

USING THE LUMINESCENT DYES FOR THE ASSESSMENT OF LIPOSOME TRANSPORT PROPERTIES AS THE BORON (^{10}B) CARRIER FOR BORON NEUTRON CAPTURE

R. Mukhamadiyarov^{1,2*}, A. Tsygankova³, V. Kanygin^{2,3}

¹Federal State Budgetary Institution “Research Institute for Complex Issues of Cardiovascular Diseases”, Kemerovo, Russia

²Budker Institute of Nuclear Physics, Novosibirsk, Russia

³Novosibirsk State University, Novosibirsk, Russia

Abstract. High effectiveness of Boron Neutron Capture Therapy (BNCT) makes actual the investigation aimed at creating transport systems for the targeted delivery of boron-containing agents. Liposomes are currently among promising boron carriers for BNCT. Existing liposomal technologies allow changing their properties by changing the particle diameter, surface charge, lipid composition, and the presence of vector molecules on the surface of the liposome membrane. The structure of liposomes can include in their composition hydrophobic and lipophilic boron-containing agents at the same time, which increases the content of boron atoms in them. Methods for rapid assessment of dynamic absorption and localization of substances delivered in their composition are required for experiments to improve liposomal drug delivery systems. The method of labeling the lipid membrane of liposomes and their internal contents is a great interest in view of the presence of a large number of various luminescent dyes and highly efficient methods for assessing their intracellular localization (confocal microscopy). By using the method of the rapid freezing of tissues and the preparation of cryosections from them makes it possible to perform an express assessment of the liposomes transport properties for a high volume of samples. The blue region of the spectrum for labeling liposomes did not use in our experiments leaving it for the nuclear dyes (Hoechst 33342, DAPI). Nile red was used for labeling liposomal membranes (excitation/emission maxima ~552/636 nm), PKH-26 (excitation/emission maxima ~551/567 nm), TopFluor PC (excitation/emission maxima ~495/503 nm). High molecular dextran derivatives FITC-Dextran (excitation/emission maxima ~495/520 nm), Rhodamine B isothiocyanate–Dextran (excitation/emission ~570/590 nm) were used for labeling internal water core liposomes. The combination of the proposed luminescent labels allows us to determine the localization of the labels of liposomes delivered in the lipid and aqueous phases selectively and makes it possible to extrapolate these data with respect to hydrophilic and lyophilic boron-containing agents. The remaining free region of the spectrum lying in the far-red spectrum allows using it for determining the localization of liposomes in certain organelles, for example, mitochondria (MitoTracker Deep Red for mitochondria, Liso Tracker Deep Red for lysosomes, etc).

Keywords: Boron Neutron Capture Therapy, liposomal transport, fluorescent labeling, liposomal targeting

1. INTRODUCTION

The creation of targeted liposomal delivery systems of Boron-containing liposomes to cancer tissues, cells and organelles is one of the problems of modern Boron Neutron Capture Therapy (BNCT). BNCT makes it possible to destroy tumor cells selectively without damaging normal cells [1-3]. BNCT is based on the nuclear capture of epithermal neutrons with the formation of alpha particles and ^7Li nuclei that cause radiation damage to cells [4-6]. Only cells containing ^{10}B are exposed to radiation damage due to the short trajectory of these particles (9-10 μm), which is approximately equal to the cell diameter.

For a high therapeutic efficacy of BNCT, it is necessary to create a high gradient of boron concentration between the tumor and healthy tissues. For this purpose, it is necessary to develop ^{10}B directional transfer systems in the tumor [7-9].

These studies require solving the problem of detection of the precise localization of substances delivered with liposomes. The complexity of this problem is associated with the invisibility of liposomes in the light microscope because of the small size, lack of color and autoluminescence. A possible way of solving this problem is to visualize the liposomes by including fluorescent dyes in their composition [10]. In the study with a fluorescent or confocal microscope, colocalization of liposomal dyes and dyes for differential staining of intracellular organelles may serve as evidence of the presence of liposomes in this compartment. For these studies, an important prerequisite is an adequate choice of dyes for labeling liposomes and organelles. We investigated the methodological approaches of the study of intracellular localization of the liposomes in the cells using vital dyes for labeling liposomes and intracellular organelles. Using the rapid freezing of target organs and the preparation of cryosections from them makes it possible to perform an express assessment of the

* rem57@rambler.ru

liposomes transport properties for a large volume of material.

