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Rsearch Article

Synthesis and *In vitro* Evaluation of Doxorubicin loaded Polymeric Nanoparticles on Cancer Cells

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Abstract: Cancer is the second biggest mortality rate globally. Most of anti-cancer drugs are hydrophobic and when they are administered in the body, they get clear from the blood. That's why polymeric nanoparticles (NPs) have been used for delivering anti-cancer drugs to targeted sites. Biodegradable and self-assembled nature, PEG-PLGA has been used as a nanocarrier for biomedical applications. We developed PEG-PLGA NP for the doxorubicin (DOX) delivery to cancerous cells. The successful PEG-PLGA synthesis was confirmed by its ^{1}H NMR spectrum. All NPs displayed individual spherical morphology and 100 nm size range with -18.5mV zeta potential. Drug release profile showed DOX had sustained release pattern from DOX@NPs. *In vitro,* MTT assay and apoptosis analysis revealed that low-dose DOX@NPs exhibited more toxic effects on cancerous cells as compared to DOX alone. Overall results demonstrate that polymeric-based nanosystems increase the efficacy of DOX on cancer cells.

Keywords: Cancer, Chemotherapy, Doxorubicin, Drug delivery, PLGA nanoparticles

1. INTRODUCTION

Cancer is a main public health issue globally, with a growing number of cases diagnosed each year [1]. The most significant distinction between healthy and cancerous cells is their uncontrollable reproductive nature. One of the biggest challenges in the treatment of most of the diseases is getting the drug to target infectious site [2]. Cancer can be treated in a variety of ways. Treatment options are dependent on the specific cancer you have and its stage of development. One course of treatment may be sufficient for some cancer patients. However, many patients undergo multiple treatments simultaneously [3].

Chemotherapy is a type of aggressive treatment for cancer in which toxic medications are used to kill fast-dividing cancer cells. It can be used to decrease the tumor size or the tumor cells number in the body, lowering the probability of spreading cancer [4]. Unfortunately, current chemotherapy has a number of limitations, the majority of which are attributable to the lack of target specificity, which results in unsatisfactory clinical outcomes. Chemotherapy kills fast-dividing cells; because normal cells divide quickly, cancerous drugs have toxic effects on normal cells such as bone marrow, hair follicles, macrophages, and digestive tract. [5]. Nanotechnology is concerned with materials that have dimensions of one-hundredth of a millimeter or less [6]. Because of its uses in biotechnology, targeted drug administration, gene delivery, and drug delivery, nanotechnology has grown in popularity in recent years [7]. Nanoparticles (NPs) with smaller sizes (in nm) can easily penetrate various cells, particularly cancer cells. Since nanocarrier has the benefit of the EPR effect in cancer and escape filtration in the spleen while still being sufficiently large to avoid absorption in the liver, a size of 100-200 nm is currently considered the ideal size for drug delivery systems [8].

Polymer nanotechnologies are crucial for overcoming obstacles to drug delivery, such as targeted therapy and the delivery of molecules

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that can't be delivered, like hydrophobic therapeutics, oligonucleotides, or RNA-interfering molecules [9]. Only a few polymers can be used as components of NPs intended to deliver drugs *in vivo*. Consider that an appropriate polymer needs to fulfill several criteria to be used in this application to better understand this [10]. It must be biodegradable or swiftly eliminated from the body so that it can be administered repeatedly without the danger of uncontrolled accumulation. It should not be immunogenic or toxic. If there are any degradation products, they must also be nontoxic and immunogenic. [11]. Polymeric NPs have lots of advantages in drug delivery, including protect therapeutic payload and biological molecules during their journey to the target site and increasing bioavailability and therapeutic index. Because of the continuous polymeric network that forms their structure, drugs can be maintained within or adsorbed onto the nanosphere surface [12]. Because it has been demonstrated that they are safe to use in the clinic. Poly (D, L-lactic-co-glycolic acid) (PLGA) based NPs are considered as the sustained release polymer system for drug delivery applications. PEG-PLGA NPs are mostly wanted because systematic clearance of pegylated polymeric NPs is lower than similar particles without PEG. PEG can enhance the pharmaceutical properties of many FDA-approved drugs. DOX is a highly effective anticancer drug that has been authorized for use against a variety of tumor types. Unfortunately, toxicities associated with anthracycline drugs, the most serious of which is heart failure, limit its long-term clinical use. Encapsulating DOX in PEGylated liposomes reduces DOX-induced cardiomyopathy but maintains anticancer activity against solid tumors [13]. Doxorubicin is a hydrophobic drug and clears in bloodstream very fast [14]. PEG is hydrophilic that's why it is encapsulated in PEG to convert it into a hydrophilic form. Polymeric micelles consist of hydrophilic and hydrophobic monomer units and are primarily amphiphilic co-polymers, an efficient drug delivery system for anticancer medications that are only weakly water-soluble. The blood circulation of the polymeric micelles is prolonged, and they concentrate more at the site of the tumor [15]. In this study, DOX is transformed into a hydrophilic condition by encapsulated in polymeric nanoparticles to improve its anticancer efficacy.

