



# Preparation and In Vitro Evaluation of 5-Fluorouracil-Loaded PCL Nanoparticles for Breast Cancer Treatment

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## Abstract

Breast cancer is a significant public health issue in both developed and developing countries. Chemotherapy is the primary method of treatment to eliminate drug-resistant cells and prevent progenitor cancer cell growth. Polycaprolactone (PCL) is an excellent candidate for sustained drug delivery due to its amphiphilic nature, semi-crystalline form, biodegradability, biocompatibility, and slow drug release profile. This significant property has motivated their relevance, particularly in the area of anticancer drug delivery applications in the various nanoaggregates. Doxorubicin (DOX) and 5-fluorouracil (5-FU) are common anti-cancer drugs used for breast cancer treatment that they can easily incorporate into PCL. In this study, we synthesized the copolymer of PCL, PCL-DOX-FU, and performed characterization tests (Fourier transform infrared (FTIR), scanning electron microscopy (SEM)). We prepared a breast cancer cell line and evaluated the viability of cultured cells using the MTT test in different treatment groups (PCL, DOX-5FU, DOX, FU) at three different time points (24 h, 48 h, 72 h), and at different concentrations (9, 25, 50, 70 µg/ml). Additionally, we determined the expression rate of Bax and BCL-2 as apoptotic activator genes in treatment groups via real-time PCR techniques. Our data confirms that there are interactions between the active groups in PCL and DOX, as well as 5-FU drugs. After 72 h, a significant decrease was observed at concentrations of 50 and 70. Furthermore, the expression levels of Bcl2 and Bax were significantly reduced in cells treated with DOX, 5-FU, and DOX-5-FU-PCL nanoparticles. Our objective was to produce a nanocarrier system by coating magnetic nanoparticles with FOX-5-FU-PCL and evaluate its impact on the survival of MDA-MB-231 breast cancer cells. Our findings demonstrate that this method was highly successful in enhancing drug delivery efficiency for cancer treatment. However, further investigations are necessary to fully understand the safety and efficacy of these nanoparticles for clinical use.

**Keywords** Doxorubicin · 5-FU · Magnetic nanoparticle · DNA damage · Drug delivery · PCL

## Abbreviations

PCL Polycaprolactone  
5-FU 5-fluorouracil  
DOX Doxorubicin

SLNs Solid lipid nanoparticles  
SiNP Silica nanoparticles  
FUTP Fluorouridine triphosphate  
FdUMP Fluorodeoxyuridine monophosphate  
FdUTP Fluorodeoxyuridine triphosphate  
TS Thymidylate synthase  
MTT 3-(4,5-dimethyl thiazol-2-yl)2,5-diphenyl-tetrazolium bromide)  
DMSO Dimethyl sulfoxide  
FTIR Fourier transform infrared  
SEM Scanning electron microscopy  
CDKs Cyclin-dependent kinases  
Bcl2 B-cell lymphoma 2  
Bax Associated X protein  
PEG Poly(ethylene glycol)

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## 1 Introduction

Chemotherapy is a common cancer treatment that involves the use of anticancer drugs. However, these drugs can have drawbacks and side effects on healthy cells. Doxorubicin, which belongs to the anthracycline and antimetabolite classes, and 5-fluorouracil, an analog of uracil, are conventional anticancer drugs that were commonly used in breast cancer treatments. However, their effective doses can present serious side effects. Recent advances in the field of nanotechnology opened the door to new approaches in the development of diagnostic and therapeutic tools. Due to their distinctive physical, chemical, and biological properties, nanomaterials are widely employed as a promising approach for drug and gene delivery, as well as effective diagnostic and biomedical imaging [1, 2]. Favorable circulating nanosystems have been designed to enhance the specificity of conventional chemotherapy and detect early precancerous and malignant lesions in cancer cell lines [3–5]. Nanotechnology has contributed significantly to the field of medicine, particularly in gene therapy, tissue engineering, and magnetically induced hyperthermia [6, 7]. Nanomaterials were designed with various chemical and physical properties in size, shape, surface charge, and surface chemistry according to the slightly target cells [8, 9]. In addition, there is a high demand for appropriate delivery systems with high efficiency, low toxicity, low risks, and low costs. Among promising nanoscale drug carriers, solid lipid nanoparticles (SLNs), liposomes, polymeric nanoparticles, silica nanoparticles (SiNP), magnetic nanoparticles, and micelles have proved abundant potential clinical impacts [10–15]. Investigation on biodegradable and biocompatible polymers as particulate carriers in the pharmaceutical and medical fields revealed that these carriers lead to preventing metastasis, enhancing drug accumulation in tumors, and increasing cellular uptake [16–18]. Recently, the technique of “drug-polymer blending” can help to combine two hydrophobic and hydrophilic drugs in polymers such as docetaxel and cisplatin [19, 20].

