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Enhancement of the stability of BCNU using self-emulsifying drug delivery systems (SEDDS) and in vitro antitumor activity of self-emulsified BCNU-loaded PLGA wafer

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Abstract

The main purpose of this study was to develop self-emulsifying drug delivery systems (SEDDS) for the improvement of the stability of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) after released from poly (D,L-lactide-co-glycolide) (PLGA) wafer and to evaluate its in vitro antitumor activity against 9L gliosarcoma cells. The in vitro stability test of BCNU was characterized by the BCNU amount in phosphate buffered saline (PBS, pH 7.4) at 37 °C. SEDDS increased in vitro half-life of BCNU up to 130 min compared to 45 min of intact BCNU. Self-emulsified (SE) BCNU was fabricated into wafers with flat and smooth surface by compression molding. In vitro release of BCNU from SE BCNU-loaded PLGA wafer was prolonged up to 7 days followed first order release kinetics. Beside, the cytotoxicity of SE BCNU-loaded PLGA wafer against 9L gliosarcoma cells was higher than intact BCNU-loaded PLGA wafer which is more susceptible to hydrolysis. SE BCNU degraded much more slowly than the intact BCNU in PLGA matrix at 25 °C. These results strongly suggest that the self-emulsion system increased the stability of BCNU after released from PLGA wafer. From these results, it could be expected that the penetration depth of BCNU could be improved in brain tissue using self-emulsion system.

Keywords: Brain tumor; Self-emulsifying drug delivery system (SEDDS); 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU)

1. Introduction

The localized, controlled delivery of anticancer agents using biodegradable polymeric implant is an alternative to the systemic administration of

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chemotherapeutic agents for treating malignant brain tumors. Gliadel® made of poly 1,3-bis(*p*-carboxyphenoxy)propane-sebacic acid copolymer (polyanhydride, PA) and 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine, BCNU) as a chemotherapeutic agent has been commercialized in treating malignant brain tumors (Dang et al., 1996; Sampath and Brem, 1998). However, the conventional therapies cannot extend the median survival of patients significantly because these tumors tend to recur within 2 cm of treated site (Chasin et al., 1990).

In our previous study, we applied BCNU-loaded poly(D,L-lactide-co-glycolide) (PLGA) wafer to study for brain tumor, because PLGA has been approved for drug delivery use by the Food and Drug Administration (FDA). Long-term delivery of BCNU (Seong et al., 2002), the BCNU release behavior with the changes of various dimension of wafer (Chae et al., 2004) and additives (An et al., 2002a,b; Lee et al., 2003), cytotoxicity against various tumor cell lines (Seong et al., 2003; Lee et al., 2004) and in vivo anti-tumor activity were investigated. But it has been reported that BCNU presents very short penetration distance because it gets drained out of the system before being able to diffuse to any appreciable distance (Wang et al., 1999).

BCNU is known to cross the BBB due to its low molecular weight and lipophilicity (Paoletti, 1984). However, its effectiveness was hindered by both dose-limiting side effects and relatively short half-life (<20 min, in plasma) (Loo et al., 1966). The exposure of BCNU released from matrix is very brief; therefore, the penetration depth is very short (2–3 mm in brain tissue). Improving the permeation depth of BCNU in brain tissue is very important for treating brain tumor effectively. One of the ways to improve the penetration depth of BCNU is enhancing the stability of BCNU released from matrix against hydrolysis in vitro and in vivo.

One of the technological resources used to improve the permanence of drugs more largely at the site of action, in this case, is the use of emulsions. The self-emulsified drug delivery system (SEDDS) prepared from oil, a surfactant, and possibly one or more hydrophilic solvents or co-surfactants (Holm et al., 2003; Kang et al., 2004) when exposed to aqueous media under condition of gentle agitation, such as body fluid and blood (Tarr et al., 1987; He et al., 2003) to retard exposure of BCNU from aqueous media. SEDDS were mainly used to improve the bioavailability of poor water soluble drug compounds (Humberstone and Charman, 1997; Kommuru et al., 2000). Moreover, the drug dissolution into oil components was diffused without any activity in the mean time of forming emulsion, because of the presence of a surfactant, which can adsorb on the oil droplet surface (Dickinson and Matsumura, 1994).

