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RESEARCH ARTICLE

Preparation and in-vitro evaluation of PU- PCL films containing doxorubicin and ezetimibe on the prostate cancer cell line PC3

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ABSTRACT

One of the most potentially hazardous diseases, prostate cancer has a high morbidity and mortality rate. Polymeric matrix drug-eluting implants have become widely employed, and modeling their behavior is becoming more and more prominent. It is always difficult to achieve effective drug delivery and release of it into specific tumor sites. One of the most significant purposes of this investigation, is the enhancement of the anticancer effects of prostate cancer treatment by co-delivering anticancer multi-drugs with PU-PCL films. The films were recognized utilizing SEM (scanning electron microscopy) while the material was being characterized. In addition, the MTT assay and flow cytometry (Annexin V/PI staining) have been employed to assess cell viability at various times. A dialysis approach was used to investigate the drug release characteristics of DOX and Ezetimibe in films in vitro for 5 days. To optimize pharmacokinetic profiles and reduce systemic toxicity induced by drugs, we loaded polymeric PU-PCL films with ezetimibe (EZ) and doxorubicin (DOX). Co-delivery of EZ and DOX via film-carrier demonstrated improved anticancer effects when compared to free drug delivery. The co-delivery of DOX and EZ drugs by PU-PCL films improved anticancer effects while reducing systemic toxicity, suggesting clinical usage of drug-resistant prostate cancer therapy.

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INTRODUCTION

With 1,276,106 new cases and 358,989 deaths reported in 2018, prostate cancer was ranked as the second most frequent malignant disease in men after lung cancer (3.8% of all male cancer deaths) [1-3]. The average prostate cancer evaluation age is 66 years, and both prevalence and death rates rise with ageing. the men with African American nationalities have an increased prevalence rate in comparison with white men (with 158.3 new cases detected per 100,000 males), and the death rate is nearly twice as high in former comparison [4].

As prostate cancer appears to be in its early stages, there may be no symptoms and the disease is frequently benign, require not much treatment [5, 6]. However, difficulty urinating, increased frequency, and nocturia are common complaints, all of which are symptoms of prostatic hypertrophy. Because one of the most typical sites for bone metastatic morbidity is the axis skeleton, patients with advanced stages of the illness may have urine retention and back discomfort [7, 8]. The majority of prostate malignancies have been recognized by prostate tissue usually creates the glycoprotein prostate-specific antigen (PSA > 4 ng/mL), or PSA, in response to elevated plasmatic levels. However, the biopsy of tissue has become the gold standard

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for determining the presence of cancer because it has been found that men without cancer have higher PSA levels [9].

Chemotherapy, radiation therapy, and surgery are the three most frequently used prostate cancer treatments. These methods, however, can cause nausea, weight loss, heart poisoning, hair loss, liver and kidney damage, and high blood cholesterol [10, 11]. The widespread application of polymers is due to their usefulness in producing appropriate drug release patterns from carriers as well as their biodegradability or nonbiodegradability and biocompatibility characteristics [12, 13]. Aside from killing effectiveness, another factor to evaluate is medicine dispersion [14, 15]. In drug delivery systems, soluble polymers with enhanced permeability and retention effects, as well as pH-controlled drug release, could efficiently facilitate drug aggregation in tumor sites, as a result of which the anticancer activities are improved and systemic toxicity is minimized in vivo [16, 17].

Polymeric materials are frequently employed in implantable technologies and serve a significant role in the delivery of therapeutic medicines to specific tissues [10, 18]. Numerous polymeric materials have been explored as coatings and membranes for drug-eluting stents. These components can be divided as biodegradable or non-biodegradable substances [19, 20]. Non-degradable polymers have advantages for DESs due to the former's polymer covering degradation could lead to stent migration, stricture recurrence, or stent obstruction from malignant tumor in growth [21]. Polymeric materials employed in DES coatings poly(ethylene terephthalate) (PET), polyurethane, poly (tetrafluoroethylene) (PTFE), polysiloxanes and non-vascular stents [21-23]. One of the most frequently used polymers in the pharmaceutical industry is polysiloxanes. Due to their low toxicity, excellent thermal and oxidative stability, high resistance to irradiation degradation, and compatibility, which make them suitable for the manufacturing medical supplies that include catheters, drains, stents, cardiac, and aesthetic implants [24].

