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EVALUATION OF INTRACELLULAR DISTRIBUTION OF LIPOSOMES USING THE METHODS OF FLUORESCENCE AND CONFOCAL MICROSCOPY

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Abstract

This study is aimed at examining luminescence and confocal microscopy potential in determining intracellular localization of luminescent labeled substances incorporated into liposomes.

The study shows the data of different experiments on cell cultures and laboratory animals where liposomal labels were used to examine liposome-cell interaction and intracellular distribution of the substances incorporated into liposomes. It was demonstrated that if luminescent labels were chosen adequately it was possible to simultaneously assess the localization of liposomal labels in several subcellular structures. The results of the studies showed the efficacy of luminescence and confocal microscopy for intracellular liposomal substances localization.

Key words: *liposomes, luminescence labeling, intracellular delivery*

1. INTRODUCTION

Structural features of liposomes turn them into almost ideal mean for delivery of various drugs to cells and tissues (Goyalet. al., 2005; Torchilin, 2005). Existing technologies allow creating liposomal formulations of various biologically active substances and obtaining preparations with increased bioavailability, stability, biodegradability and low toxicity (Doijadet. al., 2009; Maureret. al., 2001).

Current research in the field of nanomedicine is aimed at creating new liposomal drugs which provide selective delivery to specific intracellular organelles. Depending on the desired pharmacological effect these liposome formulations may be targeted to mitochondria, nuclei, endoplasmatic reticulum, and lysosomes (Thekkedath, 2012; Boddapatiet. al., 2008; Elbayoumi, Torchilin, 2010; Pollocket. al., 2010). Importantly, delivery of biologically active substances to certain organelles may multiply the therapeutic effect of the drug (Torchilin, 2006; Yuet. al., 2012).

A mandatory condition for this kind of study is the correct approach for determining the localization of the liposomes and substances delivered with the liposomes. The problem is that the conventional liposomes are invisible in the light microscope because of the size beyond the limits of the optical resolution, and also due to lack of color. Moreover, biochemical composition of these liposomes does not differ from other lipids within the cell. In addition, liposomal lipids may be extracted by

dehydration of the samples during the standard preparation of the material for histological and electron microscopy analyses.

A possible solution of this problem is the inclusion of specific markers into the liposome. These markers should ensure visibility of liposomes and should not affect their properties. Also, such markers should be firmly associated with the structure of liposomes, and must not show any toxic effect.

The presence of markers in tissues, cells, and subcellular organelles may indicate the level of absorption of liposomes by these structures. Currently, fluorescent labels are commonly used as liposomal markers (Ishidaet. al., 2002, Kampet. al., 1997, Sherpofet. al., 2001). Modern methods which based on fluorescence and confocal microscopy provide determination of intracellular localization of even single molecules.

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

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

2. MATERIALS AND METHODS

Normally, animal cells have a very low self-luminescence. Therefore, we used different dyes for staining of subcellular structures (Jonson, Spence, 2010). It is essential to choose the correct fluorescent dye as a label. Optimally, this label should possess bright luminescence. Moreover, the spectra of excitation and emission of the dyes should be different.

Modern manufacturers offer a wide selection of different dyes. In our study, we used the following dyes:

1. Hoechst 33342, the vital dye for selective staining of DNA with the spectrum very close to the most common nuclear dye DAPI.

2. Mito Traker Deep Red, the vital dye for selective staining of mitochondria.

3. PKH 26 (Sigma), lipophilic vital dye which is used for selective staining of cell membranes. In our study, we used PKH 26 to stain the membranes of the liposomes. Therefore, the glowing in the red region of the spectrum, which is essential for PKH 26, indicates the presence of the substance in the lipid part of the liposome.

4. FITC-dextran, a water-soluble dextran (molecular weight of 4500) labeled with fluorescein isothiocyanate. Since this dye does not penetrate biological membranes, it was used in our study for labeling the internal water-soluble content of liposomes.

5. DAPI, the most common dye for selective staining of nuclear material. DAPI staining is the standard procedure for staining of the nuclear chromatin and it gives an overview of the structure of the cell as a whole.