In objective of this study, SEDDS of BCNU was developed for enhancing the stability of BCNU. The self-emulsified (SE) BCNU was incorporated into PLGA wafer as a new polymeric implant. In vitro release pattern of BCNU from SE BCNU-loaded PLGA wafers and cytotoxicity according to droplet size and stability of the SE BCNU-loaded PLGA wafer against 9L gliosarcoma cells were assessed.

2. Materials and methods

2.1. Materials

BCNU was purchased from Sigma Chem. Co. (St. Louis, MO, USA) and stored at −20 °C until use. PLGA (50:50 mole ratio of lactide to glycolide) having molecular weight of 8000 g/mole (Resomer®, RG 502H) was purchased from Boehringer Ingelheim (Germany). Tributyrin was purchased from Sigma Chem. Co. Cremophor RH 40 was purchased from BASF (Germany). Polyglycolyzed glycerides (Labrafill M-1944CS) were obtained from Gattefosse (Westwood, NJ, USA). Methylene chloride (MC, Tedia, Japan), methanol (Junsei, Japan) and acetonitrile (HPLC grade, Hurdick & Jackson, USA) were used as purchased. All other chemicals were reagent grade. Deionized water was prepared by a Milli-Q purification system from Millipore (Molsheim, France). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and trypsin/1,2-ethylenediaminetetraacetic (EDTA) were purchased from Gibco BRL® (Grand Island, NY, USA). Sodium bicarbonate, amphothericin penicillin G sodium and 1, 2-cyclohexanediaminetetraacetic acid (CDTA) were purchased from Sigma Chem. Co. Sulforhodamine B (SRB), TRIZMA® base and trichloroacetic acid (TCA) were also purchased from Sigma Chem Co. T-25, T-75, 6-well and 96-well plates

Table 1
Composition of SEDDS formulations

Formulation	Composition (w/w, g)			
	Solubilizer Tributyrin	Surfactant Cremophor RH 40	Cosurfactant Labrafil 1944	BCNU
a_s	–	–	–	
b_s	0.15	0.03	–	
c_s	0.15	0.03	0.15	
d_s	0.15	0.10	–	0.01
e_s	0.15	0.10	0.15	
f_s	0.15	0.15	–	
g_s	0.15	0.15	0.15	

were purchased from Falcon Co. (Lincoln Park, NJ, USA).

2.2. Preparation of SE BCNU and SE BCNU-loaded PLGA wafer

A series of SEDDS were prepared in each of the seven formulas (Table 1) with BCNU, oil, surfactant and cosurfactant. Briefly, BCNU and oil were poured in glass vials, and then the mixture was mixed by vortex-mixing until BCNU perfectly dissolved. Surfactant and cosurfactant were accurately weighed into glass vials, respectively. Then the components were mixed by gentle stirring and vortex-mixing.

SE BCNU and PLGA as a composition of Table 2 were mixed, sieved and molded into wafers using Carver Press (MH-50Y CAP 50 tons, Japan) at 20 kgf/cm^2 for 5 s at 25 °C. The wafers were 3 mm × 1 mm in size with a flat surface and stored at 0 °C until use. In Tables 1 and 2, the control SE BCNU and wafer contained SE BCNU were indicated to (a_s) and (a_w).

2.3. In vitro stability test

SE BCNU and intact BCNU were immersed respectively into PBS (pH 7.4) at 37 °C. At a scheduled time, the specific amount of PBS solution was retrieved and BCNU was extracted with acetonitrile. All samples were analyzed using HPLC system equipped with a Model P-2000 pump, a Model AS-3000 autosampler and a Model UV-1000 UV detector at 248 nm (Thermo Separation Products, Fermont, CA, USA). The column used was μ-Bondapak^{TM} C_{18} (3.9 mm × 300 mm, Waters, Milford, MA, USA). Mobile phase was composed of deionized water and methanol (6:14 v/v) mixture and flow rate of that was adjusted 1.0 ml/min.