PCL is a saturated aliphatic biodegradable polymer used in the development of tissue-engineering scaffolds and other biomedical applications [21]. PCL NP has significantly been loaded with diverse drug molecular DOX, 5-FU, curcumin, paclitaxel, and gemcitabine. Besides, magnetic nanoparticle carriers can be used for target therapy by external magnetic and avoid the problems caused by traditional chemotherapeutics. The investigations have shown that injecting anticancer drugs with magnetic properties into a patient's body can help control the drug release at the site of tumor cells by using a magnetic field. Alexiou et al. have reported that magnetic nanocarriers do not have side effects on other normal cells. The magnetic coating helps to increase the stabilization of nanoparticles in the bloodstream by reducing the immediate clearance of the carriers by the RES [22–25].

The antimetabolite, 5-FU, has a fluorine atom at the C5 position of the pyrimidine ring that fluorine atom was seen in uracil. A different active metabolite can be seen from 5-FU in diverse cells like fluorouridine triphosphate (FUTP), fluorodeoxyuridine monophosphate (FdUMP), and fluorodeoxyuridine triphosphate (FdUTP). The 5-FU has a wide range of biological effects and acts as a genotoxic agent due to the incorporation of the ribonucleotide FUMP into RNA and DNA metabolism due to thymidylate synthase (TS) inhibition or direct incorporation of FdUMP into DNA. 5-FU induces G1/S phase cell cycle arrest and leads to cell apoptosis. Studies on cytotoxicity showed that 5-FU-loaded PCL nanoparticles reduce side effects, compared with conventional anti-cancer drugs with efficient doses and shelf life [26].

Doxorubicin has been isolated from *Streptomyces* paucities. This anticancer drug can have a significant effect on diverse malignant cells. One of the properties of DOX is the inhibition of the biosynthesis of DNA and RNA through intercalation into DNA and the release of free radicals. In addition, doxorubicin showed a dual capacity in inhibiting collagen production and proliferation at the level of the cell cycle [27].

In this research, we found that the combination of a novel DOX and 5-FU covering with magnetic nanoparticles has anti-tumor potential loaded in PCL biodegradable polymers against breast cancer [28].

## 2 Materials and Methods

Doxorubicin (DOX) and 5-fluorouracil are analogs of uracil (5-FU) purchased from Medical Science Pharmaceuticals, Tabriz, Iran. The breast cancer cell line MDA-MB-231 was obtained from Pasteur Institute, Iran. Stannous-2-ethyl-hexanoate ( $\text{Sn}(\text{Oct})_2$ ) (Aldrich, St. Louis, USA, CAS. 301100), 3-caprolactone (98% purity) (Aldrich, St. Louis, USA, CAS. 502443), 3-(4,5-dimethyl thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), RPMI-1640 medium, streptomycin and penicillin G, and fetal bovine serum (FBS) were provided by Sigma-Aldrich. Biotrade kit for extracting RNA, QuantiTect SYBR Green Real-time PCR (RT-PCR) Kit, and cDNA synthesize kit were purchased from Takara. In this research, the cellular cytotoxicity was investigated with an MTT assay. The chemical structure of the prepared material was evaluated by using Fourier transform infrared (FTIR) spectroscopy (Bruker, Tensor 27) and  $^1\text{H}$ NMR spectra (Bruker AM 300.13 MHz spectrometer) in  $\text{CDCl}_3$  solvent. The shape and size of surface morphology were detected by SEM measurements (VEGA/TESCAN).

### 2.1 Synthesis of PCL Copolymer

The copolymer was prepared by ring-opening polymerization of 3-caprolactone in the presence of  $\text{Sn}(\text{Oct})_2$  as a catalyst. First, 3-caprolactone (4 g) in a round-bottom flask was

heated at 120 °C under a nitrogen atmosphere. In the next step, Sn(Oct)<sub>2</sub> (0.01 mmol) was added, and the polymerization reaction was performed with stirring for 6 h. Finally, the obtained viscous liquid was cooled, dissolved in dichloromethane, and re-precipitated by the addition of diethyl ether. The purification of the mixture was done twice.

## 2.2 Characterization of the PCL Copolymer and PCL-DOX-5-FU

Fourier transform infrared spectroscopy (FT-IR) (Bruker, Tensor 27) was used to detect the chemical structure of PCL and PCL-DOX-5-FU. The surface morphology of PCL-DOX-5-FU was determined by SEM.

Doxorubicin and 5-fluorouracil were loaded in PCL and covered with magnetic nanoparticles. First, DOX (5 mg) and 5-FU (5 mg) were dissolved in 2-ml DCM. And in the second step, Fe<sub>3</sub>O<sub>4</sub> (15 mg) and PCL copolymer were dissolved in 5- and 10-ml DCM, respectively. The prepared PVA solution for increasing the stability of the complex was added to the combined whole being prepared solutions and exposed to ultrasound irradiation for 2 min. The provided solution is centrifuged at 7000 rpm twice. Finally, the obtained precipitate was freeze-dried.

## 2.3 Cell Culture

The breast cancer cell line (MDA-MB-231) was purchased from the Pasteur Institute of Iran. The cells were cultured in RPMI1640 with 10% FBS and 1% antibiotic (streptomycin and penicillin G) and then grown at 37 °C in a 5% CO<sub>2</sub>.

