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LUMINISCENT LIPOSOME LABELING TECHNOLOGY WITH PKH-26 SOLUTION AND ABSORPTION AND DISTRIBUTION ASSESSMENT OF LIPID MULTILAMELLAR VESICLES AND SMALL LIPOSOMES IN LIVER FOLLOWING INTRAVENOUS ADMINISTRATION

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Abstract

This study examined the possibility to form liposomal compositions containing PKH-26 fluorescent dye and to use those compositions in order to assess absorption and tissue distribution of the liposomes in rat liver.

Liposomal compositions consisted of egg lecithin and cholesterol (Sigma) in a 7:5 ratio, respectively. The dye was incorporated into the lipid layer while preparing multilamellar vesicles with the extruder.

The obtained labeled liposomal compositions allowed to quantitatively assess how the vesicles are absorbed by the liver and visualize the dye distribution in different liver cells. The study presents the data on the absorption and distribution of the dye in the liver, according to the size of the lipid vesicles, 1, 2 and 24 hours after the intravenous administartion.

Key words: *luminescent labeling, absorption of the liposomes, visualization of the liposomes, liposomal distribution in the cells, PKH -26, liver*

1. INTRODUCTION

If conventional liposomes are administered intravenously, they are absorbed mainly by the liver cells within 60-120 min. (see for example Freise et. al., 1980, Ishida et. al., 2002, Scherphof et. al., 1983, Wu et. al., 1998). This property of conventional liposomes can be used for biologically active agents delivery into an organ by incorporating them into phospholipid vesicles. However, the liver consists of various types of specialized cells, which, to the different extent, take part in liposomal absorption.

In order to get the expected pharmacology effect the issue of biologically active agents delivery to a certain type of cells becomes important. Conventional liposomes are absorbed from the blood predominantly by reticuloendothelial cells (Freise et. al., 1980, Scherphof et. al., 1983), but a range of publications show that liver parenchymal cells can also play a significant role in the liposomal absorption (Ishida et. al., 2002, Sherpof et. al., 2001, Wu et. al., 1998).

The results are different, probably, due to the fact that different size, surface and composition of the conventional liposomes can significantly influence the rate of their absorption and distribution in the cells (Adrian et. al., 2005, Erion et. al., 2005, Sherpof et. al., 2001, Torchilin 2005).

A range of experiments showed that incorporating specific components, which are capable of binding with certain cell receptors, into the lipid layer and modifying the lipid composition, size and surface energy charge allows to form the liposomes, which have a high affinity to a certain type of cells: liver parenchymal cells, sinusoid endothelial cells, stellar cells and reticuloendothelial cells (see for example Adrian et. al., 2005, Erion et. al., 2005, Kamp et. al., 1997).

The quantitative assessment of the liposomal absorption and distribution are associated with serious technical issues. Liposomes are invisible through the optical microscope due to their size and the absence of color. Liposomes do not have any biochemical difference from the lipids of the surrounding tissues. Standard material preparation for histology and microscopy implies the extraction of liposomal lipids during the samples dehydration.

A possible solution for this problem is incorporating special dying substances into the liposomes, which can allow their visualization and the possibility of a quantitative assessment. These marker substances should not alter the properties of the liposomes, be incorporated into the liposomal composition and should not have any toxic effect. Then the presence of the dye in tissues, cells and subcellular organells shows the liposomal absorption by those structures. Luminescent and radioactive substances can be used as liposomal markers.

In our experiments, the vital dye PKH -26 was used as a luminescent label of liposomal lipids. The dye has a bright stable luminescence in the red area of the spectrum, tightly binds to the lipid membrane and does not migrate form cell to cell. The cell luminescence is proportional to the amount of the absorbed dye (Oh et. al., 1999). The use of liposomal compositions with PKH-26 allows to quantitatively assess the same samples for the total absorption of the liposomes into the organ tissues, on the one hand, and visually assess the cryostate sections for the distribution of the liposomes in different cell elements of the organ under study, on the other hand.

The aim of the study is to form liposomal compositions containing fluorescent PKH-26 dye and their use for the absorption and distribution assessment of the liposomes in the tissues in an experiment with the rat liver.