Another common biomedical substance is polyurethane. As a result of their structural variety and remarkable elasticity, compliance, biocompatibility, durability, and fatigue resistance, they have been applied for developing biomedical devices consisting of as tissue engineering scaffolds, artificial organs, and drug delivery systems [25, 26]. A particular kind of segmented copolymer made up of soft and hard domains, polyurethanes provide flexibility while the hard domains provide mechanical strength [27]. Polyurethanes has been used in vaginal rings, implants, coatings, and stents in the past as controlled drug delivery methods [28-30]. Because of their biodegradability and high drug delivery efficiency, PU polymers are gaining popularity [31-33]. In recent years, PU polymer has also been used to make films in recent years [34]. It is interesting that no comparative examinations evaluating the physicochemical and releasing properties of various polymers in one investigation have been done, even though the PU-PCL polymers utilized in our work are frequently applied for DES [35, 36].

Doxorubicin is a chemotherapy drug used to treat different types of cancer. For example, breast, stomach, lung, ovarian, thyroid, and prostate cancer, has issues such as poor solubility, rapid excretion, limited stability, and a lack of choice [37, 38]. Furthermore, the drug, because of properties such as high lipophilicity and a long half-life in the body, increases the dose to treat the fact that these factors may increase side effects and damage to healthy cells in the body [39]. It is critical to use effective methods of targeted drug delivery at the target site (tumor) for this purpose in order to reduce drug side effects, reduce the dose, and target delivery to cancerous tumors [40- 42]. Schilling et al. investigated different concentrations of doxorubicin in polylactic acid-polyethylene glycol (PLA-PEG) nanofibers, which resulted in DOX being distributed in the center of the fibers and decreasing the release rate by increasing the amount of DOX in the fibers [40, 43]. Doxorubicin is also loaded into polymers for cancer treatment [44, 45].

One of the most successful treatments is combination chemotherapy, which mixes two or more distinct drugs [46, 47]. Prostate cancer has also been treated with doxorubicin, both by alone and in conjunction with other cancer therapies [48-50]. Adults with prostate cancer who have hypercholesterolemia, or high cholesterol, are at an increased risk of heart disease and stroke [51, 52]. Several studies have found that elevated cholesterol accelerates the growth of prostate cancer. [53].

According to one study, the cholesterol-lowering medicine ezetimibe inhibited the development of prostate cancer in the LNCaP xenograft model. The prior research revealed a

Table 1. Effectiveness of nanofibrous formulations for drug loading nanofibers

Formulation	Phamaceutics	Phamaceutics content (drug/polymer)	Phamaceutics loading efficiency
DOX/PU-PCL	DOX	14/100	97%
EZ/PU-PCL	EZ	10/100	98%
DOX/EZ/PU-PCL	DOX	7/100	99%
DOX/EZ/PU-PCL	EZ	5/100	98%

relationship between lower blood cholesterol and slower growth of tumors and less angiogenesis in mice administered ezetimibe while consuming a high-cholesterol, high-fat diet. As a consequence, Ezetimibe has been approved for the treatment of prostate cancer.

On the other hand, New study evidence indicates that prostate cancer can develop at the cellular level as a result of DNA damage, dysregulated cell division, mitochondrial dysfunction (Bax and Bcl2 unbalance), excessive activity of growth factor pathways, and formation of reactive oxygen species (ROS). According to reports, ROS causes cellular damage and a sizable rise in tissue apoptosis and necrosis [54-59].

As a result, we used a PU-PCL polymer to carry doxorubicin (DOX) and ezetimibe (EZ). The solvent casting approach was used to make drug-loaded PU-PCL films and blank PU-PCL films for consistent administration of DOX and Ez and the treatment of prostate malignancies.

