Journal of Drug Delivery Science and Technology 66 (2021) 102867

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Development of curcumin and docetaxel co-loaded actively targeted PLGA nanoparticles to overcome blood brain barrier

Indrit Seko [a], Hayrettin Tonbul [b], Ece Tavukçuoğlu [c], Adem Şahin [d], Sedenay Akbas [a], Hamdullah Yanık [c], Süleyman Can Öztürk [c], Gunes Esendagli [c], Mansoor Khan [e], Yilmaz Capan [a,f,*]

[a] Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey
[b] Department of Pharmaceutical Technology, Faculty of Pharmacy, Inonu University, Malatya, Turkey
[c] Department of Basic Oncology, Cancer Institute, Hacettepe University, Ankara, Turkey
[d] Department of Pharmacy Service, Vocational School of Health Services, Bilecik Seyh Edebali University, Bilecik, Turkey
[e] Rangel College of Pharmacy, Texas A&M University Health Science Center, College Station, TX, USA
[f] Department of Pharmaceutical Technology, Faculty of Pharmacy, Lokman Hekim University, Ankara, Turkey

ARTICLE INFO

Keywords:
PLGA Nanoparticles
Blood brain barrier
Combination drug delivery
Polysorbate 80

ABSTRACT

The aims of this study were to develop and characterize curcumin (CCM) and docetaxel (DTX) co-loaded poly lactide-*co*-glycolide (PLGA) nanoparticles (NPs) to overcome blood brain barrier. The cytotoxicity of the obtained curcumin and docetaxel co-loaded polysorbate 80 coated PLGA NPs were studied in U87 glioma cells and bEND.3 endothelial cells. The IC50 values are determined for both cell lines. *In vitro* release profile of the optimized formulation approximately 27% of DTX was released in the 1. hour and after a steady controlled release the DTX released percentages plateaued after 48. hour. *In vitro* curcumin release profile had a more controlled released by releasing less than 8% in the 1. hour and plateaued after 48. hour at approximately 78% curcumin released. Polysorbate 80 coated DTX-CCM-PLGA NPs showed no cytotoxicity and had better uptake in bEND.3 cells than uncoated DTX-CCM- PLGA NPs. The combination of CCM and DTX in PLGA nanoparticles showed a significant increased cytotoxic activity compared to CCM and DTX solutions, CCM loaded PLGA NPs and DTX loaded PLGA NPs. Moreover, in vivo biodistribution studies show that polysorbate 80 coating significantly improve brain penetration. Polysorbate 80 coated CCM and DTX loaded PLGA Nanoparticles can be potentially useful in the treatment of glioma by increasing the delivered quantity of drug in the brain through blood-brain barrier.

1. Introduction

Nanoparticles (NPs) are colloidal particle consisting of natural or synthetic materials which range in diameter size between 1 nm and 1.000 nm [1]. Although the size range of NPs is wide, most of the nanoparticles designed and synthesized nowadays are less than 200 nm in diameter. Due to their larger surface area to volume ratio, NPs exhibit special physical, chemical and biological feature compared to larger particles. It provides better pharmacokinetics and biodistribution for low availability, unstable and high toxicity drugs. NPs can be optimized by controlling the particle size, polydispersity (PDI), surface properties and drug release rate to ensure drug targeting and designed controlled drug release.

Conventional antineoplastic agents demonstrate dose limiting serious adverse effects due to limited targeting abilities. Docetaxel (DTX) has been shown to be effective in the treatment of gliomas using local delivery methods [2]. In order to successfully achieve a high local DTX concentration and limit cytotoxic activity adverse effects during intravenous administration, DTX may encapsulated by poly D,L-lactide-*co*-glycolide (PLGA) polymeric NPs. PLGA NPs are biocompatible and biodegradable carrier systems that are widely used as sustained drug release application [3]. The clinical success of DTX has been limited by multidrug resistance (MDR) [4] which restricts it long term and high dose usage. Combining DTX with another antitumor and chemosensitizing agent can increase the effectivity and reduce the adverse effects of DTX. Curcumin (CCM) is a well-tolerated polyphenol compound originating from the rhizome of *Curcuma Longa* plant and shows chemoprotective activity [5–8]. Curcumin has demonstrated in

* Corresponding author. Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 06100, Ankara, Turkey.
E-mail addresses: indritseko@yahoo.com (I. Seko), hayrettin.tonbul@inonu.edu.tr (H. Tonbul), ycapan@hacettepe.edu.tr (Y. Capan).

https://doi.org/10.1016/j.jddst.2021.102867
Received 1 June 2021; Received in revised form 2 September 2021; Accepted 20 September 2021
Available online 22 October 2021
1773-2247/

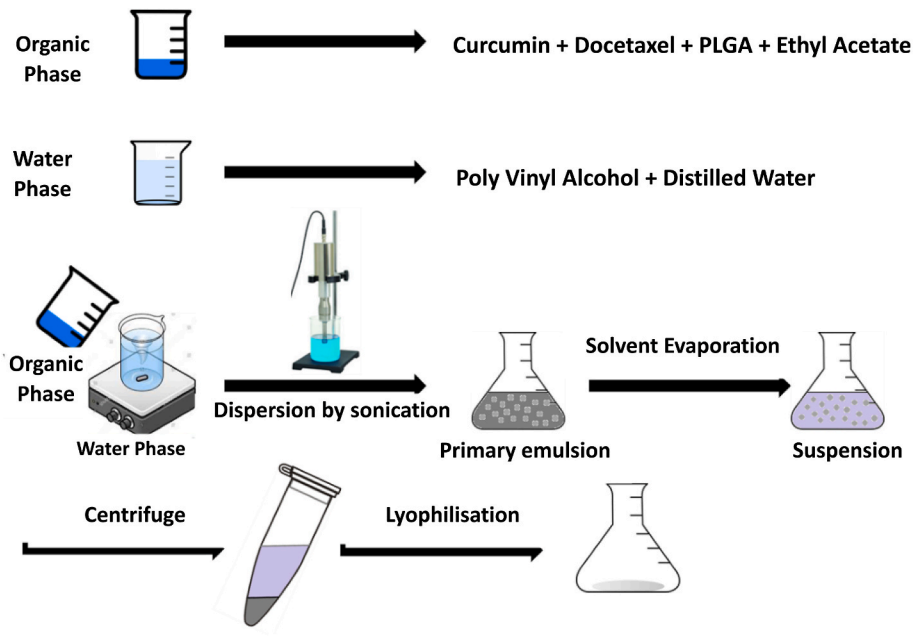

Fig. 1. The preparation of docetaxel and curcumin loaded PLGA nanoparticles.

preclinical and animal studies anticancer activity by various action mechanism associated with cell proliferation and metastasis inhibition, induction of apoptosis, chemosensitizing and progenitor cell proliferation inhibition [9–11]. The oral bioavailability of curcumin is less than 1%, subsequently the bioavailability can be enhanced by encapsulating in PLGA NPs [12].

The aim of this study is combining docetaxel with highly tolerable curcumin which has chemosensitizing effect it would decrease the effective dose administered during treatment and obtain an actively targeted co-delivery system with polysorbate 80. By encapsulating both DTX and CCM in the biocompatible PLGA NPs coated with polysorbate 80 would protect the drugs from the rapid clearance from the blood and increase the uptake by the endothelial cells in the brain capillaries which is also proved by in vitro uptake cell studies and in vivo by showing accumulation of the NPs using in vivo imaging system.

