JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Release profile and cytotoxicity study of modified doxorubicin nanoparticles

Sumanta Sen[1*], Biswajit Mukherjee[2], Gurudutta Pattnaik[3], Manoj Kumar Pani[1]

[1]Biju Patnaik University of Technology, Rourkela, Odisha-769015, India

[2]Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

[3]Centurion University of Technology& Management, Bhubaneswar, Khordha, Odisha, India

Abstract

The nanoparticles can be developed into smart drug delivery systems to enhance therapeutic efficacy by manipulating the size, surface characteristics, and selective polymer material. In the present study, we developed nanoformulations using PLGA and investigated the release profiles of encapsulated doxorubicin in different concentrations. **Methods:** Fluorescence microscopy investigated cellular uptake and localization of nanoparticles in cells. MTT assay, Acridine orange/ethidium bromide (AO/EB) staining, DAPI Staining, and DCFDA staining were performed for the in-vitro anti-cancer evaluation of the synthesized nano-formulation against MCF-7 human breast cancer cell line. The results revealed that PLGA nanoparticles of Doxoribicin demonstrated an IC_{50}value of 0.906 µg/ml for MCF-7 breast cancer cell line and extended-release of thedrug. Doxorubicin can be formulated into PLGA nanoparticles for extended delivery and improved efficacy as a chemotherapeutic agent.

Keywords: Doxorubicin, MTT assay, MCF-7, Cytotoxicity

Introduction

Nanoparticles are novel colloidal drug delivery systems, with particle sizes ranging from 10 to 1000 nanometers, known for their promising results in controlling drug delivery andenabling site-specific drug delivery, thereby reducing the dose and decreasing toxicity[1]. Poly (lactic-co- glycolic acid) (PLGA) is an excellent polymer for drug delivery applications due to its nontoxicity, biocompatibility, and nonimmunogenicity[2]. It is approved by the US food and drug administration (USFDA) for human use as surgical sutures, implantable devices, and drug delivery systems. Depending on the ratio of lactides to glycolides used for polymerization, different forms of PLGA are available: PLGA(85:15), PLGA(75:25), PLGA(65:35), and PLGA(50:50)[3].

Doxorubicin is a chemotherapeutic agent used to treat cancers such as breast cancer, bladder cancer,Kaposi's sarcoma, lymphoma, and acute lymphocytic leukemia[4]. The success of the treatment can be improved by formulating it into controlled drug delivery as it would offer long- term sustained exposure and moderate drug concentrations in the body. This would further improve patient compliance due to the reduced dosage frequency. The present study developed PLGA-based nanoparticles of doxorubicin for its extended drug release and investigated their therapeutic efficacy in breast cancer cell lines.

Material and methods

Doxorubicin was a gift sample from Sun Pharmaceuticals, Gujarat, India. PLGA ratio 65:35 was procured from Sigma-Aldrich India Pvt. Ltd, Bengaluru, India. Polyvinyl Alcohol was purchased from SD Fine-Chemicals, Mumbai, India. Dichloromethane (DCM) was purchased from Merck life science Pvt. Ltd, Bengaluru, India. Pluronic F-127 was purchased from Sigma- Aldrich IndiaPvt. Ltd, Bengaluru, India.

Preparation of drug-loaded nanoparticles using PLGA

The biodegradable polymer poly (D,L-Lactide-co-glycolide) based nanoparticle is prepared by multiple emulsification and solvent evaporation method[5]. The required amount of PLGA was taken in a beaker and dissolved in DCM. A specific percentage of PVA solution containing the drug was prepared.Theaqueous phase containing PVA, and drug was added dropwise into the oil phase and homogenized with a high-speed homogenizer with a specific speed for 4 minutes.The primary emulsion (w/o) formed was added dropwise into the aqueous phase containing PVA with a specific concentration and homogenized at the same speed for 6 minutes to form a multiple emulsion (w/o/w).The prepared emulsion was placed on a magnetic stirrer and stirred for 12 hours at room temperature to evaporate organic solvent such as DCM.The nanoparticles were washed thrice using double distilled water by cooling centrifugation at 4°C at 16000 rpm for 40 minutes.Particles were then freeze-dried (by pre-freezing at – 20°C overnight and lyophilizing at – 40°C for 12 hours) in a lyophilizer and stored at 4°C.

