Tumor Biol. (2016) 37:2703–2708
DOI 10.1007/s13277-015-4106-7

ORIGINAL ARTICLE

Enhanced anti-tumor effects of doxorubicin on glioma by entrapping in polybutylcyanoacrylate nanoparticles

Yuebin Zhang[1] · Jia Yu[2] · Lifeng Zhang[1] · Jiabin Cai[1] · Doute Cai[1] · Chengjie Lv[1]

Received: 22 May 2015 / Accepted: 20 September 2015 / Published online: 24 September 2015

Abstract For effective therapy for glioma, it is essential for chemotherapeutics to pass the blood-brain barrier to target glioma cells with little side effects to surrounding normal cells. In this study, we prepared doxorubicin-polybutylcyanoacrylate nanoparticles (Dox-PBCA-NP) and assessed its inhibition effects on glioma both in vitro and in vivo. Dox-PBCA-NP was prepared using the emulsion polymerization method. The size and size distribution of nanoparticles were measured by Malven laser mastersizer and the morphology was observed under transmission electron microscope. Drug loading (DL) and entrapment efficiency (EE) of doxorubicin in the nanoparticles were measured by UV spectra. The proliferation of C6 glioma cells was detected by MTT assay, and cell cycle was analyzed by flow cytometry. The expression of telomerase was detected by immunocytochemical analysis. The anti-tumor efficiency of Dox-PBCA-NP was assessed in C6 glioma intracranial implant rat model. The average diameter of NP-Dox was 120 nm, DL was 10.58 %, and EE was 87.43 %. We found that the cytotoxicity of Dox-PBCA-NP was lower than Dox in vitro. In vivo, Dox-PBCA-NP could transport more Dox into tumors compared to contralateral control, and the life span was longer than Dox. Moreover, Dox-PBCA-NP had less cardiotoxicity than Dox. Taken together, our results suggest that Dox-PBCA-NP exhibits better therapeutic effects against glioma and fewer side effects and is a potential nano-scale drug delivery system for glioma chemotherapy.

Keywords Polybutylcyanoacrylate nanoparticles · Doxorubicin · Glioma · Cardiotoxicity

Introduction

Glioma is the most common intracranial malignant tumor, and its prognosis is poor due to the existence of the blood-brain barrier (BBB), which hinders the effective targeting of chemotherapeutic agents to glioma [1]. To improve therapy for glioma, it is essential for chemotherapeutics to pass BBB and enter into the brain to target tumor cells with little side effects to surrounding normal cells [2]. Doxorubicin (Dox) is widely used for the treatment of breast, cervix, prostate, lung, and ovarian cancers. Although intratumoral injection of Dox improved glioma survival ratio [3], Dox can hardly pass the BBB, which limits its clinical utility on glioma treatment. The binding of adriamycin to polybutylcyanoacrylate nanoparticles can increase the ability to penetrate the cell membrane. Furthermore, nanoparticles with the surface modified by bioactive ligands could deliver a large amount of agents into the tumor [4, 5].

To improve targeted chemotherapy of glioma, in this study, we prepared doxorubicin-polybutylcyanoacrylate nanoparticles (Dox-PBCA-NP) and assessed its anti-glioma effects both in vitro and in vivo.

✉ Chengjie Lv
boy_zyb@qq.com

[1] Department of General Surgery, Children's Hospital Zhejiang University School of Medicine, No. 57 Zhugan Lane, Hangzhou, Zhejiang 310003, China

[2] Department of ICU, Children's Hospital Zhejiang University School of Medicine, Hangzhou 310003, China

Materials and methods

Preparation of NP and Dox-encapsulated NP

The Dox-PBCA-NP was prepared using the emulsion polymerization method [6]. Dextran70 (Sigma) was dissolved in double-distilled water with pH 3.0 to get aqueous phase, and butylcyanoacrylate (BCA, Guangzhou Baiyun Med Co.) was dissolved in dichloromethane with the ratio of BCA to CH2C12 1:3 to get the oil phase. The oil phase was slowly dropped into water phase at a speed of 20 ml/h and stirred at 600 rpm at room temperature for 2 h. The blank polybutylcyanoacrylate nanoparticle (PBCA-NP) was obtained when colloidal solution was filtered with 0.45-um vertical containing glass funnel. To get Dox-encapsulated NP, Dox was added to PBCA-NP and stirred for 2 h.