2.4. Determination of drug content in the wafer and in vitro release study

SE BCNU and intact BCNU in PLGA wafer were assayed by HPLC in order to determine drug content in the wafer. SE BCNU and intact

Table 2
Composition of 3.85% SE BCNU-loaded PLGA wafer

Formulation	Composition (w/w, %)				
	SEDDS			Drug	PLGA
	Tributyrin	Cremophor RH 40	Labrafil 1944	BCNU	8 K g/mole
a_w	–	–	–		96.15
b_w	6.42	1.28	–		
c_w	3.5	0.7	3.5		
d_w	4.62	3.08	–	3.85	88.45
e_w	2.89	1.92	2.89		
f_w	3.85	3.85	–		
g_w	2.57	2.56	2.57		

BCNU-loaded PLGA wafer were dissolved respectively in 2 ml of MC. PLGA was precipitated by adding 18 ml of methanol and after centrifugation, 100 μl of aliquots of supernatant were analyzed by HPLC.

Release of BCNU from PLGA wafer was monitored for a period of incubation in PBS, pH 7.4 at 37 °C. Wafers were individually placed in 20 ml of PBS with constant shaking at 110 rpm. At specific time following incubation wafers were retrieved and freeze-dried for 48 h. Wafers were dissolved respectively in 2 ml of MC. After precipitation of PLGA with methanol, 20 μl of aliquots of supernatant were analyzed by HPLC. Due to the instability of BCNU in the release test media, the amount of BCNU released into PBS was calculated by the amount of BCNU remained in the wafer after scheduled release test period.

2.5. Storage stability of SE BCNU from SE BCNU loaded PLGA wafer

SE BCNU and intact BCNU-loaded PLGA wafer were stored for 50 days at 25 °C to evaluate storage stability of each sample. And only SE BCNU-loaded PLGA wafer was stored for 90 days at different temperature.

2.6. Emulsion size analysis of SE BCNU

The each sample of fabricated SE BCNU for size measuring was diluted with distilled water in a volumetric flask and gently mixed by inverting the flask. The emulsion size and its distribution of SE BCNU were measured by electrophoretic light scattering (ELS-8000, Photal, Otsuka Electronics, Tokyo, Japan).

2.7. Cell culture

Nine litres of gliosarcoma cells grown in DMEM medium was added antibiotics (10 μg/ml of amphotericin and 10 U/ml of penicillin G sodium) and supplemented with 10% (v/v) FBS. The cells were cultured in 95% air/5% CO_2 atmosphere at 37 °C in a humidified incubator and dissociated with 0.05% trypsin–EDTA in case of transferring or dispensing before experiment.

2.8. Preparation of PKH26-labeled cells

Nine litres of gliosarcoma cells were labeled with PKH26 red fluorescent cell linker kit (Sigma Chem. Co., St. Louis, MO, USA) and measured cell proliferation of attached cells. Briefly, cells were concentrated by centrifugation, resuspended and washed two times in serum free medium. The number of viable cells were determined using a trypan blue exclusion assay, enumerated using a hemocytometer (Reichert Co., USA) and resuspended in serum-free medium at concentration of 2×10^7 cells/ml. Cells were labeled with PKH26 (4×10^{-6} M) at 25 °C for 2–5 min in incubator. Two millilitres of FBS was added to stop the labeling reaction, and then labeled solution was removed by centrifugation. Finally, cells were washed twice in complete medium by centrifugation and resuspended to the desired concentration. All centrifugation steps were done at $400 \times g$ for 10 min.