2. MATERIALS AND METHODS

2.1. Synthesis of the Block Co-polymer (PEG-b-PLGA)

Ring-opening polymerization approach was used to synthesize PEG-b-PLGA with little modification in the previous methodology [16]. mPEG-OH (500 mg, 0.09 mmol) was mixed in toluene (60 mL) and was treated with azeotropic distillation for 6 hours using a Dean-Stark apparatus. After cooling under nitrogen to room temperature, lactic acid (LA) (1.0 g, 6.8 mmol), glycolic acid (GA) (0.22, 1.8 mmol), and tin (II) ethoxyhexanoate (20.0 mg) were heated to 130 °C for 8 hours in a glove box (water and oxygen contents 0.1 ppm). In chloroform, the resultant polymer was dissolved then precipitated and dried in cold diethyl ether. The white solid product (PEG-b-PLGA) achieved (900 mg, 68%) was analyzed using ^{1}H-NMR spectroscopy.

2.2. Preparation of Nanoparticles

For DOX@NPs preparation, 10 mg of PEG-b-PLGA and 10 µl of DOX (10 mg/mL in DMSO) were mixed in 1 mL of DMSO under constant stirring. After adding ultra-purified water (5.0 mL), the solution was stirred for another 30 minutes. After that, DMSO was then removed by dialysis membrane method. Newly prepared DOX@NPs solution was dialyzed with membrane MWCO of 14 kDa against distiller water for 24 hours. After that NPs solution was centrifuged to remove the un-encapsulated drug and polymeric debris from the supernatant.

2.3. Characterization of Nanoparticles

Mean diameter and surface charge measured by Dynamic light scattering (DLS). The size distribution profile and morphology of DOX@NPs were analyzed by transmission electron microscopy (TEM).

2.4. DOX Encapsulation Efficiency and Loading Content

For encapsulation efficiency and DOX loading content, newly prepared 10 mg of DOX@NPs were dissolved in DMSO (5 mL). To remove polymeric debris, solution was centrifuged at 16000 rpm,

15 °C for 30 min and washed twice with fresh DMSO. After that pellet was dissolved in 1 ml DMSO and analyzed for DOX content at λ max value of 485 nm by UV spectrophotometer.

The drug loading content (DLC) and encapsulation efficiency (EE) were quantified with belove mentioned equations:
DLC= (DOX weight in the NPs) / (Total NPs weight) × 100%
EE= (encapsulated DOX on NPs / total DOX added to polymer) × 100%

2.5. Drug Release Profile

To determine the release pattern, the dialysis membrane of 14 kDa was used. Drug-loaded nanoparticles (15 mg) were dissolved in phosphate buffer saline (PBS) (1mL) at pH 7.4 and immersed in the dialysis bag in 30 mL of PBS while being stirred at 110 rpm and 37 °C. After preset intervals, 1 mL of PBS was collected from the immersed solution and replaced with fresh PBS. The concentration of released DOX was quantified using a DOX standard curve developed with known concentrations. DOX release curve was drawn by plotting the cumulative % DOX release against preset time interval.

