

**METHODOLOGICAL APPROACHES TO EVALUATION
OF INTRACELLULAR DISTRIBUTION OF LIPID AND AQUEOUS PHASE
OF LIPOSOMES USING FLUORESCENT PROBES**

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Abstract

Creation of targeted liposomal delivery systems of biologically active substances to the tissues, cells and organelles is one of the topical problems of cellular medicine. These studies require solving the problem of detection of the precise localization of substances delivered with liposomes. Complexity of this problem is associated with invisibility of liposomes in the light microscope because of the small size, lack of colour and autoluminescence. A possible way of solving this problem is to visualize the liposomes by including in their composition fluorescent dyes. In the study with 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. In this work, we investigate the methodological approaches of the study of intracellular localization of the liposomes in the cells using vital dyes for labeling liposomes and intracellular organelles. On specific examples, we demonstrate that the technology of the assessment of intracellular distribution of fluorescence using labeling of hydrophilic and hydrophobic parts of the liposomes and determination of the colocalization of these labels and organelle-specific fluoresce is effective and can be used in experiments aiming to create liposomal formulations for targeted drug delivery.

1. INTRODUCTION

Over the next decade, one of the widely accepted strategies in the pharmaceutical industry will remain design of the most effective drugs with a minimum of side effects. A promising approach in solving this problem is to develop targeted drug delivery systems among which great importance belongs to liposomal formulations (Fan, Zhang, 2013; Levchenko, Hartner, Torchilin, 2012; Rani, 2013).

Structural features of liposomes make them effective means of drug delivery into cells and tissues (Goyal et. al., 2005; Torchilin, 2005; Rani, 2013). Current nanotechnologies enable targeting liposomes to specific cells and tissues, encapsulate various biologically active substances in the liposomes and produce preparations with enhanced bioavailability, low toxicity and sustainable to biodegradation (Doijad et. Al., 2009 ; Kulkarni, Yadav, Vaidya, 2011 ; Maurer et. al., 2001).

Recent researches have been aimed at the design of liposome formulations which are targeted not only to particular type of cells but also to specific intracellular organelles. Depending on the desirable pharmacological effect, liposomal compositions targeted to mitochondria, nuclei, endoplasmic reticulum or lysosomes can be produced (Thekkedath, 2012; Boddapati et. al., 2008; Elbayoumi, Torchilin, 2010 ; Pollock et. al., 2010).

In the study of targeted liposomal drug delivery, it is important to make an adequate choice of methods for determining both the localization of the liposome themselves and substances incorporated in them.

The main problem in the evaluation of intracellular liposome localization in medical and biological studies is invisibility of the liposomes under the light microscope due to their small size and lack of color. A possible solution of this problem can be inclusion into the liposomes labeling compounds which can provide visualization and quantitative analysis. These compounds should not affect the properties of the liposomes, be robustly affiliated in their composition, have no toxic effect. The presence of these labels in tissues, cells and subcellular organelles allows determining the presence of the liposomal components. Fluorescent dyes may serve as the liposome labeling compounds (Ishida et. al., 2002 , Kamp et. al., 1997 , Sherpof et. al., 2001).

Fluorescent dyes for liposomes and organelles chosen according to the specific aims of the studies can ensure detailed determination of intracellular localization of even single molecules.

Each fluorescent dye has its own properties including a number of parameters: the object or the "staining" structure, fluorescent intensity, different peaks of excitation and emissions. Thus, the optimal choice of the combination of fluorescent dyes for liposome labeling, selection of their optimal concentration and time of exposure, methods of evaluation of particular label localization are important in the studies with several fluorescent dyes.

Aim: to develop a detailed protocol of the study of uptake and intracellular distribution of liposomes using the fluorescent and confocal microscopy.

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 of the researcher's interest and liposomes. After interaction of the cells with the labeled liposomes, localization of each fluorescent dye is defined 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.

A number of aspects should be taken into account while selecting a combination of fluorescent probes.

Nowadays, the biomedical market offers a wide range of fluorescent probes for cellular studies. This includes dyes which are able to label selectively nuclei, lysosomes, mitochondria and endoplasmic reticulum (Jonson, Spence, 2010).

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 (Verma et. al., 2005).

