Polyborane-encapsulated PEGylated Liposomes Prepared Using Post-insertion Technique for Boron Neutron Capture Therapy

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Abstract: PEGylated liposomes are one of the useful boron carriers for boron neutron capture therapy (BNCT). Recently, a method of adding PEG after liposome formation (post-insertion) was reported. In this study, we prepared polyborane-encapsulated PEGylated liposomes for BNCT with half the amount of DSPE-PEG of the conventional method using post-insertion technique (post-PEG liposomes), and their usefulness were evaluated in comparison with conventional PEGylated liposomes (pre-PEG liposomes). From the results of physicochemical property measurements, it was confirmed that particle size distributions, surface charge densities, and fixed aqueous layer thicknesses of these liposomes were equivalent. In vitro cytotoxicity and cell uptake tests were also carried out using B16 melanoma and RAW264.7 cells. Polyborane solution and bare liposomes were used for comparison. From the results of these tests, we confirmed that post-PEG liposomes and pre-PEG liposomes have the same influence of PEGylation. To evaluate biodistribution properties at 24 h post-administration, these liposomes and polyborane solution were injected into the tail veins of tumor-bearing mice. Boron concentration and tumor/blood ratios of PEGylated liposomes were 73.2-77.6 µg/g of tumor tissue and 5.5-5.8, respectively. From these results, it was found that by using post-insertion technique, liposomes for BNCT having same effect as the liposome prepared using the conventional method can be prepared with half amount of DSPE-PEG.

Key words: post-insertion, PEGylated liposomes, polyborane, biodistribution, boron neutron capture therapy

1 Introduction

Research on liposomes as drug carriers for cancer treatment has been widely conducted. Liposomes are closed vesicles formed of phospholipids which are basic components of biomembrane. They can encapsulate the lipophilic drug within the lipid bilayer and the hydrophilic drug within the aqueous core. In addition, it is also possible to incorporate both types of drugs into the same lipidosome formulation1. PEGylation of liposomes has been frequently used to efficiently deliver drugs to tumors. PEGylation has the ability to decrease uptake by reticuloendothelial system, prolong blood retention, decrease degradation by metabolic enzymes, and reduce protein immunogenicity2-3). Strategies utilizing this are known to be useful for passive and active targeting to tumor cells2-4). In the conventional PEG modification method, PEG lipid is added before liposome formation (pre-insertion). However, this method has the problem that PEG is also modified on the membrane surface facing the internal aqueous phase of the liposome5-8). Therefore, when preparing liposomes using PEG lipids, or modifying ligands to PEG on the liposome surface, more PEG lipids or ligands are required compared with the original design to obtain the expected effect. This problem becomes more serious when expensive materials are used, and the increased cost makes it difficult to develop and commercialize drugs for cancer treatment8). In addition, it was reported that hydrolysis of PEG lipids was promoted when pH-gradient method was used to encapsulate drug in liposomes5,6). In this method, to effectively dissolve and encapsulate the drug in the internal aqueous phase of liposomes, the internal aqueous phase is adjusted to pH conditions at which the drug becomes water-soluble, and a pH gradient is generated between the outer aqueous phase and the inner aqueous phase. Recently, a method of adding PEG after liposome formation (post-insertion) was studied. In this method, since PEG is modified only on the mem-
brane surface facing the outer aqueous phase of the liposomes, the amount of PEG lipid or ligands required is smaller than that of pre-insertion, and by modifying PEG under physiological pH conditions, it is possible to suppress its decomposition.

PEGylation is particularly important in treating cancer using boron neutron capture therapy (BNCT). BNCT is a cancer therapy using nuclear reactions between boron-10 (10B) and thermal neutrons. Since the high linear energy transfer particles have limited path lengths in tissue (5-9 μm), the destructive effects of high-energy (~2.3 MeV) particles generated in BNCT (α particles and recoiling lithium-7 nuclei) are limited to boron-containing cells. For BNCT to be successful, it is essential that 10B atoms are selectively delivered and accumulated in the interstitial space of tumor tissue and/or the intracellular space of tumor cells. Studies on the PEGylation of formulations or the modification of ligands to PEG chains on the formulation surface to efficiently deliver the drug to tumor cells have been reported. However, little has been reported on the influences of pre-insertion and post-insertion in boron delivery.