2. Experimental

2.1. Materials

Docetaxel and curcumin were donated by ILKO Pharmaceuticals, Turkey. PLGA (lactide:glycolide ratio of 50:50, acid and ester terminal groups, 7–17 kDa of molecular weights), dichloromethane, polyvinyl alcohol (PVA), polysorbate 80, acetonitrile, ethanol, 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT), methanol, formic acid, and phosphate-buffered saline (PBS) tablets were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin–streptomycin, L-glutamine, and Dulbecco'smodifiedEagle'smedium (DMEM) were purchased from BioChrom AG (Berlin, Germany). All chemicals were either extra pure or chromatography grade.

Table 1
Chromatographic conditions for curcumin and docetaxel assay.

Parameter	Curcumin	Docetaxel
Column type (Stationary phase)	Zorbax C18, Agilent 150 × 4.6 mm (ODS)	Zorbax C18, Agilent 150 × 4.6 mm (ODS)
Flow rate (ml/s)	1.0	1.2
Column temperature (°C)	45	45
Wavelength (nm)	425	230
Analysis time (minute)	15	10
Injection volume (μL)	10	10
Mobile Phase	Acetonitrile: 5% Acetic Acid (40:60)	Acetonitrile:Water (45:55)

2.2. Preparation of docetaxel and curcumin loaded nanoparticles

PLGA nanoparticles were prepared by single emulsion preparation method [13] and preliminary studies was conducted to obtain optimum formulation parameters. Firstly, the organic phase was formed by dissolving of 42,5 mg PLGA, docetaxel and curcumin in 1.1 mL ethyl acetate. The aqueous phase (10 mL) was formed by dissolving 1% of the PVA. The organic phase was added on the aqueous phase, slowly and continuously by stirring at 550 rpm on a magnetic stirrer and right after, the emulsion was sonicated with an ultrasonic probe at 25% power, 5× periods, under an ice-water bath for 1 min (6 times with 10 s intervals). The organic phase was allowed to evaporate with continuous stirring for 24 h. After the organic solvent has been evaporated, the PLGA nanoparticles are precipitated by centrifugation at 14,500 rpm for 30 min and the supernatant is stored for drug quantification analysis and the precipitation was washed 3 times with distilled water. Finally, NPs were suspended in a solution containing 1 ml of 5% (w/w) mannitol and frozen at −60 °C and lyophilized (Fig. 1).

2.3. Nanoparticle coating and polysorbate 80 assay method

The lyophilized nanoparticles were mixed for 30 min in 10 ml of 1% polysorbate 80 (w/v) solution for the coating process. To remove the residual polysorbate 80, the sample was centrifuged at 14,500 rpm for 15 min and the supernatant was stored. The remaining polysorbate 80 coated PLGA nanoparticles were washed twice with 10 mL of distilled water and the supernatant was stored again. To find the amount of adsorbed polysorbate 80 on the PLGA nanoparticle surface, the remaining supernatants were assayed for polysorbate 80. The UV spectrum of the polysorbate 80 solution was taken (between 190 and 380 nm) and the maximum wavelength was observed at 234 nm. Absorbance of 0.015%, 0.031%, 0.062%, 0.125% and 0.25% polysorbate 80 (w/v) solvents at 234 nm wavelength were measured and the calibration curve plotted. Amount of polysorbate 80 was determined by using the calibration curve.

2.4. Drug assay and chromatographic conditions

Analytical methods were developed for determination of curcumin and docetaxel by high performance liquid chromatography (HPLC) (Agilent UV 1200, CA, USA), UV and visible region detection method. The HPLC-UV device consists of a 4-channel, automatic pump, automatic sampler, column thermostat, UV-GB detector and a C18 column (Zorbax C18, Agilent, CA, USA). Chromatographic conditions of both drugs are summarized in Table 1. The detection wavelength for curcumin was 425 nm under visible spectrum and for docetaxel was 230 nm under UV spectrum, therefore there was no overlapping between drugs in chromatograms. The prepared samples were first degassed and then filtered using 0.22 μm pore sized polytetrafluoroethylene (PTFE) filters to avoid any syringe to clog. All the mobile phases as well were of chromatographic quality, were degassed and filtered with 0.22 μm pore sized PTFE filters before use. Both assay methods were validated according to ICH Validation Of Analytical Procedures (Q2).

2.5. Particle size distribution and zeta potential

The particle size, polydispersity (PDI) and zeta potentials of the nanoparticles were investigated by dynamic light scattering (DLS) method using the 173° angle (Zetasizer Nano ZS Malvern Instruments Ltd., Worcestershire, UK). Firstly, lyophilized nanoparticles were suspended in pure water and the measurements were performed at room temperature. Mean nanoparticle size, PDI and zeta potential measurements were repeated three times. Z-average mean value was used as parameter to measure particle size.

2.6. Fourier transform infrared spectroscopy

In order to evaluate the state and any possible interaction of curcumin, docetaxel and PLGA in the formulation, blank PLGA nanoparticle, curcumin-docetaxel loaded PLGA nanoparticle and polysorbate 80 coated curcumin-docetaxel loaded PLGA nanoparticle were examined with Fourier transform infrared (FTIR). Scanning with Cary 630 FTIR Spectrometer (Agilent, CA, USA) at a resolution of 4 m^{-1} in the range of 750–4000 cm^{-1} wave number was performed 10 times for each sample.

2.7. Scanning Electron Microscope

Scanning Electron Microscope (SEM) was used to examine the surface properties and circularity of the nanoparticles displayed. An accurate measurement was made taking into account the properties of both the nanoparticles and the instrument. The Quanta 400F high resolution field emission SEM (FEI, OR, USA) instrument with 10 kV acceleration voltage was used. Nanoparticles were placed on carbon bands and spray-coated with gold-palladium mixture at 3–5 nm thickness.

2.8. Transmission Electron Microscope

Transmission Electron Microscope (TEM) was used to examine the size and size distribution of the nanoparticles displayed. Suspended PLGA nanoparticles were dropped onto the grid and dried at room temperature. The dried samples were examined with Tecnai G2 Spirit Biotwin (FEI, OR, USA) in the range of 20–120 kV.

2.9. Encapsulation efficiency

The amount of encapsulated drug was calculated by subtracting the amount of unencapsulated drug at the end of the nanoparticle centrifugation from the amount of curcumin and docetaxel added during preparation to calculate the encapsulation efficiency (the amount of drug encapsulated inside and adsorbed at the surface the nanoparticle). The amount of drug from the nanoparticles washed three times during preparation was also included. Quantitation of both curcumin and docetaxel was repeated three times.

Encapsulation efficiency was determined according to the formula below:

$$\text{Encapsulation efficiency (\%)} = (\text{Amount of drug in nanoparticles} / \text{Amount of drug in formulation}) \times 100$$

2.10. In vitro drug release

90% of the amount of curcumin deteriorates rapidly in buffer systems under neutral-base pH (pH 7.0–7.4) [14,15]. In human blood and in PBS (pH 7.4) medium containing 10% foetal bovine serum (FBS), less than 20% curcumin deteriorated within 1 h, and after 8 h of incubation, more than 50% curcumin remained stable [14]. 0.1% polysorbate 80 was added to the release medium to increase the solubility of curcumin and docetaxel in the solution and to fulfil the sink condition [16]. To assess the amount of curcumin and docetaxel released in vitro, the same amount of nanoparticle was dispensed into ependorf tubes in 10 mL medium of phosphate buffered saline (PBS) containing 0.1% (w/v) polysorbate 80 and 10% FBS. The eppendorf tubes were maintained at 37 °C and shaken in a water bath at a rate of 100 rpm. Samples were taken at predetermined time points as 1st, 2nd, 4th, 8th, 12th, 24th, 48th, 72nd and 96th hour (3 eppendorf tubes containing the same amount of NPs for each time period) and centrifuged at 14,500 rpm for 30 min. The supernatant taken at each time point was filtered with a 0.22 μm PTFE filter and the amount of drug released was calculated by curcumin and docetaxel quantification by HPLC. After each sample was taken for each time period they were discarded.