2. MATERIALS AND METHODS

The experiments were conducted on mature Wistar male rats weighing 300-330g in accordance with the principles of the European Convention (Strasbourg, 1986) and the WHO Helsinki Declaration (1996).

2.1. Preparation of labeled multilamellar vesicles

Multilamellar vesicles (MLV) were obtained using Heidolph (Germany) rotational evaporator. First, egg lecithin (Lipoid, Germany) and cholesterol (SIGMA-Aldrich) solution in chloroform in a 7:5 ratio was put into the 0.5-L flask. PKH-26 (SIGMA-Aldrich) was then added (lecithin to dye ratio 15000:1). The solvent was being evaporated under vacuum at 40 ^{o}C until its smell disappeared. The film of lipids on the walls of the flask was then hydrated with 0.9% NaCl solution and shaken to obtain multilamellar vesicles. The obtained suspension was frozen and stored under -80 ^{o}C. The lipid concentration was 100 mg/ml in the obtained suspension. Negative contrast electron microscopy showed that MLV obtained after thawing were polymorphous and their size varied from 600 to 3000 nm.

2.2. Obtaining liposomes

Liposomes were prepared by forcing suspensions of lipids through polycarbonate filters with the LIPEX extruder (Lipex Biomembranes Inc., Canada).Previously obtained multilamellar vesicles were forced through the extruder with Costar polycarbonate filters (USA) with a prespecified pore size.

In order to obtain 100 nm liposomes the vesicles were 10 times forced through the extruder with two 100 nm filters. 50 nm liposomes were obtained from 100 nm liposomes by forcing them 15 times through the extruder with two 50 nm filters.

2.3. Administering the rats with the liposomes and obtaining the material

Labeled multilamellar vesicles and liposomes were intravenously administered 25 mg/kg. 1, 2 and 24 hours after the administration the rats were excluded from the experiment, their liver was taken out, weighed, 3 pieces from different lobes were taken for research. 10 rats were experimented on in both groups. Liposome-free samples were used in order to make a calibration curve. All the samples had been frozen in the liquid nitrogen and stored in a low-temperature (-80 ^{o}C) fridge before the research was conducted.

2.4. Quantitative assessment of liposomal absorption and distribution in the liver tissues using luminescent dye

0.2 g of the tissue was taken from every sample and homogenized frozen in a porcelain jar with liquid nitrogen. After the nitrogen was evaporated 2 ml of the Folch solution (chloroform and methanol in a 2:1 ratio) was added into the jar and thoroughly grinded and the liquid from the jar was poured into a volumetric centrifuge tube. Then the Folch solution was added to the remaining substance and the procedure was repeated. The content of the tubes was closed and centrifuged and the supernatant was used to measure the concentration of the dye. Varian Cary Eclipse (Australia) spectrofluorimeter was used to measure the fluorescence with the wave length of the excitation light being 551 nm and 567 nm for the emission.

The quantitative assessment was done by the previously made calibration curve. In order to do that the liposomes were added to the control liver samples in the concentrations making up 0.5%, 1.0%, 1.5% and 2% of the concentration administered. This was done at the stage of while obtaining the homogenate. Then the above-described dye extraction procedure and luminescence measurement were done. The obtained results were used to form the calibration curve. The relationship between the luminescence and the concentration of the administered liposomes was linear. The calibration curve was used to measure the concentration of the liposomes in every weight. The data on one and the same liver sample were averaged and the percentage of absorbed liposomes in relation to the total organ mass was calculated.

2.5. Histology studies

The tissue samples from every liver were attached to the Micron HM 525U (Thermo, Germany) cryostat microtome table for making sections of 14 um. The sections were air-dried during not less than 60 min. and studied in the Axio Imager A.1 (Carl Zeiss, Germany) microscope. The images of one and the same sites were done in translucent and fluorescence modes with luminescent filters in the red area of the spectrum.

2.6. Statistical processing of the data

Statistical data processing was done with Statistica 7.0 software package. The samples were first checked for the normality of distribution. The mean value and standard deviation were calculated. Statistical significance of intergroup differences was assessed by Student's t test.