## 2.4 Cell Viability (MTT Assay)

We evaluated cell viability and toxicity of PCL, DOX-5-FU-PCL covered by Fe<sub>3</sub>O<sub>4</sub>, and free DOX and 5-FU in breast cancer cell line MDA-MB-231. This consists of different concentrations (9, 25, 50, 75 µg/ml) of drug-loaded polymer, pure drug, and the polymer was prepared in fresh cell growth medium and administered into the 96-well microplates including 5 × 10<sup>3</sup> cells in each well in different three times. Then, the desired time and incubation MTT solution were provided with 2 mg/ml concentration and added to each well. After 4-h incubation, the MTT solution was removed from each well, and buffer Sorenson (5 µl) and DMSO (150 µl) were in the wells. The microplate was shaken for 5–8 min. The absorption rate of each well was read with an Elisa plate reader (Multiskan MK3, Thermo Electron Corporation, Minneapolis, MN) at a wavelength of 570 nm and calculated cell viability percentage [25, 29].

## 2.5 qRT-PCR

In the process, we investigated apoptotic gene expression in MDA-MB-231 cell lines. The MDA-MB-231 breast cancer cell line was treated with a dose of 75 mg/ml of both DOX-5FU-PCL with magnetic nanoparticle and pure DOX, pure 5-FU, and non-treated cells as control cells placed in incubator for 48 h with 37 °C, 5% CO<sub>2</sub>.

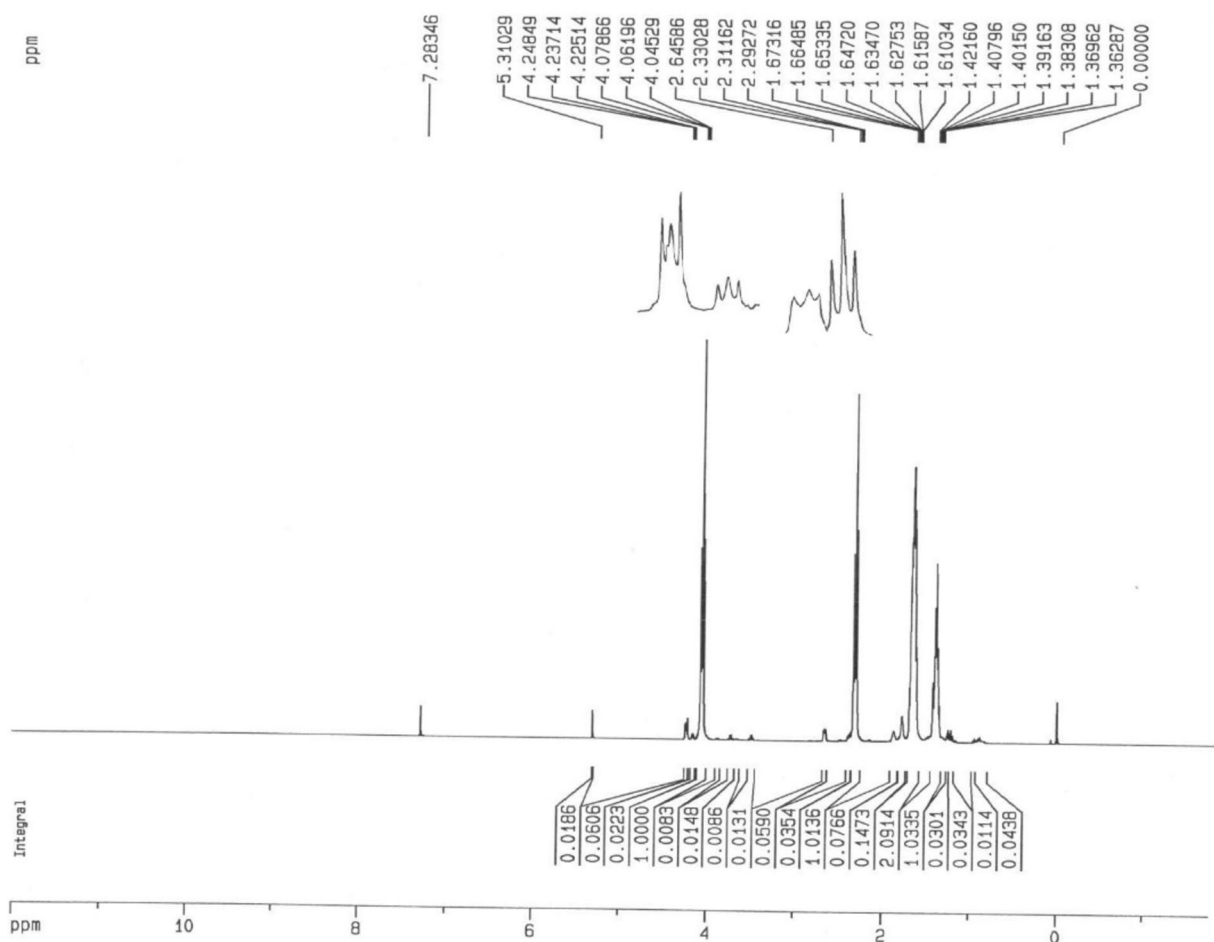
The total RNA was extracted from each well, treated with DOX-5FU-PCL with magnetic nanoparticles and pure DOX, pure 5-FU, and non-treated cells using a Biotrade kit, and RNA concentrations were quantified by nanodrop, then cDNA synthesis was done by Takara kit that can change RNA samples to cDNA; for each reaction sample, 1 µg/µL total RNA was used. Each reaction sample was prepared containing 1 µL of oligo dT primer, 10 µL of nuclease-free water, 4 µL of 5× reaction buffer, 1 µL RiboLock RNase inhibitor, 2 µL of dNTP Mix, and 1 µL of RevertAid M-MuLV Reverse Transcriptase. The mixture of the reactions was incubated under the following conditions: 37 °C, 15 min (reverse transcription); 85 °C, 5 s (inactivation of reverse transcriptase with heat treatment); 4 °C. A JenaBio-science Master SYBR Green on a Rotor gene 6000 (Corbett, Mortlake, NSW, Australia) is a Real-time system that by this system, Bcl-2 and Bax gene expression in MDA-MB-231 breast cancer cells can be evaluated. The GAPDH gene was used as an internal control. We analyzed the results of real-time PCR with the ΔΔCt method [28, 30].

