Research Article

Chemical Review and Letters
journal homepage: www.chemrevlett.com
ISSN (online): 2645-4947 (print) 2676-7279

Preparation and *in-vitro* evaluation of PCL–PEG–PCL nanoparticles for doxorubicin-ezetimibe co-delivery against PC3 prostate cancer cell line

Mina Yousefnezhad[1], Mirzaagha Babazadeh[1, *], Soodabeh Davaran[2,3, *], Abolfazl Akbarzadeh[4], Hamidreza Pazoki-Toroudi[5]

[1]*Department of Chemistry, Tabriz Branch, Islamic Azad University, Tabriz, Iran*
[2]*Department of Medicinal Chemistry, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran*
[3]*Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran*
[4]*Department of Medical Nanotechnology, Faculty of Advanced Medical Science, Tabriz University of Medical Sciences, Tabriz, Iran*
[5]*Physiology Research Center and Department of Physiology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran*

ARTICLE INFO

Article history:
Received 21 January 2024
Received in revised form 16 February 2024
Accepted 22 February 2024
Available online 22 February 2024

Keywords:
Doxorubicin
ezetimibe
co-delivery
PCL-PEG-PCL nanoparticles
prostate cancer
combination therapy

ABSTRACT

In the recent decade, the design and engineering of novel drug delivery systems based on biodegradable nanoparticles using biocompatible polymers like poly (ε-caprolactone)/poly(ethylene glycol)/poly(ε-caprolactone) triblock copolymer (PCEC) attracted many attentions. These nanocarriers have shown high potentials in enhancing treatment efficiency and minimizing the side effects of drugs. Besides, combination therapy has become a potential approach for cancer treatment with synergistic impacts. For the first time, we investigated co-delivery of the antitumor drug, doxorubicin (DOX), and ezetimibe (EZ) as a cholesterol uptake-blocking drug with PCEC on prostate cancer cell line (PC3). The PCEC was synthesized by ring-opening polymerization of ε-CL initiated by PEG2000. The obtained copolymer was characterized by Fourier transform infrared spectroscopy (FT-IR), proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, and gel permeation chromatography (GPC). In this study, DOX and EZ were encapsulated within PCEC by double and simple emulsion techniques, which led to the preparation of DOX@PCEC, EZ@PCEC, and DOX+EZ@PCEC nanoparticles. The size and morphology of the developed nanoparticles were analyzed by field emission scanning electron microscopy (FE-SEM). Also, the particle size and zeta potential of the drug-loaded PCEC nanoparticles were determined by dynamic light scattering (DLS) analysis. The release behavior of DOX and EZ from nanoparticles at two pH values and temperatures was evaluated. The cytotoxicity of nanoparticles was demonstrated by MTT assay using PC3 prostate cancer cell line. Based on the MTT assay results, PCEC copolymer exhibited negligible cytotoxicity on the growth of the PC3 cell line. Therefore, PCEC is a biocompatible and suitable nano-vehicle for this study. Moreover, the cytotoxic activity of all formulations was dose-dependent. The cytotoxic effect of DOX+EZ@PCEC nanoparticles against PC3 cell line was higher than single drug@PCEC nanoparticles. All data confirmed that the EZ as a cholesterol-lowering drug showed a synergistic effect in combination with DOX as an anticancer drug. Finally, the obtains results showed a successful formulation of DOX+EZ@PCEC nanoparticles with high efficiency in prostate cancer treatment.

∗ Corresponding authors.; e-mail: babazadeh@iaut.ac.ir; davaran@tbzmed.ac.ir,
https://doi.org/10.22034/crl.2023.390623.1213

1. Introduction

According to the American Cancer Society, the most significant number of deaths are related to lung, prostate, and colorectal cancers in men and lung, breast, and colorectal cancers in women [1-3]. Moreover, almost 17000 patients were recognized with prostate cancer (PCA) in the US in 2019. Chemotherapy, hormone therapy, tumors targeting therapy, radiation therapy, and surgery are practical cancer treatment choices [4]. Among these mentioned treatment strategies, chemotherapy is widely used. However, many side effects are accompanied by nausea, vomiting, fatigue, pain, mouth ulcers, nerve damage, and skin reactions. A co-delivery of different therapeutic agents might be a promising strategy in chemotherapy that provides an additive or a synergistic effect [5].

In recent decades, nanotechnology has attracted lots of attention in various fields, specifically drug delivery to tumor tissue. Nanocarriers such as nanoparticles, micelles, and dendrimers can decrease the side effects of antitumor drugs like toxicity, burst release, and damage to healthy tissue. Moreover, they improve solubility, stability, biodistribution, therapeutic efficiency, and site-specific therapeutic agents' delivery. Drug-loaded nanocarriers provide a controlled release of drugs and improve their bioavailability and pharmacokinetics. Polymeric nanoparticles have a unique potential in carrying both hydrophilic and hydrophobic drugs [6, 7]. Biodegradable polymers are among the most suitable polymers used to prepare drug delivery systems, convert to individual monomers, and are removed from the body through normal metabolic pathways [8]. Poly(caprolactone) (PCL) is a hydrophobic semi-crystalline polymer known as a biodegradable and biocompatible polyester [9]. The non-toxicity and high permeability of PCL have made it a prominent candidate to prepare drug delivery systems and tissue engineering. However, it has a slow degradation rate [10]. Poly(ethylene glycol) (PEG) is another common polymer with non-toxicity, high biocompatibility, non-antigenicity, and high immunogenicity, used in drug delivery systems. Therefore, it is often opting to incorporated with PCL [11].

Utilization of drug carriers like nanoparticles, micelles, or dendrimers, loaded with multiple drugs, resulted in enhanced cytotoxicity and further apoptosis of cancer cells at a low dosage of chemotherapeutic agents. These outcomes show the high potential of such systems in chemotherapy [12]. Many studies have been carried out to evaluate these systems' efficiency as drug delivery systems for cancer therapy. Doxorubicin (DOX), one of the most effective chemotherapeutic agents, is an anthracycline drug, which has been used alone or in combination therapy for different types of cancers such as breast, prostate, lung, ovarian, and leukemia [13].

In a study by Wang *et al.* [5], co-delivery of curcumin and DOX incorporated in methylated PEG-PCL (mPEG-PCL) micelles resulted in improved efficiency of systemically administered chemotherapeutic agents in mice with lung cancer. In another study by Danafar *et al.* [14], DOX-conjugated mPEG-PCL micelles loaded with curcumin were evaluated *in-vitro* as a co-delivery system for cancer therapy. The cytotoxicity assay results showed enhanced death of A549 cells than free drugs. Pang *et al.* [15] prepared an engineered drug delivery system based on PEG-PCL diblock copolymer as carriers for DOX. This drug delivery system was modified by folic acid to provide targeted delivery to the tumor site. The results demonstrated improved accumulation of the drug in tumor tissue and high cancer therapy potentials.

As reported by mentioned studies, nanocarriers' utilization as delivery systems for multiple drugs has shown promising potentials for treating different types of cancer, including PCA [16]. PCA is accompanied by the accumulation of high cholesterol levels in prostate tissue [17]. Ezetimibe (EZ) is a white crystalline powder soluble in ethanol, methanol, and acetone. It is a class of lipid-lowering compounds that limits intestinal cholesterol absorption by binding to its extracellular loop and consequently decreases low-density lipoprotein cholesterol (LDL-C) [18]. Scientific research shows that EZ could reduce prostate tumor volume by preventing cholesterol accumulation in prostate tissue [19, 20].

In this study, we prepared biodegradable PCL/PEG/PCL copolymer (PCEC)-based nanoparticles as potential carriers for co-delivery of DOX and EZ. The advantage and novelty of this research is synergistic effects of these two drugs. For the first time, we investigated co-delivery of DOX as an antitumor drug and EZ as a cholesterol uptake-blocking drug with PCEC on a prostate cancer cell line (PC3).