The formulations are listed in **Table 1.**

Table 1. Doxorubicin-PLGA formulations using different ratios if the drug and polymer

Formulations	**PLGA : DRUG**	**Speed of Homoginization**	**Stabilizer Used**
NPF1	30 : 2	12000	Pluronic – 127 (0.5% w/v)
NPF2	30 : 2	14000	Pluronic – 127 (0.5% w/v)
NPF3	20 : 2	16000	Tween-80
NPF4	20 : 2	21000	Tween-80

Evaluation and characterization of doxorubicin-loaded nanoparticles

Drug release study

To measure the drug release of prepared nanoparticles at the different time points, 5 mg of nanoparticles were suspended in 1 ml phosphate buffer saline (PBS) pH 7.4 in prelabeled microcentrifuge tubes and kept in an incubator shaker (Somex incubator Shaker) at 37°C with constant shaking at 72 rpm after short vortexing. Formulations were processed in triplicate, and samples were kept for a specific period. At any particular time point, only the sample for analysis was removed from the shaker, centrifuged at 15000 rpm for 30 min at 4°C, and the drug from the supernatant was analyzed at a wavelength of 480 nm with a UV-Visible spectrophotometer (Beckman Instruments).

In vitro cellular uptake study

Confocal laser scanning microscopy was used to visualize the uptake of the polymeric nanoparticles within the cancer cells. For fluorescence imaging of cellular uptake, MCF-7 cells using method suggested by Mani etal.[6].

Cytotoxicity study

MTT assay for Anti-Cancer activity in MCF-7 breast cancer cells

We performed the preliminary in-vitro anti-cancer evaluation of the synthesized nano- formulation against the MCF-7 human breast cancer cell line using the MTT assay[7].The cells were grown in 96-well plates at a density of 1.5 × 104 cells/well.Doxorubicin was added at various concentrations into the wells. At 48 h after treatment, the medium in the wells was replaced with 100 µL/well of medium containing 0.5 µg/µL MTT and incubated for 4 h. Subsequently, the medium was removed, and 100 µL DMSO was added to each well to dissolve the formazan crystals. The absorbance of the samples was measured at650 nm.

Acridine orange/ethidium bromide (AO/EB) staining

The AO/EB staining assay was performed as suggested by Liu et al. to differentiate between live and apoptotic cells. AO dye permeated the live cells' cell membrane and stained the nuclei green,while EB dye can permeate the disintegrated membrane.[8]

4′,6-diamidino-2-phenylindole (DAPI) nuclearstaining

4′,6-diamidino-2-phenylindole(DAPI),the blue-fluorescent DNA stain, was used to enhance fluorescence by ~20-fold upon binding to AT regions of dsDNA and visualizing the nuclear feature after treating the cells with doxorubicin. [9]

2', 7' dichlorodihydrofluorescein diacetate (DCFDA) staining

The anti-cancer agents generally induce ROS formation inside the cancer cells and cause oxidative damage to the cell. DCFDA (2', 7' dichlorodihydrofluorescein diacetate)staining was used to quantify the intracellular ROS levels.[10]

Results and Discussion

Evaluation and characterization of doxorubicin-loaded nanoparticles

Of the four formulations, the Doxorubicin-loaded nanoparticles (DNP) of NPF3performed best as they showed an average diameter of 120±9.8nm, with a polydispersity index (PDI) of 0.330±14.3 and zeta potential of -10.8±0.008. This formulation showed the maximum drug loading of 8.33 ± 0.15 and the highest entrapment efficiency of 91.63 ± 0.3%

Drug release study

The comparative release of doxorubicin as a pure compound and when formulated into PLGA nanoparticles is given in **Figure 1.**

The figure demonstrated that more than 99% of drugs getreleased immediately after administration when tested as a pure compound. However, an extended release was observed when doxorubicin was formulated as PLGA nanoparticles with NPF1 and NPF2, showing the drug release over a period of 2 days and NPF3 and NPF4 over the period of 4 days.