3. RESULTS

The data on Figure 1 demonstrate that the absorption of the liposomes by the liver tissues depends on their size. All the liposomes had similar dynamic changes in the concentrations of the luminescent dye. The maximum concentration of the luminescent dye in the liver tissues was observed 1 hour after the administration of the lipid vesicles. The highest degree of vesicles absorption (about 80%) was observed if multilamellar vesicles were injected, the absorption degree was less with the injection of 50 nm liposomes and much less with the injection of 100 nm liposomes. 2 hours after the administration of all types of liposomes the decrease in the dye concentration was observed in the liver. By that period the maximum decrease in the luminescence was observed for multilamellar vesicles (almost 50% decrease compared with 1-hour values). The minimum decrease in the luminescence at a 2-hour timepoint was observed for 100 nm liposomes compared with 1-hour timepoint. 50 nm liposomes had an intermediate decrease. 24 hours after the lipid vesicles administration all the groups showed a significant increase in the liver dye concentration. A significant increase ($p<0.05$) in the liposomal dye concentration was also seen. The analysis of the images of cryostate liver sections showed that after the administration of labeled lipid vesicles there was bright tissue fluorescence in the red area of the spectrum, which was similar to PKH-26 fluorescence (Fig. 3-10). These results also showed the liposomal absorption by the liver tissue. The luminescence of the dye was enough to visualize the organ histology. The liver lobes and their trabecular structure were seen very clearly. The blood vessels were dark with light fluorescence in the areas where significant accumulation of formed blood elements was observed.

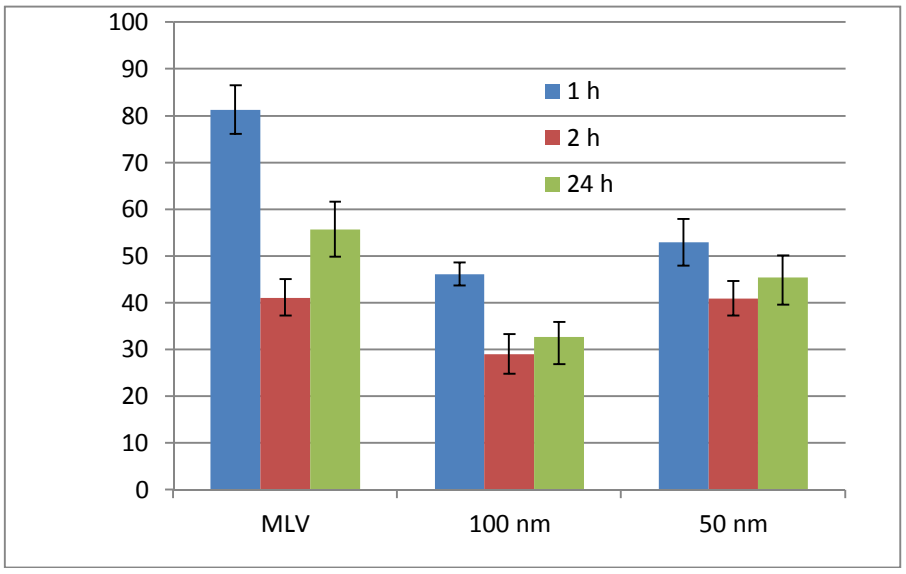


Fig.1. The absorption of the liposomes (% of the administered vesicles concentration) by the experimental rat liver, according to the size, and further changes in the concentration of the PKH-26 dye incorporated into the lipid vesicles.