## 3 Results

### 3.1 Characterization of PCL-PEG Covering Magnetic Nanoparticles

The structure characterization of the prepared PCL-PEG-PCL copolymers was followed by FTIR and <sup>1</sup>HNMR analyses. The typical PCL-PEG absorption bands at 1000–1240 cm<sup>-1</sup> and 1740 cm<sup>-1</sup> were assigned to the C–O, C–C, and C=O carbonyl stretching of bonds. Stretching bands at 2938 and 2869 cm<sup>-1</sup> were attributed to the –CH group from an aliphatic chain. For pure PCL, there is a slightly shifted peak at 1014 cm<sup>-1</sup>, which could be attributed to the C–O and C–C stretching groups. The presence of the same adsorption bonds in pure PCL and PCL-PEG revealed the absence of possible intermolecular interactions (Fig. 1).

The <sup>1</sup>H NMR spectrum of the prepared PCL-PEG-PCL copolymer is shown in (Fig. 1) The presence of a sharp peak at 3.65 ppm could be associated with the methylene protons of –CH<sub>2</sub>CH<sub>2</sub>O– in the PEG components of the triblock copolymer. Peaks at 4.23 and 3.82 ppm could be related to the methylene protons of the –O–CH<sub>2</sub>–CH<sub>2</sub>– group. The methylene protons of



**Fig. 1**  $^1\text{H}$  NMR spectrum of PCL

$-(\text{CH}_2)_3-$ , and the methylene proton of  $-\text{OCCH}_2-$  in PCL units appeared at 1.40–1.65 ppm and 2.32 ppm, respectively (Fig. 2).

### 3.2 SEM

Scanning electron microscopy (SEM) determined the shape and size of DOX-5-FU-PCL covering magnetic nanoparticles. According to the SEM results (Fig. 3), the size of DOX-5-FU-PCL covering magnetic nanoparticles yielded 28~71 nm.

### 3.3 MTT Assay

MTT assay was used for evaluating the cytotoxicity of the PCL and PCL-5-FU-DOX, pure DOX, and pure 5-FU, the combination of pure DOX and 5-FU. In this study, *in vitro*, cytotoxicity of different doses (9, 25, 50, 70  $\mu\text{g}/\text{ml}$ ) from PCL and PCL-5-FU-DOX, pure DOX and pure 5-FU, and