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

The nuclei were stained using Hoechst 33342 (Sigma-Aldrich) which represents a contrasting probe for nucleic acids. This probe becomes fluorescent as a result of binding to double-stranded DNA molecule. This dye is specific for adenine-thymine nucleotide pair.

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 (Sigma-Aldrich) is lipophilic and vital. It can be used for labeling of both cellular and liposome membranes. Fluorescence of this dye is observed in the red region of the spectrum.
- 2) TopFluor PC is green fluorescent lipophilic dye with a molecular weight of 909.6 . It is suitable for labeling of liposomal membranes.

Labeling of the internal aqueous phase:

- 1) FITC-Dextran (Sigma - Aldrich) 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 labeling of 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 region of the spectrum.

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

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
FITC-Dextran (Sigma –Aldrich)	aqueous phase of liposomes	495	520

Selected probes have different peaks of excitation and emission. Therefore, in case of optimal choice of the filter, multiple labels on a single sample can be visualized.

Two combinations of fluorescent probes were selected based on comparison of excitation and emission and taking into account that the labels must be present in liposomes, nuclei and mitochondria. In both cases, nuclei and mitochondria were labeled with Hoechst 33342 and MitoTracker Deep Red, respectively. However, the combinations of liposomal fluorescent dyes were different. In the first case, lipid phase of the liposomes was labeled with PKH- 26 whereas FITC-Dextran was used for labeling of aqueous phase. In the second case, lipid phase was labeled with TopFluor PC. Both nuclei in two combinations of fluorescent probes exhibited violet-blue fluorescence (346 nm) and labeled mitochondria had deep red fluorescence (644 nm). In the first combination of dyes, lipid phase of liposomes exhibited red fluorescence (551 nm), whereas the aqueous phase had green fluorescence (495 nm). In the second combination of dyes, lipid part exhibited green (495 nm) fluorescence.

2.2. Cell cultures and preparation of fluorescent solution

Experiments were performed on cultured rat fibroblasts. Cells were seeded on coverslips in six-well plates. The cells were grown until their adhesion to the coverslips. Then the wells were gently washed with cell culture medium to remove non-adherent cells. Adherent fraction was used in further experiments. All manipulations with the cells were performed directly in the plates with addition of the fluorescent dyes and labeled liposomes. Procedures with the live cells were performed in a CO₂ incubator. When the experiment was completed, the cells were fixed with 4 % formaldehyde in PBS and rinsed three times for 5 min in PBS. Then the coverslips with cells were removed from the wells transferred onto glass slides and immersed in 30 µl of mounting medium. The nitrocellulose polish was applied along the edges of the coverslips. In two hours, slides were ready for viewing under a fluorescent microscope.

Mowiol 4-88 (MOWIOL Mounting Media) was used in our study. For preparation of the medium, 2.4 g of Mowiol 4-88 was added to 6 g of glycerol. The mixture was gently stirred with a glass rod and 6 ml of deionized water was added. The resulting mixture was constantly stirred for several hours at room temperature. Then, 12 ml Tris (pH 8.5) was added and the mixture was heated to 65°C with continued stirring until the maximal dissolution of the precipitate. The solution was centrifuged at 3000g for 20 min, placed into the plastic tubes and stored at -20°C. The medium was defrosted directly before use.

The obtained samples were analyzed under the fluorescent microscope AxioVision Z.1 (Carl Zeiss) using X40 and X100 objectives and the following filter sets: Filter Set 49 for Hoechst 33342; Filter Set 09 for FITC-Dextran and TopFluor PC; Filter Set 15 for PKH- 26 and MitoTracker Deep Red.

2.3. Fluorescent staining of intracellular organelles

In addition to the correct choice of the fluorescent probes, optimal concentration and the exposure time of each dye are important in the study involving a combination of different dyes in order to obtain similar fluorescent intensity of the probes.

This article presents a detailed protocol for all manipulations with the cells necessary for achieving the goals.

Fluorescent staining of nuclei

For labeling the nuclei, the fluorescent dye Hoechst 33342 (Sigma-Aldrich) was used. It should be noted that Hoechst 33342 is incompatible with phosphate buffer.

For preparation of the stock solution, 10 mg of Hoechst 33342 was diluted in 1 ml of deionized water.