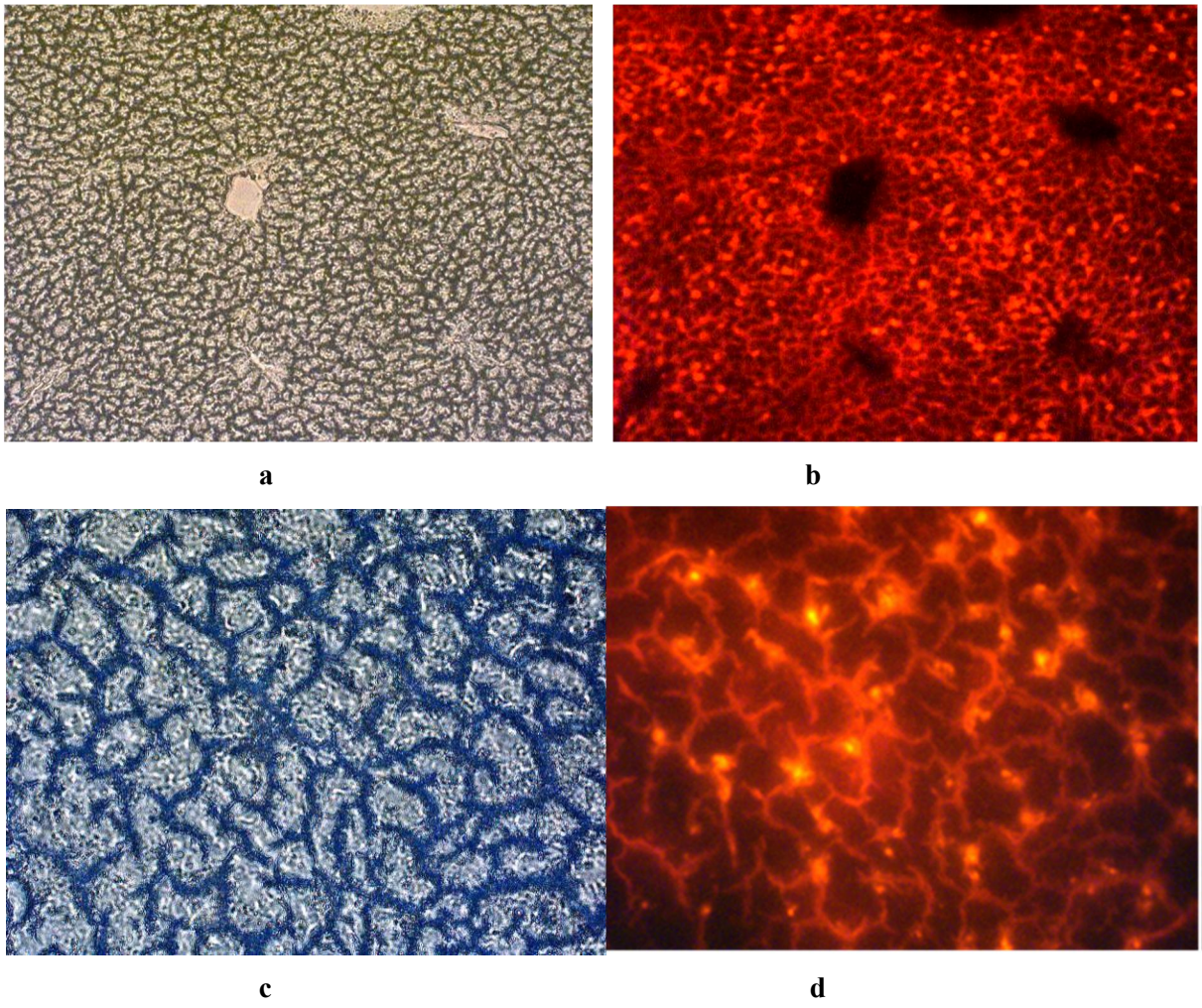


Fig. 2. Microimages of the rat liver 1 hour after the intravenous administration of multilamellar vesicles. Left, translucent mode, right, fluorescence mode. Enlargement - a, b - x 100, c, d – x 400.