2. Materials and Methods

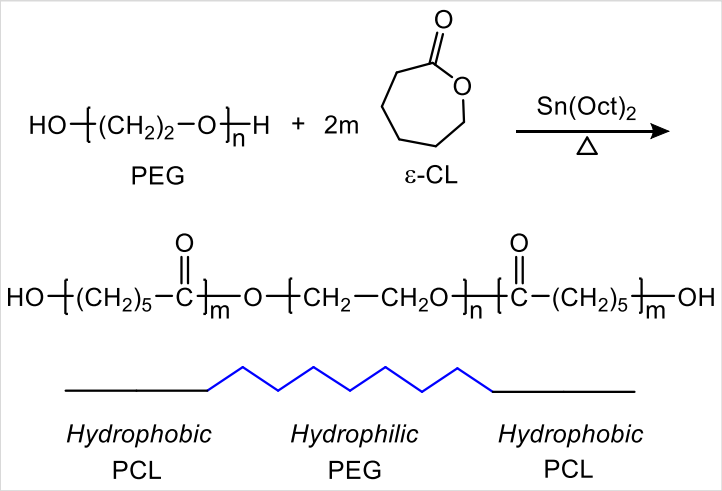
2.1. Materials

Dimethyl sulfoxide (DMSO), dichloromethane (CH_2Cl_2), poly (ethylene glycol) (MW=2000), Tin (II) 2-ethyl hexanoate (stannous octoate, $Sn(Oct)_2$), ε-caprolactone (ε-CL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Materials used in biological protocols including, Roswell Park Memorial Institute 1640 growth

medium (RPMI) and trypsin, were purchased from Gibco BRL Life Technologies (Ireland). Fetal bovine serum (FBS) was obtained from Bioidea Co. Human prostate carcinoma cell line (PC3) was obtained from Pasteur Institute of Iran (Tehran, Iran). DOX salts and EZ were purchased from EBEWE Pharmaceutical Co. (Austria) and Cipla USA Inc., respectively.

2.2. Preparation of PCL-PEG-PCL copolymer

The PCL-PEG–PCL triblock copolymer (PCEC) was prepared by ring-opening polymerization of PEG and ε-caprolactone (ε-CL) in the presence of $Sn(Oct)_2$ as the catalyst [21, 22]. PEG and CL monomer with a ratio of 1:10 were transferred to a three-neck round-bottom flask and heated in a bath of silicone oil with a temperature of 130°C under a nitrogen atmosphere for 10 min. A thermometer was placed inside the oil bath to precisely control the temperature during the whole process. $Sn(Oct)_2$ (1%) was added to solution, heated at 150°C under the stirring condition for 7 h, and then cooled to room temperature (25°C). Afterward, the mixture was dissolved in DCM for 20 min, and then poured into an excess amount of cold diethyl ether as a non-solvent. Finally, the precipitated copolymer was collected, washed with the same non-solvent for several times, and dried under vacuum at 25°C for 48 h.



Scheme 1. Scheme of the PCL-PEG-PCL copolymer synthesis mechanism.

2.3. Preparation of drug-loaded PCEC nanoparticles

Nanoparticles were prepared using both double emulsion ($W_1/O/W_2$) [23] and simple emulsion (O/W) methods [24].

2.3.1. Preparation of EZ-loaded PCEC nanoparticles (EZ@PCEC)

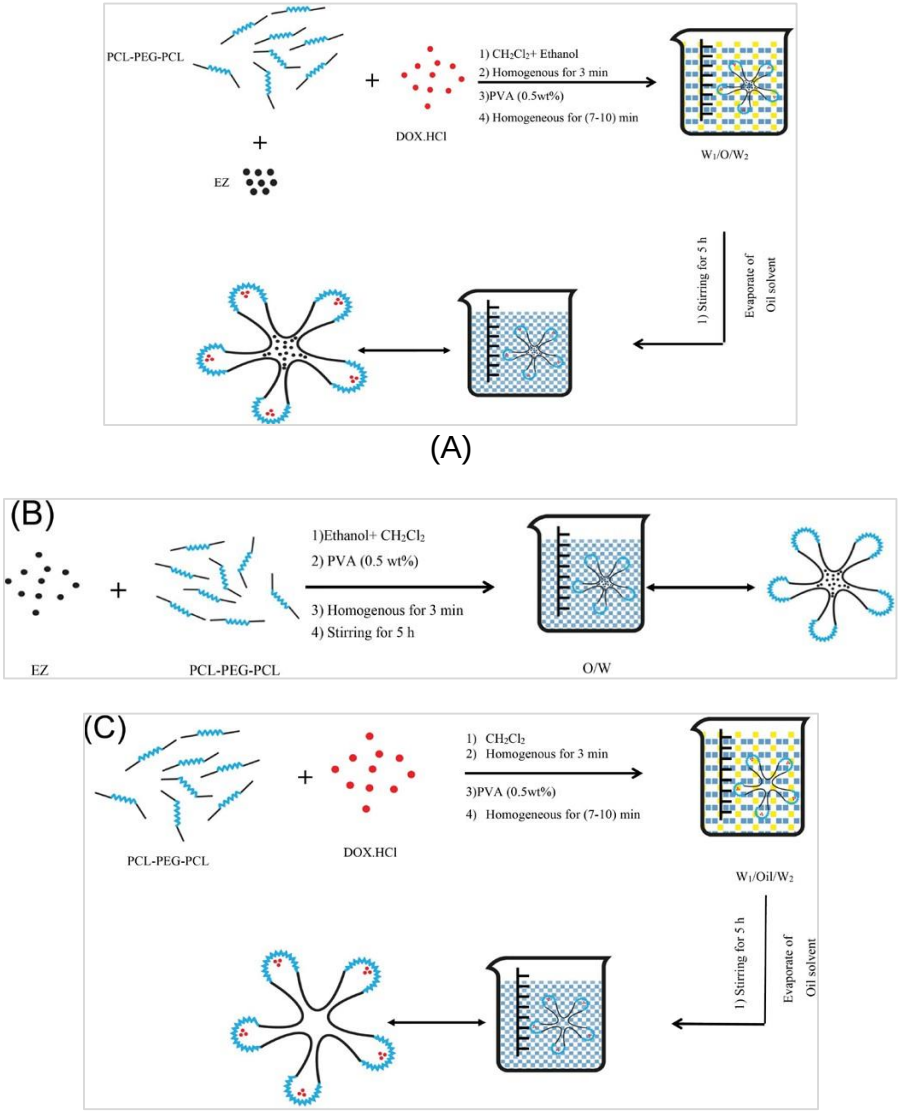
EZ-loaded PCEC nanoparticles were prepared using a simple emulsion technique. For this purpose, 10 mg of EZ and 100 mg of PCEC were weighed and dissolved in 700 µl and 2ml of ethanol and dichloromethane, respectively. Then, the solution was poured into a 30 ml aqueous solution of polyvinyl alcohol (PVA) 0.5 wt% and mixed at 12000 rpm for 3 min. The solution was stirred at room temperature for another 5 h to evaporate the dichloromethane. The prepared nanoparticles were separated by centrifugation at 9000 rpm for 20. The collected nanoparticles were dried by freeze-drying, and the supernatant was utilized to measure the encapsulated drug concentration (Scheme 2B).

2.3.2. Preparation of DOX-loaded PCEC nanoparticles (DOX@PCEC)

An aqueous solution of (DOX. HCl) (2000 ppm:5ml) was added to the organic solution (Oil) of 100 mg copolymer in 2 ml dichloromethane. In order to avoid DOX decomposition in the presence of light, the suspension was kept in the dark condition. The first emulsion (W_1/O) was prepared by homogenization (Silent Crusher M, Heidolph Instruments GmbH, Schwabach, Germany) at 11000 rpm for 3 min. Then W_1/O emulsion was added to a 30 ml aqueous PVA solution (0.5wt %; W_2), and the mixing was continued at 13000 rpm for 7 min to make $W_1/O/W_2$ emulsion. The $W_1/O/W_2$ emulsion was stirred at room temperature for 5 h to evaporate the organic phase. Nanoparticles were separated using a centrifuge, and the supernatant solution was utilized to measure the concentration of the encapsulated drug. Nanoparticles were collected and dried by freeze-drying (Scheme 2C).

2.3.3. Preparation of DOX+EZ-loaded PCEC nanoparticles (DOX+EZ@PCEC)

DOX+EZ-loaded PCEC nanoparticles prepared using double emulsion method. For this purpose, an aqueous solution of (DOX. HCl) (2000 ppm:2.5ml) was added to the organic solution (Oil) containing 100 mg triblock copolymer and 5 mg of EZ in 2 ml dichloromethane and 400µl ethanol, respectively. The first emulsion (W_1/O) was prepared by homogenization at 11000 rpm for 3 min. The further preparation procedure follows the protocol explained in the previous section. The ratio of drugs in EZ+DOX@PCEC was 1:1 (Scheme 2A).