Brief description of the dyes and stained structures is shown in Table 1.

Table 1. Fluorescence dyes used in the study for staining of liposomes and intracellular organelles.

Fluorescence dye	Stained structure	Maximum excitation, nm	Maximum emission, nm
Hoechst 33342 (Sigma-Aldrich)	DNA (nuclei)	346	460
DAPI (Dihydrochloride (Millipore)	DNA (nuclei)	375	450
FITS-Dextran (Sigma-Aldrich)	Internal contents of the liposomes	495	520
PKH 26(Sigma-Aldrich)	Lipid part of the liposomes	551	567
MitoTracker Deep Red (Molecular Probes)	Mitochondria	644	655

These dyes have different spectra of absorption and emission which allow their separate determination on the same preparation using the appropriate filters. Thus, we used Hoechst 33342 for staining of DNA, Mito Tracker DeepRed for staining of mitochondria, FITS-Dextran for staining the internal contents of the liposomes and PKH 26 for staining the lipid part of the liposomes.

2.1. Preparation of labeled liposomal compositions

In our study we used two types of liposomes, namely, (1) liposomes containing the label in the membrane only and (2) liposomes containing the label both in the membrane and inside the liposomes.

2.1.1. Preparation of multilamellar labeled vesicles and liposomes containing the label in the lipid membrane.

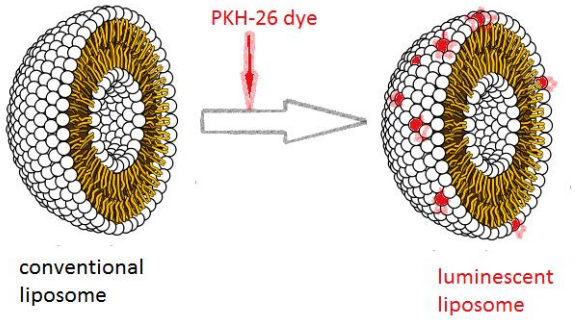


Fig. 1. Preparation of liposomes containing a label in the lipid membrane.

Staining was conducted using a lipophilic membrane dye PKH-26 (Sigma-Aldrich).

Multilamellar vesicles (MLV) were prepared using a rotary evaporator Heidolph (Germany). A solution of egg lecithin (Lipoid, Germany) and cholesterol (SIGMA-Aldrich) at a molar ratio of 7:5 was prepared in chloroform. Thereafter, the PKH-26 dye was added to the solution in a molar ratio of 1:15000.

The solvent was then evaporated in a flask under the working vacuum at 40°C. The lipid film on the walls of the flask was hydrated with 0.9% NaCl solution and then shaken to form multilamellar

vesicles. The lipid suspension was then frozen and stored at 80°C. The quantity of lipids in the resulting suspension was 100 mg/ml. Negative staining on the transmission electron microscope (JEM-100C, «JEOL», Japan) showed that obtained MLV were polymorphic, and their size varied between 600-3000 nm.

In order to obtain liposomes of small diameter, the MLV were passed through an extruder (LipexBiomembranes Inc., Canada) ten times using polycarbonate filters («Costar», USA) with a pore diameter of 100 nm .

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

The simultaneous presence of two different dyes in different parts of the liposome can identify the substance introduced both in the lipid membrane of liposomes and inside the liposome.

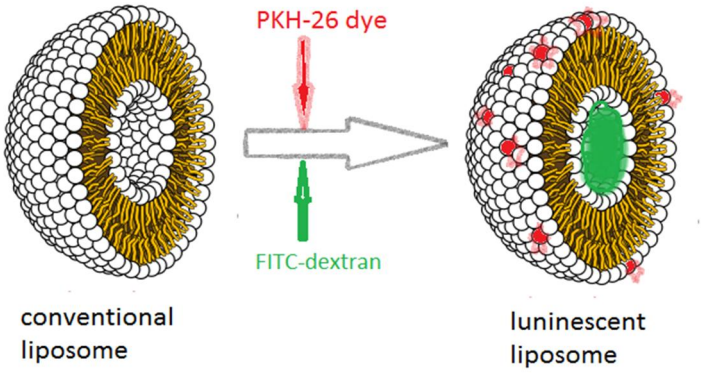


Fig. 2. Preparation of liposomes containing label in the lipid membrane and also inside the liposomes.