2.9. Cytotoxicity test

Experimental cells were plated on 6-well flat-bottomed plates with each well at a density of 2×10^4 cells/well and incubated for 24 h at 37 °C in the CO_2 incubator. Cells were counted in a hemacytometer using the trypan blue exclusion method. After cells were attached on 6-well flat-bottomed plates, SE BCNU and intact BCNU were directly added to cell culture medium of 10 ml. The cells were incubated with the wafers for 3 h at 37 °C in the CO_2 incubator. SE BCNU and intact BCNU-loaded PLGA wafers were also directly added to cell culture medium of 10 ml, respectively. And then, cells were washed with PBS after removing the wafers and the culture medium. Removed wafers were passaged into the 6-well flat-bottomed plate incubated the cells for 24 h at a density of 2×10^4 cells/well, repeatedly. Cell morphology was observed after 3 days treatment. Cells were observed by microscopy system (Nicon TE-300) and photographed by microscope attached camera (Nicon F70).

The cytotoxicity following the above-mentioned treatments was evaluated by the SRB assay which measures whole culture protein content as an index of tumor cell viability. The SRB assay is an indirect measure of cell density or number of living cells attached to the culture plate. Briefly, the culture medium was aspirated and cells were fixed with 10% cold TCA. Plates

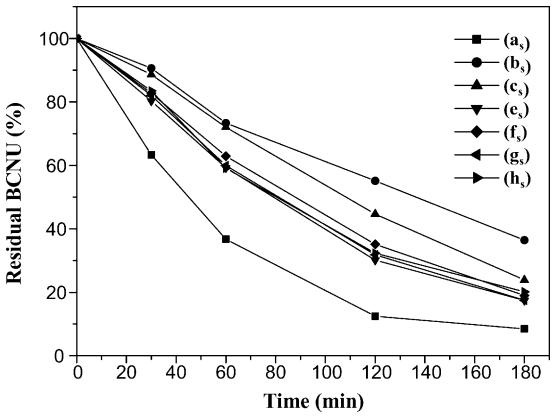

Fig. 1. The degradation kinetics of intact BCNU and SE BCNU in PBS at 37 °C: (a_s) control (intact BCNU).

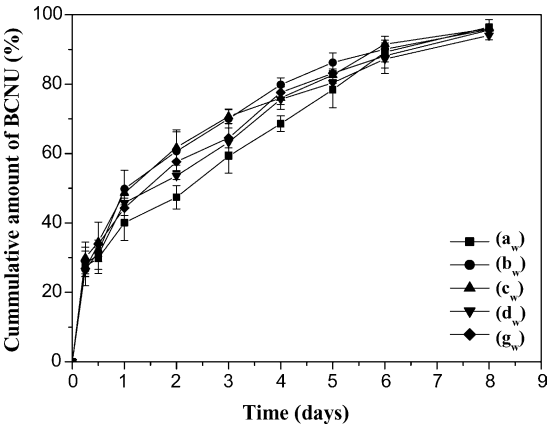

Fig. 2. The release profiles of intact BCNU and SE BCNU from PLGA wafer in PBS at 37 °C: (a_w) control (intact BCNU).

were incubated for 1 h at 4 °C and then washed several times with tap water to 25 °C for 30 min with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid. SRB was removed and cultures were rinsed several times with 1% (v/v) acetic acid to remove unbound dye. Residual wash solution was removed and plates were air-dried. The bound dye was then solubilized with 2 ml of 10 mM unbuffered TRIZMA® base solution (pH 10–10.5). After solubilizing, the absorbance was measured with a SpectraMax 250 microplate reader (Molecular Devices, Menlo Park, CA, USA) at a wavelength of 520 nm. The results of two independent experiments were expressed as treatment over control (T/C) values; cell viability (%) = $T/C \times 100$. All experiments were performed in triplicate.