2.6. Cytotoxicity Assay

HepG2 and MDA-MB-231 cells (5000 cells/ well) were plated in 96 well plates respectively and cultured at 37 °C for 24 hours. Afterward, cells were incubated with different concentrations (from 3.15 to 50 g/mL of DOX) of DOX@NPs and free DOX respectively. Cells that had not been treated served as a control. After 24 hours, an MTT assay was carried out on cells cultured in 96 well plates to compare the proliferative potential of different groups on HepG2 and MDA-MB-231 cell lines. After washing the cells with PBS, they were incubated for 2 hours in a 500 µl complete medium containing 25 µl of MTT (10 mg/mL) solution. MTT is converted into purple formazan in alive cells, which is then solubilized with 10% sodium dodecyl sulphate (SDS) and checked the absorbance at 570 nm using a microplate reader. Triplicates were run for each treatment.

This equation calculates the percentage cell viability:

% viability = Mean OD Sample / Mean OD blank × 100.

2.7. Apoptotic evaluation by p53 ELISA

Quantification of apoptotic protein level in the post-treated cells was measured by p53 ELISA (Zokeyo, China) kit according to the manufacturer's protocol. Initially, 50 µL of lysate from different treatment groups was added in triplicate along with standard solution in particular wells. After the addition of HRP-conjugated reagent (100 µL), plate was incubated for at 37 °C 1 hour. The HRP-conjugated reagent was removed by washing with wash buffer three times. After washing, Chromogen A and Chromogen B solutions (50 µL each) were added to all wells and incubated for 15 min at 37 °C. After that 50 µL of stopping solution was added in each well and reading was measured at 450 nm.

3. RESULTS AND DISCUSSION

3.1. Characterization of Polymer

The successful PEG-PLGA synthesis was confirmed by its ^{1}H NMR spectrum (Fig. 1). The *d, l*-lactide peaks appear at 5.21 (m, nH, CH) and 1.5 (m, 3nH, CH_3) and glycolide peaks of PLGA was found at 4.72 (s, 2nH, CH_2). The PEG peak has appeared at 3.2 (m, 4nH, OCH_2CH_2).

3.2. Physicochemical Characterization

Hydrodynamic diameter of newly synthesized NPs was measured by DLS. At pH 7.4, the hydrodynamic diameter of DOX@NPs was approximately ~115nm (Fig. 2A). The zeta potential (mV) of DOX@NPs was found to be -18.5 mV.

DOX@NPs showed size distribution was approximately 100 nm and displayed individual spherical morphology as captured by TEM (Figure 2B). Based on UV spectrometry analyses, the DOX loading content and encapsulation efficiency of NPs were 9.5 ± 0.07 µg/mg and 96.13 ± 4.05 % respectively.

3.3. *In vitro* Release Study

To evaluate drug loading efficiency and drug release profile, standard curve was obtained.

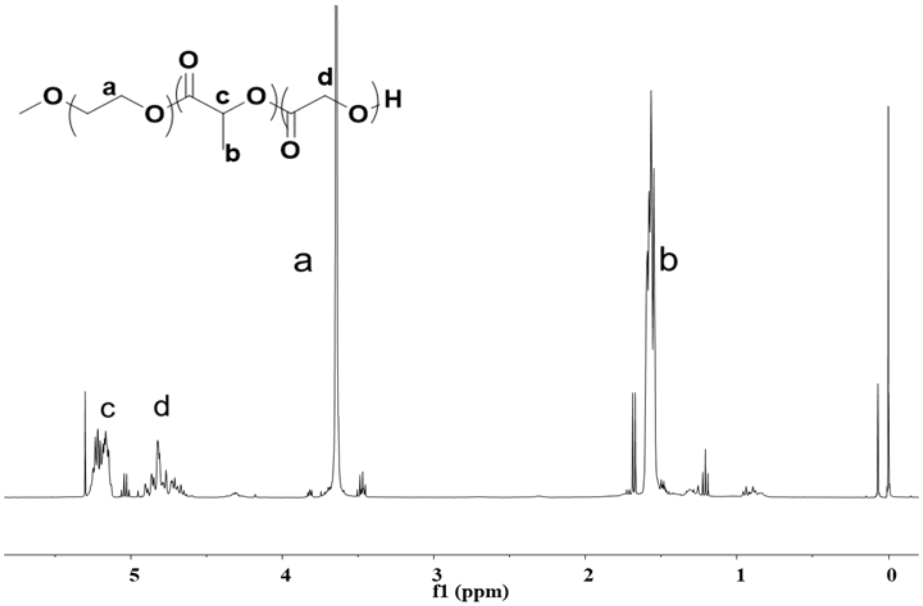

Fig. 1. [1]H NMR spectra of co-polymer (PEG-PLGA). Peak "a" shows a functional group of PEG while peaks "b", "c" and "d" show functional groups of PLGA.