For staining the cells in the wells of the plate, 0.6 μ l of the stock solution was added to the culture medium and the mixture was gently stirred. The volume of the medium in the wells was 3 ml. Thus, the dye concentration was 0.002 μ g/ml. Cells were incubated in the Hoechst 33342 labeling solution for 20 min at 37°C.

Fluorescently labeled nuclei of isolated fibroblast are shown in Figure 1. The nuclei exhibit bright blue fluorescence. Heterochromatin is well visualized in the nucleus under x40 and x100 objectives.

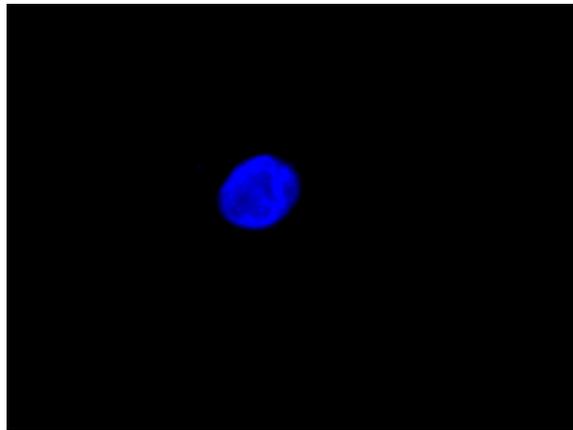


Fig. 1. Distribution of the fluorescent dye Hoechst 33342 in the fibroblast nucleus

Fluorescent staining of mitochondria

For preparation of the stock solution of MitoTracker Deep Red, 1 mg of the dye was dissolved in 1 ml of high quality dimethylsulfoxide.

This probe is sensitive to blood oxidases and, therefore, they are not recommended to be used with complete medium.

Mitochondrial staining followed the nuclei staining. The cells were rinsed 3 times for 5 minutes with culture medium without fetal bovine serum. Then, 0.6 μ l of the stock solution of MitoTracker Deep Red was added to the cells immersed in 3 ml of fresh medium. The cells were incubated with MitoTracker Deep Red for 30 min at 37°C. The results of fluorescent labeling are shown in Figure 2. Separate discrete structures exhibiting bright red fluorescence are observed in the periphery of cells. The number of such structures increases greatly in the central region of the cells and individual mitochondria become undistinguishable in this area.