Modern methods based on fluorescence and confocal microscopy provide determination of intracellular localization of even single molecules.

However, despite the variety of existing fluorescence labels for research, individual selection of dyes for labeling liposomes and subcellular organelles is required.

In our study, we used fluorescence and confocal microscopy to determine the intracellular localization of substances included in luminescent-labeled liposomes, which were introduced to cell cultures and laboratory animals.

The aim: assessment of uptake and distribution of liposomes in tumor cells in experiments.

2. EXPERIMENTAL PROCEDURE

2.1. Selecting a combination of fluorescent probes for the study

The principle of determining the intracellular localization of liposome is based on fluorescent labeling of intracellular structures and liposomes. After the interaction of the cells with the labeled liposomes, localization of each fluorescent dye is determined using the fluorescent or confocal microscopy. The colocalization of labeled intracellular organelles and liposomal dyes will indicate the presence of liposomes in the specific compartment.

In these experiments, the correct choice of luminescent dyes is necessary, taking into account the possibility of intravital coloration and luminescence spectra. Currently, there is a wide range of fluorescent probes for cellular research. This includes dyes for selective dying of nuclei, lysosomes, mitochondria, and the endoplasmic reticulum.

Since liposomes may contain biologically active substances both in their lipid and inner aqueous parts, the study of their transport capacity may include separate labeling for lipid and aqueous phases.

Since we used live cells in our study, the preference was given to vital dyes.

The nuclei were stained using Hoechst 33342 (Sigma-Aldrich) and DAPI (Molecular Probes) which represents a contrasting probe for nucleic acids.

Mitochondria were selectively labeled using MitoTracker Deep Red (Molecular Probes).

One of the following dyes may be used to label lipid part of the liposomes:

1) PKH 26 is lipophilic and vital. It can be used for the labeling of both cellular and liposome membranes. Fluorescence of this dye is observed in the red region of the spectrum.

2) TopFluor PC is a green fluorescent lipophilic dye with a molecular weight of 909.6. It is suitable for the labeling of liposomal membranes.

3) Nile Red can be used for the labeling of both liposome membranes. Fluorescence of this dye is observed in the red region of the spectrum.

Dyes of the internal aqueous phase:

1) FITC-Dextran is water-soluble dextran with a molecular weight of 4500 conjugated with fluorescein isothiocyanate. It is impermeable for phospholipid membranes and therefore is optimal for the labeling of the internal aqueous phase of the liposomes. Fluorescence of this dye in the cell corresponds to the localization of soluble substances delivered by the liposomes. It is non-toxic and suitable for vital staining. Fluorescence of this dye is observed in the green part of the spectrum.

2) Rhodamine B-Dextran is water-soluble dextran with a molecular weight of 4500 conjugated with Rhodamine B isothiocyanate. It is impermeable for phospholipid membranes and therefore is optimal for the labeling of the internal aqueous phase of the liposomes. Fluorescence of this dye in the cell corresponds to the localization of soluble substances delivered by the liposomes. It is non-toxic and suitable for vital staining. Fluorescence of this dye is observed in the red part of the spectrum.

The characteristics of the fluorescent dyes are presented in Table 1.

2.2. Lipids and chemicals: 1,2-Distearoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol))-2000] (PEG2000-DSPE), diethyl ether and chloroform were purchased from Sigma Aldrich.

Enriched with ^{10}B isotope, mercaptoundecahydrododecaborate (BSH) was obtained from (Katchem, Czech Republic).

2.3. Preparation of labeled liposomal formulations

In our investigation, we used two types of liposomes: (1) liposomes containing the label only in the membrane and (2) liposomes containing the label in the membrane – and inside the liposomes.

2.3.1. Preparation of liposomes containing the label in the lipid membrane

Liposome Preparation with BSH and Nile Red.

Liposomes were prepared according to the reverse-phase evaporation (REV) method. 1 g DSPC, 0.4 g DSPE-PEG 2.000, 0.254 g cholesterol was dissolved in 21 ml of chloroform-diethyl ether mixture (1:1 by volume). Nile Red, PKH 26 or TopFluor PC in the amount of 1.5 mg was used as a lipid tag. Then 12 ml of 125 mM BSH was added to the solution.