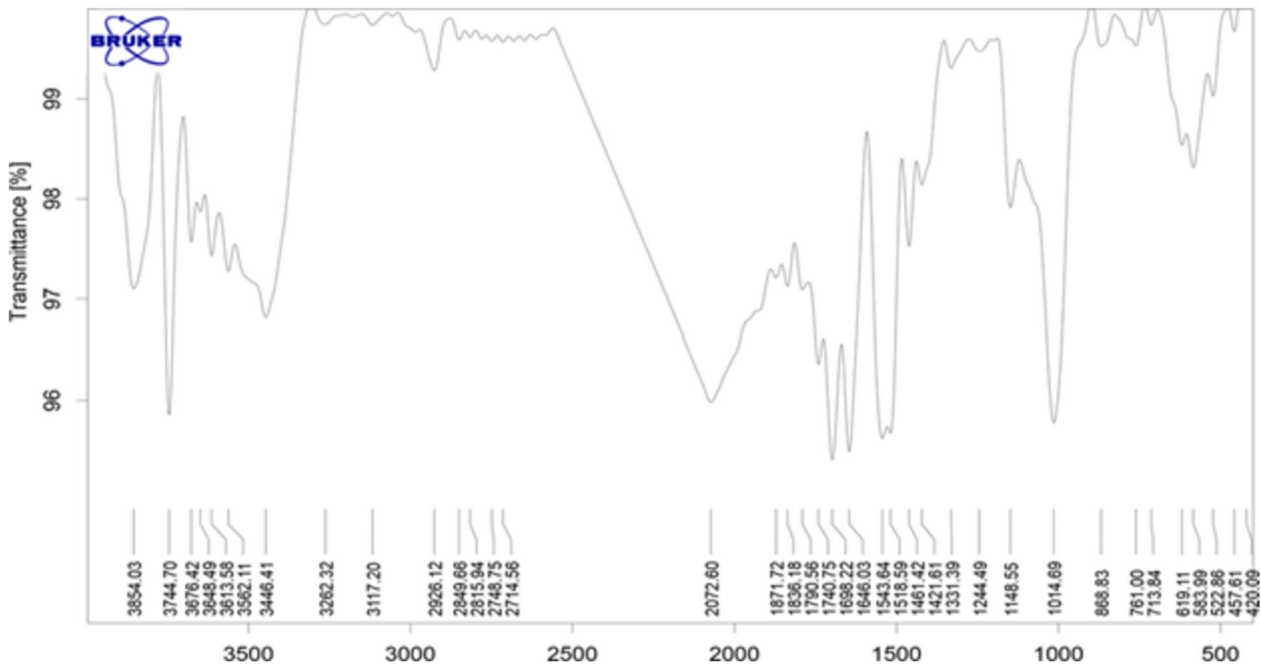
the combination of pure DOX and 5-FU for 24, 48, and 72 h in MDA-MB-231 breast cancer cells was investigated. According to the obtained results, the efficacy of chemotherapeutic agents on the cancer cells depends on dose and time manner. We observed that PCL is a biocompatible polymer and can be used as a drug delivery system (Figs. 4, 5, 6).

### 3.4 Real-Time PCR

The cell cycle is controlled via cyclin-dependent kinases (CDKs). Low- and high-level expressions of cyclin or inhibition of cyclins lead to disruption of the normal regulation of the cell cycle. Bcl2 and Bax are regulators of apoptosis whose gene expression levels were measured by real-time PCR.

The Bcl-2 gene is an anti-apoptotic protein with apoptosis-blocking effects; downregulation of autophagy by Bcl-2



