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Elemental Boron-10 Nanoparticles Synthesized by Laser Fragmentation for Boron Neutron Capture Therapy: *In Vitro* Experiments

A. I. Kasatova[a,b], K. S. Kuzmina[b], D. A. Kasatov[b], E. V. Barmina[a,c], K. O. Aiyyzhy[c], P. A. Kotelnikova[a,d], M. S. Grigoryeva[a], D. S. Petrunya[a,e,*], E. L. Zavjalov[f], S. Yu. Taskaev[a,b], and I. N. Zavestovskaya[a,e]

[a] *Lebedev Physical Institute, Russian Academy of Sciences, Moscow, 119991 Russia*
[b] *Budker Institute of Nuclear Physics, Siberian Branch, Russian Academy of Sciences, Novosibirsk, 630090 Russia*
[c] *Prokhorov General Physics Institute, Russian Academy of Sciences, Moscow, 119991 Russia*
[d] *Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, 117997 Russia*
[e] *National Research Center Kurchatov Institute, Moscow, 123182 Russia*
[f] *Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences, Novosibirsk, 630090 Russia*
**e-mail: d.petrunya@lebedev.ru*

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Abstract—Elemental boron-10 nanoparticles synthesized by laser fragmentation of micropowder in isopropanol are used for the first time as a boron-containing agent for boron neutron capture therapy. The study is conducted on human cell cultures U87, BT474, and BJ-5TA. It is found that these nanoparticles are nontoxic to all three cell cultures at boron-10 concentrations required for successful boron neutron capture therapy. The cell cultures are pre-incubated with nanoparticles and then irradiated for 30 min with a beam of epithermal neutrons generated by the VITA accelerator source (BINP SB RAS, Novosibirsk). Using in vitro study results (MTT assay and clonogenic analysis), an enhancement of the therapeutic effect of boron neutron capture therapy is observed.

Keywords: boron neutron capture therapy, elemental boron nanoparticles, laser fragmentation, cell cultures

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1. INTRODUCTION

GLOBOCAN—the cancer incidence database of the International Agency for Research on Cancer and the World Health Organization (WHO)—estimated the incidence of 20 million new cases of malignant neoplasms (MNs) in 2022 worldwide. Malignant neoplasms are responsible for 9.7 million deaths in 2022 [1] and are among the top three most common causes of death in people aged 30–69 in 177 of 183 countries [2].

Boron neutron capture therapy (BNCT) is a method of radiation therapy based on the selective destruction of tumor cells. The therapy uses a nonradioactive isotope boron-10, which is administered in the form of various compounds and irradiated with a flux of epithermal neutrons. The boron-10 neutron capture reaction results in the formation of a lithium nucleus and an α-particle, which are characterized by high energy release and a short range, which allows tumor cells to be locally destructed [3]. This method has been used in clinical trials at nuclear reactors and accelerator-based neutron sources and has demonstrated positive results [4]. @Fig 1

Currently, boronphenylalanine (BPA) is used in clinical practice. This drug does not have high selectivity for accumulation in tumor tissues, and is also characterized by variability in absorption by tumors of the same histological type. This is why the search continues for boron delivery agents that would meet such characteristics as absence of toxicity, concentration greater than 20 μg/g, tumor/blood and tumor/healthy environment ratio of 3 : 1 or more, maintenance of sufficient concentration in the tumor throughout the entire irradiation period, and rapid elimination of the drug from surrounding tissues and organs [5]. Currently, development of delivery drugs based on various nanosystems is underway, including