MATERIALS AND METHODS

Materials

Tetrahydrofuran (THF) and poly [4,4 methylene bis (phenyl isocyanate)-alt,4 butanediols (propylene glycol) polycaprolactone (PU-PCL), Doxorubicin hydrochloride, ezetimibe, and phosphate buffer saline (pH 7.4, PBS)were supplied from Sigma-Aldrich (Aldrich, USA) and Merck (Merck, Germany), respectively.

DOX and ezetimibe-loaded film repair and Phamaceutics loading efficiency

A solvent casting method was used to create blank and DOX/EZ-loaded PU-PCL films. In order to produce blank films, PU-PCL was completely dispersed in THF (4 mL) in a sealed vial before being heated at 40°C for 24 hours. The solution was put into a Petri dish (30 mm in diameter), and the solvent was evaporated in an oven at 40° C for 1 hour to produce blank film with an average thickness of 300 mm. DOX/EZ-loaded films were made in a variety of DOX and EZ concentrations (Table 1). Drug-loaded films were made in the same way as blank films, except that before

casting, drugs dissolved in THF (2 mL) were added to the polymer solution.

The spectroscopy of UV-visible at 266 nm absorbance has been employed to assess drug loading and release. The drug loading efficacy of DOX, EZ, and DOX/EZ-loaded films has been evaluated by dissolving the produced nanofibers in THF for 2 hours.

The **pharmaceutical compound** loading efficiency (DLE%) was calculated utilizing the formulas below:

$$DLE\% = \frac{Actual\ drug\ content}{Initial\ drug}\ 100\%$$

Particle size analysis and morphology research

SEM (Czech) was used to examine the morphology of films. The Fourier transform, the FTIR (infrared spectroscopy), interesting interactions between the polymers and the polymer structure were all used to characterize the pharmaceuticals. By depositing polymer solutions in dichloromethane on KBr windows and recording the results on an FTIR spectrometer (USA), the samples' IR spectra were scanned in the 400–4000 cm-1 range. The ^{1}HNMR spectra were collected utilizing a Bruker Avance III-400 MHz superconducting NMR spectrometer (400.1 MHz for 1H), employing DMSO-d6 with approximately 5% (w/v) of concentration as the for ^{1}HNMR and around 20% (w/v) for ^{13}CNMR. All spectra have been evaluated at 298 K.

Study of the drugs' release

Drug release examinations and in vitro kinetics

A dialysis approach was used to investigate the drug release properties of DOX and EZ in films at the in vitro method. In a nutshell, dialysis bags were loaded with 2 mg of DOX or EZ in PBS with drug-laden PU-PCL films. The dialysis bags were agitated at 120 rpm in a 50 ml tube containing 20 ml of PBS and 2 ml of FBS (37 °C and pH = 5.0 or 7.4) for 20 minutes. The goal of this study is to obtain repeatable results by keeping variables like buffer volume, initial amount of drugs/PU-PCL