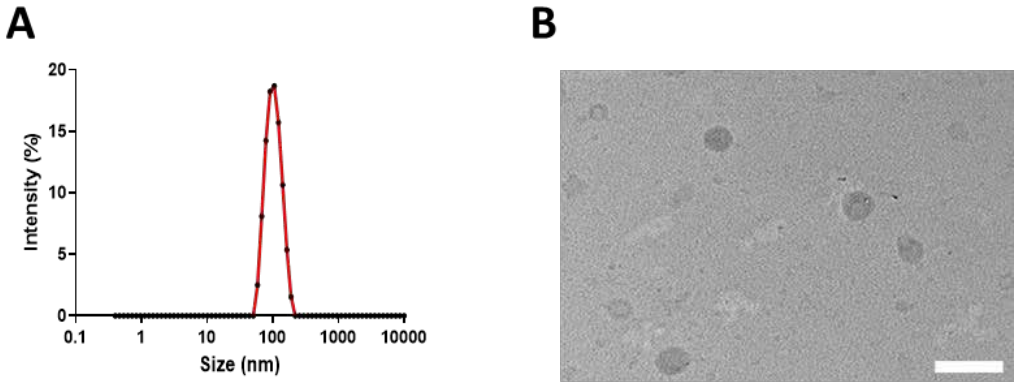

Fig. 2. Physicochemical characterization of the DOX@NPs **(A)**. Hydrodynamic diameter of DOX@NPs at physiological pH 7.4 in PBS solution. **(B)** TEM image of DOX@NPs. The bar size is 200 nm.

Different concentrations of DOX (0.03125µg/ml to 0.00024375µg/ml) were checked using a UV spectrophotometer at 485 nm and a standard curve (Fig. 3A).

The DOX release behavior from NPs was studied shown in Figure 3B. The DOX@NPs had a significantly longer period of sustained release. During the first six hours, about 40% of the drug was released, and in 48 hours, 98% of the DOX was released. As a result, the results show that DOX@NPs have more consistent drug-release and controlled-release properties.

3.4. Cytotoxicity Study of DOX@NPs

HepG2 and MDA-MB-231 cells were utilized to evaluate the *in vitro* cytotoxic effect of DOX@NPs by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Figure 4A and B show the cellular cytotoxicity of free-Dox and DOX@NPs at equivalent concentrations of loaded Dox. The viable cell percentage was measured by an MTT assay. Both Free-Dox and DOX@NPs showed a significant decrease in cell viability in dose dose-dependent manner against both MDA-MB-231 and HepG2 cells. Auspicious results were observed

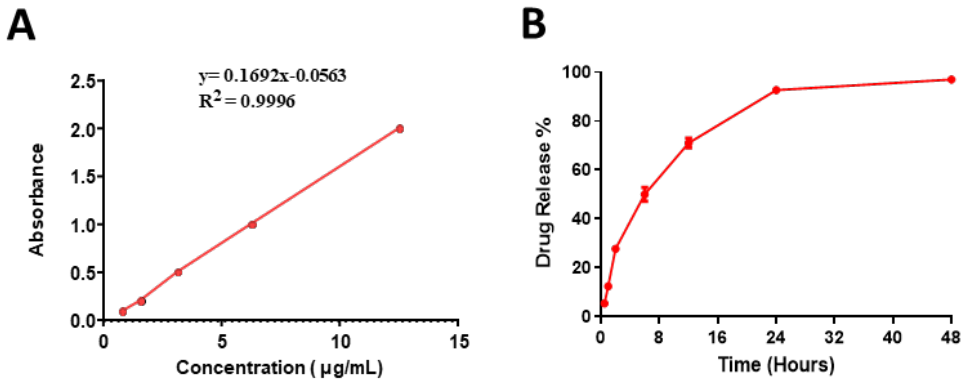

Fig. 3. *In vitro* release study. **(A)** Standard curve for free DOX in DMSO was determined at 485 nm UV wavelength. **(B)** DOX release profile of the DOX@NPs at pH 7.4 at 37 °C. DOX release was observed after regular time intervals.