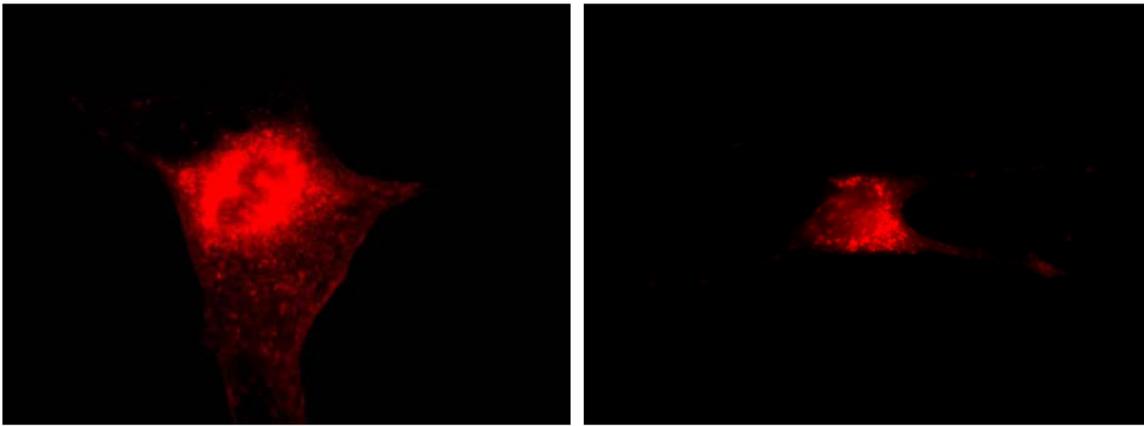


Fig.2. MitoTracker Deep Red – labeled mitochondria of rat fibroblast

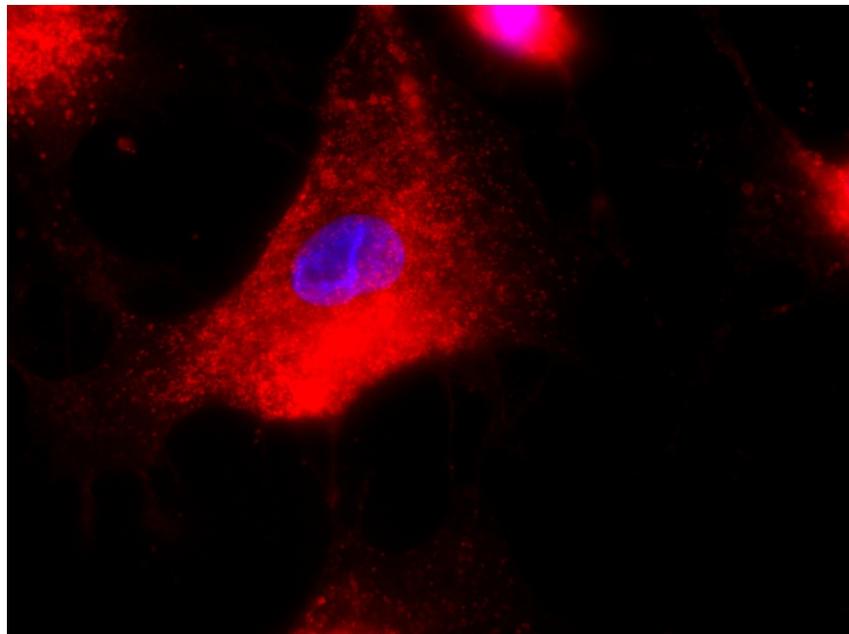


Fig.3. Fibroblast cells showing the colocalization of Hoechst and MitoTracker Deep tracers

2.4. Experimental protocol for evaluation of uptake and intracellular distribution of labeled liposomes

In order to avoid possible influence of Hoechst and MitoTracker Deep Red on the uptake of liposomes, this step of the experiment was performed before applying the above mentioned dyes.

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 according to the above mentioned staining procedure.

Below are examples of implementation of the described technique.

2.5. Examples

Example 1. The intracellular distribution of liposomes with PKH26-labeled lipid and FITC-Dextran-labeled internal aqueous phase (Fig. 4).

