Articles

Development and estimation of the adherent cell culture flask with acoustic window film for ultrasound irradiation to glioblastoma cells

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I. Introduction

In recent years, transcranial magnetic resonance-guided focused ultrasound (tcMRgFUS)[1–6] surgery has been investigated and applied in the clinical setting. This treatment has an advantage over existing therapies, such as radiotherapy, in that it is minimally invasive and can be applied repeatedly. However, when brain tumor cells are coagulated and necrotized with this therapy, the surrounding normal brain tissue may be affected negatively. At worst, it is possible that brain functions are affected due to the induction of an inflammatory reaction in response to necrosis. Therefore, we have considered the induction of apoptosis in brain tumor cells by ultrasound exposure as a therapeutic approach[7]. Using this strategy, it would be possible to suppress the damage to normal brain cells since apoptosis does not induce an inflammatory response. We propose that this can be an effective treatment for high-grade malignant brain tumors with an indistinct boundary between tumor and normal tissue.

To develop this therapy, we have examined the effect of ultrasound irradiation on human glioblastoma cell lines. However, when commercial polystyrene (PS) cell culture flasks with a thickness of approximately 2 mm are employed as containers of brain tumor cells exposed to ultrasound, there is a problem in that ultrasound energy is reflected by the surface of the flask as it has a different specific acoustic impedance from the water outside of the flask and the culture medium inside the flask and the estimation of the acoustic pressure of the ultrasound wave which the cells were exposed became ambiguous. Multiple reflections between the upper and the lower surface of the flask bottom with 2 mm thick raises the temperature of the cell culture flask and adversely affects the cell culturing.

In addition, it is necessary to ensure that the entire bottom of the flask is included within the ultrasound beam completely. This is because all cells on the bottom surface of the culture flask are exposed to ultrasound. If the bottom end of flask body and the acoustic window film for cell culture

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for ultrasound exposure are bonded with adhesive, the cell culture surface of the film is contaminated and its toxicity may adversely affect cell culture. We previously reported that development and estimation of brain tumor cells culture flask with acoustic window film for ultrasound exposure[8–16] at Japanese Journal of Applied Physics paper by Iwashiro *et al*.[8]

We had to develop a proprietary cell culture flask with PET film acoustic window to culture adherent cells and expose them to ultrasound.

To address these issues, we will present a handmade adherent cell culture flask with a polyethylene terephthalate (PET) film bottom (acoustic window) for ultrasound irradiation.

In this study, we examined the welding conditions (contact load and welding time) of an ultrasound welding machine for the purpose of developing cell culture flasks for ultrasound irradiation experiments. We presented these results at the 39th symposium on Ultrasonic Electronics 2018.[17]

II. Materials and methods

2.1. Cell culture

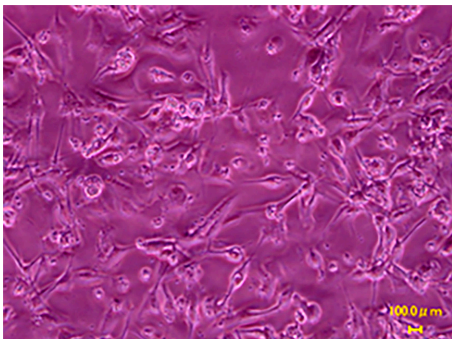
We used the YKG-1 glioblastoma cell lines (JCRB® Responsible BANK) in this study. Glioblastoma is classified as a grade IV astorocytoma by the World Health Organization, and has the highest incidence among all primary brain tumors. The 5 year survival rate of patients with glioblastoma is 7 %[18–26].

YKG-1 cells were cultured as a monolayer on the bottom surface of a commercial cell culture dish (surface area of 1000 mm^2). Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum and 1 % penicillin-streptomycin was added to the cell culture dish. The cells were cultured at 37°C in a humidified atmosphere with 5 % CO_2 in an incubator. A typical phase contrast microscope image of YKG-1 cells is shown in ***Fig. 1 (a)***. A proliferation curve of YKG-1 cells is shown in ***Fig. 1 (b)***. The measured doubling time of YKG-1 cells in our laboratory was 23 h.

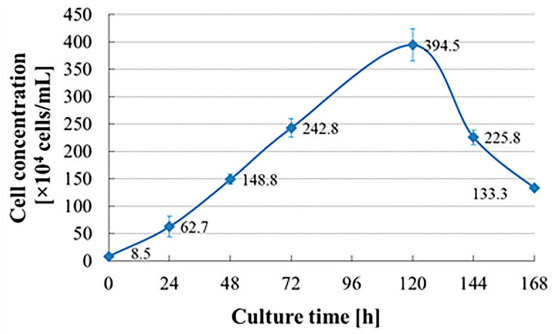
2.2. Ultrasound welding

The following characteristics are required for an adherent cell culture flask with an acoustic window film for ultrasound irradiation of glioblastoma cells; 1) minimally interference to the propagation of ultrasound waves into the flask;, 2) high cell adhesion; and 3) low toxicity to the cells.