in MDA-MB-231 cells because of a significant decrease in IC_{50} values of DOX@NPs (12.5 µg/mL) as compared to free DOX (30 µg/mL). At the highest concentration, cell viability was reduced to 10 % for the DOX@NPs while free DOX, which was > 35%. Similar pattern was found in HepG2 cells, IC_{50} of DOX@NPs against HepG2 cells was much lower (10.7 µg/mL) in comparison with free DOX (25 µg/mL). As shown by IC_{50}, the anti-proliferation ability of the formulations followed the order: DOX@NP > DOX. The findings indicate that DOX@NPs appear to have a higher antitumor effect than free DOX.

3.5. Apoptotic Analysis

The apoptosis in post-treatment groups along with the control group was assessed by a p53 ELISA kit. The level of p53 was quite low in the control group and a similar low apoptotic effect was found in PEG-PGLA treated cells which confirmed the biocompatibility of co-polymer. Moreover, the prevalence of apoptosis in DOX@NPs was higher than that of free DOX in both cells which was in line with cytotoxicity MTT results. These findings revealed that the enhanced cytotoxic effect and apoptosis induction of DOX@NPs were accredited to higher internalization and sustained DOX release, which could efficiently inhibit cancer cell proliferation.

4. DISCUSSION

Cancer is a leading cause of death globally, approximately 9.5 million people died due to cancer in 2020. The incidence of cancer is increasing worldwide, due to multiple reasons, such as population aging, changes in lifestyle, and environmental factors [17]. However, the cancer survival rate is also increasing, because of early

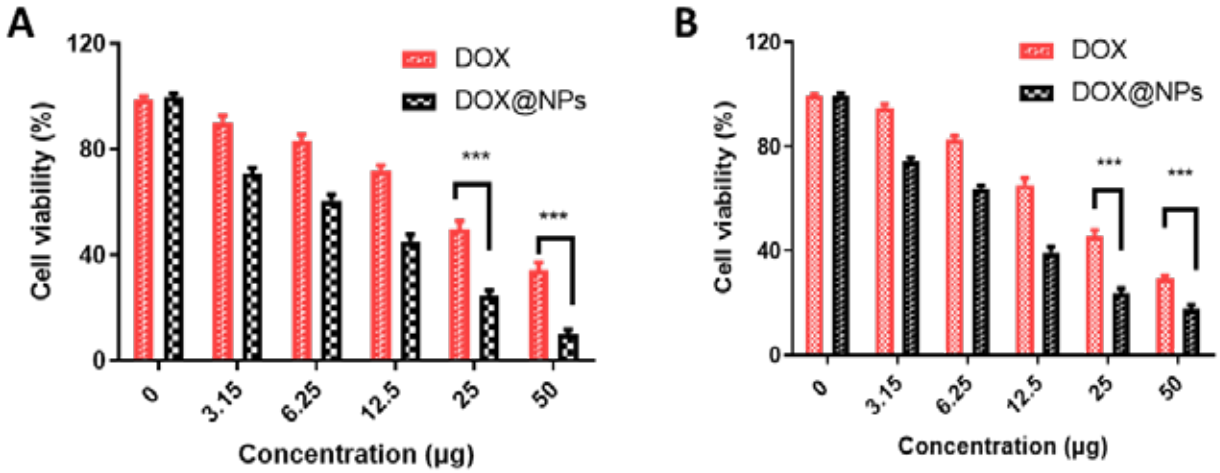

Fig. 4. Cytotoxic effect of DOX@NPs on MDA-MB-231 **(A)** and HepG2 **(B)** cells evaluated by MTT assay.