Scheme 2. (A) Preparation of EZ+DOX-loaded PCL-PEG-PCL NPs using double emulsion method, (B) preparation of EZ-loaded PCL-PEG-PCL NPs using single emulsion method, and (C) preparation of DOX-loaded PCL-PEG –PCL NPs using double emulsion method.

2.4. Characterization of prepared triblock copolymer and nanoparticles

The obtained copolymer was characterized through Fourier transform infrared spectroscopy (FT-IR, Tensor 270/Bruker, Germany). Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy (in CDCl3) was recorded on an ultra-shield 400 spectrometer (Bruker, Germany) at 400 MHz. The molecular weight and polydispersity of PCEC copolymer were determined using gel permeation chromatography (GPC, Shimadzu LC-20A). The sample was dissolved in tetrahydrofuran (THF) at a concentration of 1-2 mg/2ml for this purpose. At a rate of 1.0 ml/min, THF was eluted. The external and column temperature was kept at 35°C. The size and morphology of the drug-loaded PCEC nanoparticles were determined by field emission scanning electron

microscopy (FE-SEM) (MIRA3 FEG-SEM/TESCAN). Dried nanoparticles were mounted on a tape, coated with a thin layer of gold, and images were obtained at the voltage of 15 kV. The particle size and zeta potential of the drug-loaded PCEC nanoparticles were determined by dynamic light scattering (DLS) analysis (zetasizer nano ZS90, Malvern Instrument, Uk).

2.5. Drug encapsulation efficiency and loading capacity

Ultraviolet-visible (UV-Vis) spectrophotometer (PU 8620/PHILIPS) was used to calculate the encapsulation efficiency (EE) and loading capacity (LC) of prepared EZ and DOX-loaded nanoparticles at two wavelengths of 228 nm and 480 nm, respectively. The EE and LC were calculated using the following equations:

$$\text{EE \%} = \frac{\text{total drug} - \text{drug in supernatant}}{\text{total amount of the drug}} \times 100$$

$$\text{LC \%} = \frac{\text{mass of drug in nanocarriers}}{\text{mass of nanocarriers}} \times 100$$

2.6. In vitro drug release

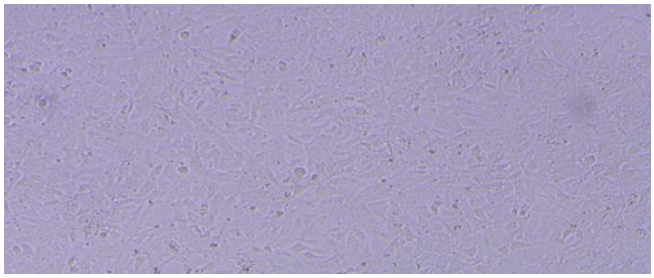
The release of DOX and EZ was investigated according to the sample and separate (SS) method [25] as follow: 4 mg of each drug-loaded nanocarriers (DOX@PCEC, EZ@PCEC, and DOX+ EZ @ PCEC nanoparticles) were dispersed in the release medium containing 2 ml PBS and ethanol 96% with the ratio of 60:40 at two different pH values of 5.6, and 7.4. The samples were placed in an incubator under gently stirring at various temperatures (40°C and 37°C) for particular time intervals. The supernatant was taken out at pre-determined time intervals to measure the amount of released drug and replaced with the same volume of fresh PBS to keep the sink condition. The absorbance peak of released DOX and EZ were measured by UV-Vis spectrophotometer at 480 and 228 nm, respectively. The absorbance intensity converted to drugs' concentration by interpolating in their calibration curves equation calculated separately at two pH values. The drug release experiments were done in triplicate, and average data was reported. The cumulative release of drugs was calculated using the following equation:

$$\text{Cumulative drug release (\%)} = \frac{\text{Ci} \times V + \sum C(i-1) \times Vs}{\text{m}} \times 100$$

where C_i is the concentration of drug in the release medium at the time *i*, *V* is the total volume of release solution, *Vs* is the sample volume, and *m* is the mass of drug encapsulated in nanocarriers.

2.7. Cell Culture

The PC3 cell line was cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin/streptomycin 1% (Scheme 3). The cells were treated with different concentrations of drug-loaded PCEC nanoparticles and free drugs. The same volumes of the medium, without drug-loaded PCEC nanoparticles or free drugs, were added to the 96-well plate as a control group. The culture was maintained in a 95% air humidified atmosphere containing 5% CO_2 at 37°C for 72h [26].



Scheme 3. Image of cultured PC3 cell line under invert microscope.

2.8. Cytotoxicity assay

The prostate PC3 cell line was cultured in an RPMI-1640 culture medium containing 10% FBS, 10 ml penicillin /streptomycin, 2 mg sodium bicarbonate, and incubated in a 95% air humidified atmosphere containing 5% CO_2 at 37°C in sterile flasks. MTT assay was conducted to evaluate the cytotoxicity of free DOX, EZ, and DOX+EZ and drug-loaded nanoparticles (DOX@PCEC, EZ@PCEC, and DOX+EZ@PCEC). PC3 cells were suspended in culture medium and seeded in two different 96-well plates in triplicate at a density of 10^4 cells/well for 24h. Then, free DOX, EZ and DOX+EZ, and drug-loaded nanoparticles of DOX@PCEC, EZ@PCEC and DOX+EZ@PCEC with different drug concentrations (0, 0.39, 1.56, 3.12, 6.25, 12.5, 25, 50 and100 µg/ml) were incubated with PC3 cells for 48h. Moreover, the cells were treated with blank nanocarriers with different PCEC copolymer concentrations to investigate the biocompatibility of nanocarriers. Cell-free wells without treatment were used as controls, and wells contained a cell-free medium was used as a blank of Elisa Reader (Sunrise Instruments, Tekan). After 48h, the cell medium was taken out, and the wells were rinsed with sterilized PBS solution twice. Then, 150 µL fresh culture medium and 50 µL MTT solution was added to each well. Plates were kept in the dark to prevent decomposition of MTT in the presence of light and incubated at 37°C for another 4 h. Afterward, the medium containing MTT was removed from each

well, and replaced by 200 µl DMSO and incubated for 20 min to dissolve formed blue formazan crystals. After shaking for 5 min, the cell viability was determined using Elisa Reader at 570 nm with a reference wavelength of 630 nm. All tests were done in triplicate. The following equation was used to convert OD to the percentage of live cells:

$$\text{Cell Viability}(\%) = \frac{\text{OD(test)}}{\text{OD (control)}} \times 100$$

where OD (test) and OD (control) are the mean absorbance value of tested groups and control groups (without any treatment), respectively.

2.9. Combination effect analysis

The combination index (CI) values were calculated utilizing CompuSyn v.1 software [27] according to Chou-Talalay equation given below [28]:

$$CI_X = \frac{D_1}{(IC_x)_1} + \frac{D_2}{(IC_x)_2}$$

CI_X was utilized to assess the synergistic effect of DOX and EZ combination against PC3 cells in vitro, where $(IC_x)_1$ and $(IC_x)_2$ are the IC_x of EZ-loaded and DOX-loaded nanoparticles, respectively. D_1 and D_2 are the concentration of EZ and DOX in the dual drug-loaded nanoparticles at the IC_x value.

2.10. Dynamic light scattering (DLS) technique

The particle size and zeta potential of the drug-loaded PCEC nanoparticles were determined by dynamic light scattering (DLS) analysis.

2.11. Statistical analysis

Graph Pad Prism 8 (Graph Pad Software, Inc., La Jolla. GA) was employed for statistical analysis. Single-factor analysis for variance (ANOVA) was utilized to evaluate the statistical significance of the results. All the samples were analyzed in triplicates and are expressed as the means ± SD for n=3. P-value determined the level of significance. $p<0.05$ (*) is supposed to be statistically significant, $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) are regarded as highly significant.