An acoustic window film that does not interfere with ultrasound propagation should be employed for transmission of ultrasound into the cell culture flask without reflection. By using a thin polymer film with a thickness of less than 1/100 of the wavelength of the ultrasound wave, the propa-



(a) Phase-contrast microscopic image of YKG-1 glioblastoma cells.



(b) Proliferation curve of YKG-1 glioblastoma cells

Fig. 1 YKG-1 glioblastoma cell line (JCRB® Responsible BANK)

Table 1 Material properties of the acoustic windows films

Material (name of article)	Thickness [mm]	Density [g/m^3]	Acoustic velocity [m/s]	Acoustic impedance [MRayl]	Transmission coefficient [%]
Commercial PS cell culture flask	2	1.05	2340	2.46	85.77
PS film (OPS®)	0.025	1.05	2340	2.46	99.88
PET film (Lumirror®)	0.025	1.4	2337	3.27	99.67

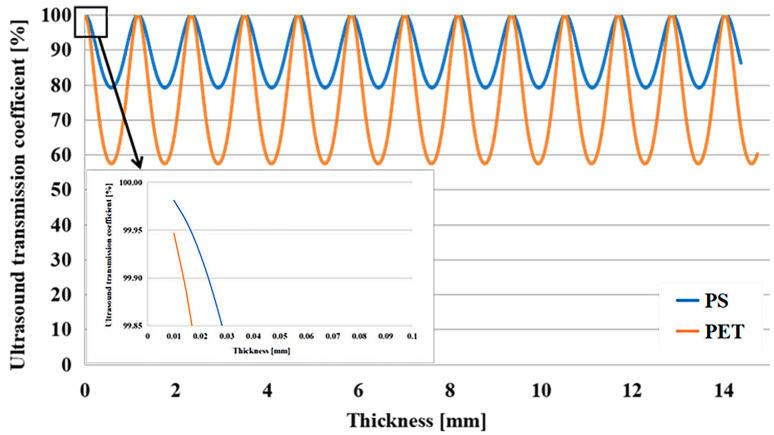


Fig. 2. Ultrasound transmission coefficients (sound pressure) of culture flasks under normal conditions.

gation of ultrasound waves is not blocked. Therefore, a cell culture flask for ultrasound irradiation was fabricated with thin polymer film acoustic windows at the top and bottom ends of a hollow cylindrical plastic pipe.

The material properties of the thin polymer films for the acoustic windows of the flask and the calculated ultrasound transmission coefficient (sound pressure) for the film of acoustic window are shown in ***Table 1*** and ***Fig. 2***. This ultrasound transmission coefficient was calculated theoretically.

From previous research, a PET film (Toray Industries, Inc. Lumirror® film, Thickness 25 μm) with excellent cell adhesion ability was selected for use in the cell culture flask for ultrasound irradiation[27–33]. A hollow cylindrical PET pipe with an outer diameter of 10 mm, wall thickness of 1.5 mm, and height of 12 mm was used for the body of the culture flask[17]. We could not perform ultrasound welding of acoustic window films to the flask body when the end faces perpendicular to the longitudinal direction axis of the PET pipe flask body were polished. Therefore, we tapered the end of the flask body in order to improve welding by focusing ultrasound energy generated by the ultrasound welder on the bonding surface.

Ultrasound welding generates heat due to friction at the end surface of culture flask by the repeated application of ultrasound vibrations and compression. Furthermore, ultrasound shock waves soften, melt, and weld the materials of flask body and acoustic window film. Ultrasound welding requires no adhesives, and the materials can be welded in a short period of time. Therefore, ultrasound welding is considered to have low cytotoxicity.

PET which is the same material as the acous-

Table 2 Settings conditions of the ultrasound welding machine.

Manufacturer	Model	Oscillating frequency	Power	Press drive system	Welding pressure	Contact load	Welding time
Seidensha Electronics Co., Ltd.	Sonopet Σ-620S Σ-P30S	28.5 kHz	600 W (max 800)	Air cylinder	10～510 N	0.09 s, 0.11 s, 0.13 s, 0.15 s, 0.2 s (Contact load : Fixed at 50 N)	10～70 N (Welding time : Fixed at 0.15 s)

tic window film is used for the flask body. Both the flask body and the acoustic window film were welded using an ultrasound welding machine (Sonopet Σ-620S/Σ-P30S; Seidensha Electronics Co., Ltd.). The condition of output power, contact load and welding time of the ultrasound welding machine were examined. We controlled two items of contact load and welding time and we measured the other items. In ultrasound welding, the inside of the workpiece is heated and the temperature rises due to friction of the welding surface by ultrasound vibration and compression repeated by applied to the material itself of the workpiece. Furthermore, the material of the workpiece is softened, melted and welded by ultrasound vibration. Ultrasound welding requires no adhesive and can be welded in a short time. Therefore ultrasound welding is considered to have low cytotoxicity for the tumor cells under test in this study.

We examined the optimum output power, contact load (maximum load applied to the welding target within one stroke of ultrasound welding), and welding time. (The welding time means the time for contract between the tool horn and target for welding.) Contact load was assessed from 10 to 70 N, and welding time was assessed at 0.09, 0.11, 0.13, 0.15, and 0.20 s. First, the welding time was fixed to 0.15 s, and the optimum contact load was examined. Then, the contact load was fixed to 50 N, and the optimum value was examined. The settings of the ultrasound welding machine are shown in ***Table 2***.