The main aim of the present study was to investigate the utility of polyborane-encapsulated PEGylated liposomes prepared using post-insertion technique (post-PEG liposomes) in boron delivery for BNCT. 2-[(Dicarba-closo-dodecaborane) succinic acid (Fig. 1) synthesized from 1,2-dicarba-closo-dodecaborane was used as a boron carrier. The liposomes were compared with non-PEGylated polyborane-encapsulated liposomes (bare liposomes) and PEGylated polyborane-encapsulated liposomes prepared with pre-insertion technique (pre-PEG liposomes). In vitro toxicity and cellular uptake tests were performed on polyborane-encapsulated liposomes. The effectiveness in cancer treatment using BNCT was assessed by biodistribution study in tumor-bearing mice.

![Fig. 1](image)

2-[(Dicarba-closo-dodecaborane) succinic acid.

2 Experimental Procedures

2.1 Materials

Boron standard solution (boron concentration: 1000 mg/L), cholesterol (C_{27}H_{46}O, purity ≥ 90%), and glycine (purity ≥ 99%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phosphotungstic acid hydrate was purchased from Alfa Aesar (Ward Hill, MA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). Dulbecco’s modified Eagle medium (DMEM, containing 1.0g/L glucose, with L-glutamine and sodium pyruvate) and lecithin from egg yolk was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Sunbright DSPE-020CN (DSPE-PEG, N-(carboxyl-methoxy-polyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt, Mw: 2000) was purchased from NOF Corp. (Tokyo, Japan). 2-[(4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was purchased from Dojindo Molecular Technologies, Inc. (Mashiki, Japan). Isoflurane for the animals was purchased from Mylan Inc. (Pittsburgh, PA). All other chemicals were of the highest grade commercially available.

2.2 Preparation of polyborane-encapsulated liposomes

2-[(Dicarba-closo-dodecaborane) succinic acid encapsulated liposomes (bare liposomes) were prepared using the Bangham method and the remote loading. Lipid mixtures of lecithin from egg yolk (0.60 mmol) and cholesterol (0.40 mmol) were dissolved in 10 mL of chloroform in an eggplant flask, and chloroform was evaporated under reduced pressure. After a thin film was formed in the eggplant flask, it was further dried under reduced pressure for 24 h. The thin film was hydrated with Glycine-NaOH buffer (pH 10.6) to obtain a liposome suspension. Liposomes with a mean volume diameter of 100 nm were prepared using a Lipex Extruder (Northern Lupid Inc., Burnaby, Canada) and polycarbonate membranes (Nuclepore Track-Etched Membranes, Whatman Inc., Florham Park, NJ). To obtain liposomes of desired size, the sample was passed through three types of polycarbonate membranes (pore sizes: 800, 200, and 100 nm) five times each. The mixtures of 2 mL of the prepared liposome suspension and 2 mL of HEPES buffered saline (pH 7.4) were centrifuged at 60,000 rpm for 30 min at 4°C (Himac 80WX, Hitachi Koki Co. Ltd., Tokyo, Japan). After removal of the supernatant fluid, the precipitated liposomes were redispersed in 2 mL of HEPES buffered saline (pH 7.4). 2-[(Dicarba-closo-dodecaborane) succinic acid (0.25 mmol) was added to the suspension, and it was encapsulated in liposomes by incubation at 65°C for 30 min. To remove residual 2-[(dicarba-closo-dodecaborane) succinic acid, the sample was centrifuged at 60,000 rpm for 30 min at 4°C. After removal of the supernatant fluid, the precipitated liposomes were redispersed in 2 mL of HEPES buffered saline (pH 7.4), and polyborane-encapsulated liposomes with a mean volume diameter of 100 nm (bare li-
PEGylated Liposomes Prepared Using Post-insertion Technique for BNCT