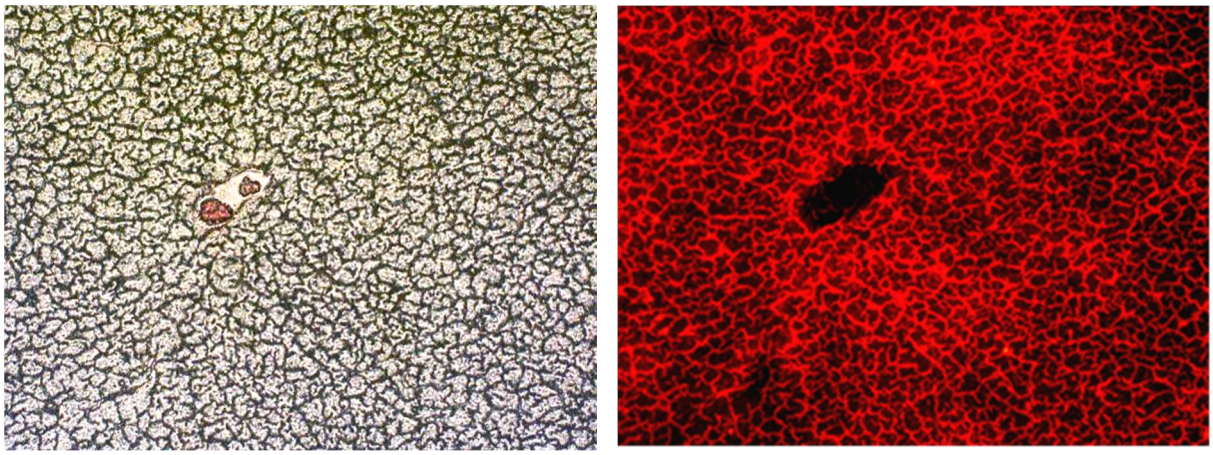


Fig. 3. Microimages of the rat liver 2 hours after the intravenous administration of multilamellar vesicles. Left, translucent mode, right, fluorescence mode. Enlargement x 100.

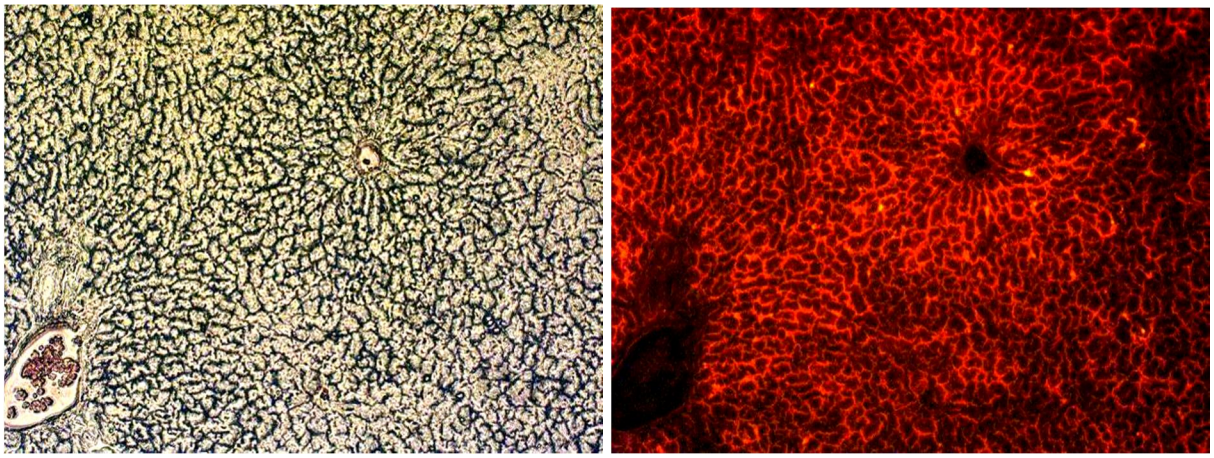


Fig. 4. Microimages of the rat liver 24 hours after the intravenous administration of multilamellar vesicles. Left, translucent mode, right, fluorescence mode. Enlargement x 100.