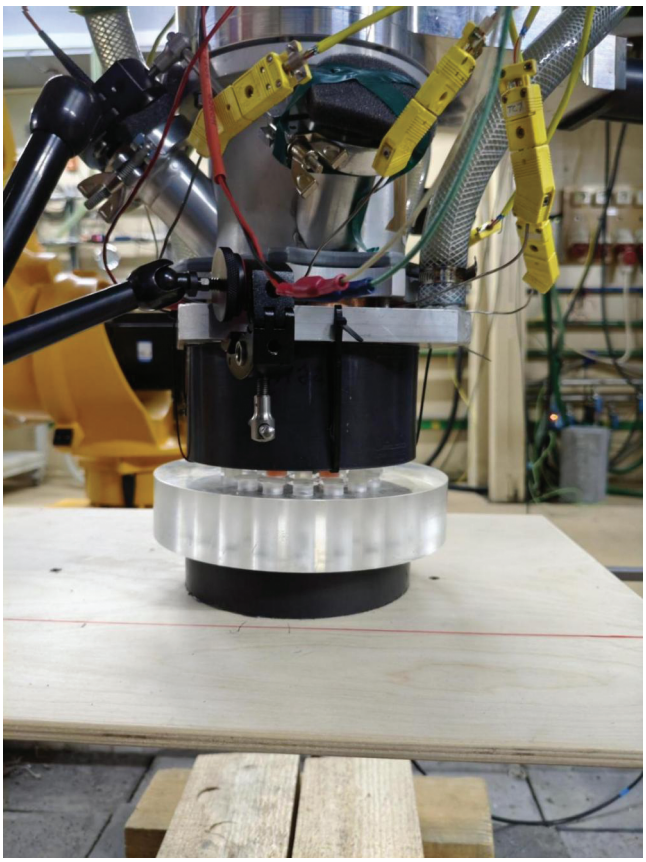

Fig. 1. Geometry of the *in vitro* experiment: positioning of the phantom with cells of the "irradiation without NPs" and "irradiation with NPs" groups in the neutron field.

liposomes, antibodies, protein conjugates, aptamers, etc. [6]. The use of nanoparticles (NPs) in targeted radionuclide therapy technologies and binary radiation therapy methods is a promising area that can improve efficiency and reduce side effects and the impact of ionizing radiation on healthy organs [7–9]. Boron-based NPs are actively used as sensitizers for binary hadron therapies: proton beam therapy [10–12] and therapy based on use of thermal and epithermal neutron beams [13]. In comparison with molecular boron-containing drugs for BNCT, designed to play this role [14], elemental boron NPs carry the maximum number of atoms that effectively interact with the radiation.

In this work, we studied elemental boron NPs obtained by laser fragmentation in isopropanol of boron micropowder enriched with the boron-10 isotope by 85%. Nanoparticles with a high content of ^{10}B were used for the first time in *in vitro* BNCT experiments. Previously, we demonstrated low cytotoxicity and high efficiency of BNCT using amorphous and crystalline boron NPs obtained by laser ablation and fragmentation of natural boron micropowder (with a ^{10}B content of only 20%) [13].

2. EXPERIMENTAL

2.1. Description of Nanoparticles

Boron NPs were obtained by laser fragmentation of boron-10 micropowder in isopropanol in a flow cell. An Nd:YAG laser (EKSPLA NL210-SH, Ekspla Vilnius, Lithuania) was used as a radiation source; it had the following characteristics: wavelength of 1064 nm, pulse duration of 4 ns, pulse energy of 2.8 mJ, and pulse repetition rate of 1 kHz. Boron concentration was 100 μg/ml, and the volume of the working fluid was 70 ml. Laser radiation was moved using a galvano-optical system at a speed of 100 mm/s and focused with an F-Theta objective ($F = 10$ cm). The calculated diameter of the laser beam in the waist was 50 μm. The fragmentation time of the suspension was 60 min. As a result of the dynamic scattering analysis, we found that laser fragmentation of boron micropowder for 60 min allows boron NPs with sizes from 40 to 200 nm to be produced. To synthesize NPs with a size of 50 nm, the supernatant was collected

after centrifugation for 5 min at 3000*g*. The emphasis on achieving such an average size was due to the planned biological applications of elemental boron NPs, since the size of the NPs greatly affects the efficiency of their cellular internalization and the duration of circulation in the bloodstream [15, 16].