2.3 Preparation of polyborane-encapsulated PEGylated liposomes

We prepared two types of polyborane-encapsulated PEGylated liposomes using pre-insertion and post-insertion. In pre-insertion method, lipid mixtures of lecithin from egg yolk (0.50 mmol), cholesterol (0.40 mmol), and DSPE-PEG (0.10 mmol) were dissolved in 10 mL of chloroform in an eggplant flask, and polyborane-encapsulated PEGylated liposomes (pre-PEG liposomes) were prepared in the same manner as described in section 2.2. In post-insertion method, lipid mixtures of lecithin from egg yolk (0.55 mmol) and cholesterol (0.40 mmol) were dissolved in 10 mL of chloroform in an eggplant flask, and chloroform was evaporated under reduced pressure. After a thin film was formed in the eggplant flask, it was further dried under reduced pressure for 24 h. The thin film was hydrated with Glycine-NaOH buffer (pH 10.6) to obtain a liposome suspension. Liposomes with a mean volume diameter of 100 nm were prepared using a Lipex Extruder and polycarbonate membranes. To obtain liposomes of desired size, the sample was passed through three types of polycarbonate membranes (pore sizes: 800, 200, and 100 nm) five times each. The mixtures of 2 mL of the prepared liposome suspension and 2 mL of HEPES buffered saline (pH 7.4) were centrifuged at 60,000 rpm for 30 min at 4°C (Himac 80WX). After removal of the supernatant fluid, the precipitated liposomes were dispersed in 2 mL of HEPES buffered saline (pH 7.4). DSPE-PEG (0.05 mmol) was added to the suspension, and the sample was incubated at 65°C for 1 h to obtain PEGylated liposomes. Then, 2-(dicarba-closo-dodecaborane) succinic acid (0.25 mmol) was added to the PEGylated liposome suspension, and it was encapsulated in liposomes by incubation at 65°C for 30 min. To remove residual 2-(dicarba-closo-dodecaborane) succinic acid, the samples were centrifuged at 60,000 rpm for 30 min at 4°C. After removal of the supernatant fluid, the precipitated liposomes were redispersed in 2 mL of HEPES buffered saline (pH 7.4), and polyborane-encapsulated PEGylated liposomes with a mean volume diameter of 100 nm (post-PEG liposomes) were obtained.

2.4 Physicochemical properties of polyborane-encapsulated bare and PEGylated liposomes

The mean volume diameter and the size distribution of the prepared liposomes were determined using a dynamic light scattering system (ELSZ-1000ZS, Otsuka Electronics Co., Ltd., Hirakata, Japan), which measures the scattered light that is generated when laser light is irradiated onto particles that are in Brownian motion, at 25°C. Also, zeta potentials of liposomes were measured in HEPES buffered saline (pH 7.4) with an ionic strength of 0.154 M at 37°C. The data were analyzed by using Ohshima’s electokinetic theory for a spherical colloidal particle, and the surface charge density was obtained using the following formula.

\[ \sigma = \frac{e \varepsilon_0 \kappa k T}{e} I \]

where \( e \) is the elementary electric charge, \( \varepsilon_0 \) is the relative permittivity of the solution, \( \kappa \) is the permittivity of a vacuum, \( k \) is the Boltzmann constant, and \( T \) is the absolute temperature. The ion pairing efficiency was given by

\[ I = 2 \sinh \left( \frac{\varepsilon \sigma}{2 k T} \right) \left( \frac{2}{\kappa a} \right) \left( \frac{\epsilon}{4 k T} \right)^2 \left( \frac{\varepsilon}{2 k T} \right) \left( \kappa a \right)^2 \sinh^2 \left( \frac{\varepsilon \sigma}{2 k T} \right) \]

where \( \zeta \) is the zeta potential, \( \kappa \) is the Debye-Hückel parameter, and \( a \) is the particle radius.