3. Results and discussion

3.1. In vitro stability test and release study of SE BCNU

Degradation kinetics of intact BCNU and SE BCNU were shown in Fig. 1. Stability tests were performed through the residual amount of BCNU by detecting BCNU amount remained in PBS. In these results, half-life of intact BCNU (Fig. 1 (a_s)) was 45 min. Beside the SE BCNU composed of oil and surfactant and/or cosurfactant increased the half-life of BCNU from 80 min to 130 min (Fig. 1 (b_s)). Therefore, we suggested that the oil system is partly protecting BCNU against the hydrolysis decomposition (Constantinides, 1995; Tuleu et al., 2004) after release from matrix. The content of tributyrin and Cremophor RH40 in SE systems can influence to the stability of SE BCNU. Moreover, cosurfactant was not important for increasing the stability of BCNU.

Fig. 2 shows the release profiles of BCNU from intact BCNU and SE BCNU-loaded PLGA wafers. Drug contents of intact BCNU and SE BCNU-loaded PLGA wafers were observed from 95 to 105% and it could confirm that SE BCNU was homogeneously dispersed in PLGA matrix. The release rates of BCNU from the all wafers followed the 1st order release kinetics. Moreover, the release rates of SE BCNU-loaded PLGA wafers were faster than intact BCNU-loaded PLGA wafers because it was considered that the SE BCNU covered with oil phase had more disperse than intact BCNU into aqueous phase. It could also be explained that the penetration rate of water into the matrix was retarded with increasing the PLGA contents due to the hydrophobic property onto matrix surface (Table 2).

Table 3
Storage stability of BCNU-loaded PLGA wafer at different temperature

Temperature (°C)	Decomposition (%)	Duration of storage (days)
−18	4.6	90
4	8.1	
25	36.2	
37	54.1	

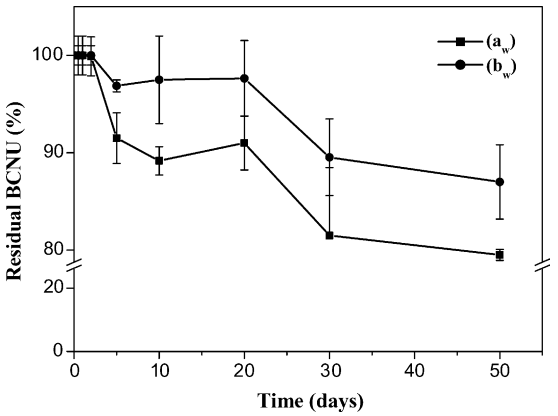

Fig. 3. Storage stability of intact BCNU and SE BCNU in PLGA wafer at 25 °C: (a_w) control (intact BCNU).

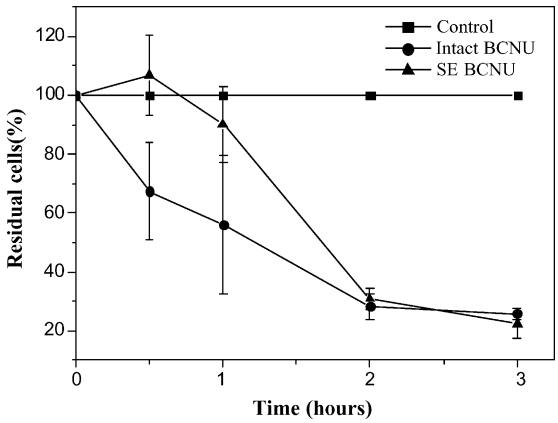

Fig. 5. The cell viability of intact BCNU and SE BCNU in PBS at 37 °C: (a_s) 30 min, (b_s) 1 h, (c_s) 2 h and (d_s) 3 h.