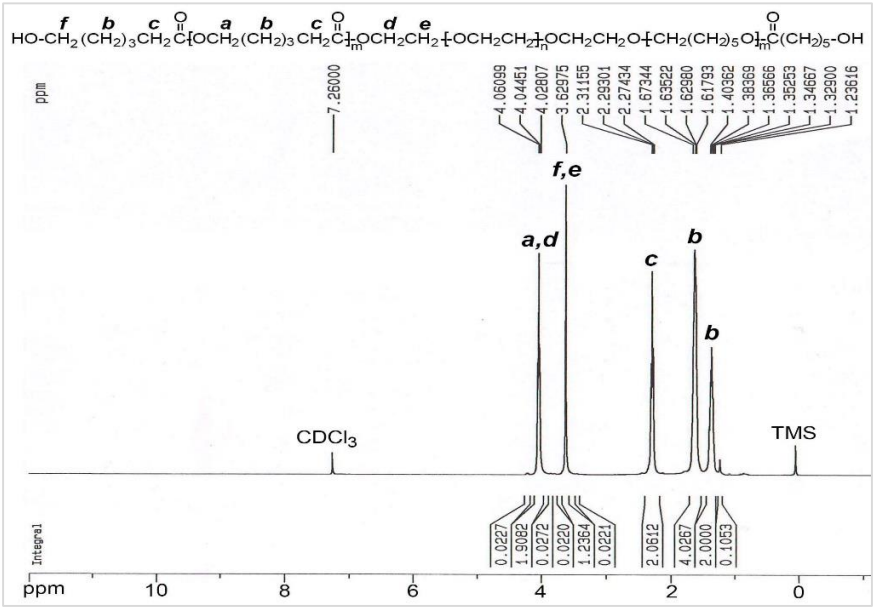
3. Result and discussion

3.1. Preparation and characterization of PCEC nanoparticles

PCEC triblock copolymer was synthesized by ring-opening polymerization of ε-CL initiated by PEG2000 (Scheme 1). Both DOX as an anticancer drug and EZ as an agent capable of inhibiting cholesterol uptake were loaded separately and combined into the obtained co-polymeric nanoparticles by double emulsion and simple emulsion methods. The compounds were characterized by ^{1}H-NMR, FTIR, GPC, FE-SEM and DLS.

3.1.1. ^{1}H-NMR analysis

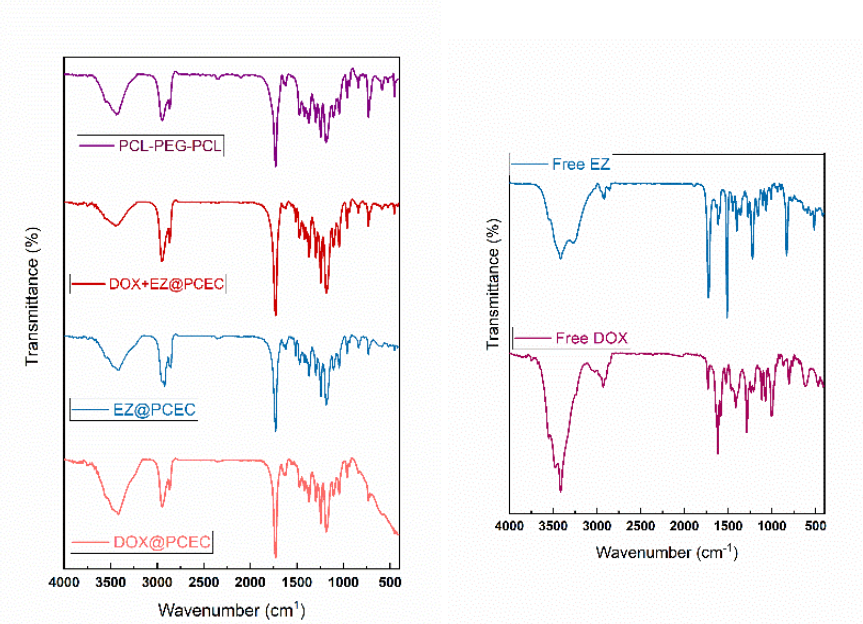
In ^{1}H-NMR spectrum of PCL-PEG-PCL copolymer (Scheme 4), methylene protons of $(CH_2)_3$, CH_2CO, and CH_2OOC in PCL chains were associated with peaks at 1.38(b), 1.63(b), 2.31(c), and 4.04(a) ppm, respectively. The methylene protons of PEG segments are responsible for the sharp peak at 3.62(e) ppm. The methylene protons of $COOCH_2$ in the PEG end unit were attributed for the weak peaks at 4.04(d) [29].



Scheme 4. ^{1}H-NMR spectrum of PCL-PEG-PCL copolymer.

3.1.2. FT-IR spectroscopy

The FT-IR confirmed the formation of the PCL-PEG-PCL copolymer, DOX+EZ@PCEC, EZ@PCEC, DOX@PCEC nanoparticles, free EZ and free DOX respectively (Scheme 5). In the spectrum of PCL-PEG-PCL copolymer, the absorption peaks at 1727 cm^{-1} belonged to C=O stretching vibrations of the ester carbonyl group. The peaks that appeared at 1188-1297 cm^{-1} are dedicated to the C-O-C stretching vibrations of the $-O-CH_2-CH_2$ repeated units present in the PEG structure and the -COO- bands stretching vibrations, respectively. The absorption peaks at 2869 cm^{-1} and 2945 cm^{-1} belong to the C-H aliphatic stretch. The peak at 3437 cm^{-1} is due to the terminal hydroxyl group (-OH) in the copolymer [29]. However, in comparison with the spectrum of drug-loaded nanoparticles, we couldn't see any observable difference in the appearance of the spectrum after the loading of drugs, except for increasing the intensity of the characteristic carbonyl peak. Similar reports in other studies suggest that the drug was localized and entrapped within the nanocarrier [30].



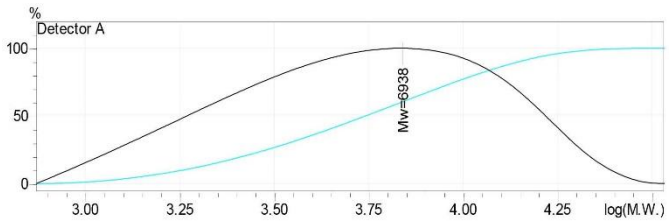
Scheme 5. FT-IR spectra of PCL-PEG-PCL copolymer, DOX+EZ@PCEC, EZ@PCEC, DOX@PCEC, free EZ and free DOX.

3.1.3. Gel permeation chromatography (GPC)

GPC is a size exclusion chromatography technique used to determine the prepared triblock copolymer's molecular weight. The molecular weight of compounds was calculated based on molecular weight of polystyrene standard (Table 1, Scheme 6).

Table 1. Molecular characteristics of copolymer

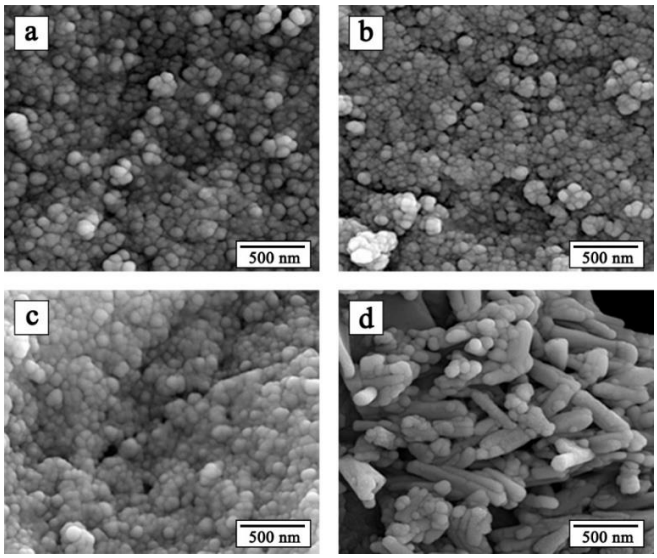
Copolymer	Cl:EG feed	M_n	M_w	M_w/M_n
PCL-PEG-PCL	10:1	3953	6938	1.75



Scheme 6. GPC spectrum of PCL-PEG-PCL copolymer.