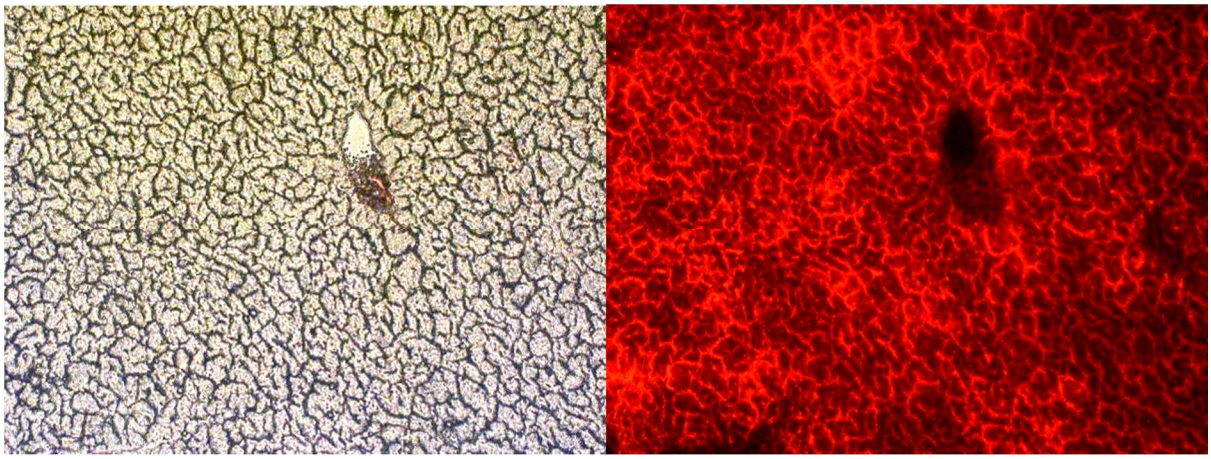


Fig. 5. Microimages of the rat liver 1 hour after the intravenous administration of 100 nm liposomes. Left, translucent mode, right, fluorescence mode. Enlargement x 100.

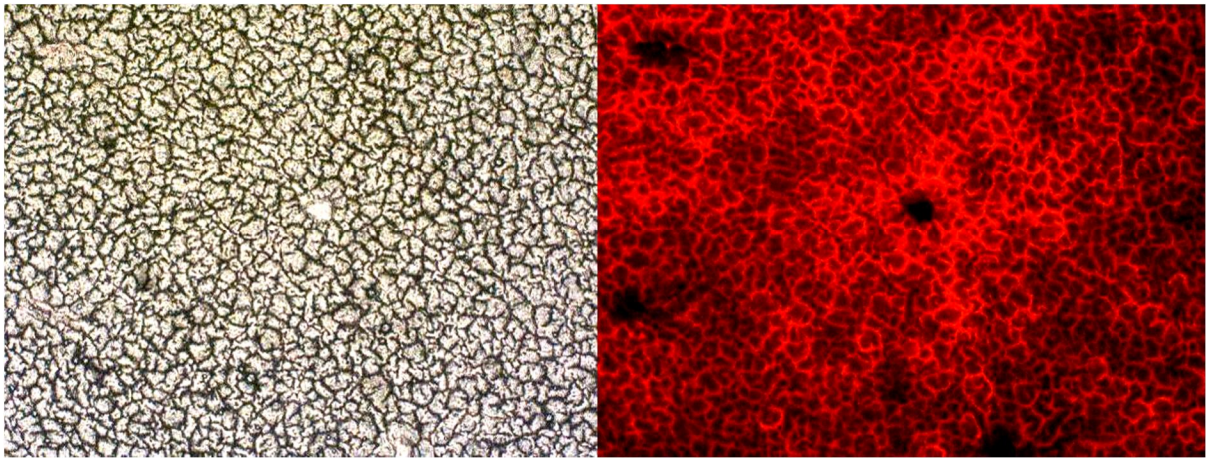


Fig.6. Microimages of the rat liver 2 hours after the intravenous administration of 100 nm liposomes. Left, translucent mode, right, fluorescence mode. Enlargement x 100.

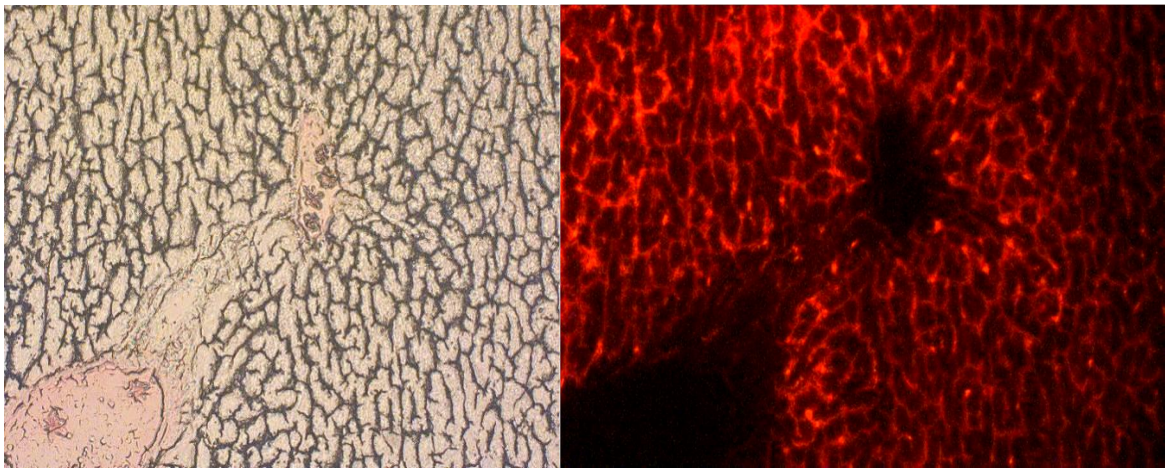


Fig. 7. Microimages of the rat liver 24 hours after the intravenous administration of 100 nm liposomes. Left, translucent mode, right, fluorescence mode. Enlargement x 100.