Characterization of NP and Dox-encapsulated NP

NP or Dox-NP was diluted with distilled water and measured by Laser diffraction particle size analyzer (Mastersizer 3000HS) to assess the size and size distribution of the nanoparticles. NP or Dox-NP suspension was diluted appropriately and dropped on the carbon film-coated copper net, stained by 2 % phosphate tungsten, dried for 30 min, and then observed under transmission electron microscope (TEM; JEM-100CX, Japan). The freeze-dried Dox-NP powder was kept for 3 months at room temperature and then was dissolved in distilled water to detect the stability.

Drug loading and entrapment efficiency of doxorubicin in the nanoparticles were measured by UV spectra at 481 nm (Unicoam UV500 ultraviolet spectrophotometer, British Thereto electronic). Drug loading ratio (DL) and encapsulation efficiency (EE) were calculated as follows [7]:

$DL\% = Dox_{total} - Dox_{supernatant}/BCA \times 100\ \%$; $EE = Dox_{total} - Dox_{supernatant}/Dox_{total} \times 100\ \%$. Finally, NP-Dox was suspended in a dialysis system and Dox released into 0.01 M PBS (pH 4 and 7) was periodically taken out, and the supernatant was analyzed for the released Dox by UV spectrophotometry.

In vitro cytotoxicity assay

C6 glioma cells were seeded in 96-well plates at 1×10^4 cells/well and cultured for 24 h, then treated with different concentrations of Dox or Dox-PBCA-NP for 24 h at 37 °C in atmosphere with 5 % CO_2. The cytotoxicity of Dox or Dox-PBCA-NP was evaluated by using MTT assay (Sigma). The experiments were performed in triplicate, dose-effect curves were made, and IC50 values were calculated.

Cell cycle analysis by flow cytometry

C6 cells were seeded in 6-well dishes at 1×10,000 cells/well and cultured for 24 h, then treated with Dox, Dox-PBCA-NP, or PBCA-NP for 24 h at 37 °C in atmosphere with 5 % CO_2. The cells were collected and fixed overnight in 70 % (*v*/*v*) ethanol at −20 °C. The cells were washed twice with PBS and incubated for 30 min at room temperature with DNA-staining solution (MultiScience, China) containing 10 μg/ml RNase and 50 μg/ml propidium iodide (PI, Sigma). All samples were assessed by a FACS Calibur flow cytometer (Becton-Dickinson, USA) and the data were analyzed by Multicycles software.

Immunocytochemical staining

The expression of telomerase was measured by immunocytochemical method. Briefly, poly-L-lysine covered coverslips was placed in 24-well dishes, and then C6 cells were seeded in each well at a density of 10^5 cells/well and cultured for 24 h. Then cells were treated with Dox, Dox-PBCA-NP, or PBCA-NP for 24 h at 37 °C in atmosphere with 5 % CO_2. The cells were fixed for 30 min at room temperature by paraformaldehyde, then washed twice with PBS and assessed by EnVision immunohistochemical staining kit (Shanghai, Dako Denmark A/S). The percentage of positive cells was calculated according to the instruction.

Biodistribution of Dox in the brain of glioma-bearing rats

Animal studies were approved by the Institutional Animal Care and Use Committee of Zhejiang University. The rats were anesthetized with 10 % chloral hydrate (0.4 g/kg), and 1×10^6 C6 cells were injected into right caudate nucleus on a stereotactic apparatus [8, 9]. Ten days after xenograft, ten rats were randomly divided into two groups in which free Dox or Dox-PBCA-Dox (dose of Dox 3 mg/kg) was injected through tail vain, respectively. Three hours later, the rats were sacrificed and the brain tissues were collected and washed by cold PBS to remove the surface blood. The left caudate nucleus (normal brain tissue) and right caudate nucleus (tumor tissue) were resected from the surrounding tissue under the operating microscope. After the homogenization of the tissues, the concentration of Dox in the tissues was analyzed by HPLC (Ex, 480 nm and Em, 580 nm) as described previously [10].