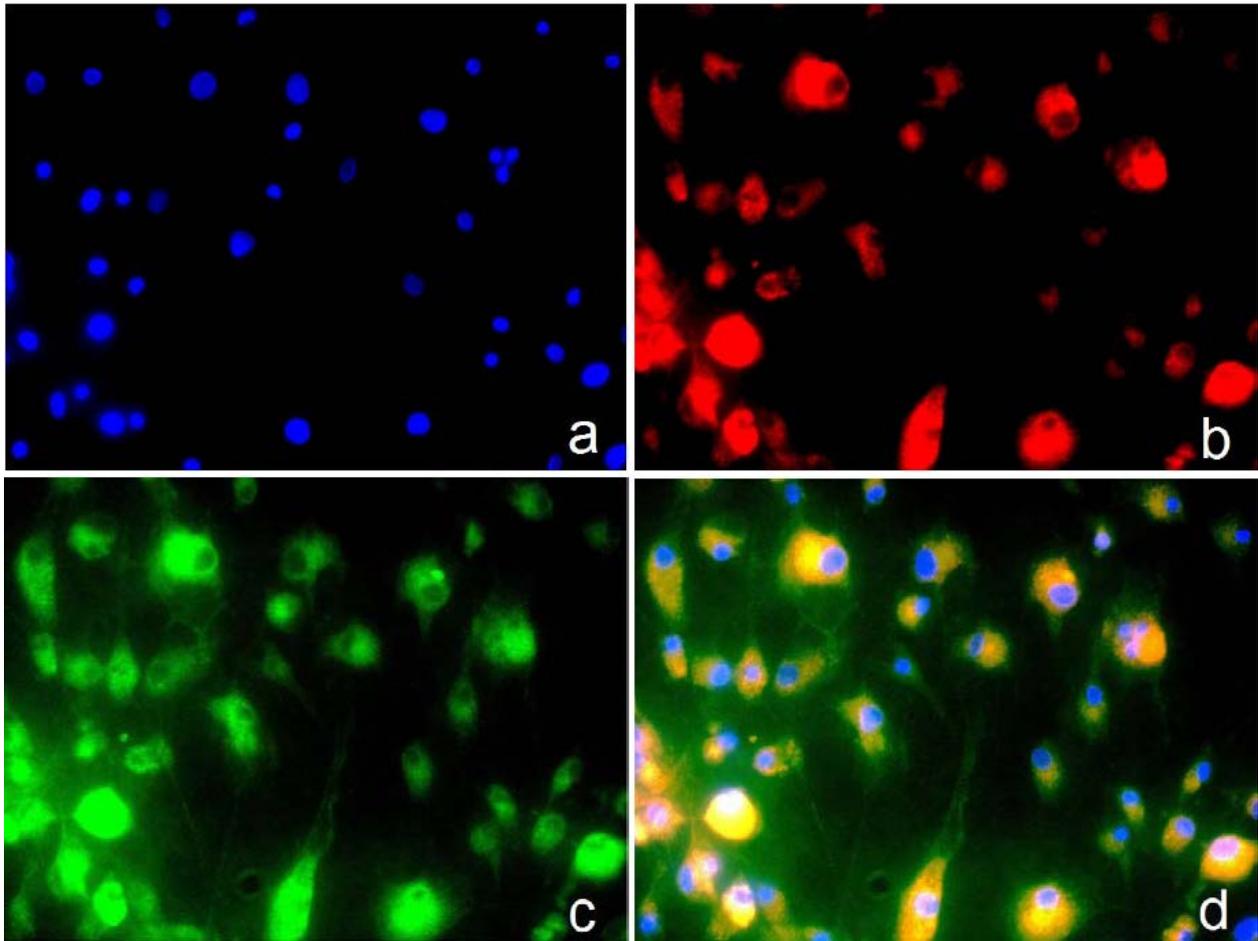


Fig. 4. The intracellular distribution of liposomes containing both PKH- 26 and FITC-Dextran used as lipophilic and hydrophilic probes, respectively. a - distribution of Hoechst 33342, b – distribution of PKH- 26, c – distribution of FITC-Dextran, d – merged images. Hydrophilic and hydrophobic tracers have been detected in the same cell compartments. This implies that liposomes penetrate into the cells unchanged. In case of liposome rupture, the dyes would have been localized to different intracellular structures.

Unilamellar cationic liposomes, $d=60$ nm, were used. After the incubation of cells with liposomes, nuclei were stained using the fluorescent dye Hoechst and the histological slides were prepared. Under the fluorescence microscope, bright red fluorescence of the lipid dye (Fig. 4B) and green fluorescence of FITC-Dextran (Fig. 4C) were observed in the cytoplasm of the fibroblasts. This means that both lipid and aqueous parts of the liposomes penetrate the cells. Analysis of superposition of the images revealed colocalization of lipophilic and hydrophilic dyes. This implies that at least a part of liposomes retain their structural integrity in the cytoplasm. However, weak and diffuse green fluorescence was detected within the cytoplasm indicating the presence of a small portion of the internal aqueous part of the liposomes.

Example 2. The intracellular distribution of liposomes with internal aqueous phase labeled with FITC-Dextran.

After incubation of cells with liposomes, the nuclei and mitochondria were stained using fluorescent dyes Hoechst 33342 and MitoTracker Deep Red, respectively. Multilamellar cationic liposomes, $d= 800-1200$ nm, were used.

Analysis of colocalization of the dyes revealed changes of fluorescence in the area of nuclei and mitochondria. These results show that large multilamellar cationic liposomes interact with the nuclei and mitochondria inside the cells.