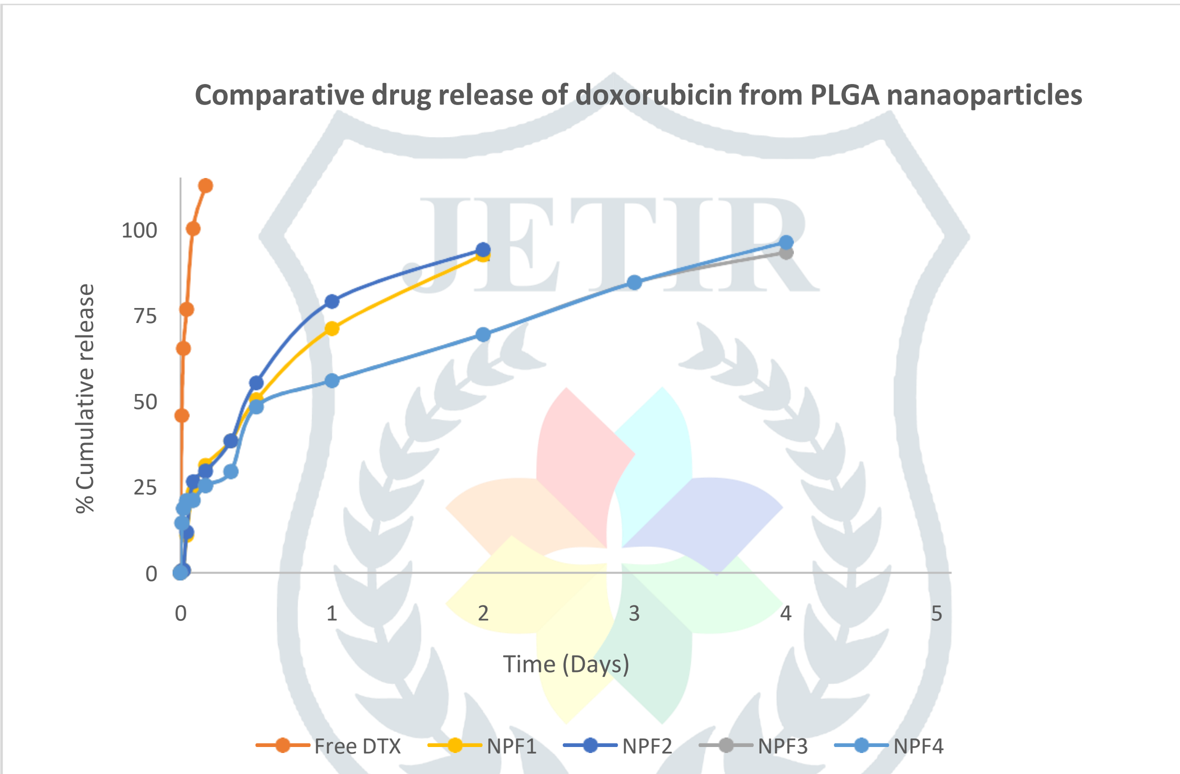

Figure 1. Cumulative release of doxorubicin from different formulations

Cellular uptake study through FITC

The results of cellular uptake of the drug are exhibited in figure 2. The green dots in Figure **2B** demonstrates the cellular uptake of the doxorubicin-loaded nanoparticles in MCF-7 cells.