Survival curves

Forty glioma-bearing rats were randomly divided into four groups (n=10), on the 7th day post xenograft. Animals in control group were administered with physiological saline. Animals in PBCA-NP group were administered with PBCA-

NP at dose of 0.5 mg/kg. Animals in other two groups were treated with free Dox or Dox-PBCA-NP via tail vein at a dose of 1.5 mg/kg Dox, respectively. Administration was made every 2 days with a total of three doses per rat. All rats were maintained to monitor the survival curves. Kaplan-Meier survival curves were plotted for each group.

Serum assay

On the 5th day after the last administration, 0.5 ml of blood was collected from the rat's tail. Serum level of aspartate aminotransferase (AST) was measured via automated chemistry analyzer with reagent kits (Micro lab 300, Merck).

Statistical analysis

Each experiment was performed in triplicate with results expressed as mean±standard error (SE). The Student's test was used. Survival data were presented using Kaplan-Meier plots and analyzed using a log-rank test. Statistical significance was set at $P<0.05$.

Results

Characterization of NPs

TEM micrographs demonstrated that NP and Dox-NP vesicles were generally round with a diameter 105.3±25.1 and 120.5± 30.8 nm, respectively (Fig. 1a, b). No obvious adhesion was found. DL of Dox in the Dox-PBCA-NP was about 10.58 % with EE above 87.43 %. Zeta potential of NP or Dox-PBCA-NP ranged from −8 to −10 mV. We took freeze-dried powder of Dox-PBCA-NP kept at room temperature for 3 months and dissolved it in distilled water; no obvious sediment was found and there were no significant changes in the size, shape, DL, and EE. The in vitro release analysis demonstrated that releasing rate of Dox-PBCA-NP was higher under acidic condition at 37 °C (Fig. 1c). Similarly, the degradation rate of PBCA-NP was higher under acidic condition (Fig. 1d).

In vitro cytotoxicity of NPs

The cytotoxicity of Dox, PBCA-NP, and Dox-PBCA-NP in C6 cells was evaluated by MTT assay. Even at the highest concentration (1 mg/mL) of NPs, the viability of C6 cells was over 95 %. The cytotoxicity of Dox or Dox-PBCA-NP was concentration dependent. IC50 (Dox concentration of 50 % inhibition) of Dox and Dox-PBCA-NP was 52.45 and 64.21 g/mL, respectively (Fig. 2). Moreover, there was a significant difference in the viability between C6 cells treated with Dox and C6 cells treated with Dox-PBCA-NP ($P<0.05$).

The effects of NPs on cell cycle progression of C6 cells

In the control and PBCA-NP-treated groups, there was no significant difference in cell cycle progression between the two groups ($P>0.05$, Table 1). In Dox and Dox-PBCA-NP-treated groups, the number of cells in G1/G0 phase increased while the number of cells in S phase decreased significantly, indicating the inhibition of cell proliferation. At the same concentration of Dox, Dox had stronger inhibition on C6 cell proliferation compared to Dox-PBCA-NP ($P<0.05$).

The effects of NPs on telomerase expression in C6 cells

Immunocytochemical analysis showed that the a positive rate of telomerase staining in blank control group and PBCA-NP group was 93.5±2.4 and 95.1±1.8 %, respectively. There was no significant difference between the two groups ($P>0.05$). In Dox and Dox-PBCA-NP groups with Dox concentration of 10 μg/ml, the positive rate of telomerase staining was 55.2± 0.8 and 69.7±1.3 %, with a significant difference between these two groups ($P<0.05$, Table 2).

Biodistribution of Dox in the brain of glioma-bearing rats

To evaluate the anti-glioma efficacy of Dox-PBCA-NP in vivo, the glioma-bearing rats were injected with the different agents via tail vain on the 10th day post intracranial implantation. Dox concentration in normal brain tissue (left caudate nucleus) and tumor tissue (right caudate nucleus) were detected by HPLC. The results showed that Dox concentration was much higher in tumor tissue than normal brain tissue in Dox-PBCA-NP group ($P<0.01$, Fig. 3). Furthermore, Dox concentration of tumor tissue in Dox-PBCA-NP group was much higher than that of Dox groups ($P<0.05$, Fig. 3).