3.1.4. FE-SEM analysis

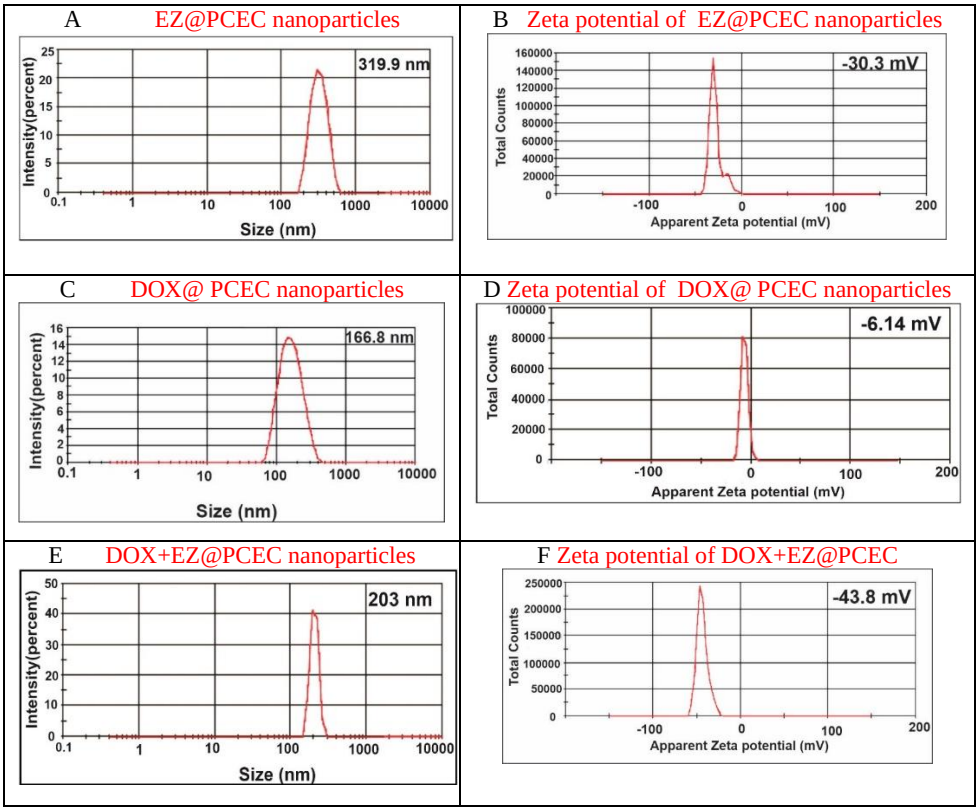
The size and morphology of the drug-loaded PCEC nanoparticles were determined by FE-SEM. Scheme 7 (a-d) demonstrates images of EZ@PCEC, DOX@PCEC, DOX+EZ@PCEC nanoparticles, and PCEC copolymer, respectively. The evaluation of FE-SEM images confirmed the formation of nano-sized nanoparticles. The measurements showed the average size of nanoparticles to be 82±23.80 nm, 59.7±18.7 nm, and 67.6±23.8 nm for EZ@PCEC, DOX@PCEC, and DOX+EZ@PCEC nanoparticles, respectively. While the mean size of blank PCEC copolymer were 201±83.5 and without round morphology. As a result, the size of the nanoparticles decreased significantly compared with blank PCEC copolymer, which was approximately in the same range reported by other studies [31].



Scheme 7. FE-SEM images of (a) EZ@PCL-PEG-PCL nanoparticles, (b) DOX@PCL-PEG-PCL nanoparticles, (c) EZ+DOX@ PCL–PEG–PCL nanoparticles, and (d) PCL-PEG-PCL copolymer before drug loading.

3.1.5. Dynamic light scattering (DLS) technique

The particle size distribution and zeta potential of the drug-loaded PCEC nanoparticles in distilled water were determined by dynamic light scattering (DLS) analysis. According to Scheme 8, DLS measurement showed a monomodal size distribution of around 319.9, 166.8 and 203 nm hydrodynamic diameters and negative zeta potential (-30.3, -6.14 and -43.8) mV for EZ@PCEC, DOX@PCEC, and DOX+EZ@PCEC nanoparticles, respectively. In addition, negatively charged NPs are often more resistant to plasma macromolecular protein adsorption and are easier to disperse in the bloodstream compared to positively charged ones, which favors in vivo drug delivery.



Scheme 8. Hydrodynamic size distribution and zeta potential of (A,B) EZ@PCEC, (C,D) DOX@PCEC, and (E,F) DOX+EZ@PCEC nanoparticles.

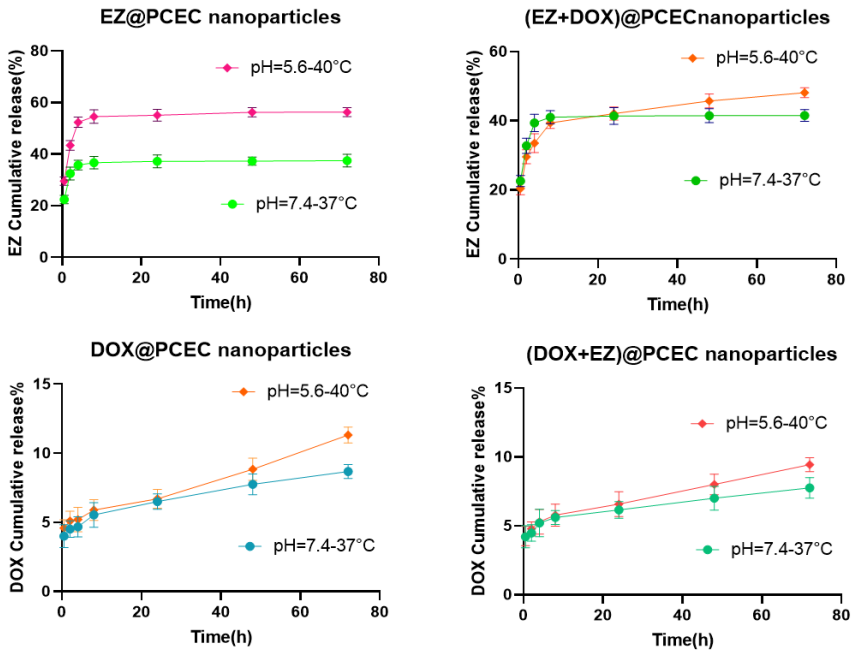
3.2. Encapsulation efficiency and in vitro drug release

The simple and double emulsion technique was used to prepare DOX and EZ-loaded nanoparticles. The feeding ratio of each drug to nano-carriers was 1 to 10. The loading capacity of DOX+ EZ@PCEC for DOX and EZ was obtained 3.5% and 3.2%, respectively. At the same time, this measurement for DOX and EZ in DOX@PCEC and EZ@PCEC was calculated to be 6.0% and 9.6%, respectively. Drug encapsulation efficiency for DOX and EZ in DOX+EZ@PCEC was 70 and 64%, respectively. Moreover, drug encapsulation efficiency of DOX@PCEC and EZ@PCEC nanoparticles was obtained 60% and 96%, respectively (Table 2).

Table 2. Encapsulation efficiency and loading capacity

Formulation	EE% (DOX: EZ)	LC% (DOX: EZ)
DOX@ PCEC	60	6
EZ@PCEC	96	9.6
DOX+EZ@PCEC	70:64	3.5:3.2