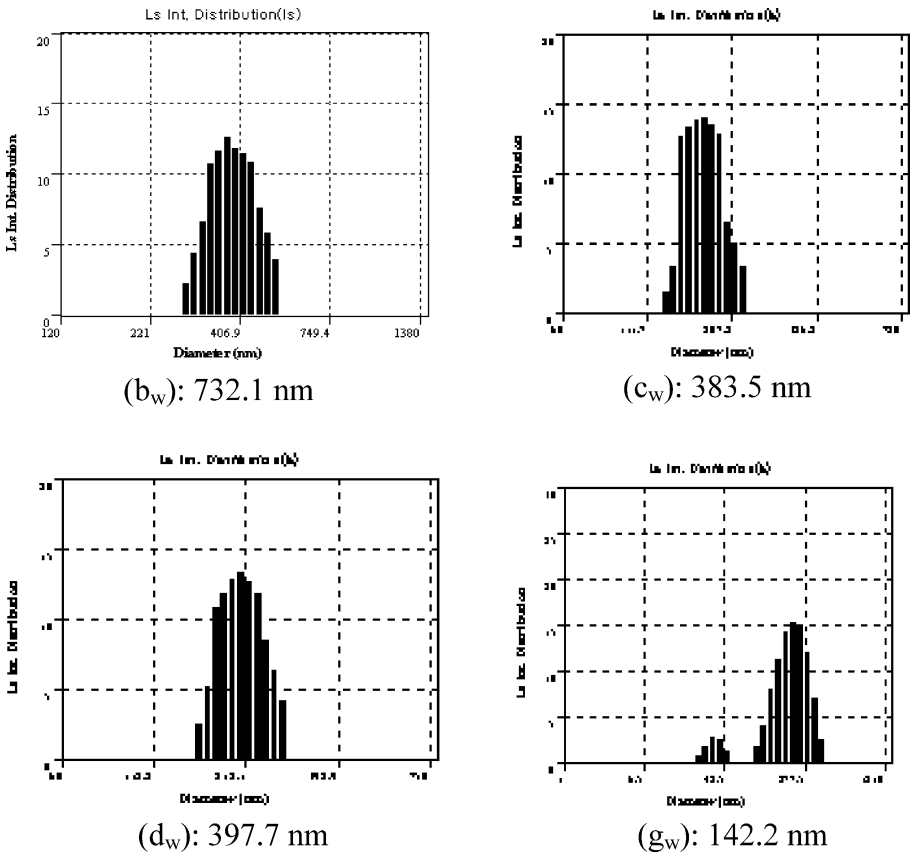

Fig. 4. The emulsion size of SE BCNU in aqueous emulsion system by ELS.

3.2. Storage stability studies

Storage stability of BCNU-loaded PLGA wafer about different temperature such as −18, 4, 25 and 37 °C was assayed by residual amount analysis for 90 days. Storage stability of BCNU preserved by PLGA increased, but showed rapid decomposition above the 25 °C (Table 3). Storage stability of intact BCNU and SE BCNU in PLGA wafer at the 25 °C was shown in Fig. 3. All wafers were stored at 25 °C for 50 days and assayed for their drug content. SE BCNU was more slowly degraded than the intact BCNU in PLGA matrix: intact BCNU and SE BCNU showed the decomposition of 20 and 10% at the 25 °C after 50 days, respectively. Therefore, BCNU was partly protected to heat decomposition by oil system in PLGA matrix. In these results, we confirmed that BCNU was very sensitive to temperature and the SE BCNU using emulsion system increased stability for storage and temperature.