The effects of NPs on the survival of glioma-bearing rats

The survival of glioma-bearing rats from saline, PBCA-NP, free Dox, and Dox-PBCA-NP group was 13–27, 14–28, 14–25, and 17–30 days, respectively. The rank of median survival time was Dox-PBCA-NP (24 days)>NP (20 days)>saline (19.5 days)>Dox (19 days). By log-rank test, the median survival time of Dox-PBCA-NP group was significantly prolonged compared to the other three groups, while there was no significant difference between Dox, NP group, and saline group ($P>0.05$, Fig. 4).

The cardiotoxicity of NPs

The administration of Dox (1.5 mg/kg/time, 3 times/rat) increased serum AST levels compared to saline control or blank NP group, but the administration of Dox-PBCA-NP decreased serum AST levels significantly compared to Dox group

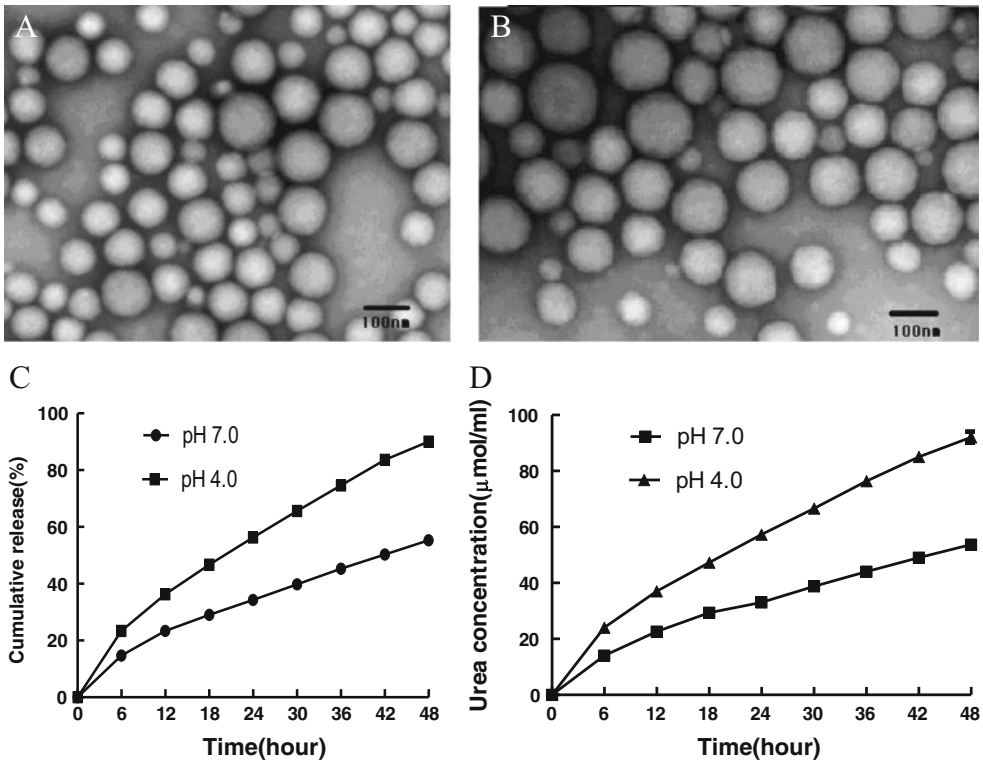

Fig. 1 Characterization of Dox-PBCA-NP. **a** TEM image of PBCA-NP. **b** TEM image of Dox-PBCA-NP. *Scale bar*, 100 nm. **c** Cumulative release (%) of Dox from Dox-PBCA-NP in medium with different pH at 37 °C. Dox concentration was calculated as described in "Materials and methods." **d** The curve of concentration of urea released by PBCA-NP. Urea is one of PBCA-NP degradation products, and the concentrations of urea in PBCA-NP solution reflect the degradation of PBCA-NP

(Fig. 5). These results showed that Dox induced cardiotoxicity manifested by significant increases in serum AST levels [11]. However, serum AST levels decreased significantly in Dox-PBCA-NP group, suggesting less cardiotoxicity after entrapping Dox into nanoparticles.