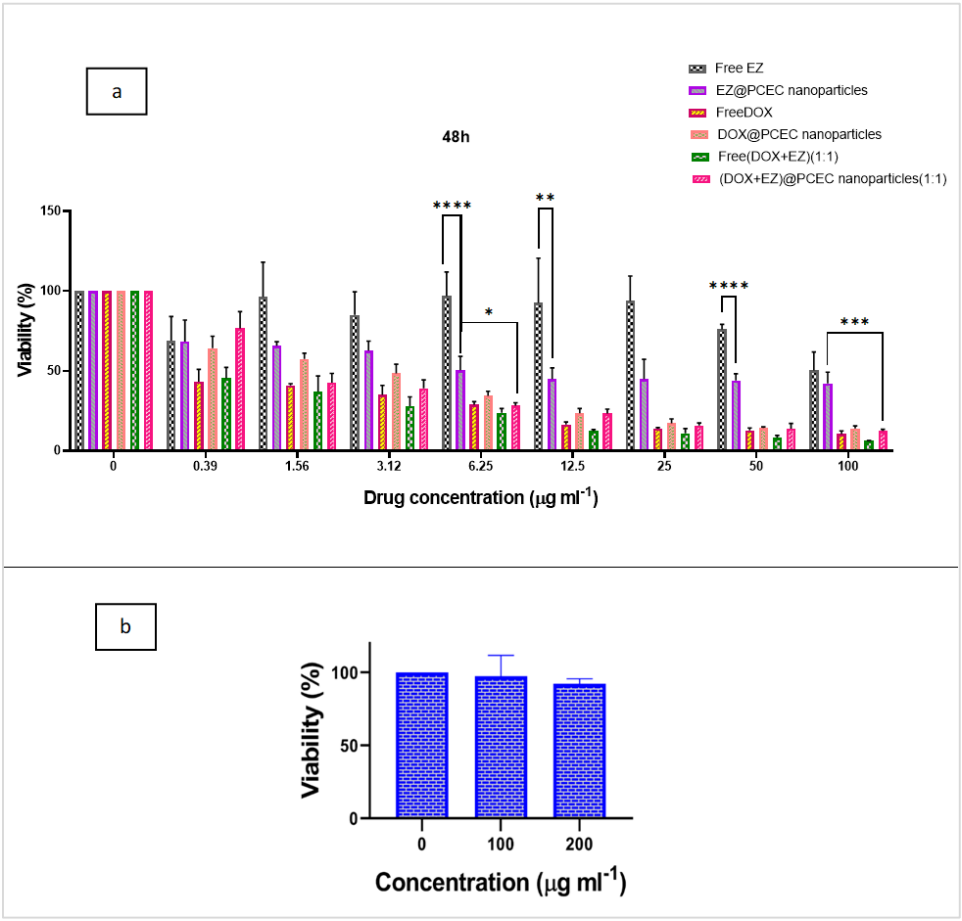
The in vitro drug release of different formulations was investigated at two pH values and temperatures at physiological condition (pH 7.4 at 37°C) and cancer tissue condition (pH 5.6 at 40°C) [32]. As shown in Scheme 9, the results indicated an initial burst release of about 54% and 39.35% in the first 8 h for EZ@PCEC and EZ+DOX@PCEC nanoparticles at cancer tissue condition (pH 5.6 at 40°C). Whereas under physiologic conditions, a sustained release was observed for EZ@PCEC nanoparticles. The release profile of EZ+DOX@PCEC nanoparticles experienced an intersection between acidic and physiologic pH, which follows a steep upward slope. At the beginning of the study, the burst release of EZ may be attributed to the drugs that were physically absorbed on the surface of the nanocarriers [33]. The total release for single EZ and DOX-loaded nanoparticles after 72 h was 56% and 11%, respectively. Dual drug-loaded nanocarriers after 72 h showed a total release of 48% and 9.4% for EZ and DOX, respectively. These results indicated that release in single drug-loaded samples was higher than dual drugs incorporated formulations. It may be attributed to the low encapsulation efficiency of EZ in EZ+DOX@PCEC NPs compared with EZ@PCEC NPs. On the other hand, the release profile of DOX and DOX+ EZ-loaded nanoparticles showed no burst release of DOX and experienced a sustained and slow release compared to the EZ. Besides, the results illustrated that the general release of DOX in the single drug-loaded form was higher than the co-delivery formulation (11% at pH 5.6). It can be attributed to the formation of high amounts of hydrogen bindings between DOX and nanoparticles. The results also proved that the total drug release in cancer conditions (pH 5.6, 40°C) is more than release under physiological conditions (pH 7.4, 37°C), which was also reported by Abedi *et al* [32]. The same result was also reported for DOX-loaded nanoparticles in which single drug-loaded nanocarriers executed higher release than dual drug-loaded ones [33].



Scheme 9. Cumulative release of ezetimibe and doxorubicin-loaded PCEC nanoparticles.

3.3. Cytotoxicity assay

MTT assay is a colorimetric method used to evaluate mitochondria activity and quantify cell proliferation or cell death. This study used MTT assay to evaluate the cytotoxic effects of PCEC formulations as biocompatible drug-loaded nanocarriers and free drugs of DOX and EZ on the PC3 prostate cancer cell line (Scheme 10a). Finally, optical absorption results were analyzed using Graf pad prism software, and inhibition concentrations (IC_{50}) for each specimen were then calculated (Table 3) [9]. The results showed dose-dependent cytotoxicity for all formulations in which the cell viability was reduced by an increase in drug concentration [26]. Cell viability in the formulations of free drugs and drug-loaded nanoparticles decreased with steep and slow slopes, respectively, except for the EZ@PCEC nanoparticles. This could be due to the different uptake mechanisms and cellular distribution of free DOX and DOX@PCEC nanoparticles, besides the release rate of DOX and EZ from nanoparticles.



Scheme 10. (a) Cytotoxicity results for the PC3 treated with different doses of free DOX, free EZ, free DOX+EZ, DOX@PCEC nanoparticles, EZ@PCEC nanoparticles, and DOX+EZ@PCEC nanoparticles for 48h. Comparison among groups was conducted by one-way ANOVA followed by Tukey's HSD analysis, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****). (b) Cytotoxicity results of PC3 treated with different doses of PCEC.

The free DOX+EZ (ratio 1:1) exhibited more significant antitumor activity compared to DOX+EZ (ratio 1:1) @PCEC nanoparticles, which could be attributed to the diffusion mechanism that free drugs use to enter the cancer cells, as well as the slow release of DOX+EZ from its polymeric carrier.

Furthermore, the comparison of cytotoxicity between DOX@PCEC, DOX+EZ@PCEC, and EZ@PCEC nanoparticles, showed a significant difference in cell viability of PC3 treated with EZ@PCEC nanoparticles in all of their concentrations.

The free DOX+EZ (ratio 1:1) exhibited more significant antitumor activity compared to DOX+EZ (ratio 1:1) @PCEC nanoparticles, which could be attributed to the diffusion mechanism that free drugs use to enter the cancer cells, as well as the slow release of DOX+EZ from its polymeric carrier.

Cytotoxicity comparison of DOX@PCEC, DOX+EZ@PCEC and EZ@PCEC nanoparticles showed a significant difference in cell viability of PC3 treated with EZ@PCEC nanoparticles in all of concentrations.

The MTT assay results demonstrated that EZ@PCEC nanoparticles with a concentration of 13.45 μg/ml had a cytotoxic effect for 50% of PC3 cells. Moreover, calculated IC_{50} showed that 0.4653 μg/mL of free DOX and 1.924 μg/mL of DOX@PCEC nanoparticles were able to compel cytotoxic effect in 50% of PC3 cells. Besides, the results revealed that 0.4021 μg/ml of free DOX+EZ and 1.543 μg/ml of EZ+DOX@PCEC nanoparticles could be followed by the death of 50% of the PC3 cell line in prostate cancer. The EZ+DOX@ PCEC nanoparticles and EZ+DOX formulations were more effective than the single free drugs and single drug-loaded nanoparticles at their most concentrations after 48h. The biocompatibility of PCEC copolymer was also confirmed using MTT assay as they did not affect the growth of the PC3 cell line (Scheme 10b).

The combination effect analysis was also calculated by CI. CI < 1, CI=1, and CI >1 show synergistic, additive, and antagonistic effects, respectively [33]. Dose-effect parameters are given in Table 4. These parameters include *m*, *Dm*, and *r*, which represents the slope of the median-effect plot (shape parameter), the dose of the median-effect (potency parameter like IC_{50}), and the linear correlation coefficient of the median-effect plot (conformity parameter), respectively.

Table 3. IC_{50} values calculated for PC3 cell line treated with free drugs and drug-loaded nanoparticles for 48h

Specimen	IC_{50}
Free EZ	-
EZ@PCEC nanoparticles	13.45
Free DOX	0.4653
DOX@ PCEC nanoparticles	1.924
Free EZ+DOX	0.4021
(EZ+DOX)@PCEC nanoparticles	1.543

Table 4. Dose-effect parameters for EZ and DOX in PC3 cell line. The data were collected from MTT assay and were subjected to the automated calculation of m, Dm, and r parameters using CompuSyne software

Specimen		m	Dm (µg/ml)	r
Free drug	EZ	0.17032	759465	0.25695
	DOX	0.39357	0.37098	0.96359
	EZ+DOX	0.49305	0.38173	0.98557
Drug @PCEC nanoparticles	EZ@NPS	0.22758	13.9774	0.94012
	DOX@NPS	0.50765	1.75958	0.97681
	(EZ+DOX)@NPS	0.54453	1.57958	0.97001

The resulted values of combination index (CI) for free drugs and drug-loaded nanoparticles at the actual experimental point, along with various effect levels (Fa) and type of effect, were calculated using CompuSyne software and presented in Table 5.