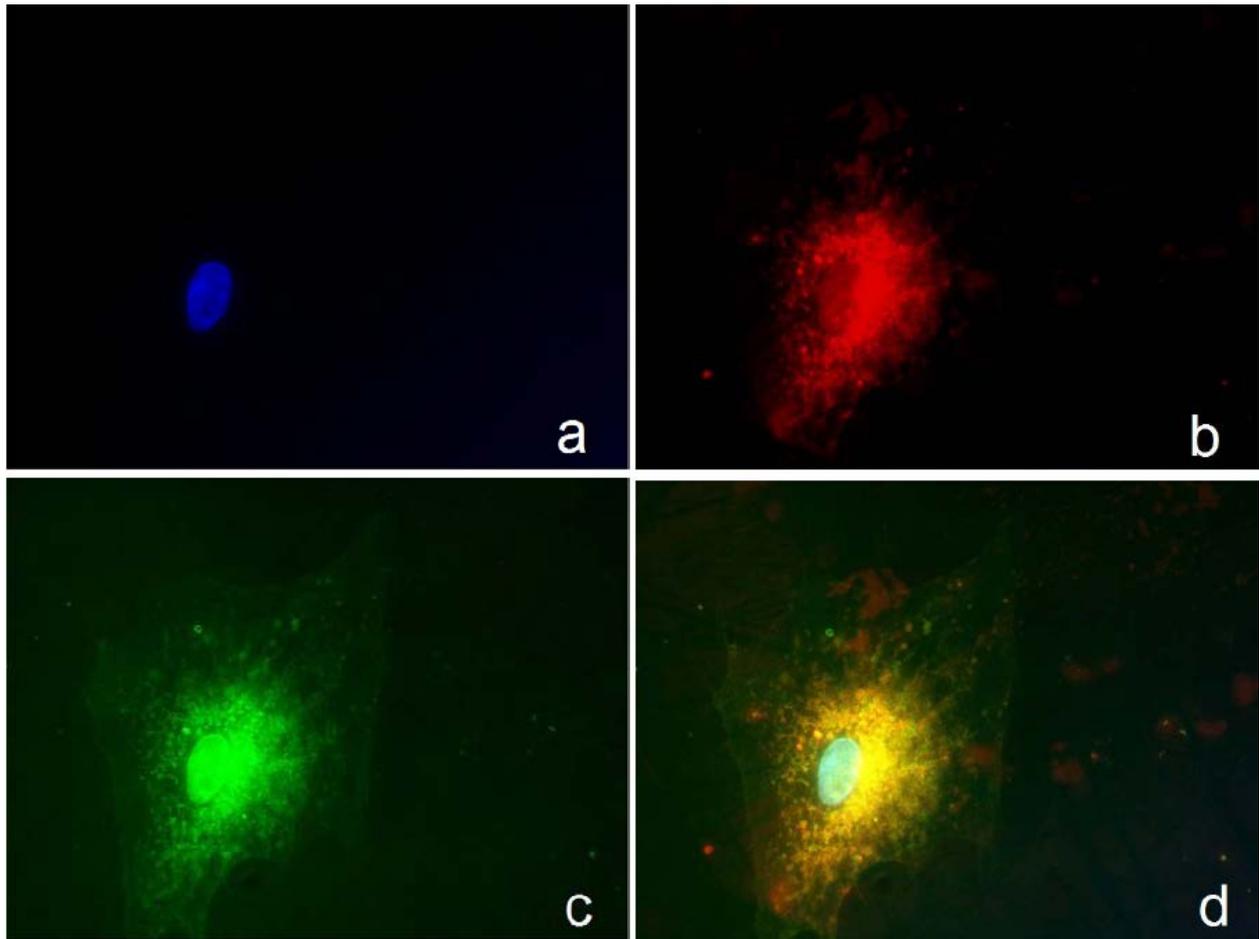


Fig. 5. Intracellular distribution of large multilamellar cationic liposomes containing FITC-Dextran in the fibroblast cell culture. A - distribution of Hoechst 33342 , B – distribution of MitoTracker Deep Red, C – distribution of FITC-Dextran, D - merged images. The change of fluorescence color indicates that a large number of absorbed liposomes interact with the nuclei and mitochondria of the cells (Panel D).

Example 3. The intracellular distribution of liposomes with TopFluor PC labeled lipid membrane, containing polyethylene glycol.