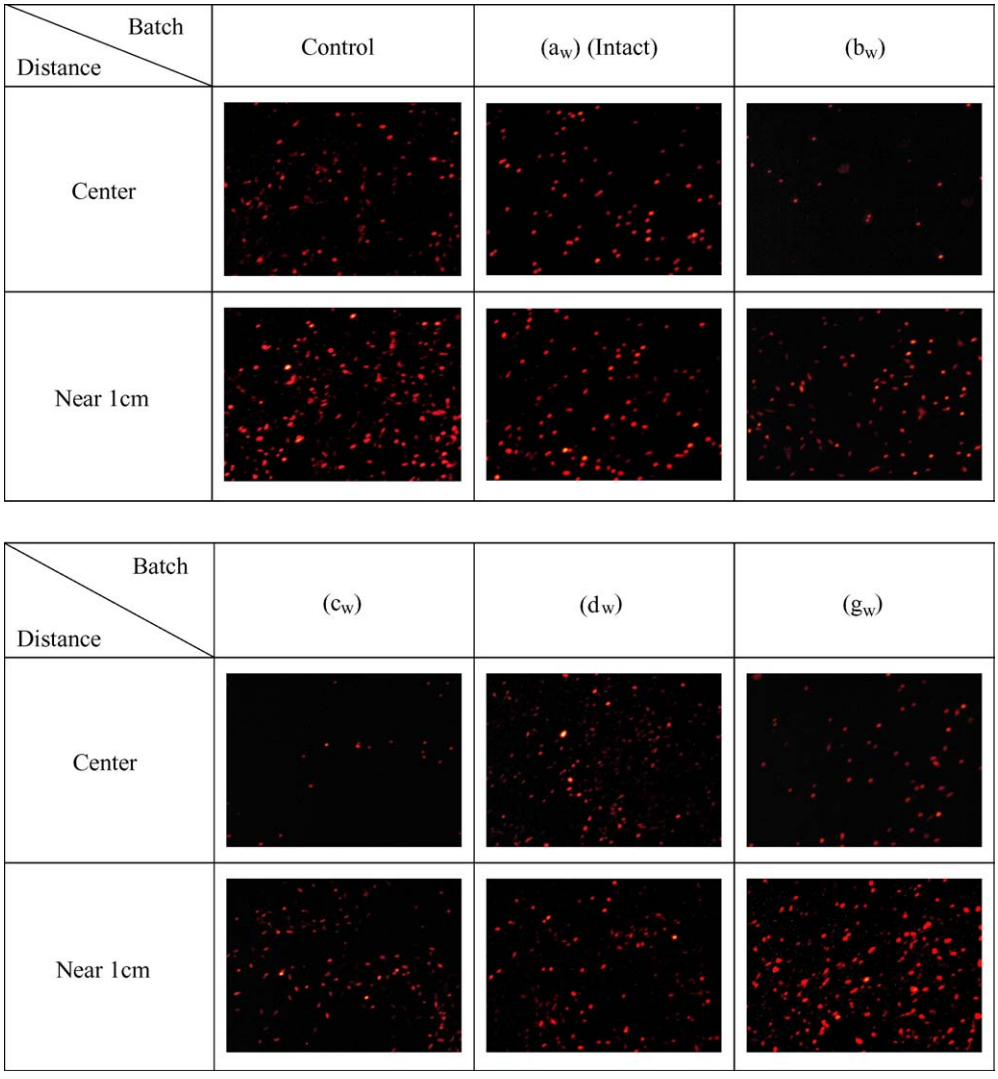

Fig. 6. In vitro morphology of 9L gliosarcoma cells after treated by intact BCNU and SE BCNU-loaded PLGA wafer for 3 days.

3.3. Emulsion size measurement of SE BCNU

The effect of the emulsion size and its distribution in proportion to the each SE BCNU formulation were shown Fig. 4. We measured emulsion size to confirm the effect on the cell viability according to change of formulation and size of SE BCNU, because more stable emulsion was obtained according to decrease of emulsion size (Gullapalli and Steth, 1996). The emulsion size of batch (b_s), constituted by tributyrin and surfactant was 732.1 nm in Fig. 4. The size of batches (c_s), (d_s) and (g_s) showed 383.5, 397.7 and 142.2 nm from increasing with amount of surfactant and co-surfactant. Therefore, the emulsion size was the greatest influenced by the variation of tributyrin rate in total amounts.