For the preparation of this type of liposomes, we used the same lipid mixture as for the first type of liposomes but at the stage of hydration, we used saline solution with the addition of FITC-dextran as a fluorescent label. Further preparation of MLVs and liposomes was similar to that described above. Extra liposomal FITC-dextran was removed by dialysis or gel filtration prior to the start of the experiment. In case of gel filtration, concentration of liposomes in the medium was monitored by light scattering method using the reference samples.

2.2. Interaction between liposomes and cells in cell cultures

In our study we used cultures of rat cardiomyocytes and epithelialcell line 804G (rat carcinoma)..

2.2.1. Staining of cell nuclei

We used two ways nuclear staining. Hoechst 33342 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 DAPI. This dye, at concentration of 3 mg/ml, was added to the culture medium and stained for 14 min.

2.2.2. Staining of mitochondria.

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

2.2.3. Absorption and distribution of liposomes in liver cells

Experiments were carried out on male Wistar rats (250-270 g), Liposomes labeled with PKH-26 were injected to the tail vein at a dose of 25 mg lipid/kg of body weight. After euthanasia, criosection of liver was performed in order to evaluate distribution of liposomes in different cells of the liver.

2.2.4. Fluorescent and confocal microscopy.

In our study, we used confocal microscope LSM 780 (Carl Zeiss, Germany) and fluorescent microscope AxioObserver Z.1 (Carl Zeiss, Germany). Each series of experiments was conducted 6 times. Typical experiment is shown on Figures 3-6.

3. RESULTS AND DISCUSSION

Prepared labeled liposomes were used for evaluation of the inclusion and intracellular distribution of liposomes in different cell types. Various combinations of labels in liposomes and cells were used.

3.1. Absorption and distribution of MLV in epithelial cell line 804G culture.

Hoechst 33342 and Mito Tracker Deep Red were used for staining of nuclei and mitochondria, respectively. Then MLV, at lipid concentration of 0.5 mg/ml, were added to the culture medium for 1 hour. Preparations were observed using a confocal microscope.

Our results clearly demonstrated that the selected dyes allow staining the nucleus and mitochondria. Importantly, the chromatin was clearly identified, and it allowed to assess the activity of the cells. Mitochondria appeared as discrete intracellular structures. The analysis of images with appropriate filters showed the presence of fluorescent dye both inside and outside cells. Spherical particles with a diameter of 800-1000 nm were observed outside the cells. Apparently, these were MLV which did not interact with the cells. Superposition of the images shown on the panels A, B, and C (Fig 3) resulted in a change of the color of chromatin inside the nucleus to light blue (Fig. 3 D) confirming the interaction between MLV and nucleic acids. We found that the cytoplasm in some parts of the cells had fluorescence of liposomes. But this was not related to the nuclei (Fig. 5). Under our experimental conditions, MLV were accumulated in the cytoplasmic and nuclear membrane inside the cell and interacted with nuclear material. Localization of fluorescence from the liposomal dye PKH 26 fully coincided with the fluoresces of the DNA-selective dye Hoechst 33342. This can be considered as a confirmation of the interaction of substances with nucleic acids. Some cells demonstrated bright fluorescence of MLV in the cytoplasm in the form of large discrete particles (Fig. 5).