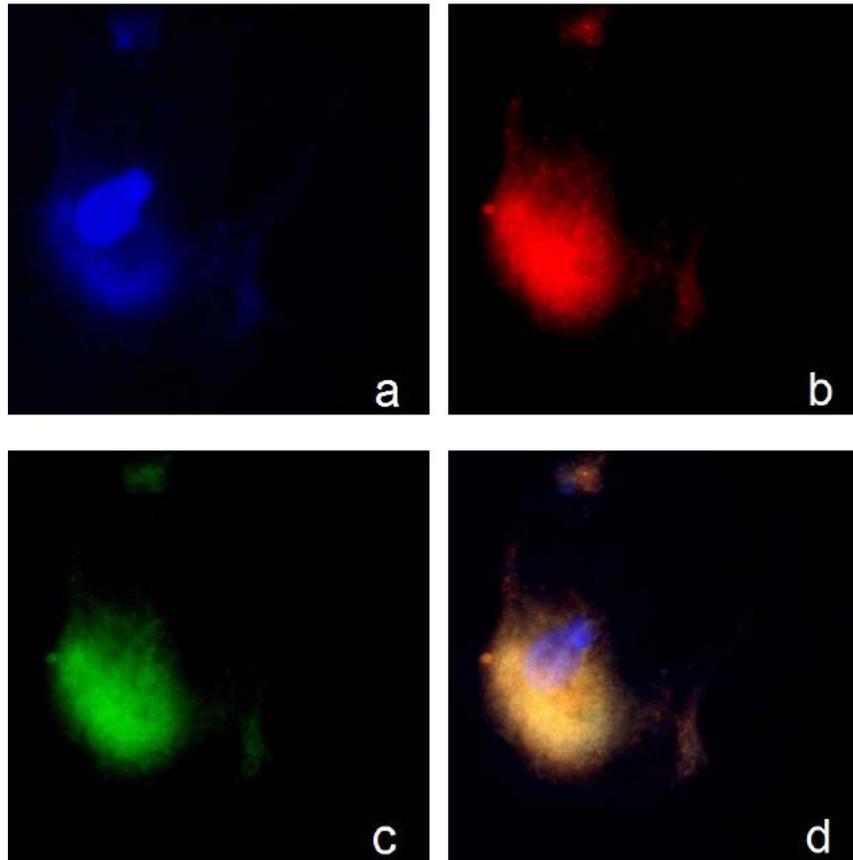


Fig. 6. The intracellular distribution of polyethylene glycol and the fluorescent dye TopFluor PC incorporated into liposomal membrane. A - distribution of Hoechst 33342, B - distribution of MitoTracker Deep Red, C - distribution of TopFluor PC, D - merged images.

The fluorescent dye TopFluor PC exhibits bright green fluorescence that allows detecting the localization of even a single molecule (Kleusch, et. Al., 2012). High fluorescent intensity of this dye enhances the sensitivity of the discussed method for liposomes with low ability to penetrate the cells, particularly liposomes with polyethylene glycol.

The analysis of colocalization of the fluorescent dyes reveals that liposomes with polyethylene glycol are also localized in the mitochondria.

Example 4. Evaluation of intracellular distribution of liposomes based on visual similarity of the stained structures.

Another way to determine intracellular localization of the liposomal structures is based on analysis of the visual similarity of the structures stained with organelles-specific dyes and structures exhibiting fluorescence of the dyes associated with liposomes. This method may be applied in case of difficulty in the distinguishing of the fluorescence spectra of labeled liposomes and stained organelles, i.e. when fluorescence spectra of dyes are similar or there is absence of narrow band filters.

Detection of localization of PKH26-labeled liposomes in the mitochondria labeled with MitoTracker Deep Red using Filter Set 15 (Carl Zeiss) can serve as an example of this method. The application of Filter Set 15 allows visualizing both fluorescent dyes but it does not allow identifying them separately. Therefore in this study, only cell nuclei were stained with Hoechst 33342 after the interaction between cells and liposomes. The cells without interaction with liposomes and containing mitochondria stained with MitoTracker Deep Red were used to perform comparison.

On the Fig. 7.B, liposomal staining is localized in the cell within separate granules. Visual similarity of the granules in this Figure and labeled mitochondria in Fig. 2 and 3 suggests that the liposomes are also localized in the mitochondria. Localization of the liposomes in the nuclei is not observed.