3.4. In vitro antitumor activity test

We investigated the effect of intact BCNU and SE BCNU on cell culture medium for 9L gliosarcoma cell for 3 h. In Fig. 5, intact BCNU was decreased with above of 30% of cells up to 30 min in comparison with control. Whereas, in the case of SE BCNU, all samples did not show cytotoxicity for the 9L gliosarcoma cells within 1 h, and affected the growth of 9L gliosarcoma cells after 2 h obviously. In addition, these results were strongly correlated with the results of intact BCNU in vitro antitumor activity study. From these results, we could confirm that cytotoxicity of SE BCNU came to similar effect to intact BCNU after 2 h, because SE BCNU was protected by oil system for longer time than 1 h. We can also suggest that SE BCNU can be acted on further distance than intact BCNU released from BCNU-loaded wafers.

Cell morphology of 9L gliosarcoma treated intact and SE BCNU-loaded PLGA wafers during 3 days in incubator were shown in Fig. 6. Because proliferation of cells was affected by incubation time from the morphology of control, it was used PKH26-labeled cells to suppress condition about growth of cells. The wafers were plated on 6-well flat-bottomed plates on which the cells were attached, and their cell morphology on 3 days was compared with control. In case of control, initial cells during 3 days were constant separate as well as nearby wafer. In the case of intact BCNU-loaded PLGA wafer, it affected the cytotoxicity nearby wafers but did not vary extensively at a short distance. On the other hand, the cell density of SE BCNU-loaded PLGA wafers as batches (b_w) and (c_w) was lower than intact BCNU. SE BCNU such as the batches (d_w) and (g_w) which were relatively low stability did not show high cytotoxicity against 9L gliosarcoma cell in this experiment.

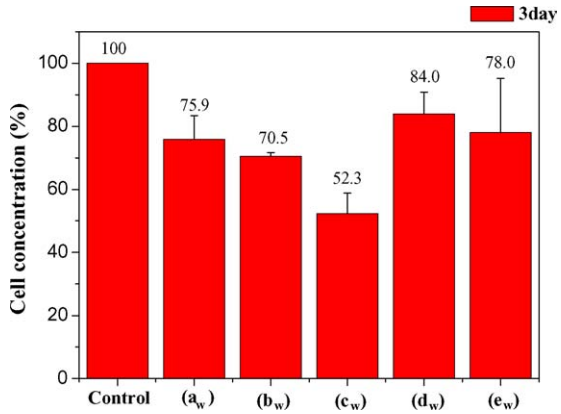

Fig. 7. In vitro antitumor activity of intact BCNU and SE BCNU-loaded PLGA wafers against 9L gliosarcoma cells.

In vitro cytotoxicity of BCNU-loaded PLGA wafers for Fig. 6 is shown in Fig. 7. This cell line was sensitive to the stability and emulsion size of released drug when it was exposed continuously to intact and SE BCNU for 3 days. In the case of PLGA wafer only (without BCNU, blank) in culture medium, all samples did not show cytotoxicity for the 9L gliosarcoma cells test period and the cells proliferate to 44.4×10^5 cells/well in forth day. Batch (a_w) such as intact BCNU-loaded PLGA wafer was dead about 25% of cells and batches (b_w) and (c_w) of SE BCNU-loaded PLGA wafers showed to higher in vitro antitumor activity, 30% and half, than intact BCNU for 3 days. Whereas, in vitro antitumor activity different SE BCNU wafers, batches (d_w) and (g_w), were similar work and/or lower than intact BCNU. As the result of that, we supposed that the size as well as stability of SE BCNU affected in the cytotoxicity against 9L gliosarcoma cell.

4. Conclusion

SEDDS composed of oil, surfactant and cosurfactant was established for the enhancement of BCNU stability. SE BCNU resulted in a significant four-fold

increase of half-life in vitro. SE BCNU degraded much more slowly than the intact BCNU in PLGA matrix at 25 °C. The release rate and cytotoxicity of BCNU from SE BCNU-loaded PLGA wafer was faster and higher than intact BCNU-loaded PLGA wafer according to the stability and emulsion size, respectively. Studies on the penetration into brain tissue and in vivo anti-tumor activity of self-emulsified BCNU-loaded PLGA wafers are now underway.

Acknowledgement

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