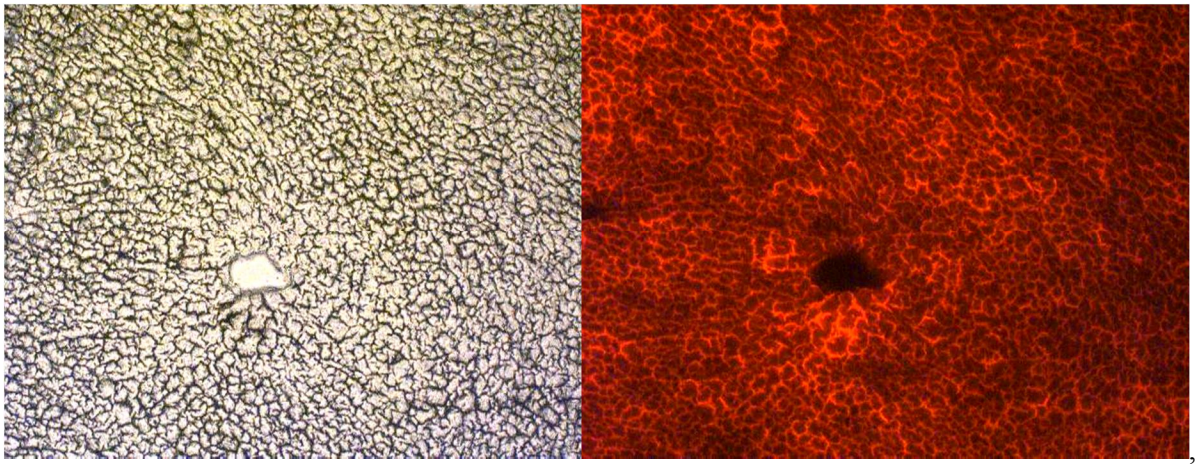


Fig. 8. Microimages of the rat liver 1 hour after the intravenous administration of 50 nm liposomes. Left, translucent mode, right, fluorescence mode. Enlargement x 100.

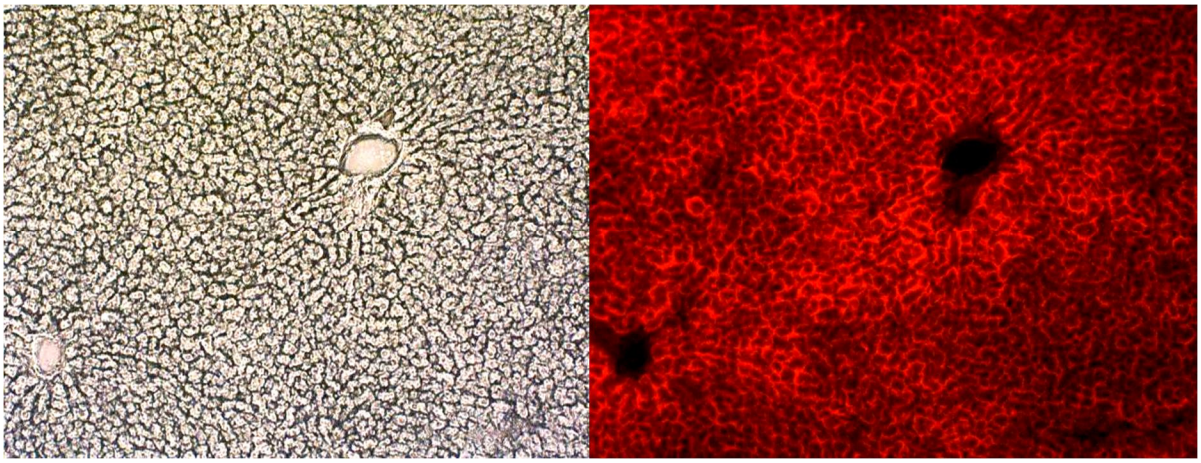


Fig. 9. Microimages of the rat liver 2 hours after the intravenous administration of 50 nm liposomes. Left, translucent mode, right, fluorescence mode. Enlargement x 100.