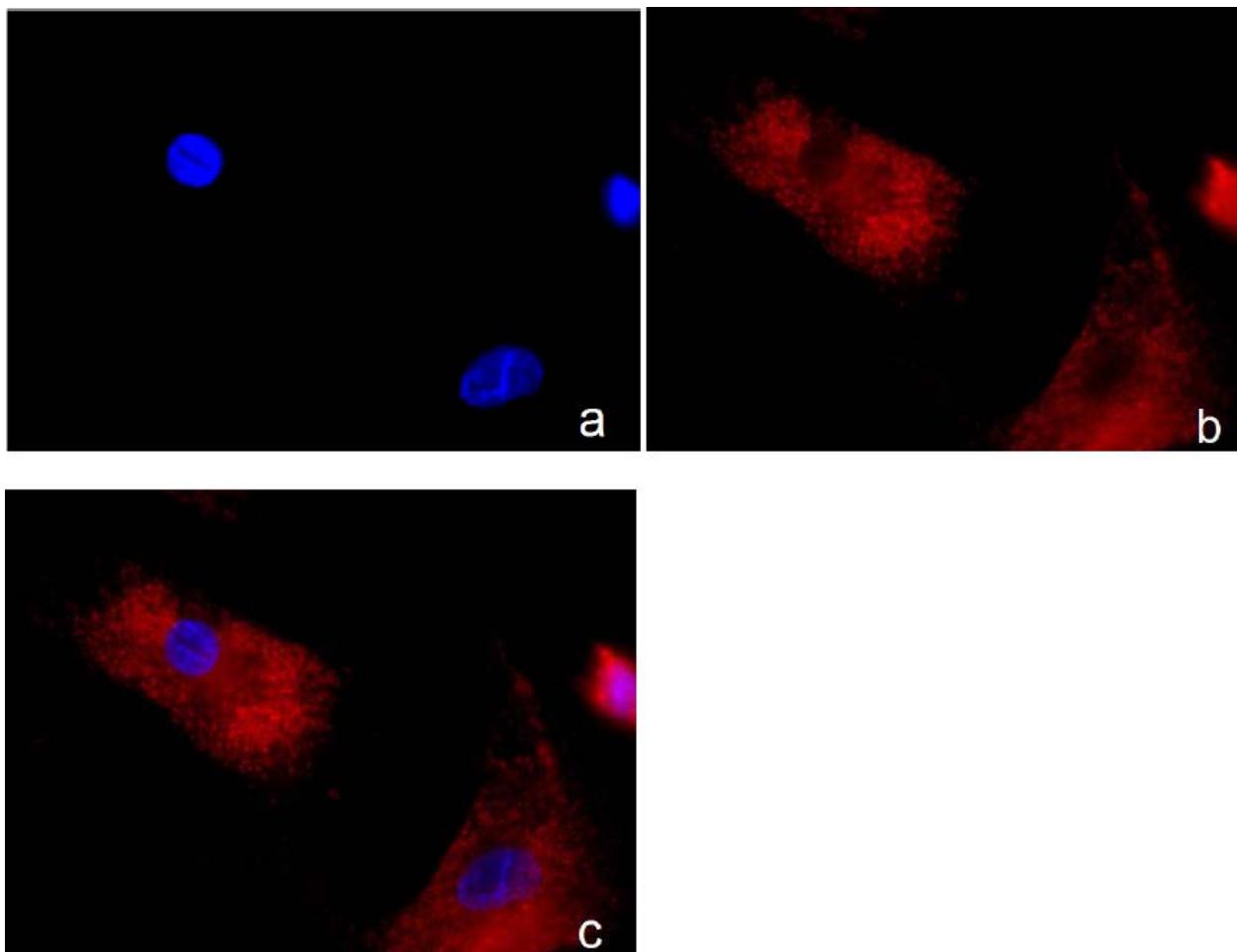


Fig. 7. Intracellular distribution of the cationic liposomes containing PKH26 in the liposomal membrane. A - distribution of Hoechst 33342, B –distribution of PKH26, C - merged images.

3. CONCLUSION

Thus, the presented technology of the evaluation of intracellular distribution by fluorescent labeling of hydrophilic and hydrophobic portions of the liposomes as well as the determination of colocalization of fluorescent labels and organelle-specific fluorescent dyes proved its efficiency and may be used in experimental design of liposomal formulations targeted to specific intracellular organelles.

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