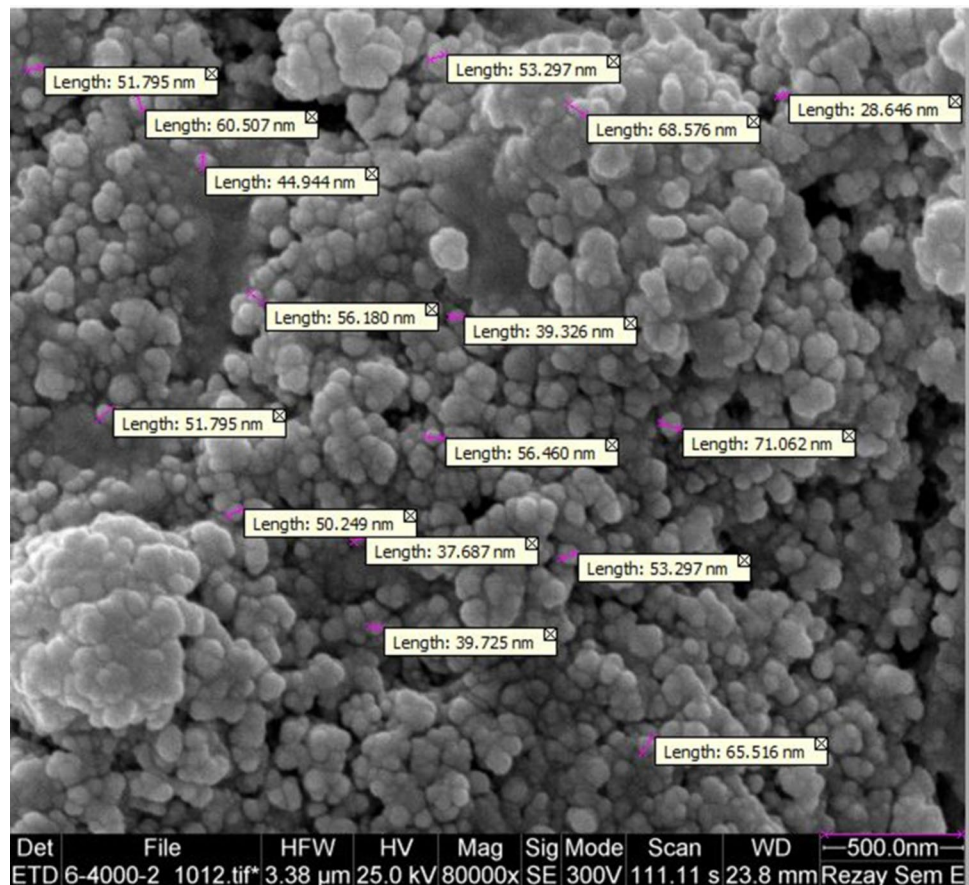


**Fig. 2** FTIR spectrum of PCL

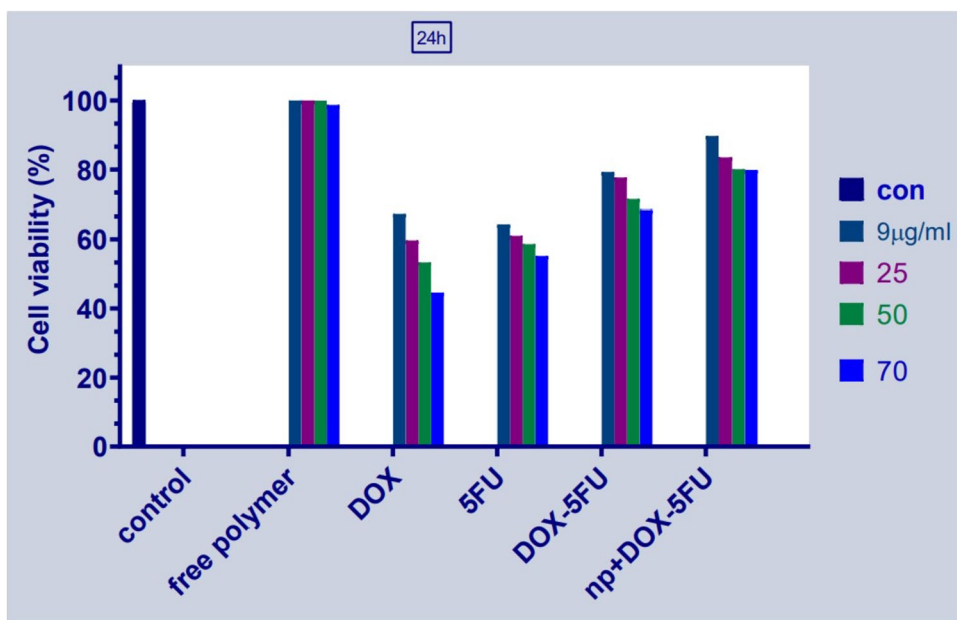
promotes MCF7 breast cancer cell growth independent of its inhibition of apoptosis. *Cell Death & Differentiation*. 18 [31],

452–464]. A key cell cycle regulator of the G1 to S phase progression is cyclin D1. Cyclin D is a potential target for cancer

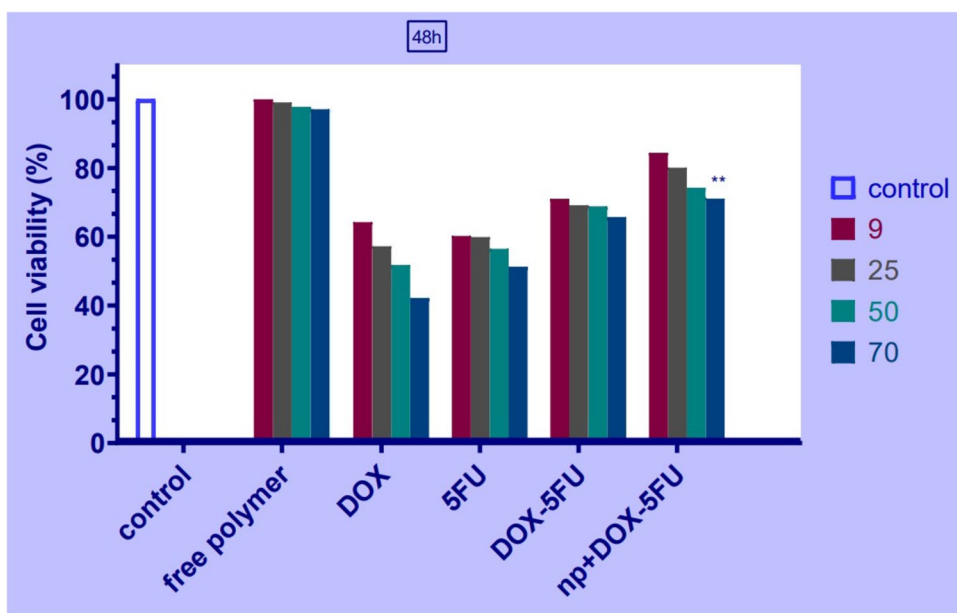
**Fig. 3** Field emission scanning electron microscopy (FE-SEM) micrograph of DOX-5-FU-PCL covering magnetic nanoparticles