The fixed aqueous layer thickness (FALT) on the liposomal membrane, which is an important factor to avoid capture by reticuloendothelial system, was also calculated from the zeta potential values. Zeta potentials of liposomes used for the calculation were measured in HEPES buffered saline (pH 7.4) with nine different ionic strengths \((I = 0.005 \text{ M}, 0.010 \text{ M}, 0.020 \text{ M}, 0.040 \text{ M}, 0.060 \text{ M}, 0.075 \text{ M}, 0.100 \text{ M}, 0.125 \text{ M}, \text{ and } 0.154 \text{ M}) at 37°C. Boron concentrations of the liposomes were determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES, ICPE-9000, Shimadzu Corp., Kyoto, Japan). A calibration curve was prepared using a boron standard solution. The boron concentration was calculated from the peak intensity observed at a wavelength of 249.773 nm using the calibration curve. All ICP-AES measurements were carried out under the same conditions. Morphological observation of the liposomes was carried out using transmission electron microscopy (TEM, H-7650, Hitachi High-Technologies Co., Ltd., Tokyo, Japan). Samples were negatively stained using a 0.2% (w/v) of phosphotungstic acid solution, and they were stored in a desiccator for 24 h to completely remove the solvent.

2.5 In vitro release study of polyborane from liposomes

To evaluate the influence of PEylation methods on drug release behavior, the release rate of polyborane from the liposomes was studied. Liposome suspensions were diluted with phosphate-buffered saline (PBS) to a concentration of 0.25 mg/mL and shaken at 100 rpm at 37°C. After 0.5, 1, 2, 4, 8, 12, and 24 h, the samples were taken and centrifuged at 60,000 rpm for 30 min at 4°C. Then, the boron concentrations in the supernatant were measured using ICP-AES, and the release rate of polyborane was calculated.

2.6 In vitro cytotoxicity tests of liposomes

Cytotoxicity tests were performed using the WST-8 assay. Cell Count Reagent SF (Nacalai Tesque Inc.) was used as a reagent, and the assay was performed according to the manufacturer’s instructions. B16 melanoma or
RAW264.7 cells, which were cultured in DMEM containing 10% FBS, were used. Briefly, 100 μL of the cell suspension was seeded in a 96-well plate at 5.0 × 103 cells/well. After culturing for 24 h at 37°C in a 5% CO2 atmosphere, 10 μL of a polyborane solution or liposomal suspensions were added and further cultured for 24 h at 37°C in a 5% CO2 atmosphere. The boron concentration of all samples was adjusted to 40 mg/L. Ten microliters of the reagent was added and color reaction was carried out for 1 h. Then, absorbance of each well was measured using a microplate reader (ARVO X4, PerkinElmer Inc., Waltham, MA) at 450 nm to calculate cell viability.

2.7 In vitro cell uptake tests of liposomes

One milliliter of the suspension of the B16 melanoma or RAW264.7 cells was seeded in a 24-well plate at 2.0 × 105 cells/well and cultured for 24 h at 37°C in a 5% CO2 atmosphere. After washing three times with PBS, 1.0 mL of the polyborane solution or liposomal suspensions were added and further cultured for 24 h at 37°C in a 5% CO2 atmosphere. The boron concentration of all samples was diluted to 40 mg/L using DMEM containing 10% FBS. Then, the boron concentration in the supernatant was measured using ICP-AES.

2.8 Biodistribution study of polyborane-encapsulated liposomes in tumor-bearing mice

Mice (ddY, 4 weeks old, male) were housed in stainless steel cages and housed under standard environmental conditions (23 ± 1°C, 55 ± 5% humidity and a 12/12 h light/dark cycle) and maintained with free access to water and a standard laboratory diet (carbohydrates 30%; proteins 22%; lipids 12%; vitamins 3%) ad libitum (Nihon Nosan Kogyo Co., Yokohama, Japan). The experiment was conducted in accordance with the Guidelines for Animal Experimentation of Tokyo University of Science, which are based on the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. B16 melanoma cells (1.0 × 106 cells in 50 μL of PBS) were subcutaneously injected into a footpad of the right hind limb of mice, and tumor-bearing mice were given after 2-3 weeks. The tumor, brain, heart, lungs, liver, stomach, pancreas, spleen, and kidneys were immediately taken from the same individual. The blood, urine, feces, and all tissues were weighed and melted using wet ashing method with nitric acid. All boron amounts of samples were determined using ICP-AES.