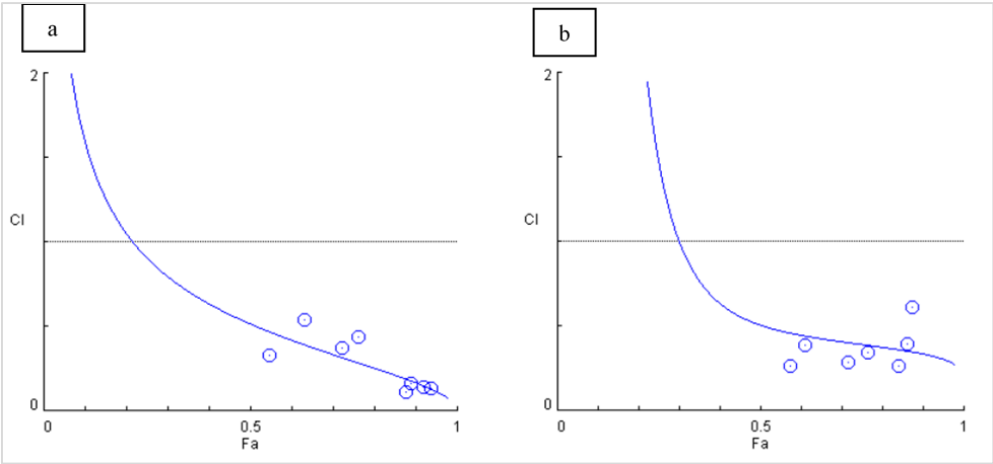
In addition, the combination index plot (Fa-CI plot) of the obtained results was depicted in Scheme 11, in which the CI values were plotted against the corresponding effect levels.

According to Chou-Talalay equation, the combination index (CI) value was calculated to be 0.45 for EZ+DOX@PCEC nanoparticles. This result indicated that EZ+DOX@PCEC nanoparticles could act synergistically with the drug ratio of 1:1 in vitro. The MTT results showed that drug-carrying nanoparticles, increased drug solubility, caused selective drug delivery, modified the drug release kinetics, and provided a prolonged sustained release of drugs. It is noteworthy that free drugs in cell culture medium could rapidly exposure their effects after being transported into cells through passive diffusion. On the other hand, the drug incorporated nanoparticles internalized the cells through the endocytosis and exhibited their anticancer activity after the drug was released from the nanoparticles. Similar cytotoxicity results were also reported in other studies by which the free drugs resulted in higher cytotoxicity than drug-loaded nanoparticles [34-36]. As a conclusion, all data confirmed that the EZ as a cholesterol-lowering drug with DOX as an anticancer drug could synergistically affect prostate cancer cells.

Table 5. The combination index (CI) values for free drug and drug-loaded nanoparticles were calculated using CompuSyne software in various concentrations

Interaction type of free drug			
Concentration (µg/ml)	Fa	CI value*	Effect type
0.39	0.54635	0.32772	Synergistic effect
1.56	0.63104	0.53768	Synergistic effect
3.12	0.72168	0.37354	Synergistic effect
6.25	0.76139	0.44171	Synergistic effect
12.5	0.87705	0.11439	Strong Synergistic effect
25	0.89087	0.16242	Strong Synergistic effect
50	0.91856	0.14285	Strong Synergistic effect
100	0.93859	0.13203	Strong Synergistic effect
Interaction type of drug-loaded NP_S			
Concentration (µg/ml)	Fa	CI value*	Effect type
0.39	0.23375	3.72114	Antagonistic effect
1.56	0.57419	0.26099	Strong Synergistic effect
3.12	0.60935	0.38511	Synergistic effect
6.25	0.71751	0.28685	Strong Synergistic effect
12.5	0.76646	0.34420	Synergistic effect
25	0.84204	0.26349	Strong Synergistic effect
50	0.86099	0.39187	Synergistic effect
100	0.87516	0.61383	Synergistic effect

*(CI> 1), (0.7< CI <1), (0.3< CI <0.7) and (CI < 0.3) indicating antagonistic, medium synergistic, synergistic, and strong synergistic effect, respectively.



Scheme 11. Combination index curves (Fa-CI plot) for (a) free drug and (b) drug@PCEC nanoparticles were plotted as a function of the fraction inhibition (Fa) of cell viability/growth by computer simulation (CompuSyn software).

4. Conclusion

In this research, PCEC was synthesized by ring-opening polymerization and characterized by ^{1}H-NMR, FT-IR, and GPC. The EZ was loaded in PCEC nanoparticles by the simple emulsion method. Moreover, DOX and a combination of DOX and EZ loaded in nanoparticles by double emulsion technique. FE-SEM evaluated the morphology and size of resulted nanoparticles. As well as, the particle size distribution and zeta potential of the drug-loaded PCEC nanoparticles in distilled water were determined by dynamic light scattering (DLS) analysis. The DOX and EZ's encapsulation efficiency was calculated, and *in-vitro* release study showed that the synthesized nanocarriers have a slow and sustained release. The cytotoxicity of nanoparticles and free drugs was evaluated by MTT assay using prostate cancer PC3 cell lines. *In-vitro* cytotoxicity assay showed that the PCEC did not affect the growth of PC3 cells; therefore, it is an appropriate and biocompatible candidate for formulating nanocarriers. The cytotoxic of the dual drugs in both free form and loaded in nanoparticles against PC3 cells was better than their single formulations. Also, the IC_{50} results showed that the EZ as a cholesterol-lowering drug and DOX as an anticancer drug incorporated in PCEC had synergistic effects on prostate cancer. The results corroborated each other and presented successful EZ and DOX formulations containing PCEC nanoparticles and their efficiency in treating prostate cancer.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics. *CA Cancer J. Clin.*, 69 (2019) 7-34.

[2] A. Arkan Majhool, M. Yakdhan Saleh, A.K. Obaid Aldulaimi, S. Mahmood Saeed, S.M. Hassan, M.F. El-Shehry, S. Mohamed Awad, S.S. Syed Abdul Azziz. Synthesis of new azo dyes of uracil via ecofriendly method and evaluation for the breast, liver and lung cancer cells in vitro. *Chem. Rev. Lett.*, 6 (2023) 442-448. (b) A. Malekhoseini, M. Montazerozohori, R. Naghiha, E. Panahi Kokhdan, S. Joohari, Antimicrobial/antioxidant and cytotoxicity activities of some new mercury (II) complexes. *Chem. Rev. Lett.*, 6 (2023) 166-182. (c) M. Sheydaei, S. Shahbazi-Ganjgah, E. Alinia-Ahandani, M. Sheidaie, M. Edraki, An overview of the use of plants, polymers and nanoparticles as antibacterial materials. *Chem. Rev. Lett.*, 5 (2022) 207-216.

[3] A. Isa, A. Uzairu, U. Umar, M.T. Ibrahim, A. Umar. 'QSAR, docking and pharmacokinetic studies 2,4-diphenyl indenol [1,2-B] pyridinol derivatives targeting breast cancer receptors. *J. Chem. Lett.*, (2024), in press. doi: 10.22034/jchemlett.2024.424800.1146

[4] K. Li, W. Zhan, Y. Chen, R.K. Jha, X. Chen, Docetaxel and doxorubicin codelivery by nanocarriers for synergistic treatment of prostate cancer. *Front. Pharmacol.*, 10 (2019) 1436.

[5] B.L. Wang, Y.M. Shen, Q.W. Zhang, Y.L. Li, M. Luo, Z. Liu, Y. Li, Z.Y. Qian, X. Gao, H.S. Shi, Codelivery of curcumin and doxorubicin by MPEG-PCL results in improved efficacy of systemically administered chemotherapy in mice with lung cancer. *Int. J. Nanomedicine*, 8 (2013) 3521-3531.

[6] J. Pan, K. Rostamizadeh, N. Filipczak, V.P. Torchilin, Polymeric co-delivery systems in cancer treatment: an overview on component drugs' dosage ratio effect. *Molecules*, 24 (2019) 1035.