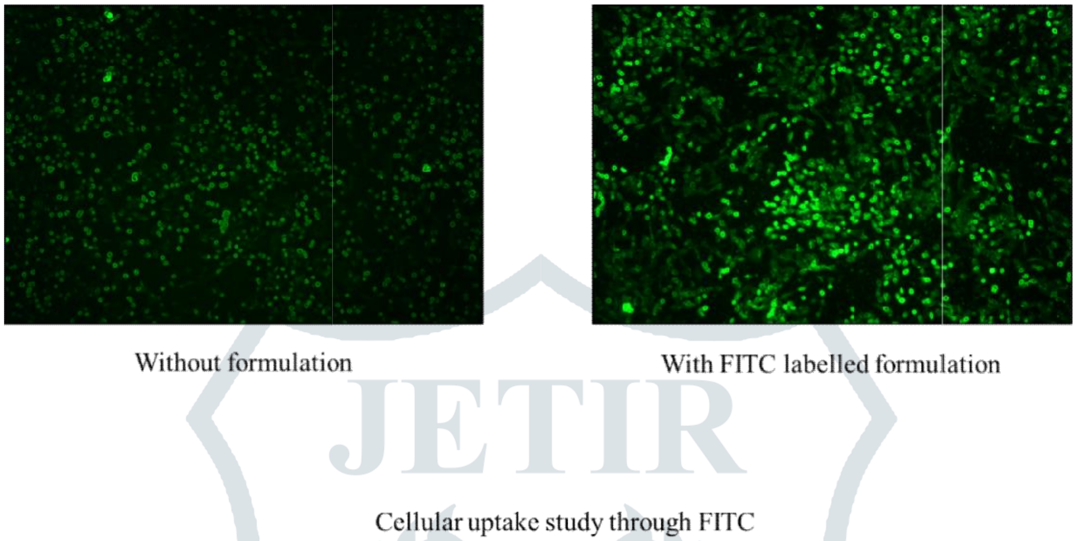

Figure 2A: Cellular uptake of pure doxorubicin; Figure 2B: Cellular uptake of doxorubicin with FTIC labeled doxorubicin nanoparticles

Cytotoxicity study

MTT assay for Anti-Cancer activity in MCF-7 breast cancer cells

MTT assay of the synthesized NP demonstrated that the IC50 value of doxorubicin was **0.906 µg/ml**, which was calculated after plotting the concentration of doxorubicin vs. the % inhibitionas presented in **Figure 3.**

Similarly, earlier studies also revealed the similar activity [11, 12].