The surface of the obtained NPs was modified with a Silane-PEG coating. For 1 ml of NP suspension (1 g/l), we added 100 μl of Silane-PEG (5 kDa) in ethanol (1 g/l), 65 μl of water, and 20 μl of 30% aqueous ammonia solution. The samples were thoroughly mixed on a vortex and sonicated. The coating was deposited at 60°C for 4 h. Then the NPs were washed from the polymer in 96% ethanol by three-fold centrifugation at 5000*g* for 15 min and transferred to water by three-fold centrifugation at 5000*g*. The size of the coated NPs was 141.2 ± 51.7 nm. The suspension remained stable in water for at least 1 month. Slow sedimentation of the largest particles was observed; after shaking the test tube, the suspension restored its properties. In the cell medium with the addition of 10% serum, the NPs were stable for at least one day; the hydrodynamic sizes of the NPs immediately, 1, 3, and 24 h after adding the medium were, respectively, $D_0 = 145.3 \pm 66.3$ nm, $D_{1h} = 146.7 \pm 58.8$ nm, $D_{3h} = 147.9 \pm 56.2$ nm, and $D_{24h} = 145.2 \pm 65.7$ nm.

2.2. Experimental Protocol

The study was performed on human cell cultures U87 (glioblastoma), BT474 (breast carcinoma), and BJ-5TA (immortalized dermal fibroblasts), which were cultured using a standard technique according to the culture passport.

To assess the cytotoxicity of elemental boron NPs, we used the MTT assay based on the colorimetric assessment of cell activity by the intensity of the reduction reaction of the tetrazolium dye MTT by NAD(P)H-dependent oxidoreductase enzymes into insoluble formazan [17]. Cells in the exponential growth phase were seeded in 96-well plates at 4×10^4 and then incubated with elemental boron NPs in a wide range of boron-10 concentrations for 24 h. Control wells were incubated in a boron-10-free medium, with the number of repetitions for each point being 5 ($n = 5$). After that, the optical density of the solutions in each well was measured using a Multiskan SkyHigh spectrophotometer (Thermo Fisher Scientific, USA). The percentage of surviving cells was calculated relative to the control group.

To conduct experiments aimed at determining the viability of cell cultures after irradiation with a neutron flux, a control group and three experimental groups were formed. The cells of the control group were not incubated with NPs and were not irradiated; the cells of the "irradiation without NPs" group were irradiated with a neutron flux without preliminary incubation with NPs; the cells of the "NPs without irradiation" group were incubated with NPs and not irradiated; the cells of the "NPs with irradiation" group were pre-incubated with NPs and irradiated with a neutron flux. In order to prepare the samples for irradiation, the cells were seeded in culture flasks with a growth surface area of 25 cm^2. After 24 h, the cells were placed in a medium containing NPs with a boron-10 concentration of 40 μg/ml of a complete culture medium and incubated for 24 h ("NPs without irradiation" and "NPs with irradiation" groups). The cells of the control group and the "irradiation without NPs" group were incubated in a medium that did not contain boron-10. Then the cells were washed with sodium phosphate buffer, removed from the plastic, precipitated by centrifugation, and transferred into cryoampoules in a volume of 1 ml (1×10^6 cells). The cryoampoules were transported in a heat-insulating container.

2.3. Irradiation

Irradiation was performed using the VITA accelerator neutron source at the BINP SB RAS [18]. The VITA accelerator neutron source consists of a tandem accelerator with vacuum insulation for producing a stationary beam of protons or deuterons, an original thin lithium target for generating neutrons as a result of the $^7Li(p,n)^7Be$ reaction, and a vertical beam formation system optimized for irradiation of specific biological objects [19]. Polyethylene with a volumetric bismuth inclusion of 72 mm in height, located between the target unit and the phantom, was used as a beam formation system. Cryoampoules with cells of the "irradiation without NPs" and "irradiation with NPs" groups were placed in the cells of a 220-mm-high polymethyl methacrylate phantom at a distance of 25 mm from the phantom center; cryoampoules with deionized water were placed in the empty cells of the phantom. The phantom was placed under the beam formation system at a distance of 10 mm, and a neutron reflector was also placed under the phantom—polyethylene with a volumetric bismuth inclusion of 30 mm in height. The phantom with cells was positioned using the ESTUN ER50B-2100 robotic arm (China). Before irradiation, the neutron flux distribution was measured by a small detector with two lithium polystyrene scintillators, one of which was enriched with boron. Irradiation was carried out for 30 min at a proton energy of 2.0 MeV to a current flu-