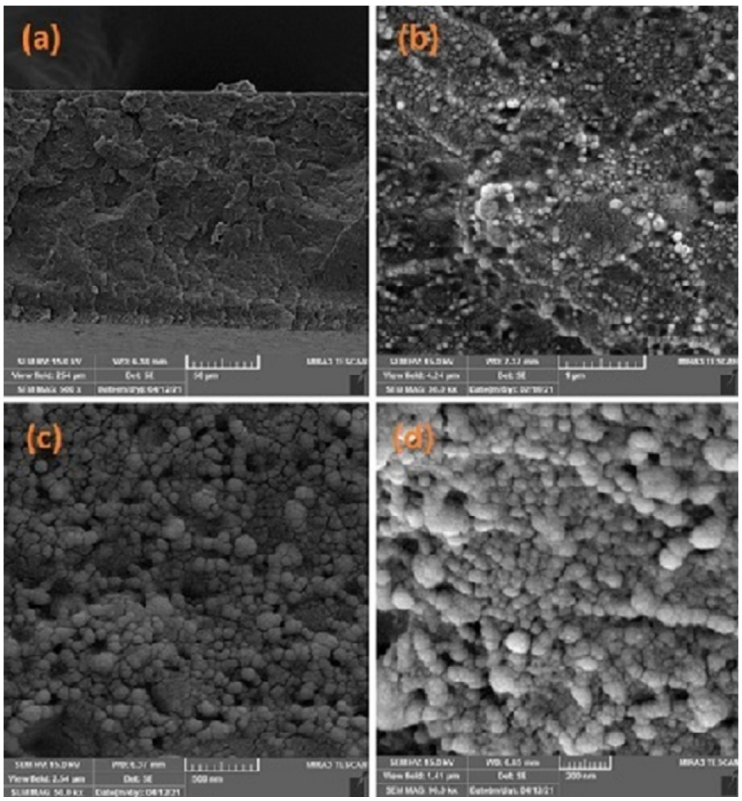

Fig. 1. SEM images of DOX/EZ-loaded PU-PCL nanoparticles.

nanofibers, and incubation conditions constant. The buffer around the dialysis bag was obtained at various times and, as the buffer level dropped, it was quickly replaced with a similar volume of fresh phosphate buffer in order to calculate drugs release. The incubation mediums required to be changed out for fresh incubation media at certain times, and 2 ml of the release media needed to be collected for further investigation. The amount of drug released was measured using a UV-Vis-NIR spectrophotometer (UV-2000, 50 Hz, Beijing). Temperature, pH, hydrophilicity, and polymer swelling, among other parameters, influence the rate of drug release in drug delivery systems. As a result, drug release in these systems may be seen as a function of polymer penetration and swelling.

Cell culture and cell toxicity assay

The cell viability has been evaluated by MTT assay. Utilizing PC3 cell lines from the Pasteur Institute of Iran (IPI, Tehran, Iran), in vitro testing for cytotoxicity was carried out. On 96-well culture plates, cells were seeded and given 12 and 48 hours to adhere. The cells were then pre-treated for two hours with various chemotherapeutic drug concentrations. After 24 and 48 hours of incubation with 10 µl of the MTT solution with 0.5 mg/ml concentration, cell growth was evaluated. Then DMSO (Dimethyl sulfoxide) was added to the liquid and vortexed for ten minutes after it had been incubated for three hours at 37 °C. After a half-hour of incubation time, Elysa read it. 570 nm was used to determine the absorbance (A).

RESULTS AND DISCUSSION

Characterizing synthesized films

Different analytical methods, including SEM, HNMR, and FTIR have been utilized to determine the chemical structure of drug-loaded films and the PU-PCL nanofibers.

As a result, the samples' FTIR bands and HNMR resonant frequency peaks present a reliable source of data and a starting point for further investigation into the link between crystallization and hard segment structure, as well as hydrogen bonding behavior, in more complex-segmented PU-PCL compositions. The SEM image and size distribution of DOX/PU-PCL films are depicted in Figs. 1. Fig.