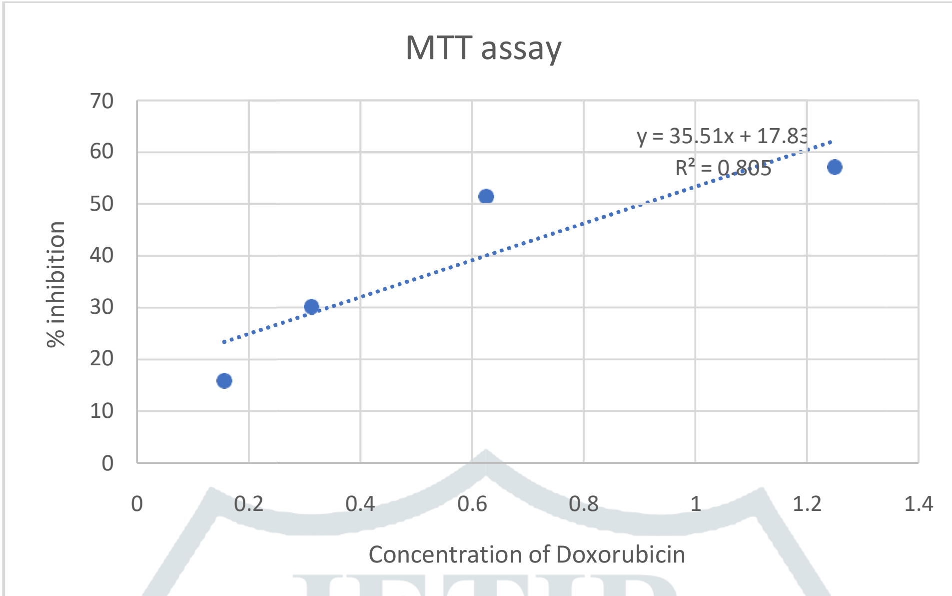

Figure 3. MTT assay of doxorubicin Nanoparticles NPF3

Acridine orange/ethidium bromide (AO/EB) staining

The AO/EB staining assay differentiated between live and apoptotic cells. AO dye permeates the live cells' cell membrane and stains the nuclei green, while EB dye can permeate the disintegrated membrane. **Figure 4** demonstrated that untreated cells had abundant healthy nuclei without the formation of any apoptotic bodies, while the doxorubicin treated cells had the abundant red stain, thus showing the formation of apoptotic bodies and disintegration of cell membrane integrity. The figure clearly depicts the initiation of apoptosis in MCF-7 cells.

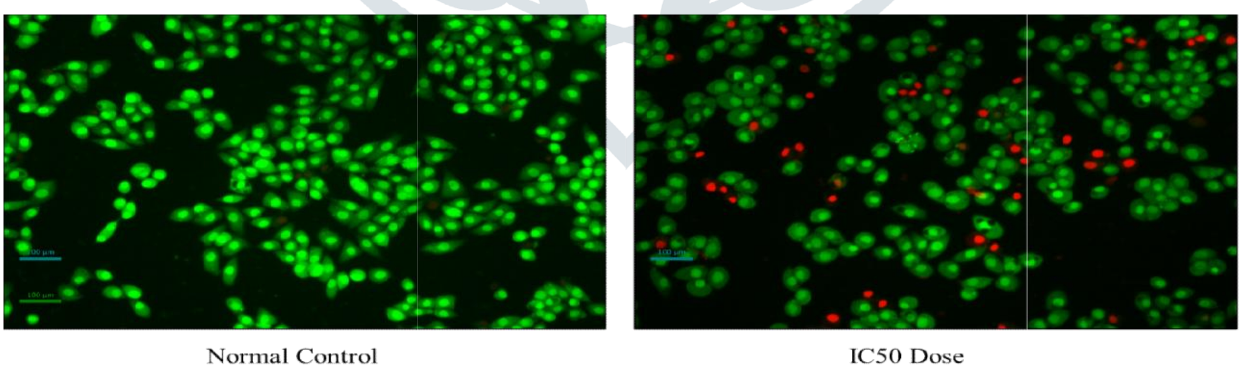

Figure 4. Acridine orange/ethidium bromide (AO/EB) staining: A: Normal control; B: Doxorubicin in IC 50 dose

DAPI staining

DAPI (4′,6-diamidino-2-phenylindole) is a blue-fluorescent DNA stain that xhibits ~20-fold enhancement of fluorescence upon binding to AT regions of dsDNA. Thus, it assists in visualizing the nuclear feature. It stains the condensed nuclei bright blue. The DAPI staining of the untreated cells showed an intact nuclear structure without any condensation, while the IC50 treated cells showed condensed nuclei with horseshoe-shaped nuclei. Hence, the figure well depicts the nuclear condensation and nuclear damage.