The emulsion was sonicated by ultrasonic disintegrator (UD-20, Poland) for 1 min and then the volume was reduced to a gel by evaporation in a rotary evaporator (Heidolph LABOROTA 4000, Germany) at 30°C under vacuum for 1 h.

The resulting gel was extruded 10 times through polycarbonate filters (Corning/Costar) with a pore diameter of 100 nm at 60 °C using the Lipex Biomembranes extruder (Canada).

2.3.2. Preparation of liposomes containing the label in the lipid membrane and inside the liposomes

In this type of liposomes, the FITC-Dextran solution was used as an aqueous medium in a concentration of 45 mg/ml.

BSH and hydrophilic dye, which were not included in the liposomes, were removed by liposome deposition and were subsequently resuspended in a 0.9% NaCl solution.

2.4. Glioma cell lines

U87 glioma cell lines were used in all cell experiments. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako) with 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

2.5. Interaction between liposomes and cells in cell cultures

In order to avoid the possible influence of Hoechst and MitoTracker Deep Red on the uptake of liposomes, exposure with liposomes preceded staining.

Various combinations of lipophilic and hydrophilic dyes were used to label liposomes.

Liposomes were added to the culture medium and incubated with cells for 2 hours at 37°C. The liposome concentration was 1 mg lipid/1 ml medium. After the incubation, the cells were rinsed in the fresh culture medium 3 times. Then nuclei and mitochondria were stained.

2.6. Mice model

A group of 8-week-old males of the Nu/J mouse line was subcutaneously injected with 10 million cells of the U87 line. After 4 weeks, the animals were intravenously injected with 0.2 ml of liposomes containing Nile Red. After 2 h and 6 h after injection of mice, euthanasia was performed and the tumor was removed. The tumor volume was controlled by measuring the length and width and then fixed in 4% buffered paraformaldehyde for 48 hours. The samples were then washed from the retainer in four shifts of 0.1 M sodium-phosphate buffer and frozen in liquid nitrogen.

These samples were used to prepare cryocutters for luminescent staining and microscopic studies.

2.7. Staining of cell nuclei

We used two ways of nuclear staining, Hoechst 33342 dye was used for vital staining. The dye in a concentration of 1 mg/ml was added to the culture medium and stained for 30 min. Fixed preparations were stained by Hoechst dye. The dye in a concentration of 3

mg/ml was added to the culture medium and stained for 14 min.

Tumor cryosection staining was performed similarly to cell culture staining.

2.8. Staining of mitochondria

The Mito Tracker Deep Red (Molecular Probes) dye in a concentration of 100 nM was added to the culture medium and stained for 30 min.

2.9. Fluorescence and confocal microscopy

In our research, we used a confocal microscope LSM 780 (Carl Zeiss, Germany) and fluorescence microscope AxioObserver Z.1 (Carl Zeiss, Germany). Each series of experiments was conducted 6 times. The typical results of experiments are shown in figures.

Table 1. Fluorescent probes used in the experiments

Fluorescent probe	Staining structure	Peak Excitation, nm	Peak Emission, nm
Hoechst 33342 (Sigma-Aldrich)	DNA (nuclei)	346	460
MitoTracker Deep Red (Molecular Probes)	mitochondria	644	655
PKH-26 (Sigma Aldrich)	lipid membrane of liposomes	551	567
TopFluor PC (Avanti Polar Lipids)	lipid membrane of liposomes	495	503
Nile Red (Molecular Probes)	lipid membrane of liposomes	552	636
FITC-Dextran (Sigma Aldrich)	aqueous phase of liposomes	495	520
Rhodamine B-Dextran (Sigma Aldrich)	aqueous phase of liposomes	570	590

3. RESULTS AND DISCUSSION

The absorption and distribution of liposomes containing Nile Red in the liposome membrane were studied on glioma cell culture at the first stage of experiments (Fig. 1). The appearance of intensive luminescence in the red region of the spectrum related to the liposomal marker confirms that liposomes are absorbed by tumor cells in high concentrations. On the

basis of the analysis of colocalization of the specific dye of cell nuclei Hoechst 33342 and liposomal marker (Nile Red) it can be concluded that the liposome part is localized only in cytoplasm and is not found inside the nuclei. Similarly, it can be expected that lipophilic B-containing agents delivered as part of liposomes will be mainly localized in the cytoplasm of cells.