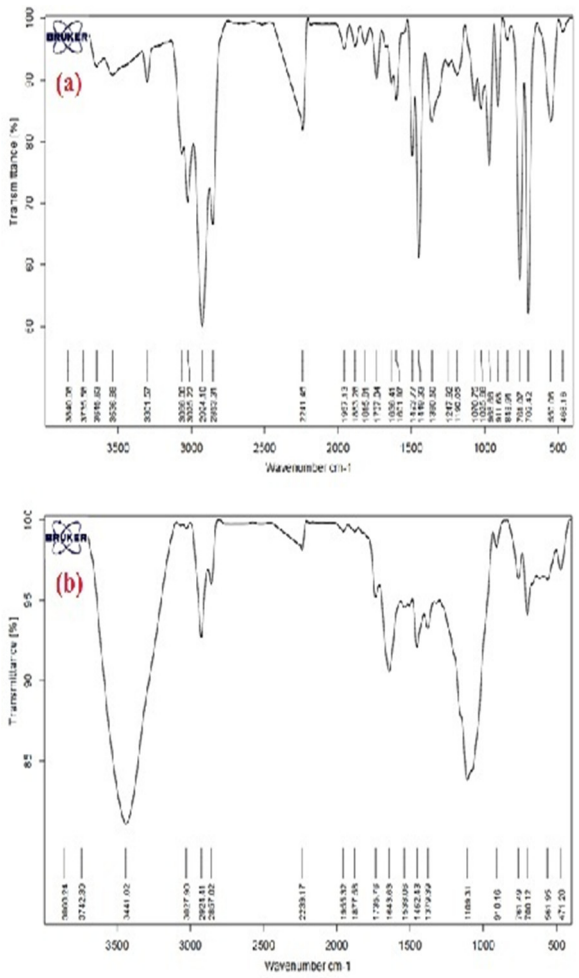

Fig. 2. The FTIR spectrum of PU-PCL film in (a). The FTIR spectrum of PU-PCL film with DOX/EZ loaded (b).

1 shows a scanning electron microscopic image of DOX/EZ/PU-PCL nanoparticles. SEM images of the blank polymeric films were also captured (Fig.1a-c).

When compared, there were no significant differences in the topography of the blank (c) and drug-loaded films (d). The SEM photos reveal that the DOX and EZ were successfully loaded into the films and that medications had no effect on the topography.

Figs. 2a and 2b show the FTIR spectra of PU-PCL films and DOX/EZ loaded PU-PCL films, respectively. The spectrum has the typical PU peaks at 1247 cm^{-1} (C-O-C stretching) and 1025 cm^{-1} (C-O stretching). Furthermore, the existence of C=C and C-C is indicated by peaks at 1449 and 1360 cm^{-1}. In addition, the N-H and C-N groups are related to a strong peak at 1493 cm^{-1} (Fig. 2a).

The existence of the C=O, or carbonyl amide group, was verified by peaks at 1728 cm^{-1}. PU peaks of 2830 and 3335 cm^{-1} were also recorded. FTIR test was performed to identify the peaks of PU-PCL, which revealed the presence of 1070 cm^{-1} (-C-O-C-), 1737 cm^{-1}, and 2853-3066 cm^{-1} (CH_2), and 3301 cm^{-1} (OH). To establish the presence of drugs in the electrospinning DOX/EZ/PU-PCL film structure, a FTIR test for PU-PCL and DOX/EZ/PU-PCL was performed, as shown in Fig. 2b. The discovery of peaks at 1643 to 1735 cm^{-1} (C=C and C=O) revealed the existence of peaks. As a result, the presence of DOX/EZ within the structure of DOX/EZ/PU-PCL films was confirmed (Fig. 2b).

The ^{1}HNMR spectrum of the PU-PCL is shown in Fig. 3. The sharp peak at 1.7 ppm attributed from the urethane functionality's CH_3 group. Aromatic hydrogens are detected in the area at 7.06 and

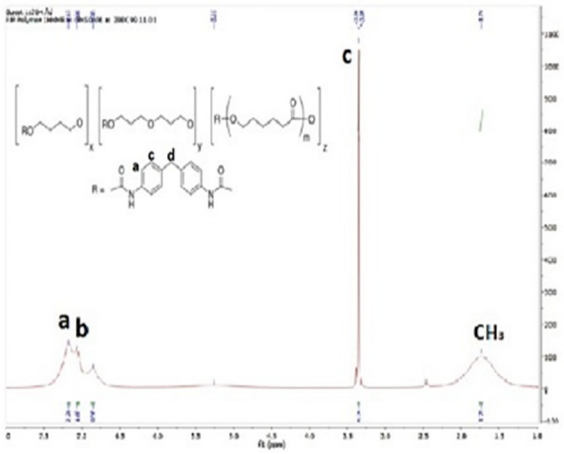

Fig. 3. The polymer PU-PCL's ^{1}HNMR spectra.

7.20 ppm, as well as C-H peaks at approximately 3.34 ppm. The 5.25 ppm peak corresponds to the bicyclic methylene proton of isosorbide.