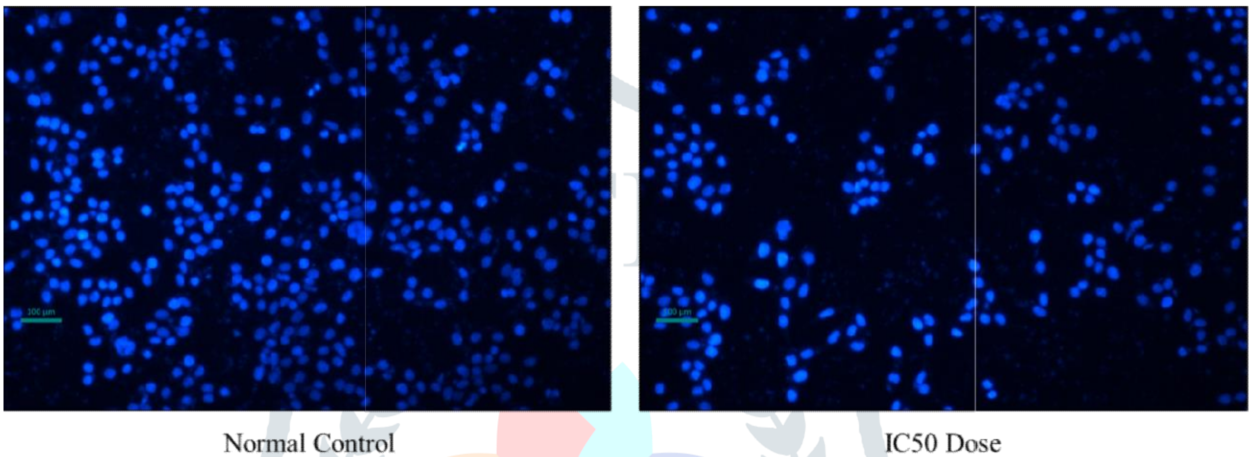

Figure 5: DAPI nuclear staining

DCFDA staining

The anti-cancer agents generally induce ROS formation inside the cancer cells and cause oxidative damage to the cell. DCFDA (2', 7' dichlorodihydrofluorescein diacetate) is a dye for ample detection of ROS. DCFDA is a non-fluorogenic molecule that gets oxidized to DCF under the presence of ROS and shows green fluorescence. The untreated cells depict no green signal and zero formation of ROS, while the IC50 treated cells have shown ample green coloured fluorescence, thus implying ROS formation in the treated cells.

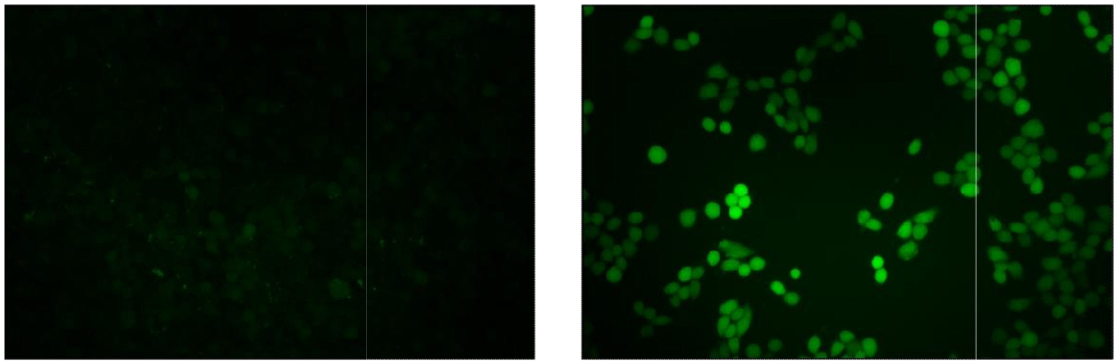

Figure 6. DCFDA staining for ROS detection

Conclusion

In vitro cytotoxicity screening using MTT assay presented formulation NPF3 as the potential carrier with an IC_{50} value of **0.906 μg/ml** in MCF-7 breast cancer cell lines. Apoptotic studies such as the observation of morphological changes, AO/EB and DAPI staining, and reactive oxidative species (ROS) generation studies revealed the induction of apoptosis by the lead compound **NPF3.** Hence, the study concludes that doxorubicin can be formulated into PLGA nanoparticles for extended delivery and improved efficacy as a chemotherapeutic agent.

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