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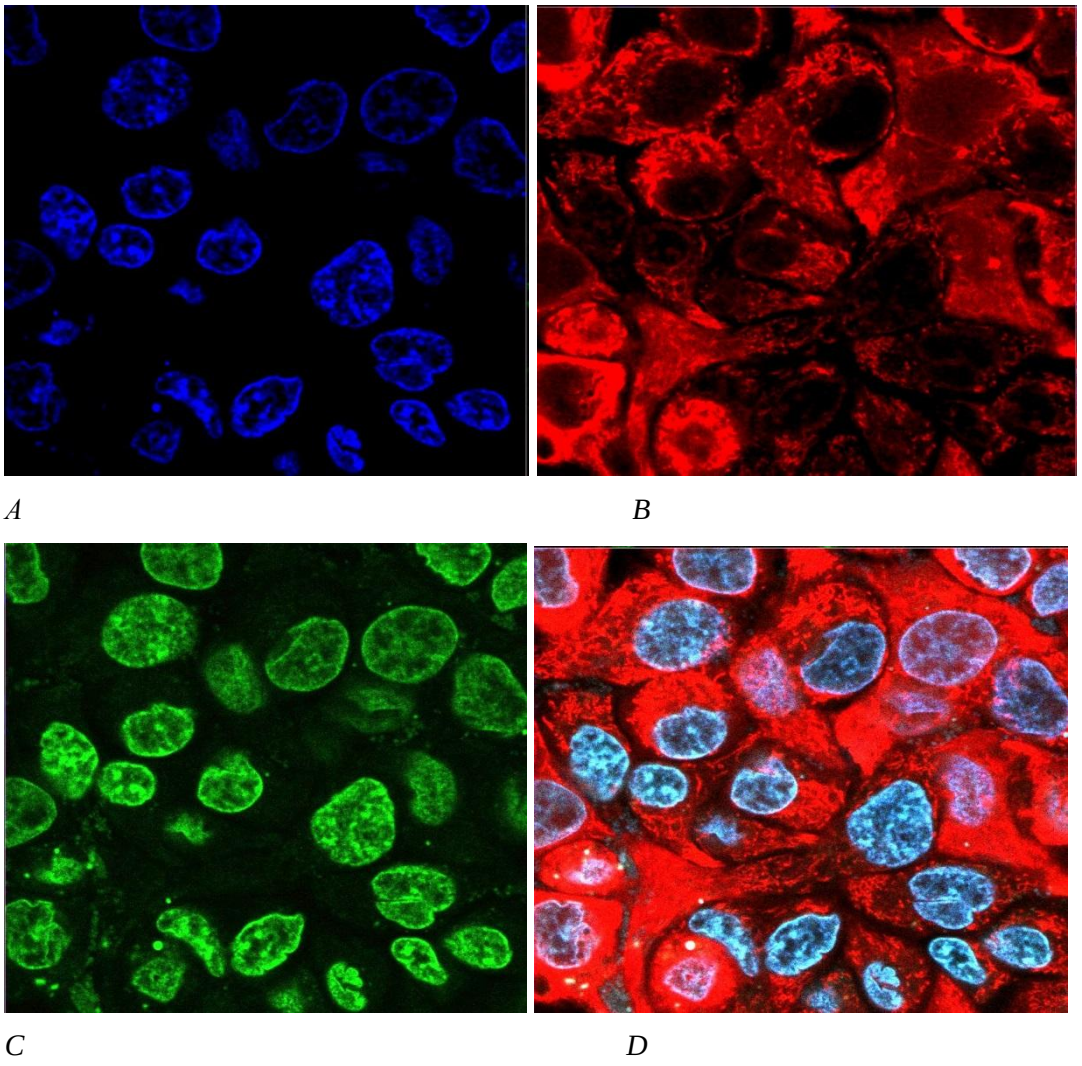


Fig. 3. Confocal imaging of epithelial cell line 804G cells after 60 min incubation with PKH-26-labeled MLV. A. Nuclei stained with PKH 26. B. Mitochondria stained with Mito Tracker Deep Red. C. Intracellular localization of PKH 26. D. Superposition of images A, B, and C showing co-localization of Hoechst 33342 and PKH 26.