2.3. Durability tests

Durability tests were performed for the fabricated cell culture flask with acoustic window films by using our 1 MHz ultrasound irradiation system. A diagram of the experimental system is shown in ***Fig. 3***. Ultrasound was applied to the culture flask with the acoustic windows for 2 min. Continuous wave output signal with peak to peak voltage of 100 mV_{P-P} from a function generator (F.G.) was amplified by using an RF power amplifier with gain of 50 dB and was applied to a transducer at frequency of 1 MHz. In this situation, applied voltage to the 1 MHz ultrasound transducer was about 40 V_{p-p}, and the measured sound pressure and acoustic intensity at the position of 10 mm

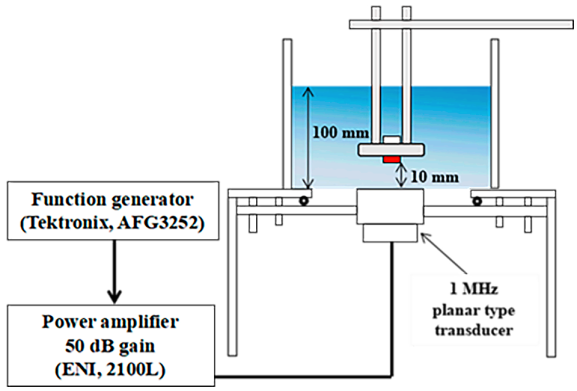


Fig. 3 Diagram of the ultrasound irradiation system for our fabricated cell flask with acoustic window films.

directly above the center of the surface of 1 MHz transducer were 230 kPa and 3 W/cm^2 respectively. The output amplified signals were applied to a transducer. The film window of the culture flask was positioned at 10 mm above the surface of the vibrating plate. We checked for leakage of a staining solution (colored liquid) from the fabricated culture flask. Temperature change in the flask was also monitored.

III. Results and discussion

3.1. Ultrasound welding

We examined the optimum welding conditions (contact load and welding time) of the ultrasound welding machine for the purpose of developing cell culture flasks for ultrasound irradiation experiments. The experimental results are shown in ***Fig. 4***.

When welding was performed with the contact load set to 40 N or more or when welding time was set to 0.15 s or more, the inside part of the flask melted, causing burrs. The blue ovals in ***Fig. 4*** indicate place where excessive welding and burr occurred. When burrs occurred, there is a concern that the cell area cannot be determined accurately. In this case, the cell culture area is discordant, and the number of cells can vary among the flasks. In addition, there is a possibility that cell growth may be affected adversely.

We found that the most favorable contact load was from 10 to 30 N, and the most appropriate welding times were 0.09, 0.11, and 0.13 s, so these conditions were examined further.

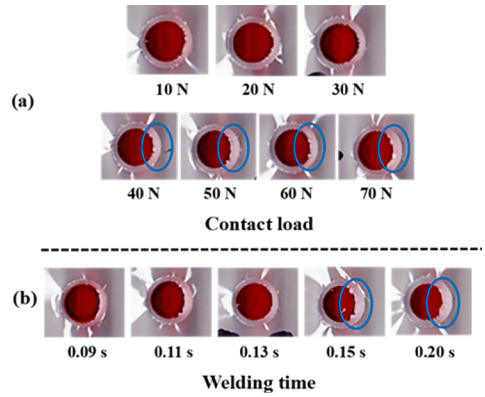
3.2. Durability test

There was no leakage of the stain solution from a cell culture flask fabricated by ultrasound welding when exposed to ultrasound with peak to peak voltage of 40 $V_{P\text{-}P}$ for 2 min. Despite exposure to a high intensity ultrasound field with the generation of acoustic cavitation, the fabricated cell culture flasks did not break. Therefore, it is considered that these flasks have sufficient durability for ultrasound exposure.

The temperature inside the flask was measured before and after ultrasound irradiation. As the flask was small, it contained a small amount of liquid, the temperature was influenced by the external temperature and flask body temperature. Thus, the establishment of a standardized experimental technique using these flasks is important when examining apoptosis.

IV. Conclusions

In this study, we examined the welding conditions (contact load and welding time) of an ultra-



(a) Relationship between contact load and welded state.

(b) Relationship between welding time and welded state.

Fig. 4. Observation of the welded state between the film and flask body by ultrasound welding.

sound welding machine for the purpose of developing cell culture flasks for ultrasound irradiation experiments.

As a results, it was found that the most favorable contact load was from 10 to 30 N, and the most suitable welding times were 0.09, 0.11, and 0.13 s. There was no leakage of the stain solution from the cell culture flask fabricated by ultrasound welding when exposed to ultrasound with peak to peak voltage of 40 $V_{P\text{-}P}$ for 2 min. Thus, we consider that these cell culture flasks have sufficient durability for ultrasound exposure.

In future, it is necessary to develop a brain tumor cell culture flask with an acoustic window for ultrasound irradiation that is not toxic to the cultured cells.

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