The accumulation of anticancer drugs in tumors could be effectively increased by loading them on film carriers, which have superior in vivo pharmacokinetic profiles and enhanced permeability and retention (EPR) effects that extend drug circulation and reduce systemic toxicity. PU-PCL films were employed as an efficient drug delivery device to load DOX with or without EZ, as well as EZ alone, for prostate cancer therapy due to its high EE, stability, and low clearance. PU-PCL biodegradation ensures that in-vivo film distribution remains safe. We put DOX and EZ onto PU-PCL films using the previously described self-assembly technique. Evaporation of the co-dissolved solution of DOX, EZ, and PU-PCL resulted in the appropriate mixing of the different components to create the DOX/EZ/PU-PCL film. The content of DOX and EZ, as well as their loading efficiencies in films, are shown in Table 1. Drug loading efficiency (DLE) of DOX, EZ and DOX/EZ loaded on nanofiber were 97, 98 and 99% respectively.

The dialysis technique was utilized to investigate the release characteristics of DOX and EZ loaded into films under varied pH settings. Fig. 4 depicts the drug release results from DOX/EZ/PU-PCL films, DOX/PU-PCL films, and EZ/PU-PCL films at pH=7.4 at 37 °C (Fig. 4a), pH=7.4 at 40 °C (Fig. 4b), and PH=5.4 at 40 °C (Fig 4c). The gradual fast release of DOX and EZ from PU-PCL films was observed during a 48 h release time. The drug molecules were found to have sustained release through the PU-PCL films for 48 hours before reaching equilibrium in vitro release kinetics after 96 hours. In particular, PU-PCL films released the loaded drugs more effectively in acidic conditions (pH = 5.7) at 40 °C than in physiological conditions (pH=7.4), indicating that the PU-PCL films can control the release of anticancer medications by sensing the pH of the tumor tissue's microenvironment (pH = 5.7), resulting in less non-specific systemic spread of toxic drugs and more specific systemic spread of toxic drugs. Drug release rates were slower for both DOX/PU-PCL films and EZ/PU-PCL films, and the maximum amounts of drugs released were for the respective DOX/EZ/PU-PCL films, Considering that DOX and EZ are simultaneously released in mixed release mode and alongside to one another.

Cell viability

To assess the extracellular toxicity of therapeutic films and drugs, the MTT method was used. The IC_{50} values for DOX/EZ drugs at 48 and 24 hour intervals was reported to be 2.34 and 2.676 g / ml, indicating their high toxicity (see Fig. 5). As illustrated in Fig. 5a and b, the cell viability is decreased upon exposure by single agents DOX or EZ at various concentrations dose and time dependently. The DOX/EZ-loaded films cytotoxic effects was found to be the highest when compared to other synthesized film formulations (Fig 5c). However, when the PU-PCL polymer was added, the toxicity increased even at low concentrations,