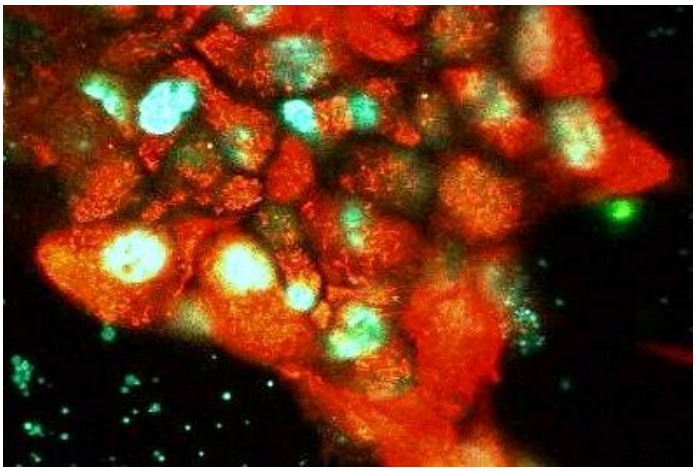


Fig. 4. Intra- and extracellular localization of MLV in cells. Confocal imaging. Superposition of images reflecting Hoechst 33342, Mito Tracker Deep Red, and PKH 26 dyes.

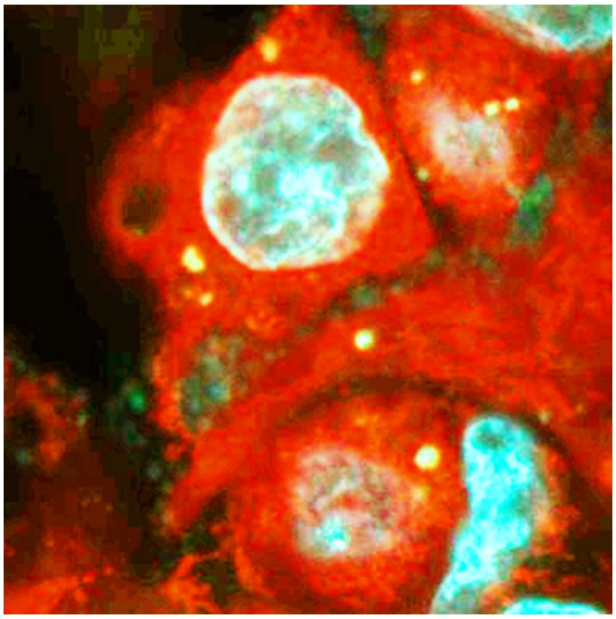


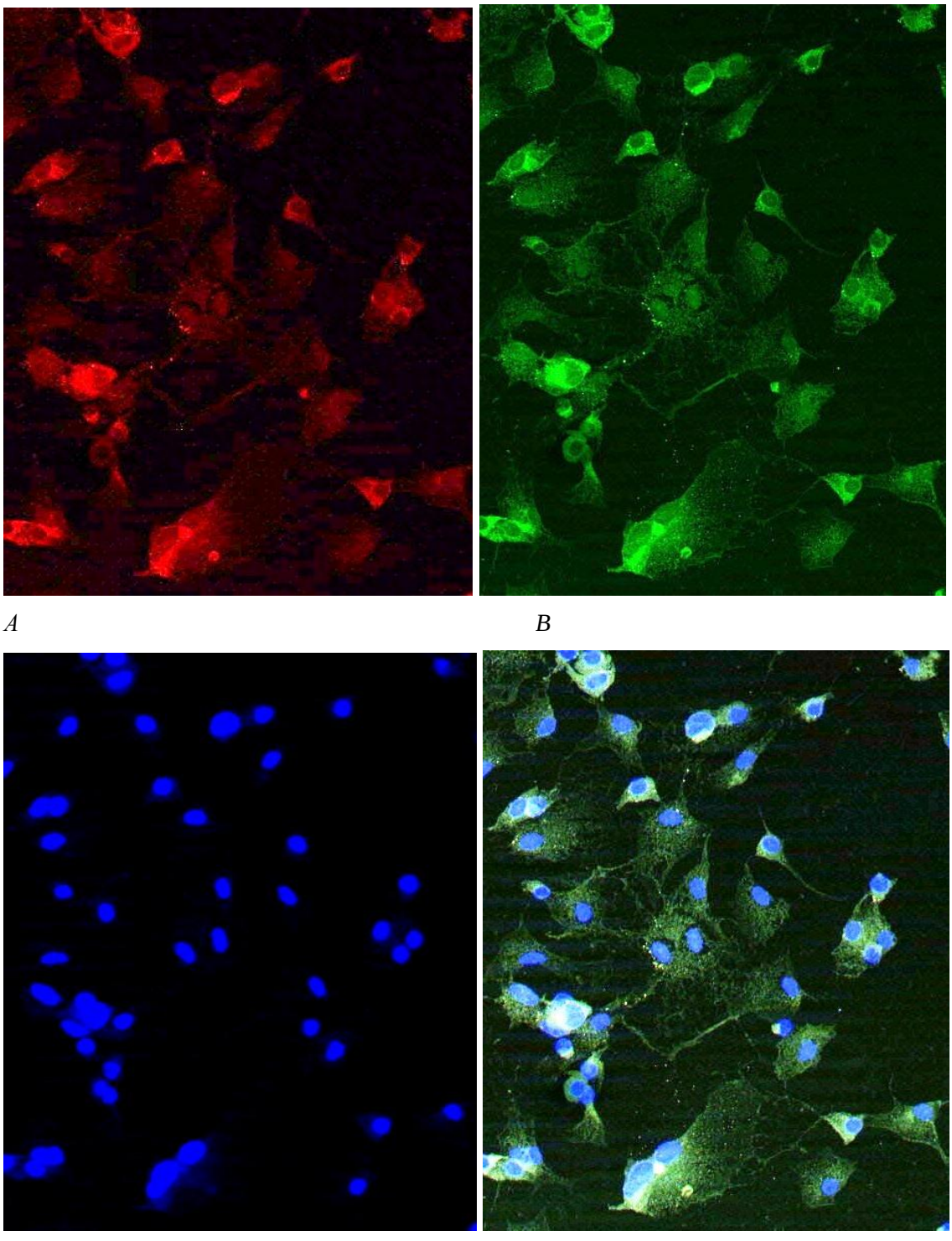
Fig. 5. Localization of MLV in cytoplasm and nuclei. Confocal imaging. Superposition of images reflecting Hoechst 33342, Mito Tracker Deep Red, and PKH 26.