2.11. Cell culture studies

bEnd.3 and U87 glioma cell lines were obtained from the American Type Culture Collection (ATCC, LGC promochem, Rockville, MD, USA). Cells were supplemented with 10% fetal bovine serum (FBS), 1% U/ml streptomycin and 1% U/mL penicillin in high glucose DMEM and incubated at 37 °C in a humidified atmosphere of 5% CO 2. The cytotoxicity was measured by MTT method. To obtain the optimal optical density for bEnd.3 and U87 cells were seeded in 96-well plates at a density of 10,000 and 12,500 cells/well, respectively, for 24 h to allow cells to adhere to the above-mentioned conditions. Blank PLGA nanoparticles should not be cytotoxic because the drug is intended to cross the BBB without causing damage to endothelial cells and show toxicity to glioma cells. Therefore, the blank PLGA NPs (both polysorbate 80 coated and uncoated), which are both the ester terminated and the acid terminated, were evaluated for cytotoxicity in bEnd.3 cells for 48 h. The effect of PLGA polymer on the toxicity of ester and acid terminal groups was also investigated. All calculations were made according to DTX cytotoxicity value ($n = 4$). IC 80 (the amount of DTX required to kill 80% of the cells) is evaluated as non-cytotoxic for blank PLGA NPs ($p < 0.05$). The polysorbate 80 coating effect on the bEnd.3 endothelial cells was also evaluated. The cytotoxicity of the curcumin and docetaxel solutions on U87 cell was determined. Cytotoxicity was measured for curcumin solution concentrations at 0.039, 0.078, 0.15, 0.312, 0.625, 1.25, 2.5, 5 and 10 μM and for docetaxel at 12.5, 25, 50, 75, 100, 125, 150 and 200 μM concentrations. IC 50 values for curcumin and docetaxel were calculated. The optimum curcumin/docetaxel ratio was determined to increase the efficacy and decrease the administered dose of docetaxel. DTX amount is fixed at IC 50 concentration and the amount of curcumin loaded into PLGA NPs is calculated according to curcumin/docetaxel ratio as 1, 2, 4, 8 and 16. Finally, cytotoxicity was compared in U87 cells with curcumin solution, docetaxel solution, blank PLGA NP, docetaxel loaded PLGA NP, curcumin loaded PLGA NP, curcumin-docetaxel loaded PLGA NP and polysorbate 80 coated curcumin-docetaxel loaded PLGA NP formulations.

Table 2
Particle size and PDI data of the optimum formulation (n = 3).

Name	Particle Size (nm)	Difference (nm)	PDI	Difference (nm)	Zeta Potential (mV)	Difference (mV)
Blank PLGA NP	128.9 ± 0.9	–	0.029 ± 0.014	–	−9.81 ± 0.57	–
CCM-DTX-PLGA NP	151.2 ± 1.6	22.3	0.057 ± 0.017	0.028	−13.67 ± 0.65	−3.87
Poly 80- PLGA NP	145.4 ± 1.0	16.5	0.077 ± 0.010	0.048	−9.68 ± 2.53	+0.13
Poly 80-CCM-DTX-PLGA NP	163.7 ± 0.9	34.8[a]	0.090 ± 0.022	0.061[a]	−12.80 ± 2.53	−2.99[a]
		12.5[b]		0.033[b]		+0.87[b]

[a] Difference from coating and drug encapsulation.
[b] Difference from coating.

To investigate intracellular uptake of PLGA NPs and impact of polysorbate 80 coating, nil red loaded NPs was used. Besides the nile red has fluorescence, it has high hydrophobic properties, like docetaxel and curcumin, and high encapsulation efficiency in PLGA NPs. Cellular uptake of nanoparticles was investigated in bEnd.3 and U87 cell lines using both flow cytometry and fluorescence microscopy.

Flow cytometry studies were carried out to quantitatively determined nanoparticle cell uptake after 30 min, 1,2, 4 and 8 h incubation period of polysorbate 80 coated and uncoated PLGA NP formulations. Mean fluorescence intensity (MFI) of the cells was measured to compare uptake quantities. To evaluate cellular uptake with fluorescence microscopy, same nanoparticle groups and same cell lines used and forumulations incubated for 4h. Then the cells were washed and harvested, cytospin preparations were obtained and fixed with 4% paraformaldehyde. Following counterstaining with DAPI the cells were examined by fluorescence microscopy (Olympus America Inc, Center Valley, PA) and the images were processed with ImageJ software.

2.12. In vivo studies

In order to evaluate ability of nanoparticles on passing blood brain barrier, in vivo studies conducted and CD1 mice were used. Prior to each procedure, ketamine i.p. at a dose of 50 mL/kg and xylazine 4 mg/kg the mice are anesthetized. A catheter is inserted into the tail vein. To determine the amount of NP penetrated into the brain by the effect of the polysorbate 80 coating a total of 7 mice will be randomly divided into 2 groups (n = 3) and the remaining 1 mouse will be used as the control group. Groups will be divided as follows:

- Control Group (the control group to be used alone to observe the effect of PBS, 0.2 mL PBS) (n = 1)
- Experimental Group 1 (Flamma 774 dye loaded PLGA NP.) (n = 3)
- Experimental Group 2 (Polysorbate 80 coated Flamma 774 dye loaded PLGA NP.) (n = 3)

All injections are administered by i.v. as a single dose from the tail vein. The volume to be given to each group was determined as 0.2 mL (dissolved in PBS solution). Since flamma 774 is a fluorescent substance, it has been used as a dye during imaging with In Vivo Imaging System (IVIS) to determine whether NPs cross the BBB and pass into the brain [17].

2.13. Statistic evaluation of the results

All experiments were repeated at least triplicate and the standard deviation was shown as "±". Minitab®17 software was used during all the calculations. *t*-student test was used for comparison of two groups, for comparison of more than two groups ANOVA was used and afterwards the Tukey's post hoc test. p-value greater than 0.05 was considered to be a significant difference between the groups.