Discussion

The anti-glioma effects mainly depend on the transport of chemotherapeutic agent across BBB to enter the glioma site [12]. Traditional strategies to overcome BBB include intracerebral injection or infusion of hyperosmotic solutions such as mannitol [13]. These approaches can increase intracranial drug levels but cause a higher risk for patients such as electrolyte disturbances, intracranial hemorrhage, or infection. With the development of materials science, nanoparticles have been prepared to absorb or encapsulate drugs. PBCA is a widely used nano-material as drug carrier with little toxicity and good biocompatible characters [14, 15]. In this study, we developed a biodegradable NP with anti-tumor agent encapsulated inside. PBCA nanoparticles polymerized butylacrylate monomer with emulsion polymerization induced by -OH group. The reaction rate and degree of polymerization are influenced by many factors. During the polymerization

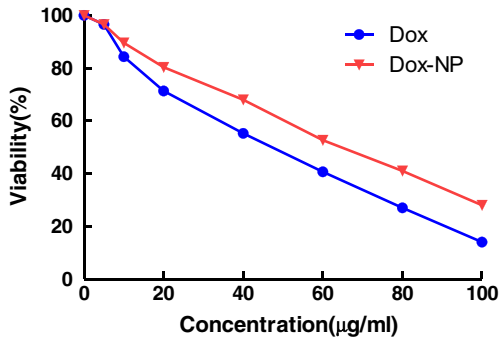

Fig. 2 In vitro cytotoxicity of Dox-PBCA-NP. The cytotoxicity of PBCA-NP and Dox on C6 glioma cells was evaluated by MTT assay. *P<0.05 Dox versus Dox-PBCA-NP

Table 1 Effects of Dox, Dox-PBCA-NP on cell cycle progression of C6 cells

Group	Percentage of cells in each phase (%)		
	G0/G1	S	G2/M
DMEM	33.2±1.2	62.3±0.2	5.0±1.3
PBCA-NP	34.5±0.8	58.1±1.6	7.3±0.2
Dox1-PBCA-NP	50.1±1.4*	42.3±0.5	7.5±0.3
Dox1 (2 μg/ml)	40.7±1.2*	51.6±0.6*	7.6±0.5
Dox2-PBCA-NP	56.2±0.8**	37.9±0.3**	5.8±0.4
Dox2 (5 μg/ml)	64.8±0.6**	30.3±0.4**	4.9±0.3
Dox3-PBCA-NP	75.7±1.5**	20.5±1.7**	3.7±0.1
Dox3 (10 μg/ml)	90.4±0.9**	5.2±1.2**	4.3±0.4

*P<0.05 compared with the control group. **P<0.01 compared with the control group

Table 2 Effects of Dox, Dox-PBCA-NP on telomerase expression in C6 cells

Dox concentration	Percentage of cells with positive telomerase expression (%)	
	Dox-PBCA-NP	Dox
2 μg/ml	86.4±3.7 %	72.3±1.6 %*
5 μg/ml	75.6±2.1 %	63.2±3.1 %*
10 μg/ml	69.7±1.3 %	55.2±0.8 %*

*$P<0.05$ Dox versus Dox-PBCA-NP

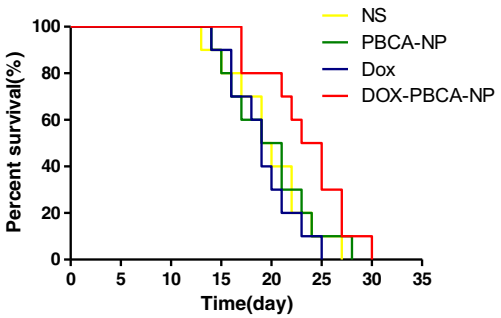

Fig. 4 Kaplan-Meier plots showing the survival of glioma-bearing rats ($n=10$)

reaction, Dextran70 acted as the stabilizer [16]. The average particle size was around 120 nm, with little polydispersity coefficient, which indicated that the nanoparticle size distribution is narrow. TEM showed that the nanoparticles had uniform particle size, smooth round surface, and little aggregation. Furthermore, after the nanoparticles were kept at room temperature for a long time, there were no obvious changes in solubility, DL, or EE.