3.2. Absorption of 100 nm liposomes by a culture of cardiomyocytes.

We evaluated inclusion and distribution of liposomes containing fluorescent label in the lipid part of liposomes, and also in the inner aqueous phase.

PKH-26 was used for staining of the liposome membrane and FITC-dextran was used for staining of the inner phase of liposomes. The diameter of the liposomes was 100 nm. Cells were incubated in the medium containing liposomes in a concentration of 0.5 mg/ml, for 6 and 24 hours at 37°C.

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A *B*

C D

Fig. 6. Intracellular distribution of 100 nm liposomes containing PKH-26 and FITC-dextran in cardiomiocytes. A. Distribution of PKH-26. B. Distribution of FITC-dextran. C. Staining with DAPI. D. Superposition of images A, B, and C.

After incubation with liposomes, the cells were fixed with 4% paraformaldehyde and stained with DAPI. Thereafter, the samples were observed in the fluorescence microscope AxioImager A.1 using appropriate filter in the Apotome mode. Fluorescence from both dyes was observed which may indicate the efficiency of intracellular delivery of lipophilic and hydrophilic substances in liposomes. Fluorescence was uneven after 6 hours of incubation; some of the observed structures had bright others weak fluorescence, indicating the accumulation of liposomes only in particular cell compartments. Interestingly, the areas of intracellular fluorescence of PKH-26 and FITC-dextran were coincided. This may imply that liposomes enter the cytoplasm without their destruction. In contrast to the experiments with MLV, we did not observe the fluorescence of nucleic acids. After 24 hours of incubation, the fluorescence was uniform in all parts of the cytoplasm. Apparently, this is due to migration of dyes during cell metabolism.

3.3. A comparative study of the distribution of MLV and liposomes in different types of liver cells after intravenous administration.

