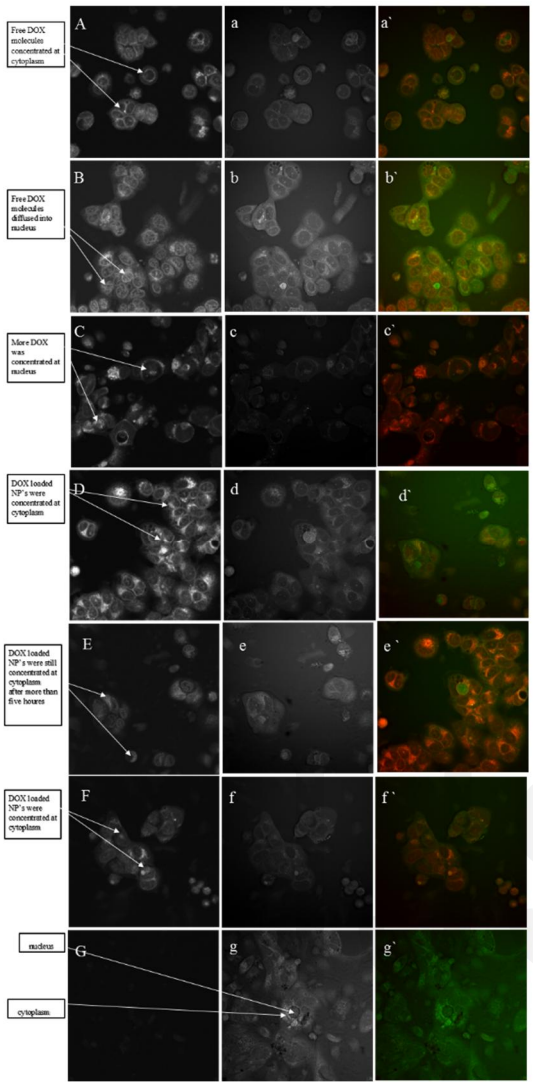

Fig. 5. TPEF and CARS images of MCF-7 cells after incubation at the equivalent DOX 0.01 mg / ml, NP 7.31 mg / ml:
A,a,a`) Free Doxorubicin (after 2h 20 min),
B,b,b`) Free Doxorubicin (after 5h50min)
C,c,c`) Free Doxorubicin (after 10 h 35min),
D,d,d`) Nanoparticles + Doxorubicin (after 2h20m)
E,e,e`) Nanoparticles + Doxorubicin (after 5h50m),
F,f,f `) Nanoparticles + Doxorubicin (after 10h35m)
G,g,g`) Control (no Nanoparticles, no Doxorubicin).

4 DISCUSSION

In order to overcome the limitations of the traditional chemotherapeutic, the drug delivery technology was design by using suitable carriers that can efficiently encapsulate anticancer drugs, overcome drug-resistance, and increase selectivity of drugs towards cancer cells while eliminating their toxicity to normal tissues. Amphiphilic block copolymer with longer hydrophilic PEG block are more hydrophilic and they can diffuse more easily in aqueous medium and give a denser hydrophilic corona thus increasing stealth properties and increase the circulation time in vivo [19].

The precipitation method was chosen to prepare the micelles of the block copolymers because the amphiphilic block copolymer micelles could not be formed in water alone because of the characteristic of the hydrophobic block, increase their physical stability, and prevent secondary aggregation (or micellar fusion). This phenomenon is very common in polymeric nanoparticle containing poly caprolactone as the hydrophobic component [20],[21]. The DOX was physically encapsulated into the copolymeric micelle due to several factors such as; the hydrophobic interaction of the drug and the PCL core, the structure of the hydrophobic core, and core–drug interaction. Hydrophilic blocks form a hydrated outer shell which may conceal the hydrophobic core preventing its quick uptake by the reticuloendothelial system (RES) and more active clearing organs such as the liver, spleen, lung, and kidneys. Therefore, the hydrated outer shell can increase the blood circulation times of the nanoparticles. The predominant characteristics of this system have been reported to reduce toxic side effects of antitumor agents, passive targeting to specific sites, solubilization of hydrophobic drugs, and stable storage of drugs, longer blood circulation, favorable biodistribution, thermal stability, and lower interactions with the RES [22].

The distinct spherical shape for polymeric micelles was formed when PEG segments are highly hydrated, and then water can cross the PEG shell freely and contact the surface region of PCL core, resulting in the swelling of PCL-PEG-PCL micelles. Movement of spheres is easier to predict due to their inherent symmetry, but non-spherical nanoparticles may align or tumble in the presence of flow [23]. Nanoparticles can escape from the circulation through openings, also called “fenestrations” of the endothelial barrier [24]. Tumor growth induces the development of neovasculature characterized by discontinuous endothelium with large fenestrations of 200–780 nm allowing nanoparticles passage [25]. During the releasing process, DOX was first released inside the hydrophobic core region of the polymeric micelles due to the attachment of DOX to PCL the core region, then, DOX diffused out from the micelle, eventually, into the incubation medium. This delay of drug release indicates the nanoparticle potential applicability in drug carrier to minimize the exposure of healthy tissues while increasing the accumulation of therapeutic drug in the tumor site. It was expected that DOX could be very slowly released in the plasma under normal physiological conditions (pH 7.4), but quickly released at the solid tumor site (pH 5.6). This indicates that envelopment of DOX in the micelles resulted in significantly sustained release of the drug, which is beneficial for drug action. This pH-dependent releasing behavior might be due to the reprotonation of the amino group of DOX and faster degradation of PCL-PEG-PCL nanoparticles at lower pH [26].

The cellular uptake of free DOX occurs through a passive diffusion mechanism whereby it may be trapped at the P-gap junction and thereby affect the normal cells while in the case of PCL-PEG-PCL / DOX, the drug has to be released in a time dependent manner from the PCL-PEG-PCL micelles before exerts its effects on the cells [27],[28].

The cellular uptake of PCL-PEG-PCL / DOX micelles was through non-specific endocytosis which may lead to reduced

effect of cytosolic free DOX for the P-glycoprotein pumping action; this mechanism of PCL-PEG-PCL / DOX micelles delivery to tumors may circumvent the effect of multidrug resistant proteins which are always present in cancer cells [29].

The cellular uptake of free DOX was faster than that of DOX-loaded micelles in MCF-7. As a small molecule, the uptake of free DOX is a dynamic process and they can freely escape from the cells. While for the DOX-loaded copolymer micelles with much larger sizes, it is likely that the prolonged circulation and passive tumor-targeting delivery process caused by the EPR effect will enhance the delivery of hydrophobic drugs into the tumor cells and once the micelles were internalized, it is not easy. The polymeric based delivery drug system may offer a successful and promising potential application for many therapeutic agent with more confidence in doxorubicin for the clinical treatment of breast cancer and for efficient intracellular delivery of hydrophobic anti-cancer drugs.

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