**Fig. 4** MTT assay of free DOX, free 5-FU, free DOX-5-FU, PCL-NP, DOX-5-FU-co-loaded PCL in MDA-MB-231 after 24 h



**Fig. 5** MTT assay of free DOX, free 5-FU, free DOX-5-FU, PCL-NP, DOX-5-FU-co-loaded PCL in MDA-MB-231 after 48 h

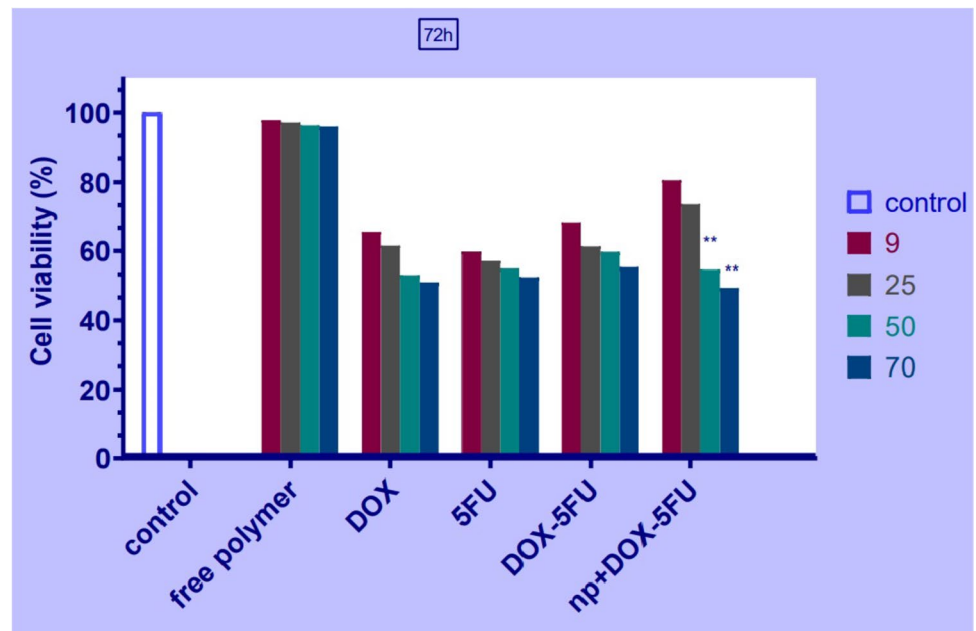


therapy. Thus, it is considered a helpful target for cancer. Moreover, cyclin D1 controls senescence, tumorigenesis, and apoptosis for the cell proliferation process. The over-expression of Bax leads to apoptosis resistance in cell lines. Here, the expression of both Bcl2 and Bax was explored for the assessment of the cytotoxic properties of fabricated nanocarrier on gene expression levels. The levels of Bcl2 gene expression and Bax were assessed through real-time PCR (Fig. 7) which exhibits that the expression level of Bax significantly reduced in both DOX and 5-FU and DOX-5-FU nanoparticle treated cells. The expression of bcl-2 was also reduced similarly by the cells treated with DOX 5-FU and DOX-5-FU nanoparticles.

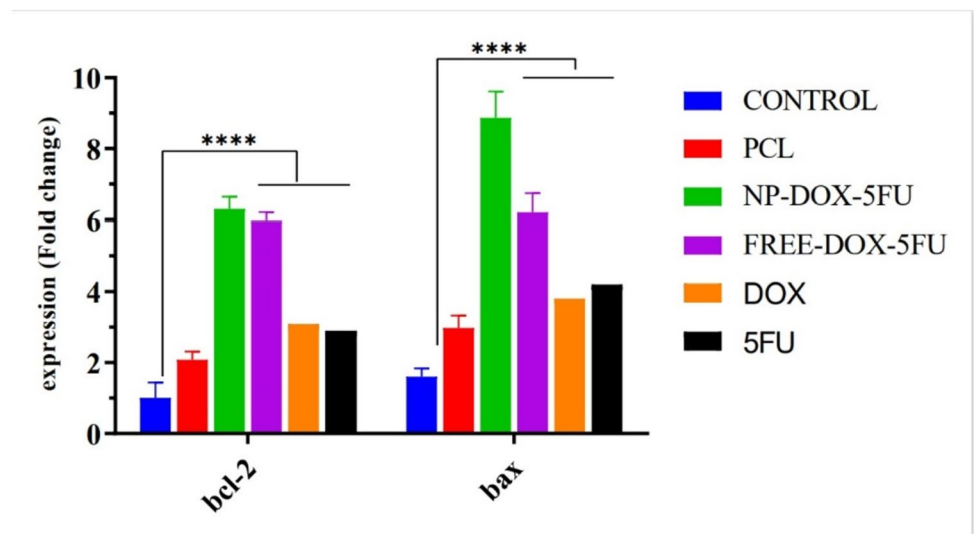
### 4 Discussion

Delivery site is significantly issued in the drug delivery system and plays an important role in effective treatment. Reactive oxygen species can induce DNA damage, and oxidative stress, and trigger apoptotic pathways of cell death and membrane damage. DOX is one of the anti-cancer drugs that interact directly with DNA and breaks double strands from each other. Its replacement in the nucleus and poison topoisomerase-II leads to DNA damage and cell death.