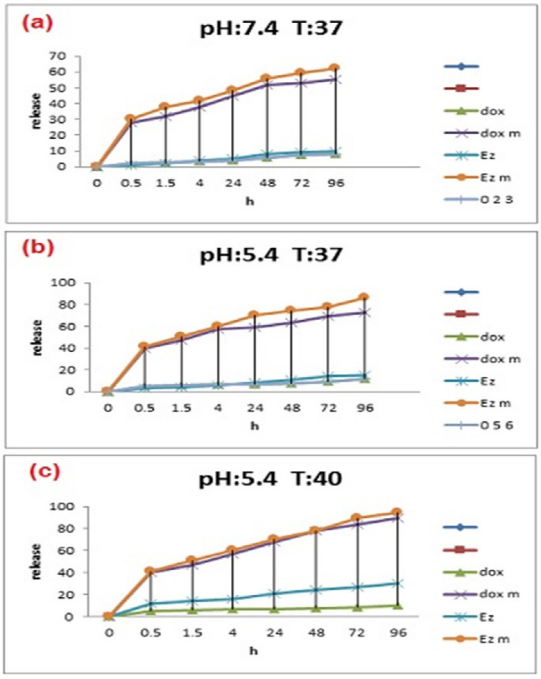

Fig. 4. (a) The drug release results from DOX/EZ nanoparticles, DOX/PU/PCL nanoparticles, and EZ/PU-PCL nanoparticles in pH=7.4 at 37 °C. (b) The drug release results from DOX/EZ nanoparticles, DOX/PU/PCL nanoparticles, and EZ/PU-PCL nanoparticles in pH=5.4 at 37 °C. (c) The drug release results from DOX/EZ nanoparticles, DOX/PU/PCL nanoparticles and EZ/PU-PCL nanoparticles in pH=5.4 at 40 °C.

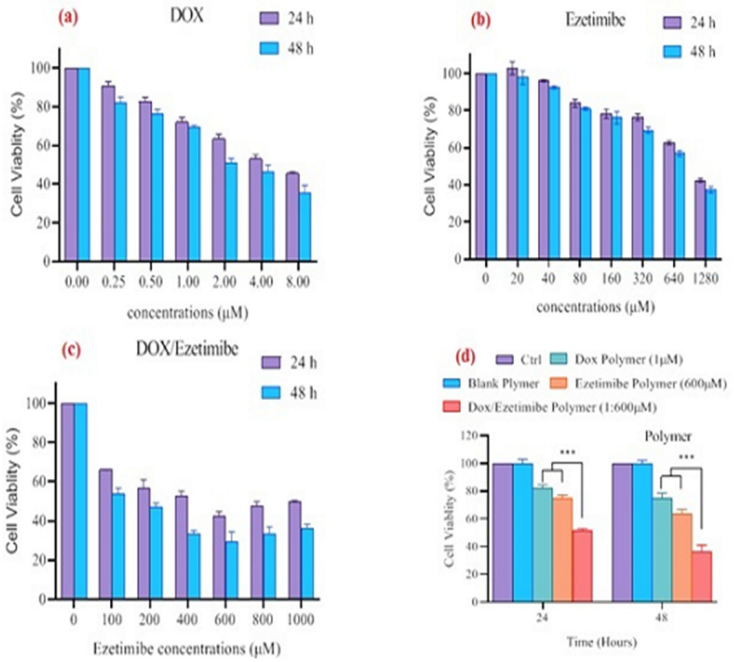

Fig. 5. The DOX and EZ cytotoxicity to prostate cancer cells has been elevated by PU-PCL polymer in vitro. (a) PC3 cancer cells' viability has been evaluated by using MTT techniques after treating with gradient doses of DOX for specific period of time (24 and 48 hours). (b) After being treated with gradient concentrations of EZ for 24 and 48 hours, the cell viability of PC3 cancer cells was assessed using MTT techniques. (c) After being treated with gradient concentrations of DOX/EZ for 24 and 48 hours, the cell viability of PC3 cancer cells was assessed using MTT techniques. (d) MTT techniques were used to examine the cell viability of PC3 cancer cells after they had been treated with gradient concentrations of DOX/EZ/PU-PCL polymer for 24 and 48 hours.

and the IC_{50} increased to 1.6 g/ml (Fig. 5d). As a result, it is possible to conclude that the toxicity of films is determined by their shape and morphology. In addition, it has been discovered to apparent toxicity at high dosages and have a minor effect on cell viability at low amounts.

CONCLUSION

DOX and EZ were loaded into PU-PCL films in this study and demonstrated efficiently delivered capacity, favorable size distribution, robust construct stability, and good biocompatibility. The polymer's DOX and EZ drug loading efficiencies were greater than 96%. Drugs were loaded onto films for controlled drug release and the treatment of PC3 cancer cells. Our findings show that the combination of EZ and the chemotherapeutic drug DOX significantly treats prostate cancer cells, which is consistent with previous research. EZ and DOX were continuously released from films for 96 hours at pH 7.4 and 5.4 at 40 °C and 37 °C, respectively. According to the results, the films of DOX/EZ/PU-PCL can be applied as a suitable drug delivery method to deliver DOX/EZ for the treatment of prostate cancer.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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