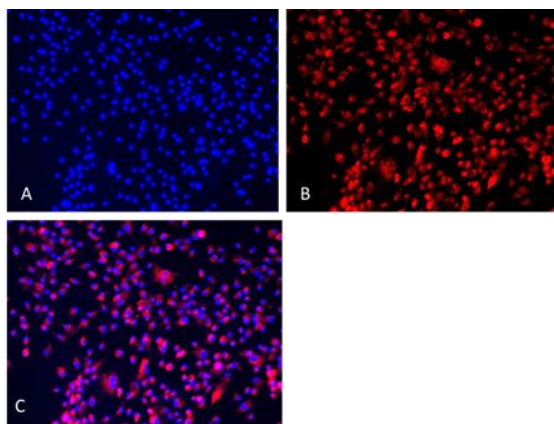


Figure 1. Intracellular localization of liposomes containing Nile Red. A - nuclei colored with Hoechst 33342, B - Nile Red luminescence, C - merged. Cell culture U87. Luminescence microscopy.

In experiments with cell incubation in an environment containing liposomes with luminescent markers in the lipid bilayer and an internal nucleus, the simultaneous presence of both dyes inside the cells was noted. However, their localization was slightly different.

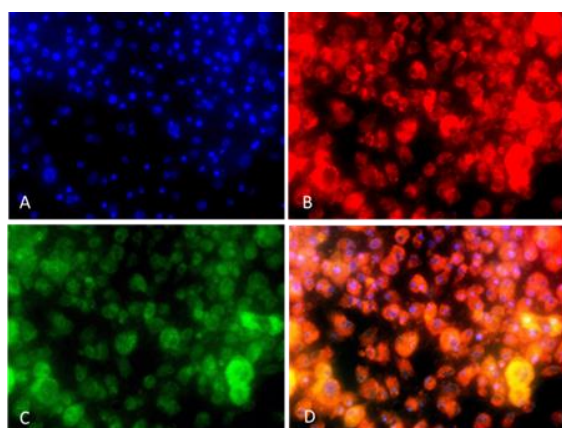


Figure 2. Intracellular localization of liposomes containing Nile Red and FITC-Dextran. A - nuclei stained with Hoechst 33342, B - Nile Red luminescence, C - FITC-Dextran luminescence, D - merged. Cell culture U87. Luminescence microscopy.

Similarly to the first variant, Nile Red luminescence was observed in the cytoplasm and the region of nuclei localization was free from this dye (Fig. 2 A, B). Luminescence localization of FITC-Dextran was close to Nile Red localization, but partially covered the area of nuclei location. The simultaneous presence of both dyes in cells indicates that liposomes are absorbed by cells

without violating their structural integrity. At the same time, the observed differences in intracellular localization of dyes can be considered as a consequence of partial destruction of liposomes in the cytoplasm, accompanied by a release of the aqueous phase of liposomes with FITC-Dextran exit from them.

It is possible to determine the subcellular localization of liposome components using the presented methodical approach. In such a variation of the research we used luminescent dyes selectively staining the required organelles. Figure 3 shows the data of such an experiment, in which MitoTracker Deep Red dye was used as a marker.

As well as in the case considered earlier, we observed a parallelism in the localization of both liposomal marks. Both dyes were in the cytoplasm and were practically absent in the areas of nuclei localization. Most of the dyes were present in the composition of cytoplasmic granular structures. These structures also demonstrated the positive staining of MitoTracker Deep Red, which indicates the presence of liposomes in the mitochondria.