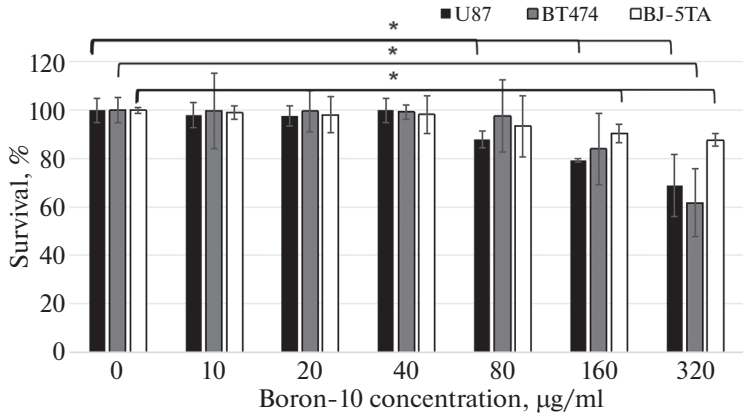

Fig. 2. Survival of U87, BT474, and BJ-5TA cell cultures after incubation with elemental boron NPs without irradiation for 24 h: MTT assay, the asterisk * denotes $p < 0.001$ compared to the control group, Mann–Whitney U test ($n = 5$).

ence of 1 mA h. Cryoampoules with cells from the control and "NPs without irradiation" groups were placed under the same conditions in a room with a natural radiation background.

To determine cell survival after BNCT and the effects of neutron flux and NPs, we performed an MTT assay and clonogenic analysis. For the MTT assay, the cells were seeded in 96-well plates at 1×10^4 [8 replicates for each experimental group ($n = 8$)] and incubated for 4 days. Survival was calculated relative to the values of the control groups, based on the optical density of each well.

For the clonogenic assay, 200 cells were seeded per well of a 12-well culture plate [4 replicates for each experimental group ($n = 4$)]. On the 10th day, the cells were fixed with a mixture of 6% glutaraldehyde and 0.5% crystal violet to stain the colonies. Colonies containing more than 50 cells were visually counted using an Olympus CKX53 light inverted microscope (Japan). The proportion of surviving cells in the experimental groups was calculated taking into account the survival in the control group.

2.4. Statistical Processing

Quantitative data were expressed as mean ± standard deviation. The obtained data were statistically processed with the STATISTICA 10.0 program, using the nonparametric Mann-Whitney U test at a significance level of $p < 0.001$.

3. RESULTS AND DISCUSSION

The cytotoxicity of elemental boron NPs in boron-10 concentrations of 10–320 μg/ml of the culture medium was determined using the MTT assay (Fig. 2) [17]. It was found that these NPs were not toxic for all three cell cultures in boron-10 concentrations up to 80 μg/ml, which indicates the safety of using NPs in concentrations required for BNCT [5]. The BT474 cell culture was the least sensitive to the effects of elemental boron NPs; a toxic effect was detected upon incubation with NPs with a boron-10 concentration of 320 μg/ml. At the same time, the survival rate of U87 statistically significantly decreased already upon exposure to NPs with a boron-10 concentration of 80 μg/ml.

Figure 3 shows the percentage of cell survival in the experimental groups relative to the control ones. Irradiation of cells in the "irradiation without NPs" group did not result in a significant decrease in survival, which indicates the safety of using the selected irradiation parameters. The survival of U87 cells incubated with elemental boron NPs and not exposed to irradiation decreased by 3.5%, while for BT474 and BJ-5TA cell lines, a decrease in survival was 2.6 and 1.5%, respectively. The survival of all cultures in the "irradiation with NPs" group used in the experiment statistically significantly decreased relative to the control and other experimental groups ($p < 0.001$).