3. Results and discussion

3.1. Characterization of nanoparticles

The docetaxel-curcumin loaded PLGA NPs developed within the scope of the study were synthesized by using primary emulsion preparation method due to hydrophobic characteristic of the active ingredients. As a result of the literature research and preliminary studies, ethyl acetate which is miscible with water was selected as organic phase solvent, PLGA used as NPs polymer due to biocompatibility properties and PVA used as emulsifying agent due to its widely usage to stabilize emulsions with uniform size distribution, small size NPs and having excellent stabilizing properties on NPs emulsion [18]. During the preliminary studies, in order to better understand the effects of PVA concentration on NP characterization properties, varies concentrations of PVA were used in formulation to determine the changes in characterization parameters. Although the particle size is relatively higher at the 1% PVA concentration, PDI values are found to be lower [19]. Ethyl

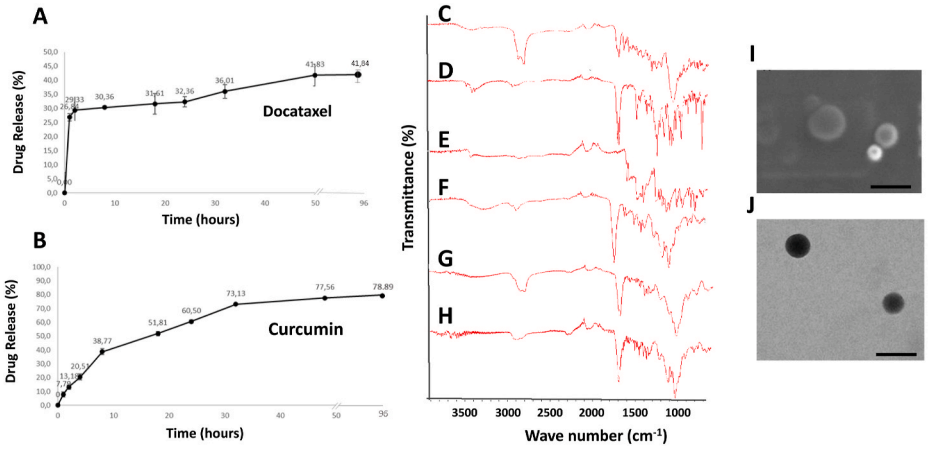

Fig. 2. Characteization of nanoparticles. In vitro drug release of **A.** Docataxel and **B.** Curcumin from polysorbate coated PLGA NPs. FTIR spectrum **C.** PLGA, **D.** Docataxel, **E.** Curcumin, **F.** Docatael-curcumin-PLGA mixture, **G.** Docataxel-Curcumin co-loaded PLGA NPs, **H.** Polysorbate 80 coated docetaxel-curcumin co-loaded PLGA NPs. **I.** SEM and **J.** TEM images of docetaxel-curcumin co-loaded PLGA NPs (scale bar is 100 nm).

Table 3
Average data on polysorbate 80 coating amounts.

PLGA Type	Absorbance (234 nm)	Adsorbed Polysorbate 80 (mg)	Coating (%)	Polysorbate 80/PLGA (mg/mg)
Acid Terminated	2048	9.3	9.3%	0.22
Ester Terminated	2038	9.7	9.7%	0.23

acetate was chosen because PLGA NPs with highly reproducible sizes and relatively narrow polydispersity were prepared [19,20]. Sound waves generated by ultrasonic probe were used to generate primary emulation. The strength, duration and intervals of the sound waves affect the NP characterization values. As a result of preliminary study and literature research, the low power, longer period and high intervals of the sound waves decreased the risk of PLGA degradation, increased the stability of NPs and the decreased PDI values [21]. As a result, the particle size, PDI and zeta potential of the optimum blank PLGA NPs formulation were found 128.9 nm, 0.029 and −9.81 mV, respectively and particle size was slightly increase to 151.2 nm when docetaxel and curcumin was co-loaded (Table 2.). After DLS analysis, SEM images were taken to examine surface morphology and TEM images to examine particle size and shape. In the images obtained from SEM and TEM analysis, the NP surface is smooth and shape is spherical (Fig. 2I and J). However, particle sizes were found to be slightly smaller compared to DLS results. In the case of DLS, theoretically, it states that when a particle passes through a liquid medium, the solvent adheres to the surface of a thin electric dipole layer. This layer affects the movement of the particle in the environment. Therefore, the hydrodynamic diameter gives information about the nanoparticle under the influence of Brownian motion, together with any coating material and a layer of solvent attached to the particle. When estimating size with SEM and TEM, this hydration layer is not available, thus only information about the particle is obtained. Due to the hydration layer formed around the PLGA NPs, the NP size obtained by DLS analysis is slightly larger than the SEM/TEM images. Similar conclusions and comments are found in the literature [22,23].

3.2. Polysorbate 80 coating

Along with a passive tumor targeting approach (i.e. EPR effect) for active tumor targeting strategies (e.g. ligand-receptor interaction), the outer surface of PLGA-based NPs has been designed with various coatings. To increase the blood circulation time, hydrophilic polymers such as polysorbate 80, PEG and poloxamers are usually coated on the surface of PLGA NPs. Polysorbate 80 assay method has been developed to examine whether the adsorption amount is dependent on PLGA terminal functional groups (ester or acid) to ensure a successful polysorbate 80 coating.

After the polysorbate 80 coating process, particles size and zeta potential were slightly increase that is considered one of the indicators for polysorbate 80 coating successfully achieved (Table 2). Moreover, no significant difference was observed in the polysorbate 80 adsorption amount values obtained for both PLGA types (Table 3). Since the ester terminate PLGA is less cytotoxic in bEnd.3 endothelial cells, in all other studies the ester terminated PLGA NP was used.

3.3. Fourier transform infrared spectroscopy

FTIR analyzes was performed to observe the possible interaction between docetaxel, curcumin and PLGA polymer, effect of active ingredients to nanoparticles and condition of polysorbate 80 coating. FTIR results are shown in Fig. 2C–H. The main peak (ester group stretching) of docetaxel was observed at 1722 cm $^{-1}$, the curcumin characteristic peak at 1628 cm^{-1} due to the stretching of C = C, and the characteristic vibration of the PLGA carboxylic acid at 1760 cm^{-1} [24,25].When looking at docetaxel and curcumin loaded PLGA nanoparticles, curcumin and docetaxel specific peaks have been significantly decreased. The curcumin and docetaxel were encapsulated almost completely to the nanoparticles, so the intensities of these peaks have been decreased. Due to the PLGA CH stretch, the characteristic peak (2885-3010 cm^{-1}) was observed at 2985 cm^{-1}, however the peak strength was decreased after polysorbate 80 coating confirming that coating took place. In the FTIR analysis with docetaxel, curcumin, docetaxel-curcumin, PLGA, docetaxel-curcumin-PLGA physical mixture and docetaxel and curcumin loaded nanoparticles, no new peaks of new functional groups that may be an indicator of chemical incompatibility have not been observed [26]. In the light of all FTIR data, it can be concluded that docetaxel and curcumin loaded PLGA nanoparticles were successfully prepared and then polysorbate 80 coating was successfully performed.

3.4. Encapsulation efficiency and in vitro drug release

HPLC results show that curcumin and docetaxel were successfully co-loaded to PLGA NPs with 99,77 ± 0,5 and 95,65 ± 3,21 encapsulation efficiency. In drug release studies, 10% FBS was added to the release medium to increase stability of curcumin, since curcumin is unstable at normal pH 7.4 PBS solution [14,15]. Both docetaxel and curcumin have a low solubility in water due to hydrophobic structures. To ensure the sink condition, 0.1% polysorbate 80 was added to the release medium. Docetaxel and curcumin release profiles of curcumin-docetaxel loaded PLGA NP formulations are shown in Fig. 2A and B. As shown, docetaxel showed an initial 'burst release' of 29.3% during the first 2 h of incubation and released 41.84% of the amount loaded within 4 days. No additional docetaxel was released after 48 h. The docetaxel drug release model matches the Higuchi model. The first burst of the docetaxel can be attributed to the adsorbed docetaxel on outermost layer surface of PLGA NP. Drug diffusion, erosion, swelling of the polymer matrix and degradation of the polymer are considered to be the main mechanisms for drug release [24]. The sustained release profile is linked to the slow degradation rate of the polymers, and therefore docetaxel release from NP is thought to depend mainly on drug diffusion and matrix erosion [27,28]. Biphasic release behavior of docetaxel from PLGA NPs has also been shown in other studies [29–31]. Curcumin, on the other hand, released 78.9% of the amount loaded within 4 days and showed a slower and sustained release profile. During the first 18 h, approximately 51.8% curcumin was released, followed by 78.9% release for 96 h. Although it is a more sustained release, it was still considered as a biphasic release graph [32].