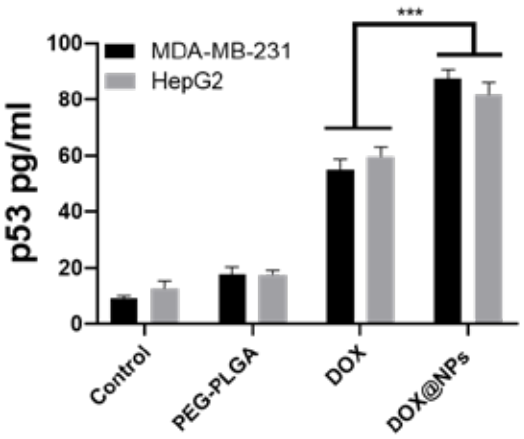

Fig. 5. Apoptosis of cancer cells was analyzed by p53 ELISA kit after DOX@NPs (20 µg/mL) treatment.

diagnosis and advancement in treatments. Multiple important habits can be done to minimize the risk of cancer, including a healthy diet, avoid smoking, getting regular exercise, maintaining a healthy weight, and regularly performing cancer screening tests [18]. Among different cancer treatment approaches, chemotherapy is one the most effective treatment approaches that directly kills the cancer cells by different chemo-therapeutics. [19]. Because of the effectiveness of DOX in a wide range of cancers, the DOX belongs to the class anthracycline is most commonly used in chemotherapy. By intercalating with DNA, doxorubicin suppresses macromolecular production. [20].

Nanoparticles (NPs) show unique features for transportation, biological activity and interaction, optical, thermal, and magnetic properties that cannot be offered at molecular and macroscale levels. The supportive entries can facilitate hydrophobic drug delivery, and protect the drug during blood circulation, controlled release, immune evasion, deep tumor tissue penetration, site-specific activation, and higher cellular uptake [6]. We investigated the delivery of hydrophobic drug by encapsulating the drug into polymeric NPs and checked *in vitro* cytotoxicity and apoptosis induction on cancer cells. The ^{1}H NMR characteristic chemical peak values of our synthesized co-polymer were similar to previous studies [21, 22] confirming the successful synthesis. The polymeric nanoparticles in this study are 100 nm in size with a spherical shape which allows them for longer *in-vivo* circulation half-life and higher intracellular uptake in cancer cells [23]. In the present work, DOX was loaded in the hydrophobic core of PLGA which gives stability to the drug, PEG is advantageous in terms of extending the blood half-life and cell membrane interaction [23].

Slow drug release from nanoparticles which leads to a sustained release of the payload over a period of time is a more effective way to kill cancer cells [24]. The release profile of DOX@NPs demonstrated that our nanosystem has sustained release properties and it was observed that about 90% drug was released in 48 h. Our sustained-release finding is in agreement with the other drug release reports which demonstrate that hydrogen bonding between drug and PLGA matrix and slow degradation rate of polymers [25, 26]. The sustain release drug delivery systems have the potential to improve efficacy, reduce toxicity, and improve patient compliance with chemotherapy [27].

A well-established method in which cancer cell lines are utilized to evaluate nano-formulations biocompatibility and determine their cytotoxicity and pharmacological response to the delivery of loaded drug [28]. Compared by the treatment of free-Dox, equivalent concentration of DOX@ NPs exerted a much stronger cytotoxic effect on both types of cancer cells. These results are in agreement with previous published reports on the anti-cancer toxicity of DOX which showed that DOX@NPs display a higher strength at lower doses than free DOX due to higher cellular uptake of DOX@NPs [29, 30]. Apoptosis is the naturally programmed cell death mechanism, which is one most applied approach for most chemotherapeutic drug and nanomedicine analyses [30]. As shown in Figure 5, the prevalence of apoptosis in cells after treatment with DOX@NPs much higher than free DOX is probably related to the fast internalization kinetics of NP drug that increases the local drug concentration in the cell [29]. Previously, it was reported that DOX caused early activation of p53 in tumor cells which was followed by caspase-3 activation and DNA fragmentation [31]. Overall, these findings indicate that DOX@NPs have considerable anticancer potential against MDA-MB-231 and HepG2 cells and can enhance the therapeutic effects of anti-cancer drugs, making them promising adjuvant therapy for breast and liver cancers.

5. CONCLUSIONS

In this study, we developed an effective nanosystem based on PEG-PLGA block co-polymer and DOX. TEM revealed that nanoparticles were well-dispersed, and spherical shape. The release profile showed that the DOX@NPs, as nanocarriers for DOX, had a good sustained-release effect. Cytotoxicity results showed that anticancer activity of DOX was significantly improved when it was encapsulated in PEG-PLGA NPs. We believe this DOX@NPs system has considerable potential for cancer treatment.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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