Dox is widely used for tumor chemotherapy, but seldom used for glioma chemotherapy because it could not overcome the BBB. Moreover, the cardiotoxicity limits its administration [17, 18]. In this study, we encapsulated Dox into PBCA-NP to overcome BBB. After being encapsulated into PBCA-NP, the release of Dox is time dependent. To exclude the possible effects of blank PBCA-NP, the cytotoxicity of PBCA-NP was evaluated and no significant toxicity was found, and it had no significant effects on the proliferation of C6 cells. Cytotoxicity of Dox-PBCA-NP was lower than Dox in vitro at the same concentration of Dox, maybe due to the slow-releasing aspect of Dox-PBCA-NP. After the injection of Dox-PBCA-NP into the vein of glioma-bearing rats, the rats showed a longer survival, in agreement with Dox biodistribution in the glioma tissue. Dox is anthracycline anti-tumor agent of low molecular weight and could not pass the BBB. After encapsulation into PBCA-NP, the solubility of Dox-PBCA-NP improved significantly and the residence time in circulation system was prolonged. Furthermore, the slow-releasing effects contributed to its long circulation.

Dox-induced cardiotoxicity is the most serious side effect of the chemotherapeutic agent. AST is an enzyme that leaks from the hearts upon tissue damage and has been used as the marker of cardiotoxicity due to the tissue specificity and serum catalytic activity. To evaluate the cardiotoxicity of Dox-PBCA-NP, we collected serum samples from glioma-bearing rats and examined serum AST levels. We found that serum AST levels were significantly lower in Dox-PBCA-NP-treated group compared with Dox-treated group. These data suggest that entrapping of Dox in PBCA-NP significantly reduces the cardiotoxicity of Dox.

In summary, we prepared Dox-PBCA-NP via emulsion polymerization method. Dox-PBCA-NP demonstrated less effective anti-tumor effect on C6 glioma cells in vitro compared to Dox, which may be due to its slow-releasing aspect. However, Dox-PBCA-NP demonstrated better therapeutic efficacy on glioma in vivo manifested by prolonged survival of glioma-bearing rats. In addition, Dox-PBCA-NP alleviated

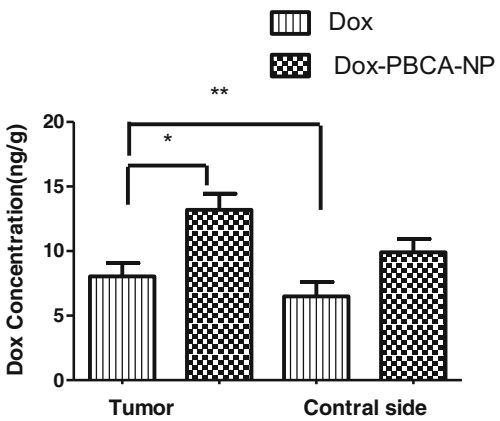

Fig. 3 Biodistribution of Dox in the brain of glioma-bearing rats. The C6 glioma cells-bearing rats received an i.v. of Dox or Dox-PBCA-Dox, Dox concentration in the tumor tissue or left caudate nucleus was detected by HPLC analysis ($n=5$). *$P<0.01$, **$P<0.05$

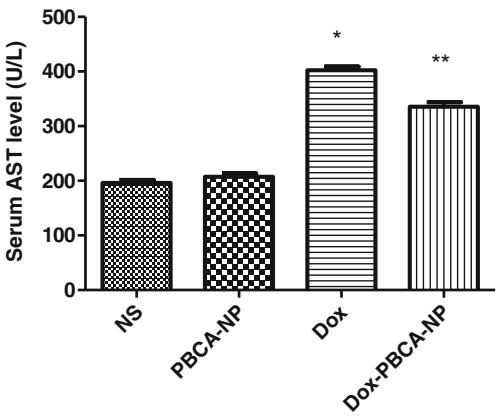

Fig. 5 Serum level of AST in glioma-bearing rats of different groups. *$P<0.05$ versus PBCA-NP; **$P<0.05$ versus Dox

Dox-induced cardiotoxicity in vivo. Therefore, Dox-PBCA-NP is a potential nano-scale drug delivery system for glioma chemotherapy.