CCM and DTX release profiles of the prepared CCM-DTX-PLGA NPs were examined for drug release compliance with zero order, first order, Higuchi, Hixson and Crowell or Korsmeyer Peppas models. The CCM release profile is described by equation Y = 11.76 + 2.387 × - 0.01773 × ^{2} and the R^2 value is 0.9652 ($p < 0.05$). As a release model, it fits the Korsmeyer Peppas model.

The DTX release profile is described by equation Y = 26.29 + 0.3909 × - 0.002346 × ^{2} and the R^2 value is 0.9017 ($p < 0.05$). It fits the Higuchi model as the emission model.

3.5. Cell culture studies

3.5.1. Biocompatibility of blank PLGA NPs

PLGA varies according to different PLA/PGA ratios and properties such as terminal groups (ester or acid) and particle properties such as particle size, shape, surface morphology, zeta potential. In the scope of this study, biocompatibility studies have been conducted to investigate the effects of the properties of PLGA terminal groups and polysorbate 80

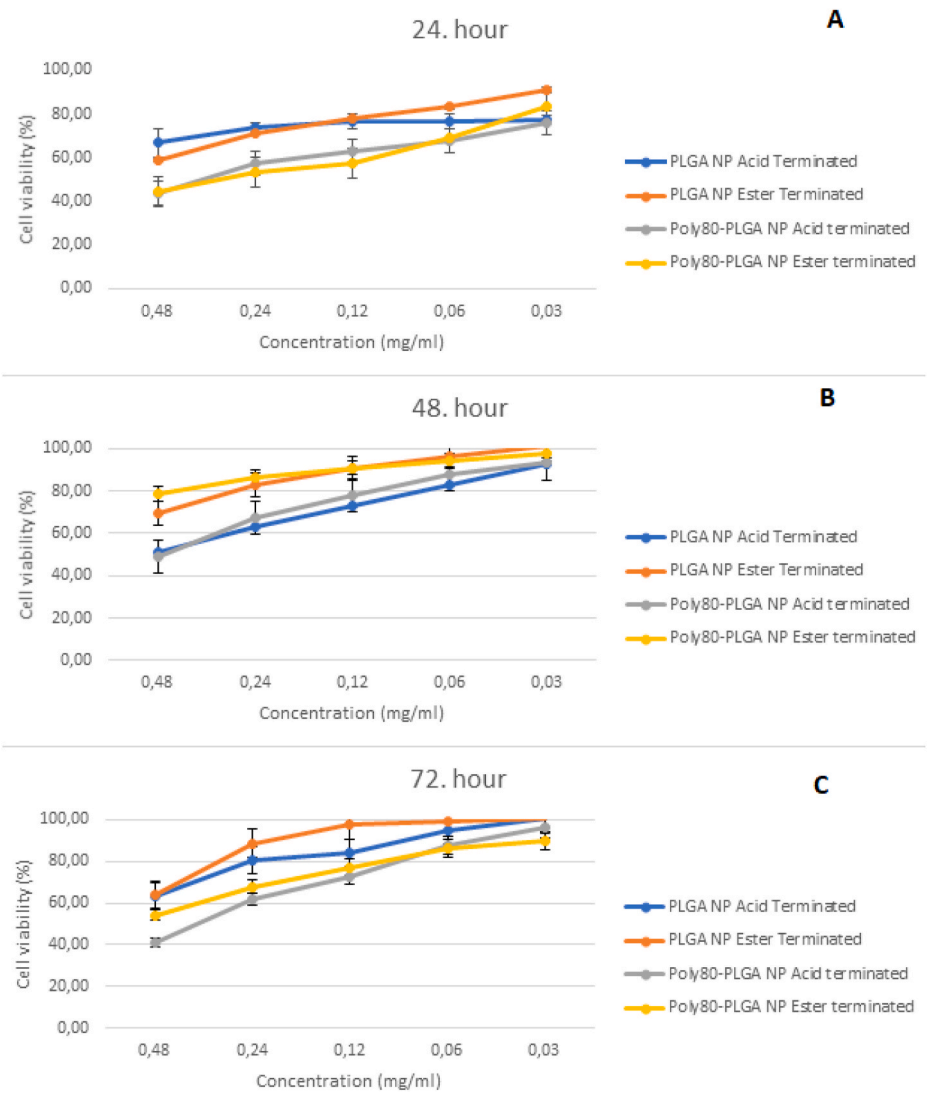

Fig. 3. Cytotoxicity values in bEnd.3 endothelial cells at **A.** 24 h, **B.** 48 h and **C.** 72 h with acid and ester terminated with polysorbate 80 coated and non-coated blank PLGA NPs.

coating on cytotoxicity in bEnd.3 mouse endothelial cells to examine the biocompatibility of the prepared PLGA nanoparticles. Acid terminal PLGA NP, ester terminal PLGA NP, polysorbate 80-coated acid terminal PLGA NP and polysorbate 80-coated ester terminal PLGA NP in various concentrations were investigated by the MTT method for cytotoxicity in various concentrations after 24, 48, and 72 h incubation. Concentrations showing viability above 80% were considered safe. Acid terminated PLGA NPs showed higher cytotoxicity than ester terminated PLGA NPs.

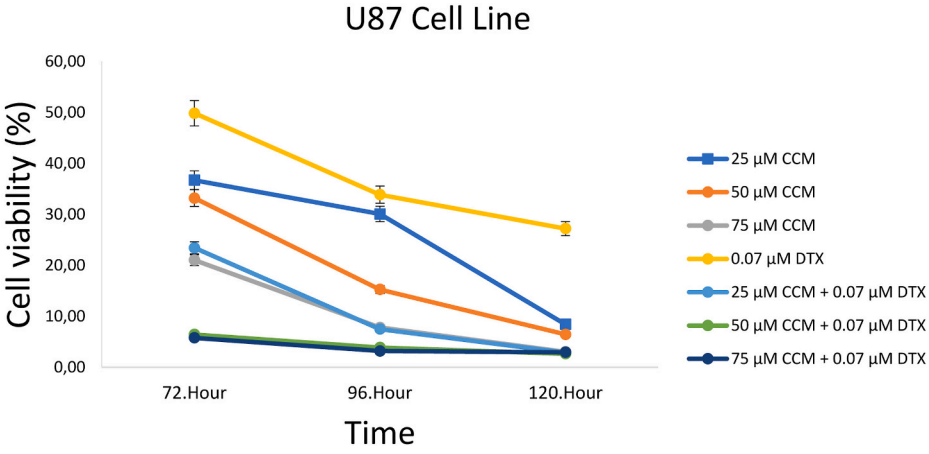

Fig. 4. The cytotoxicity of Docetaxel, curcumin and combination as solutions at various concentrations.