Figure 4 shows the results of the clonogenic analysis after irradiation of cell cultures with a flux of epithermal neutrons. This test is often used in radiobiology to determine the disruption of the proliferative activity of cells, expressed in their ability to divide, after exposure to ionizing radiation [20]. The proportion of surviving U87 cells in the "irradiation with NPs" group was 35%, which is significantly different from the result obtained in the other groups. The greatest effect was found for the BT474 cell culture, with the proportion of surviving cells being less than 1%. The obtained data are not entirely consistent with the

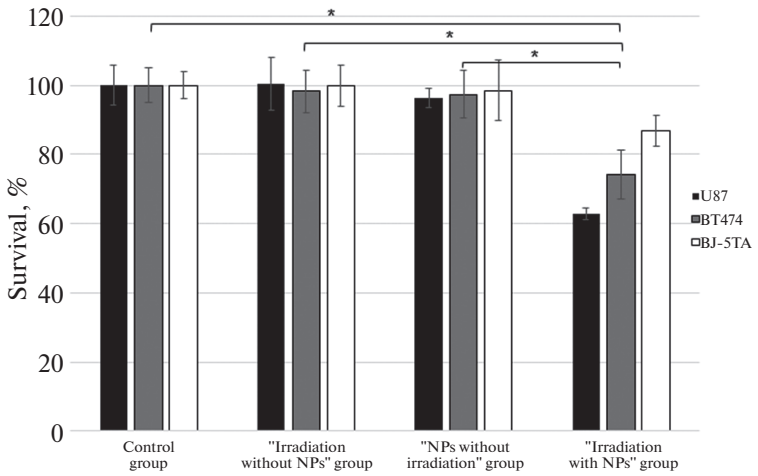

Fig. 3. Survival of U87, BT474, and BJ-5TA cell cultures pre-incubated with elemental boron NPs at a concentration of 40 μg/mL after irradiation with a neutron flux for 30 min (E_p = 2.0 MeV, I = 1 mA h): MTT assay; asterisk * denotes $p < 0.001$ in comparison with the control group, with the "irradiation without NPs" group, and with the "NPs without irradiation" group; Mann–Whitney U test (n = 8).

result obtained in the MTT assay: the survival of BT474 cells after irradiation with a neutron flux in the presence of NPs was 74%, while the survival of U87 in this test was lower and was 63%. The high survival of cells according to the results of the MTT assay is presumably due to the fact that the metabolic activity of mitochondrial enzymes in cells can be preserved even after their significant damage [21], while the clonogenic analysis allows one to determine the ability of a cell to form a colony and is a method for assessing the suppression of cell proliferative activity [22]. The BJ-5TA cells demonstrated the highest resistance to irradiation with a neutron flux in the presence of NPs, with the proportion of surviving cells decreasing down to 42% relative to the control group. Despite the different results of the two cell tests, in both cases tumor cell cultures were more sensitive to irradiation with a neutron flux in the presence of NPs than the fibroblast culture. Note that the proportion of surviving cells in the "irradiation without NPs" and "NPs without irradiation" groups decreased by no more than 5% relative to the control group.

A decrease in clonogenic activity after BNCT depended on the cell culture. In this study, tumor cells of two histological types were used: breast carcinoma and glioblastoma, as well as dermal fibroblasts. In their work, Hellweg et al. [23] noted the variability of the proportion of surviving mouse embryonic stem cells of different lines from 5 to 70% after exposure to ionizing radiation at a dose of 4 Gy [23]. Huang C.Y. et al. [24] studied the effect of BNCT on radioresistant cell cultures of hepatocellular carcinoma. It was found that the proportion of surviving cells exposed to γ-radiation at a dose of 5 Gy was 15%, while the same dose received during BNCT was lethal for 100% of the cells, which is associated with a higher relative biological effectiveness of BNCT [24]. According to a number of researchers, elemental boron NPs do not have active targeting properties and also tend to aggregate in aqueous solutions over time [6]. The use of NP conjugates with targeting biomolecules can increase the targeted delivery of compounds to tumor cells and improve the effectiveness of the therapy [25].