3 Results and Discussion

3.1 Characterization of polyborane-encapsulated liposomes

2-(Dicarba-closo-dodecaborane) succinic acid encapsulated bare, pre-PEG, and post-PEG liposomes with average sizes of approximately 100 nm were prepared. The mean volume diameters, the polydispersity indexes, and the values of encapsulation efficacy of polyborane are summarized in Table 1. Liposomes had a similar polydispersity indexes. The encapsulation efficacies of liposomes were raised by preparing the liposomes using pH gradient, which is one of the methods to actively contain polyborane in liposomes. The particle size distributions and TEM images of liposomes were shown in Figs. 2 and 3, respectively, and we confirmed that bare, pre-PEG, and post-PEG liposomes were spherical dispersed particles. Their electrophoretic mobility was also measured, and the surface charge density was obtained (Fig. 4). The surface charge densities of bare, pre-PEG, and post-PEG liposomes were −0.72 μC/m², −0.26 μC/m², and −0.20μC/m², respectively. We considered that the absolute values of the surface charge density of pre-PEG and post-PEG liposomes were reduced because PEG was present to cover a part of the charge derived from phospholipid on the liposome surface. The values of FALT on the liposomal membrane were calculated using the following formula.

\[ \text{Tumor weight} = \frac{L \times W^2}{2} \]

where \( L \) is the length (nm) and \( W \) is the width (nm). The polyborane solution or liposome suspensions (10.0 mg boron/kg, in PBS) were injected into tumor-bearing mice via the tail vein with isoflurane anesthesia, and animals were maintained in metabolic cages. The injections were well tolerated and no adverse effects were observed. After 24 h of sample administration, mice were euthanized via cervical dislocation under anesthesia and bled at inferior vena cava. The tumor, brain, heart, lungs, liver, stomach, pancreas, spleen, and kidneys were immediately taken from the same individual. The blood, urine, feces, and all tissues were weighed and melted using wet ashing method with nitric acid. All boron amounts of samples were determined using ICP-AES.

Table 1 Properties of polyborane encapsulated liposomes (n = 3, mean ± S.D.)

<table>
<thead>
<tr>
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<th>Mean volume diameter (nm)</th>
<th>Polydispersity index</th>
<th>Encapsulation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare liposomes</td>
<td>99.2 ± 30.9</td>
<td>0.08 ± 0.01</td>
<td>70.1 ± 8.6</td>
</tr>
<tr>
<td>Pre-PEG liposomes</td>
<td>104.2 ± 30.2</td>
<td>0.08 ± 0.01</td>
<td>68.5 ± 5.6</td>
</tr>
<tr>
<td>Post-PEG liposomes</td>
<td>105.1 ± 34.0</td>
<td>0.09 ± 0.01</td>
<td>67.9 ± 6.4</td>
</tr>
</tbody>
</table>

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from the values of slope derived by linear regression in Fig. 5a. As shown in Fig. 5b, modification of PEG significantly increased the values of FALT. This result indicates that the thickness of the immobilized aqueous layer was increased by the formation of the PEG hydration layer on the liposome surface. It was reported that liposomes with a FALT value of 2.5 nm significantly increased plasma and intratumoral drug concentrations compared to that of 1.0 nm. Therefore, it was suggested that the FALT values of pre-PEG and post-PEG liposomes are sufficient to improve retention in blood. From these findings, we confirmed that bare, pre-PEG, and post-PEG liposomes were prepared successfully.

3.2 In vitro release of polyborane from liposomes

The cumulative release of polyborane from liposomes at 37°C is shown in Fig. 6. There was no significant difference, and it was shown that PEGylation methods did not affect the release behavior of polyborane from liposomes. As shown in Fig. 6, 40% of polyborane was released within 1 h, and the rest showed a slow release. This result suggests that polyborane retained on the liposome surface or

Fig. 2 Particle size distribution of polyborane-encapsulated bare, pre-PEG, and post-PEG liposomes (n = 3, mean ± SD).

Fig. 3 Transmission electron microscope image of polyborane-encapsulated bare (a), pre-PEG (b), and post-PEG liposomes (c).
lipid membrane caused an initial burst, and polyborane-encapsulated as an ionic form in the aqueous phase of the liposome did not permeate the lipid bilayer membrane.  