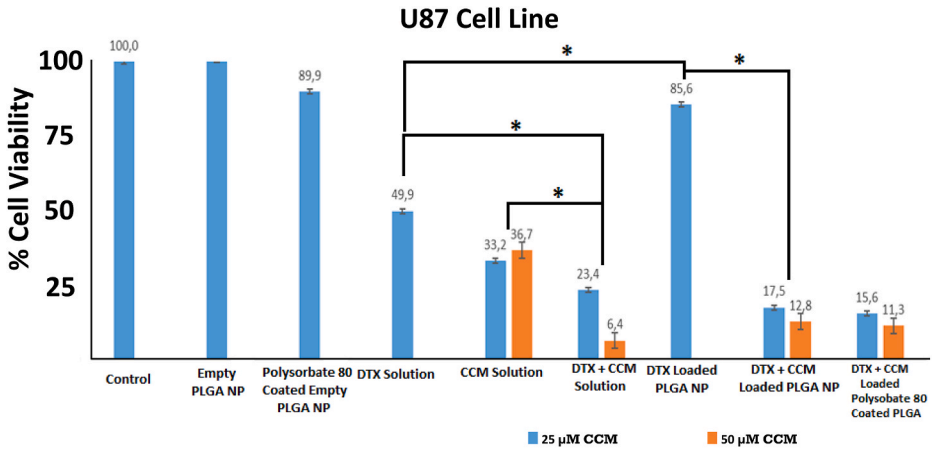

Fig. 5. Cytotoxicity values of formulations on U87 Cell line.

PLGA NPs coated with polysorbate 80 have been observed to be more cytotoxic than those without coating. Since the PLGA NPs uptake into the cell will increase after the polysorbate 80 coating, it is thought that the toxicity may have increased accordingly. At the end of the 72-h incubation period, all formulations were considered safe at 0.312 μM and lower concentrations. Ester terminated PLGA NPs used in the cell culture assays were evaluated as safe regardless of polysorbate 80 coatings at concentrations of 1.25 μM or lower. Since the amount of PLGA used in the subsequent cell culture studies will always be used below the concentration of 0.312 μM PLGA, PLGA NP containing 0.07 μM DTX will always be above 80% (Fig. 3). These results showed that, as expected, blank PLGA NPs showed a dose-dependent toxicity, but did not expose a significant toxicity at concentrations necessary for the transport of docetaxel and curcumin.

3.5.2. *Cytotoxicity of docetaxel and curcumin loaded PLGA nanoparticles*

When comparing the effectiveness of nanoparticles, IC50 values of docetaxel and curcumin solutions were primarily determined in U87

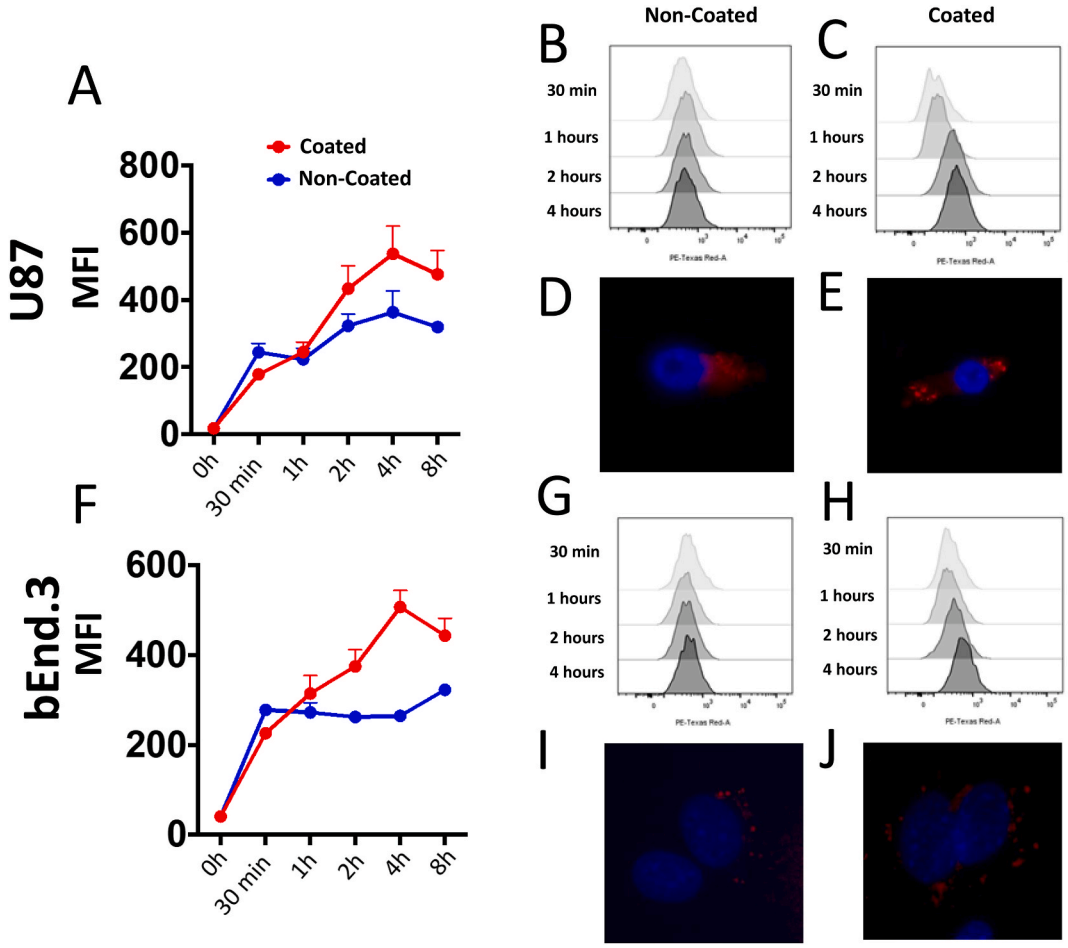

Fig. 6. Mean fluorescence intensity of polysorbate 80 coated and uncoated PLGA nanoparticles in **A.** U87 and **F.** bEnd.3 cell lines. The graphs are obtained from the histograms shown in **B. C. G.** and **H.** Fluorescence microscopy results shown in **D. E. H.** and **I.**

cells in order to decide which concentration docetaxel and curcumin will be administered. IC50 values could not be calculated up to 120 h incubation time points since viability did not decrease below 50%. After 120 h, IC 50 value of docetaxel was calculated as 5.2 nM in U87 glioma cells. On the other hand, cell viability dropped below 50% after 48 h in curcumin solution. At the end of 48th and 72 nd h, IC50 values of curcumin solution were found 71.11 μM and 40.08 μM, respectively.

To investigate the cytotoxicity of the combination of docetaxel and curcumin in U87 cells, DTX concentration was kept constant at 0.07 μM and curcumin concentration varies at 25 μM, 50 μM and 75 μM concentrations (below and above IC50 value) at 72 h, 96 h and 120 h incubation period. The cytotoxicity values in U87 cells from the lowest to the highest in order are as follows; 0.07 μM DTX solution, 25 μM, 50 μM and 75 μM CCM solutions, 0.07 μM DTX +25 μM CCM solution, 0.07 μM DTX +50 μM CCM solution and 0.07 μM DTX +75 μM. Since there was no significant difference ($p < 0.05$) between 0.07 μM DTX + 50 μM CCM solution and 0.07 μM DTX + 75 μM CCM solutions, 75 μM CCM was not loaded on the NPs (Fig. 4).