The literature describes a number of attempts to increase boron accumulation in a tumor by attaching targeting agents. For example, low-molecular compounds such as folic acid were used for delivery overexpressing FR-α pteroyl-*closo*-dodecaborate to U87 MG glioblastoma cells [26]. Short arginine peptides [27], a peptide penetrating through the cell membrane with borocaptate [28], and a gastrin-releasing peptide receptor antagonist with carborane [29] were also used for targeted delivery of boron to tumor cells. In addition, rapid proliferation of tumor cells is accompanied by a significant increase in glucose uptake, which allows the use of carbohydrates for selective accumulation of boron compounds in the tumor [30]. In this work, the surface of elemental boron NPs enriched with the boron-10 isotope by 85% was modified with a polyethyleneglycol-based Silane-PEG coating using silane chemistry methods to increase the colloidal stability of the NPs and enhance their biocompatibility [31].

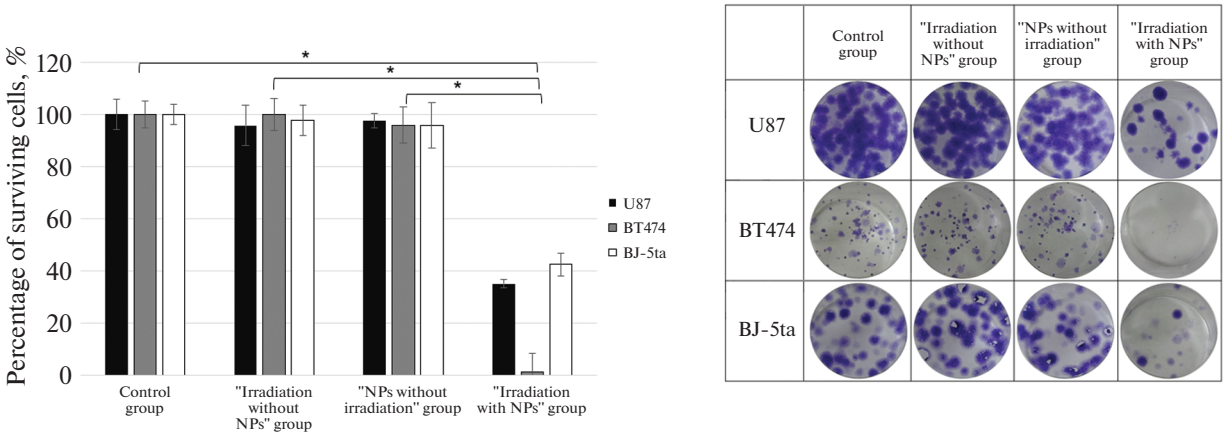

Fig. 4. Determination of the percentage of surviving U87, BT474 and BJ-5TA cells pre-incubated with elemental boron NPs after irradiation with a neutron flux for 30 min (E_p = 2.0 MeV, I = 1 mA h): clonogenic analysis; asterisk * denotes $p < 0.001$ in comparison with the control group, with the "irradiation without NPs" group, and with the "NPs without irradiation" group; Mann–Whitney U test ($n = 4$).

4. CONCLUSIONS

We demonstrate the use of elemental boron NPs obtained by laser fragmentation of micropowder in isopropanol, the surface of which was modified with a Silane-PEG coating, as an agent for BNCT. *In vitro* studies were performed on human cell cultures U87, BT474, and BJ-5TA. The MTT assay was used to assess the cytotoxicity of elemental boron NPs. It was found that these NPs are nontoxic for all three cell cultures at boron-10 concentrations required for BNCT. To determine the cell viability after exposure to a neutron flux in the presence of NPs, the MTT assay and clonogenic analysis were conducted. The cell cultures were pre-incubated with NPs and then irradiated on a VITA accelerator-type neutron source for 30 min. Irradiation of cells in the "irradiation without NPs" group with an epithermal neutron flux did not lead to a reliable decrease in survival, which indicates the safety of using the selected irradiation parameters. At the same time, in the "irradiation with NPs" group, dermal fibroblasts BJ-5TA were the most resistant to the action of the BNCT reaction products, with the percentage of surviving cells decreasing to 42% relative to the control group. The greatest effect was found for the cell culture of breast carcinoma BT474, with the percentage of surviving cells being less than 1%.

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CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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