**Fig. 6** MTT assay of free DOX, free 5-FU, free DOX-5-FU, PCL-NP, DOX-5-FU-co-loaded PCL in MDA-MB-231 after 72 h. The cell viability of MDA-MB-231 treatment with DOX-5-FU-co-loaded PCL extremity decreased after 72 h at a high dose (70  $\mu\text{g/ml}$ ). The results are presented as mean  $\pm$  SD ( $n = 3$ ). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$



**Fig. 7** Real-time PCR results after treatments DOX, 5FU, DOX-5FU co-loaded PCL-PEG NPs about bax and bcl2 after 48-h incubation time. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. control was considered significant. Results are mean  $\pm$  SD ( $n = 3$ )



The investigations showed that the complex of DOX with PEG-ley led to the extended half-shelf DOX during circulation and increased drug concentration in tumor cells. In another study, Ebrahimi and Akbarzadeh investigated the encapsulation of DOX in  $\text{Fe}_3\text{O}_4$ -modified nanoparticles with PLGA-PEG and the release of DOX from this system. Also, DOX and TRAIL with dendrimer drug delivery showed a significant therapeutic effect. In addition, investigations demonstrated another combined therapy TNF- $\alpha$  and DOX. TNF- $\alpha$  is involved in direct death of tumor cells. Investigations showed combination of two or more anticancer drugs can be effective in cancer treatment. For instance, Akbari and Akbarzadeh showed synergistic anticancer effects of TPX and MTX on the breast

cancer cell line MCF-7 and the mitochondrial apoptosis pathway activated by treating with TPX/MTX-co-loaded PLGA-PEG NPs [32]. Drug combination began in the 1980s; primary 5-fu and leucovorin were used together for colon cancer therapy. In addition, researchers reported that the consumption of oxaliplatin with 5-FU and leucovorin can upgrade the treatment pathways. In another study done by Yang, Li, and Qi et al, co-delivery of doxorubicin (DOX) and Bmi1siRNA was reported by folate receptor-targeted liposomes. The biodegradable poly (e-caprolactone) (PCL)-modified poly (ethylene glycol) (PEG) copolymer is a nontoxic, tissue-compatible polymer. These are used as excellent drug delivery systems in cancer treatment.

One investigation showed the effect of MTX and CDDP encapsulated in PCL-PEG on the A549 lung cancer cell line. This drug delivery system caused a decrease in cell viability and increased therapeutic in the A549 cell line [2, 5, 9, 18, 20, 23, 33]. Moreover, in a study that was showed to evaluate the effect of 5FU in PCL nanoparticles in the treatment of colon cancer, the toxicity study results revealed that the 5FU loaded in the PCL polymer had a greater anti-proliferative effect on colon cancer cell line compared to the drug administered alone [24, 25, 28–30, 34]. A study was conducted to investigate the effectiveness of F5, an anticancer drug loaded in PCNP nanoparticles, on different types of cancer cells. These included human colorectal carcinoma, MCF-7 (human breast adenocarcinoma), HepG2 (human hepatocellular carcinoma), and human lung carcinoma. The study found that this nanoparticle formulation could be a suitable approach in treating cancer as an active anticancer agent [35–38]. Additionally, in a recent study, superparamagnetic magnetite nanoparticles were loaded into zeolite (MZNC), and 5-fluorouracil (5-FU), an anti-cancer drug, was encapsulated within the zeolite. The study aimed to determine the cytotoxic effects of 5-FU-loaded MZNC on human gastric carcinoma (AGS) cells. Real-time cell analysis and colorimetric WST-1 cell viability assay were used to assess the effects of the treatment. Apoptosis of cells was further investigated by Annexin-V staining which indicates the loss of cell membrane integrity. The study concluded that 5-FU-loaded MZNC effectively inhibits the proliferation of AGS cells in vitro through apoptotic mechanisms. This makes it a potentially useful agent against cancer, although further animal study is still necessary to confirm its effectiveness [39–42].

## 5 Conclusions and Perspectives

Tumors often develop resistance to chemotherapy drugs, but combining different drugs can mitigate this issue. This approach has several advantages, including reduced side effects and drug dosages and increased efficacy against cytotoxicity. In this study, DOX-5-FU-PCL covering magnetic nanoparticles was provided by the double emulsion solvent evaporation process. The DOX-5-FU-PCL covering magnetic nanoparticles showed excellent cytotoxicity effect against breast cancer additionally, and the expression levels of both Bcl2 and Bax have considerably reduced in both DOX and 5-FU and DOX-5-FU nanoparticle-treated cells. According to our results, efficient drug carriers can be developed with prepared nanoparticles, which when loaded with two or more anticancer drugs can effectively treat drug-resistant tumors. Therefore, DOX-5-FU-PCL was shown to enhance anticancer activity and might be a novel anticancer agent. Although the strategy of using

nanoparticles in polymer format for cancer therapy can be a novel achievement, it needs more studies in the field of toxicity, effectiveness, and safety to be used in biomedical.

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**Author Contribution** All authors helped in performing and drafting the manuscript. The authors read and approved the final manuscript. AA conceptualized research; AA and AJ supervised research; HF performed research and analyzed data. All authors contributed to the writing and review of the manuscript.

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**Data Availability** Sharing does not apply to this article as no data sets were generated. Data analysis in the current study was performed using publicly available datasets.

## Declarations

**Ethics Approval and Consent to Participate** The ethical approval for this paper was obtained from the research ethics committee of Tabriz University of Medical Sciences) IR.TBZMED.VCR.REC.1398.436 (IR.TBZMED.VCR.REC.1398.436).

**Consent for Publication** Not applicable.

**Competing Interests** The authors declare no competing interests.

**Research Involving Humans and Animals Statement** None.

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