PLGA NPs formulations were prepared and administered to the cells contain 0.07 μM docetaxel and 2 concentrations (25 μM and 50 μM) of curcumin. At the end of 72 h, the docetaxel solution toxicity value was 49.9%, while it decreased to 23.4% and 6.4% viability in combination with 25 μM CCM and 50 μM CCM (approximately 2 folds for 25 μM CCM and 6 folds for 50 μM CCM increase in toxicity). Docetaxel solution was found to be more toxic than docetaxel-loaded PLGA NP. Likewise, 0.07 μM DTX and 25 μM CCM combined solution have higher toxicity than PLGA NPs loaded with 0.07 μM DTX and 25 μM CCM. Conversely, 0.07 μM DTX and 50 μM CCM loaded PLGA NPs have a higher toxicity than the 0.07 μM DTX and 50 μM combined solution. It is thought to be caused by slow and continuous docetaxel release from docetaxel-loaded PLGA NPs. Although there was some increase in the toxicity of the polysorbate 80 coating in U87 cells, it did not make a significant difference in terms of toxicity ($p > 0.05$) (Fig. 5).

In the literature, several studies of docetaxel and/or its derivative paclitaxel and curcumin in combination, were investigated for use in treating various cancers including brain cancer. Curcumin has been shown to have many antineoplastic properties in brain tumors like glioblastoma, such as inhibition of proliferation, inducing apoptosis, invasion and inhibiting metastasis, and reducing angiogenesis [33–35]. Curcumin not only prevents the formation of cancer cells and protects normal cells from damage due to chemotherapy, but also increases the clinical effectiveness of chemotherapy by sensitizing cancer cells to commonly used chemotherapy [36,37]. In these studies, it is evident that curcumin is a suitable candidate for overcoming the docetaxel resistance mechanism by reducing the dose of chemotherapy administered to patients. Within the scope of the study, a nanoparticular drug delivery system was designed to carry curcumin sand docetaxel, and a synergistic effect was obtained in line with the literature data.

3.5.3. Cellular uptake studies

Results for U87 and bEnd.3, respectively are shown Fig. 6A and F. No significant difference was observed for up to 1 h, after 1 h, polysorbate 80-coated PLGA NPs were found to have more uptake in both U87 and bEnd.3 cells. The highest intensity was reached in 4 h and MFI values started to decrease after this time point.

After quantification of cell uptake of the formulations by flow cytometry, considering these results, imaging studies were carried out with fluorescence microscopy by incubating the formulations for 4 h. Cell nuclei were stained with DAPI in order to determine in which region of the cell the nanoparticles are localized. The results are shown in Fig. 6D, E, 6H and 6I. Particles loaded with nile red were mostly observed in the cytoplasm of the cells. The fluorescent radiation for polysorbate 80-coated PLGA NPs was detected higher by supporting the results in the flow cytometer. These results show that polysorbate 80 coating increases the uptake of PLGA NP in bEnd.3 and U87 cell lines. In the literature, there are studies showing that the uptake of polysorbate 80 and PLGA nanoparticles in different cell can be increased in addition to the cell lines used in the study [38–42].

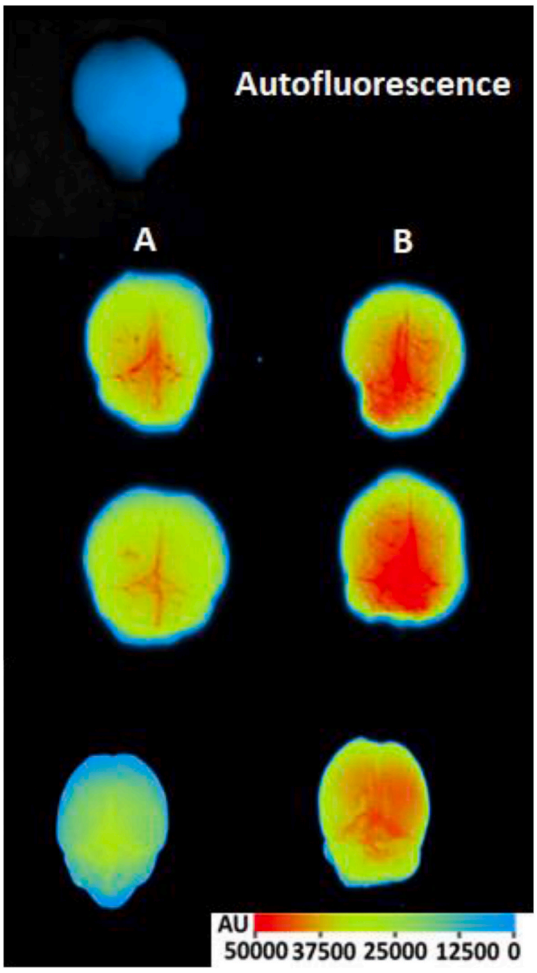

Fig. 7. Brain images of CD1 mice taken by In Vitro Imanging System at the end of the 2 nd h. **A.** polysorbate 80 uncoated flamma 774 loaded NP and **B.** polysorbate 80 coated flamma 774 loaded PLGA NPs (n = 3).

3.6. In vivo biodistribution studies

Since NP behavior in the blood, changing pharmacokinetic properties and complex BBB complex structure cannot be determined in vitro, an in vivo study is mandatory. In the results obtained during the cell culture studies, it was concluded that the polysorbate 80 coating significantly increases the cellular uptake and in order to prove this hypothesis, the efficacy of the polysorbate coating was evaluated in vivo as well. After the Flamma 74 loaded polysorbate 80 coated and uncoated nanoparticles were administered to the mice, the biodistributions of the nanoparticles were investigated by sacrificing the mice at the end of 2. hour. In the images taken after 2 h, it was found that the polysorbate-coated nanoparticles showed significantly larger amounts dye delivered to the brain tissue (Fig. 7).

4. Conclusion

Drug-loaded PLGA nanoparticles coated with polysorbate 80 (Tween® 80) provide effective transport of antineoplastic drugs used in brain tumor therapy to the brain after intravenous injection. Curcumin, decreases the dose of docetaxel used and the side effects during cancer treatment. As result, polysorbate 80 coated docetaxel and curcumin loaded PLGA NPs might be promising approach in brain glioma

treatment.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Indrit Seko: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Hayrettin Tonbul:** Writing – review & editing. **Ece Tavukçuoğlu:** Investigation. **Adem Şahin:** Writing – review & editing. **Sedenay Akbas:** Investigation. **Hamdullah Yanık:** Investigation. **Süleyman Can Öztürk:** Investigation. **Gunes Esendagli:** Conceptualization, Methodology, Resources, Writing – review & editing. **Mansoor Khan:** Writing – review & editing. **Yilmaz Capan:** Supervision, Writing – review & editing.