3.3 In vitro cytotoxicity and cell uptake efficiency of liposomes

Figure 7a shows the results of the cytotoxicity tests. The encapsulation of polyborane in liposomes tended to reduce the cytotoxicity, and particularly in RAW 264.7, which is a macrophage-derived mouse cell line, the cytotoxicity of polyborane was significantly reduced in all liposomal suspensions as compared to the polyborane solution. In addition, it was found that PEGylation methods do not affect cell viability. As shown in Fig. 7b, liposomal suspension significantly increased the uptake efficiency of polyborane in B16 melanoma and RWA264.7 cells as compared to polyborane solution. The interaction mechanism between the liposome and the cell is roughly classified into three: adsorption and binding of the liposome to the cell surface, uptake of the liposome into the cell by endocytosis, and fusion of the liposome membrane and the cell membrane. Endocytosis can be classified as phagocytosis and pinocytosis, and the mechanism for incorporating nanosized particles is mainly pinocytosis. This result indicates that the liposomes used in this study are more susceptible to cell adhesion and uptake than the simple solution. In addition, compared to bare liposomes, the uptake efficiency in RWA264.7 cells of PEGylated liposomes was reduced. It is suggested that PEG hydration layer formed on the surface of the PEGylated liposome inhibited the adsorption of the liposomes to RAW264.7 cells. In B16 melanoma cells, the number of boron atoms per cell of bare, pre-PEG, and post-PEG liposomes were $1.96 \times 10^{12}$ atoms/cell, $1.27 \times 10^{12}$ atoms/cell, and $1.33 \times 10^{12}$ atoms/cell, respectively. From this result, we confirmed that these liposomes can deliver sufficient amount of boron ($10^8$-$10^9$B atoms/tumor cell) to B16 melanoma cells for effective BNCT.
3.4 Biodistribution of liposomes

Figures 8 shows boron concentrations of blood, tumor, urine, feces, and all organs (brain, heart, lungs, liver, stomach, pancreas, spleen, and kidneys). The average boron concentrations in the tumor of pre-PEG and post-PEG liposomes reached 77.6 µg/g of tissue, and 73.2 µg/g of tissue at 24 h after injections, respectively. Moreover, these liposomes significantly increased the boron concentration in the tumor compared to bare liposomes. From this result and Fig. 7b, we considered that the retention times of these liposomes in circulation were prolonged as the PEG hydration layer formed on the surface of these liposomes inhibited adsorption to serum proteins and macrophages. It is reported that the intratumoral 10B concentration required to perform sufficient BNCT is 20-30 µg/g. Naturally occurring boron contains 19.8 10B and 80.2 11B. This intratumoral concentration can be achieved by replacing 3-4 of the 10 boron atoms in the polyborane used in this study with 10B. Tumor/blood ratio of boron concentration, which is the ratio of a boron concentration of tumor divided by that of blood, was also calculated from the results shown in Fig. 8. In the liposomal suspensions, average tumor/blood ratios reached 5.5 at 24 h after injection, whereas that of polyborane solution was 4.4. In other organs, the boron concentrations were 30 µg/g of tissue or less, and in the liver, it was less than 4 µg/g of tissue. The low boron concentration in the liver and high boron concentration in the feces suggest that polyborane hardly accumulated in the liver and rapidly migrated into the bile from the liver. The amount of polyborane excreted in urine in the polyborane solution administered group tended to be higher than in the liposomal suspensions administered group. The nanoparticles with a diameter of 100 nm are not eliminated by the glomerular filtra-
Thus, it was assumed that the polyborane released from liposomes in the blood circulation was excreted by renal excretion. From these results, it was confirmed that biodistribution of post-PEG liposomes was similar to pre-PEG liposomes.

4 Conclusion

We have prepared polyborane-encapsulated PEGylated liposomes that show equivalent efficacy with half the amount of PEG lipid used in the conventional method by using post-insertion technique. The calculated results of surface chase density and FALT showed that there was no difference in PEG modification of the liposome surface in post-PEG liposomes and pre-PEG liposomes. In vitro cell viability, in vitro cell uptake efficiency, and in vivo biodistribution of post-PEG liposomes were similar to that of pre-PEG liposomes. The results of this study will facilitate the use of post-insertion technique in research for BNCT and will contribute to the development of liposome formulations using PEG lipids and ligands.

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Conflict of Interest

The authors declare that they have no potential conflicts of interest.

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