[7] F. Moradi Kashkooli, M. Soltani, M. Souri, Controlled anti-cancer drug release through advanced nano-drug delivery systems: static and dynamic targeting strategies. *J. Control. Release*, 327 (2020) 316-349.

[8] S. Sim, N.K. Wong, Nanotechnology and its use in imaging and drug delivery: a review. *Biomed Rep.*, 14 (2021) 42.

[9] F.U. Din, W. Aman, I. Ullah, O.S. Qureshi, O. Mustapha, S. Shafique, A. Zeb, Effective use of nanocarriers as drug delivery systems for the treatment of selected tumors. *Int. J. Nnanomedicine*, 12 (2017) 7291-7309.

[10] H. Amani, H. Kazerooni, H. Hassanpoor, A. Akbarzadeh, H. Pazoki-Toroudi, Tailoring synthetic polymeric biomaterials towards nerve tissue engineering: a review. *Artif. Cells Nanomed. Biotechnol.*, 47 (2019) 3524-3539.

[11] N.V. Cuong, J.L. Jiang, Y.L. Li, J.R. Chen, S.C. Jwo, M.F. Hsieh, Doxorubicin-loaded PEG-PCL-PEG micelle using xenograft model of nude mice: effect of multiple administration of micelle on the suppression of human breast cancer. *Cancers*, 3 (2011) 61-78.

[12] H. Danafar, Preparation and characterization of PCL-PEG-PCL polymersomes for delivery of clavulanic acid. *Cogent Med.*, 3 (2016) 1235245.

[13] L. Zhang, Z. Chen, H. Wang, S. Wu, K. Zhao, H. Sun, D. Kong, C. Wang, X. Leng, D. Zhu, Preparation and evaluation of PCL–PEG–PCL polymeric nanoparticles for doxorubicin delivery against breast cancer. *RSC Adv.*, 6 (2016) 54727-54737.

[14] H. Danafar, K. Rostamizadeh, S. Davaran, M. Hamidi, Co-delivery of hydrophilic and hydrophobic drugs by micelles: a new approach using drug conjugated PEG-PCL nanoparticles. *Drug Dev. Ind. Pharm.*, 43 (2017) 1908-1918.

[15] Z. Pang, J. Zhou, C. Sun, Ditelluride-bridged PEG-PCL copolymer as folic acid-targeted and redox-responsive nanoparticles for enhanced cancer therapy. *Front. Chem.*, 8 (2020) 156.

[16] A. Tas, N.K. Cakmak, Synthesis of PEGylated nanographene oxide as a nanocarrier for docetaxel drugs and anticancer activity on prostate cancer cell lines. *Hum. Exp. Toxicol.*, 40 (2021) 172-182.

[17] M.R. Freeman, K.R. Solomon, Cholesterol and prostate cancer. *J. Cell. Biochem.*, 91 (2004) 54-69.

[18] D. Suchy, K. Tabuzek, A. Standicki, B. Okopien, Ezetimibe: a new approach in hypercholesterolemia management. *Pharmacol. Rep.*, 63 (2011) 1335-1348.

[19] S. Zivkovic, G. Maric, N. Cvetinovic, D. Lepojevic-Stefanovic, B. Bozic Cvijan, Anti-inflammatory effects of lipid-lowering drugs and supplements: a narrative review. *Nutrients*, 15 (2023) 1517.

[20] M.R. Freeman, K.R. Solomon, Cholesterol and benign prostate disease. *Differentiation*, 82 (2011) 244-252.

[21] O.G. Kocabay, O. Ismail, Preparation and optimization of biodegradable self-assembled PCL-PEG-PCL nano-sized micelles for drug delivery systems. *Int. J. Polym. Mater. Polym. Biomater.*, 70 (2021) 328-337.

[22] P. Hakemi, A. Ghadi, S. Mahjoub, F. Zabihi, H. Tashakkorian, Fabrication of PCL-PEG-PCL nanocarrier for co-loading of Docetaxel/Quercetin and assessment of its effect on growth inhibition of human liver cancer (Hep-G2) cell line. *Int. J. Nano Dimens.*, 12 (2021) 355-368.

[23] S. Fathi Karkan, S. Davaran, A. Akbarzadeh, Cisplatin-loaded superparamagnetic nanoparticles modified with PCL-PEG copolymers as a treatment of A549 lung cancer cells. *Nanomed. Res. J.*, 4 (2019) 209-219.

[24] E. Cohen-Sela, S. Teitlboim, M. Chorny, N. Koroukhov, H.D Danenberg, J. Gao, G. Golomb, Single and double emulsion manufacturing techniques of an amphiphilic drug in PLGA nanoparticles: formulations of mithramycin and bioactivity. *J. Pharm. Sci.*, 98 (2009) 1452-1462.

[25] S. D'Souza, A review of in vitro drug release test methods for nano-sized dosage forms. *Adv. Pharm.*, 2014 (2014) 304757.

[26] E.J. Orzechowska, A. Girstun, K. Staron, J. Trzcinska-Danielewicz, Synergy of BID with doxorubicin in the killing of cancer cells. *Oncol. Rep.*, 33 (2015) 2143-2150.

[27] T.C. Chou, N. Martin, CompuSyn for drug combinations and for general dose-effect analysis, PC software and user's guide: A computer program for quantitation of synergism and antagonism in drug combinations, and the determination of IC50 and ED50 and LD50 values. ComboSyn Inc Paramus, NJ, (2005), free download via www.combosyn.com upon registration.

[28] T.C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.*, 58 (2006) 621-681.

[29] E. Shakiba, S. Khazaei, M. Hajialyani, B. Astinchap, A. Fattahi, et al, Preparation and in vitro characterization of retinoic acid-loaded poly(ε-caprolactone)-poly(ethylene glycol)-poly(ε-caprolactone) micelles. *Res Pharm Sci.*, 12 (2017) 465-478.

[30] A.M. Butt, M.C.I.M. Amin, H. Katas, N. Sarisuta, W. Witoonsaridsilp, R. Benjakul, In vitro characterization of pluronic F127 and D-α-tocopheryl polyethylene glycol 1000 succinate mixed micelles as nanocarriers for targeted anticancer-drug delivery. *J. Nanomater.*, 2012 (2012) 916573.

[31] R. Feng, Z. Song, G. Zhai, Preparation and in vivo pharmacokinetics of curcumin-loaded PCL-PEG-PCL triblock copolymeric nanoparticles. *Int. J. Nanomedicine*, 7 (2012) 4089-4098.

[32] F. Abedi, S. Davaran, M. Hekmati, A. Akbarzadeh, B. Baradaran, S.V. Moghaddam, An improved method in fabrication of smart dual-responsive nanogels for controlled release of doxorubicin and curcumin in HT-29 colon cancer cells. *J. Nanobiotechnology*, 19 (2021) 18.

[33] M. Rahimi, R. Karimian, E. Mostafidi, E.B. Norouzi, S. Taghizadeh, B. Shokouhi, H.S. Kafil, Highly branched amine-functionalized *p*-sulfonatocalix [4] arene decorated with human plasma proteins as a smart, targeted, and stealthy nano-vehicle for the combination chemotherapy of MCF7 cells. *New J. Chem.*, 42 (2018) 13010-13024.

[34] M. Alibolandi, F. Sadeghi, K. Abnous, F. Atyabi, M. Ramezani, F. Hadizadeh, The chemotherapeutic potential of doxorubicin-loaded PEG-b-PLGA nanopolymersomes in mouse breast cancer model. *Eur. J. Pharm. Biopharm.*, 94 (2015) 521-531.

[35] B. Tian, Y. Ding, J. Han, J. Zhang, Y. Han, J. Han, N-Acetyl-D-glucosamine decorated polymeric nanoparticles for targeted delivery of doxorubicin: synthesis, characterization and in vitro evaluation. *Colloids Surf. B Biointerfaces*, 130 (2015) 246-254.

[36] M. Yousefnezhad, S. Davaran, M. Babazadeh, A. Akbarzadeh, H. Pazoki-Toroudi, PCL-based nanoparticles for doxorubicin-ezetimibe co-delivery: A combination therapy for prostate cancer using a drug repurposing strategy, *BioImpacts*, 13 (2023) 241-253.