References

[1] I. Brigger, C. Dubernet, P. Couvreur, Nanoparticles in cancer therapy and diagnosis, Adv. Drug Deliv. Rev. 64 (2012) 24–36.
[2] P. Sampath, et al., Interstitial docetaxel (taxotere), carmustine and combined interstitial therapy: a novel treatment for experimental malignant glioma, Journal of neuro-oncology 80 (1) (2006) 9–17.
[3] J. Vandervoort, A. Ludwig, Biocompatible stabilizers in the preparation of PLGA nanoparticles: a factorial design study, Int. J. Pharm. 238 (1) (2002) 77–92.
[4] E. Galletti, et al., Paclitaxel and docetaxel resistance: molecular mechanisms and development of new generation, Taxanes 2 (7) (2007) 920–942.
[5] C.D. Lao, et al., Dose escalation of a curcuminoid formulation, BMC Compl. Alternative Med. 6 (2006) 10.
[6] M.H. Teiten, et al., Chemopreventive potential of curcumin in prostate cancer, Genes Nutr 5 (1) (2010) 61–74.
[7] K. Wang, et al., Synergistic chemopreventive effects of curcumin and berberine on human breast cancer cells through induction of apoptosis and autophagic cell death, Sci. Rep. 6 (2016) 26064.
[8] F. Benzer, et al., Chemoprotective effects of curcumin on doxorubicin-induced nephrotoxicity in wistar rats: by modulating inflammatory cytokines, apoptosis, oxidative stress and oxidative DNA damage, Arch. Physiol. Biochem. 124 (5) (2018) 448–457.
[9] S.A. Ara, et al., Research on curcumin: a meta-analysis of potentially malignant disorders, J. Cancer Res. Therapeut. 12 (1) (2016) 175–181.
[10] M. Shakibaei, et al., Curcumin potentiates antitumor activity of 5-fluorouracil in a 3D alginate tumor microenvironment of colorectal cancer, BMC Cancer 15 (2015) 250.
[11] W.F. Taylor, E. Jabbarzadeh, The use of natural products to target cancer stem cells, American journal of cancer research 7 (7) (2017) 1588–1605.
[12] W. Liu, et al., Oral bioavailability of curcumin: problems and advancements, J. Drug Target. 24 (8) (2016) 694–702.
[13] R.L. McCall, R.W. Sirianni, PLGA nanoparticles formed by single- or double-emulsion with vitamin E-TPGS, JoVE (82) (2013) 51015.
[14] Y.-J. Wang, et al., Stability of curcumin in buffer solutions and characterization of its degradation products, J. Pharmaceut. Biomed. Anal. 15 (12) (1997) 1867–1876.
[15] J.N. Commandeur, N.P. Vermeulen, Cytotoxicity and cytoprotective activities of natural compounds. The case of curcumin, Xenobiotica 26 (7) (1996) 667–680.
[16] G.R. Valicherla, et al., Formulation optimization of Docetaxel loaded self-emulsifying drug delivery system to enhance bioavailability and anti-tumor activity, Sci. Rep. 6 (2016) 26895.
[17] R.G. Thomas, et al., Paclitaxel loaded hyaluronic acid nanoparticles for targeted cancer therapy: in vitro and in vivo analysis, Int. J. Biol. Macromol. 72 (2015) 510–518.
[18] C.T.S. Turk, et al., Formulation and optimization of nonionic surfactants emulsified nimesulide-loaded PLGA-based nanoparticles by design of experiments, AAPS PharmSciTech 15 (1) (2014) 161–176.
[19] D.L. Cooper, S. Harirforoosh, Design and optimization of PLGA-based diclofenac loaded nanoparticles, PLoS One 9 (1) (2014) e87326.
[20] H. Shadabul, et al., Suggested procedures for the reproducible synthesis of poly(d,l-lactideco-glycolide) nanoparticles using the emulsification solvent diffusion platform, Curr. Nanosci. 14 (5) (2018) 448–453.
[21] S. Haque, et al., Suggested procedures for the reproducible synthesis of poly(d,l-lactide-co-glycolide) nanoparticles using the emulsification solvent diffusion platform, Curr. Nanosci. 14 (5) (2018) 448–453.
[22] H. Tonbul, et al., Synthesis of small-sized mesoporous silica nanoparticles by experimental design and characterization for further drug delivery, Lat. Am. J. Pharm. 38 (2019) 2204–2210.
[23] H. Arzani, et al., Preparation of curcumin-loaded PLGA nanoparticles and investigation of its cytotoxicity effects on human glioblastoma U87MG, Cells 9 (5) (2019) 4225–4231.
[24] C.G. Keum, et al., Practical preparation procedures for docetaxel-loaded nanoparticles using polylactic acid-co-glycolic acid, Int. J. Nanomed. 6 (2011) 2225–2234.
[25] M. Chopra, et al., Design of curcumin loaded polymeric nanoparticles-optimization, formulation and characterization 16 (9) (2016) 9432–9442.
[26] B.P. Sahu, et al., Curcumin-docetaxel co-loaded nanosuspension for enhanced anti-breast cancer activity, Expet Opin. Drug Deliv. 13 (8) (2016) 1065–1074.
[27] P.D. Scholes, et al., Detection and determination of surface levels of poloxamer and PVA surfactant on biodegradable nanospheres using SSIMS and XPS, J. Contr. Release 59 (3) (1999) 261–278.
[28] F. Esmaeili, F. Atyabi, R. Dinarvand, Preparation and Characterization of Estradiol-Loaded PLGA Nanoparticles Using Homogenization-Solvent Diffusion Method, 2008.
[29] M.N. Koopaei, et al., Docetaxel immunonanocarriers as targeted delivery systems for HER 2-positive tumor cells: preparation, characterization, and cytotoxicity studies, Int. J. Nanomed. 6 (2011) 1903–1912.
[30] T. Musumeci, et al., PLA/PLGA nanoparticles for sustained release of docetaxel, Int. J. Pharm. 325 (1–2) (2006) 172–179.
[31] M. Senthilkumar, P. Mishra, N.K. Jain, Long circulating PEGylated poly(D,L-lactide-co-glycolide) nanoparticulate delivery of Docetaxel to solid tumors, J. Drug Target. 16 (5) (2008) 424–435.
[32] P. Verderio, et al., Intracellular drug release from curcumin-loaded PLGA nanoparticles induces G2/M block in breast cancer cells, Biomacromolecules 14 (3) (2013) 672–682.
[33] M.C. Perry, et al., Curcumin inhibits tumor growth and angiogenesis in glioblastoma xenografts, Mol. Nutr. Food Res. 54 (8) (2010) 1192–1201.
[34] H. Liao, et al., Curcumin inhibits lung cancer invasion and metastasis by attenuating GLUT1/MT1-MMP/MMP2 pathway, Int. J. Clin. Exp. Med. 8 (6) (2015) 8948–8957.
[35] C. Sobolewski, et al., Celecoxib prevents curcumin-induced apoptosis in a hematopoietic cancer, cell model 54 (10) (2015) 999–1013.
[36] Q.M. Zhou, et al., Curcumin improves MMC-based chemotherapy by simultaneously sensitising cancer cells to MMC and reducing MMC-associated side-effects, Eur. J. Cancer 47 (14) (2011) 2240–2247.
[37] L.M. Mendonca, et al., Curcumin reduces cisplatin-induced neurotoxicity in NGF-differentiated PC12 cells, Neurotoxicology 34 (2013) 205–211.
[38] S. Şimşek, et al., Brain targeting of Atorvastatin loaded amphiphilic PLGA-b-PEG nanoparticles, J. Microencapsul. 30 (1) (2013) 10–20.
[39] Y. Ling, et al., Temozolomide loaded PLGA-based superparamagnetic nanoparticles for magnetic resonance imaging and treatment of malignant glioma, Int. J. Pharm. 430 (1) (2012) 266–275.
[40] K. Tahara, H. Yamamoto, Y. Kawashima, Cellular uptake mechanisms and intracellular distributions of polysorbate 80-modified poly (d,l-lactide-co-glycolide) nanospheres for gene delivery, Eur. J. Pharm. Biopharm. 75 (2) (2010) 218–224.
[41] K. Tahara, et al., Intracellular drug delivery using polysorbate 80-modified poly(D, L-lactide-co-glycolide) nanospheres to glioblastoma cells, J. Microencapsul. 28 (1) (2011) 29–36.
[42] O. Escalona-Rayo, et al., Rapamycin-loaded polysorbate 80-coated PLGA nanoparticles: optimization of formulation variables and in vitro anti-glioma assessment, J. Drug Deliv. Sci. Technol. 52 (2019) 488–499.