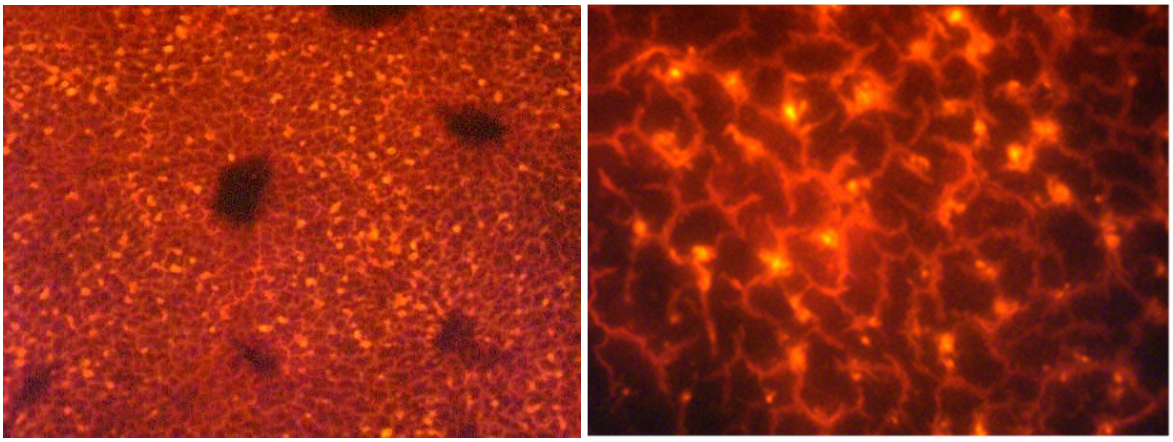
It has been shown that investigation of the absorption and distribution of liposomes *in vivo* is more difficult, because all factors of the whole organism should be taken into account. In the bloodstream liposomes can interact with proteins and lipoproteins which consequently may affect their structure and stability (Zhang, 2008). This point should be considered when creating injectable liposomal pharmaceuticals (Torchilin, 2005).

In this series of experiments we studied the absorption and distribution of MLV and PKH-26-labeled liposomes in liver cells.

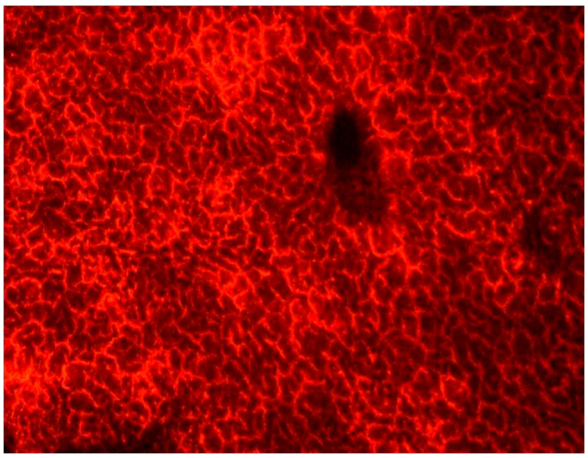
After 1 hour of intravenous administration of MLV we observed bright red fluorescence (Fig. 7). Two types of fluorescence can be distinguished in liver cells. The fluorescence of membrane and surrounding areas was observed in the prevailing share of cells. The majority of such cells were hepatic parenchyma. Color of the adjacent cells of this type was relatively uniform. Notably, introduction of liposomes into the cells of hepatic parenchyma resulted in fluorescence of the regions close to the periphery of cells. This may be due to adhesion and fusion of particles with the outer cell membrane.

After the introduction of MLV into parenchyma cells, we observed several cells with very bright fluorescence in predominantly central regions. After analysis, these cells were identified as reticuloendothelial cells. It could be suggested that MLV are recognized and devoured by reticuloendothelial cells as foreign particles. Intense fluorescence inside these cells may indicate that MLV migrate to lysosomes after endocytosis. High intensity and the large number of reticuloendothelial cells suggest that these cells make a significant contribution to the absorption of MLV.

Regarding the injection of liposomes, fluorescence was less intense and almost did not differ from the fluorescence of parenchyma cells.



A (x100) *B (x400)*



C (x100)

Fig. 7. Fluorescence imaging of liver criosection after introduction of MLV (A, B) and PKH-26-labeled liposomes (C).

4. CONCLUSION

Our findings demonstrate the efficacy of application of fluorescent and confocal microscopy for the assessment of intracellular localization of substances included in liposomes. Adequate selection of fluorescence dyes may allow simultaneous investigation of multiple subcellular structures.

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