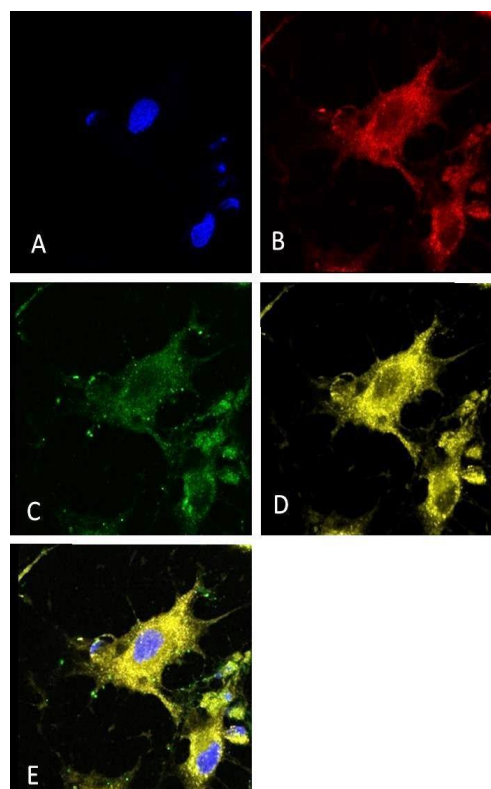


Figure 3. Localization of nuclei (A), the coloring of Hoechst 33342, liposomal lipids (B), the coloring of PKH 26, the aqueous phase of liposomes (C), FITC-Dextran, mitochondria (D), the coloring of MitoTracker Deep Red, E - merge. Cell culture U87. Confocal microscopy.

Similarly to the experiments on cell cultures, we carried out the experiments to study the localization of labeled liposomes in mice tumors after intravenous administration of the substance. In these experiments, liposomes containing Nile Red lipid layer were used. In order to take into account the possibility of the influence

of boron-containing substances on liposome uptake, borcapate was added to their composition in therapeutic dosage. According to the data presented in Fig. 4, liposomes injected into the bloodstream were found in the composition of individual tumor cells 2 hours after their injection. 6 hours after the liposome injection, the intensity of luminescence of liposomal dye in cytoplasm increased significantly, reflecting the dynamics of liposome absorption from blood flow. Intracellular localization of liposomes in experiments on animals was similar to that obtained in experiments on cells (Fig. 1).

Thus, the examined experimental scheme allows us not only to estimate the presence of liposomal labels and intracellular localization of substances introduced into liposomes but also to determine the kinetic parameters of the process of liposome absorption. Since the determination of luminescence intensity is much less labor-intensive in comparison with the quantitative determination of boron concentration, this method allows estimating the dynamics of boron absorption and accumulation in the process of its liposomal delivery. Based on these parameters, it is possible to determine the terms of the maximum therapeutic efficacy of BNCT.

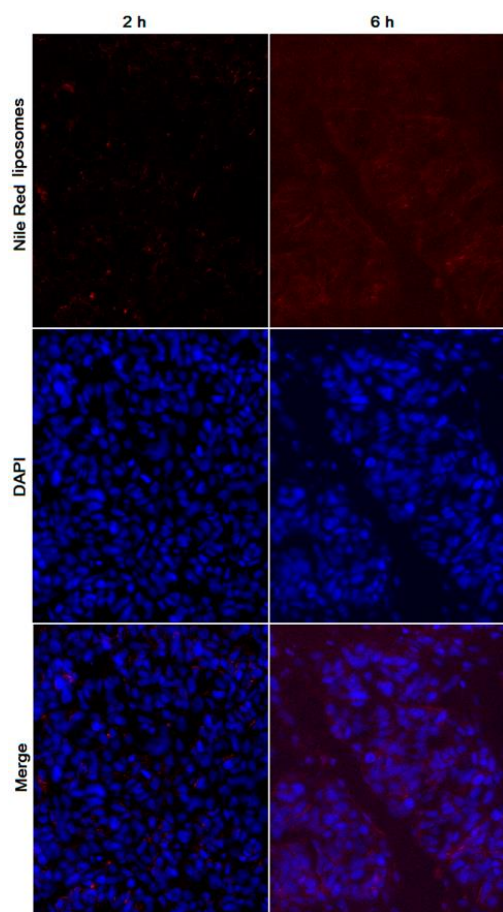


Figure 4. Fluorescence intensity of BSH PEG-liposomes labeled with Nile red in orthotopic heterotransplant tissues of glioblastoma U87 of experimental animals (SCID MICE) 2 h, 6 h after injection. Confocal microscopy.

4. CONCLUSION

The discussed technology of intracellular liposomes distribution estimation by means of separate luminescent labelling of hydrophilic and hydrophobic parts and determination of colocalization of luminescent labels has shown its efficiency. It can be used in experiments optimizing the chemical compound and size of ^{10}B containing liposomal compositions providing the boron delivery into tumor cells.

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