Compliance with ethical standards Animal studies were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Conflicts of interest None

References

1. Ricard D, Idbaih A, Ducray F, Lahutte M, Hoang-Xuan K, Delattre JY. Primary brain tumours in adults. Lancet. 2012;379(9830):1984–96.
2. Jain V, Jain S, Mahajan SC. Nanomedicines based drug delivery systems for anti-cancer targeting and treatment. Curr Drug Deliv. 2015;12(2):177–91.
3. Stan AC, Casares S, Radu D, Walter GF, Brumeanu TD. Doxorubicin induced cell death in highly invasive human gliomas. Anticancer Res. 1999;19(2A):941–50.
4. Bagherpour Doun SK, Alavi SE, Koohi Moftakhari Esfahani M, Ebrahimi Shahmabadi H, Alavi F, Hamzei S. Efficacy of cisplatin-loaded poly butyl cyanoacrylate nanoparticles on the ovarian cancer: an in vitro study. Tumour Biol. 2014;35(8):7491–7.
5. Chaudhari KR, Ukawala M, Manjappa AS, Kumar A, Mundada PK, Mishra AK, et al. Opsonization, biodistribution, cellular uptake and apoptosis study of PEGylated PBCA nanoparticle as potential drug delivery carrier. Pharm Res. 2012;29(1):53–68.
6. Tian XH, Lin XN, Wei F, Feng W, Huang ZC, Wang P, et al. Enhanced brain targeting of temozolomide in polysorbate-80 coated polybutylcyanoacrylate nanoparticles. Int J Nanomedicine. 2011;6:445–52.
7. Ebrahimi Shahmabadi H, Movahedi F, Koohi Moftakhari Esfahani M, Alavi SE, Eslamifar A, Mohammadi Anaraki G, et al. Efficacy of cisplatin-loaded polybutyl cyanoacrylate nanoparticles on the glioblastoma. Tumour Biol. 2014;35(5):4799–806.
8. Liu G, Shen H, Mao J, Zhang L, Jiang Z, Sun T, et al. Transferrin modified graphene oxide for glioma-targeted drug delivery: in vitro and in vivo evaluations. ACS Appl Mater Interfaces. 2013;5(15):6909–14.
9. Liu G, Mao J, Jiang Z, Sun T, Hu Y, Jiang Z, et al. Transferrin-modified doxorubicin-loaded biodegradable nanoparticles exhibit enhanced efficacy in treating brain glioma-bearing rats. Cancer Biother Radiopharm. 2013;28(9):691–6.
10. Mao J, Li A, Zhao L, Gao L, Xu W, Hong X, et al. PEG-PLGA nanoparticles entrapping doxorubicin reduced doxorubicin-induced cardiotoxicity in rats. Adv Mater Res. 2014;912–914:263–8.
11. Sayed-Ahmed MM, Khattab MM, Gad MZ, Osman AM. Increased plasma endothelin-1 and cardiac nitric oxide during doxorubicin-induced cardiomyopathy. Pharmacol Toxicol. 2001;89(3):140–4.
12. Li M, Deng H, Peng H, Wang Q. Functional nanoparticles in targeting glioma diagnosis and therapies. J Nanosci Nanotechnol. 2014;14(1):415–32.
13. Pardridge WM. BBB-genomics: creating new openings for brain-drug targeting. Drug Discov Today. 2001;6(8):381–3.
14. De Juan BS, Von Briesen H, Gelperina SE, Kreuter J. Cytotoxicity of doxorubicin bound to poly(butyl cyanoacrylate) nanoparticles in rat glioma cell lines using different assays. J Drug Target. 2006;14(9):614–22.
15. Ambruosi A, Khalansky AS, Yamamoto H, Gelperina SE, Begley DJ, Kreuter J. Biodistribution of polysorbate 80-coated doxorubicin-loaded [14C]-poly(butyl cyanoacrylate) nanoparticles after intravenous administration to glioblastoma-bearing rats. J Drug Target. 2006;14(2):97–105.
16. Mitra A, Lin S. Effect of surfactant on fabrication and characterization of paclitaxel-loaded polybutylcyanoacrylate nanoparticulate delivery systems. J Pharm Pharmacol. 2003;55(7):895–902.
17. Karimi G, Ramezani M, Abdi A. Protective effects of lycopene and tomato extract against doxorubicin-induced cardiotoxicity. Phytother Res. 2005;19(10):912–4.
18. Hydock DS, Lien CY, Schneider CM, Hayward R. Exercise preconditioning protects against doxorubicin-induced cardiac dysfunction. Med Sci Sports Exerc. 2008;40(5):808–17.