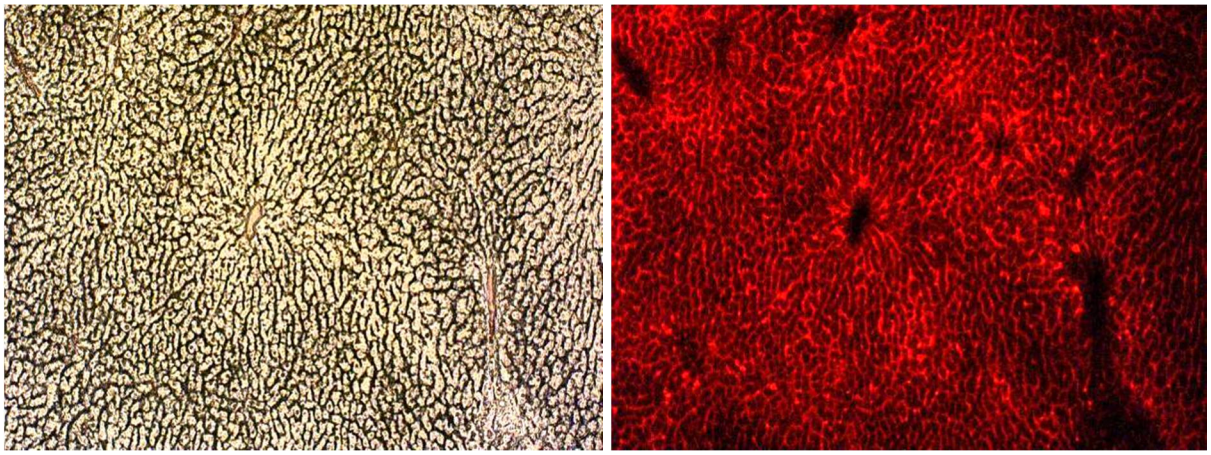


Fig. 10. Microimages of the rat liver 24 hours after the intravenous administration of 50 nm liposomes. Left, translucent mode, right, fluorescence mode. Enlargement x 100.

1 hour after the MLV administration there was bright red cell luminescence observed on liver sections (Fig. 3). Luminescent cells can be divided into two types according to their luminescence. A predominant number of cells were luminescent around the membrane. By translucent and luminescent microscopy this characteristic can be attributed to the parenchymal cells of the liver trabeculars. The adjacent cells of the same type had relatively homogenous color.

Among parenchymal cells there were some diffusely located cells with very bright luminescence. Unlike parenchymal cells the luminescence in those cells was bright and observed somewhere close to the centre. Intensive absorption of multilamellar vesicles by those cells shows their phagocyte activity, which, along with their localization, allows to differentiate them as reticuloendothelial cells.

2 hours after the MLV administration parenchymal cells still had luminescence around the membrane and visually the luminescence level was similar to the level seen at 1 hour after the MLV administration (Figure 4). The luminescence of reticuloendothelial cells significantly decreased and their visualization became poor. However, those cells could still be identified by the luminescence of

the internal cell mass. 24 hours (Figure 5) after the MLV administration parenchymal cell luminescence was the same. Reticuloendothelial cell luminescence significantly increased but its intensity varied form cell to cell: both moderately luminescent and brightly luminescent cells were observed.

1 hour after 100 nm liposomes administration liver cells were also luminescent (Figure 6). Organ histology was well visualized. The intensity of parenchymal cell luminescence was different being higher near the central vein in some liver lobes. After 100 nm liposomes administration reticuloendothelial cells had the luminescence intensity similar to that of parenchymal cells, which made it difficult to identify them properly. Parenchymal and reticuloendothelial cells were mainly different in the luminescence localization.

2 hours after 100 nm liposomes administration there were no significant alterations in the luminescent dye distribution in the liver cells (Figure 6). 24 hours after 100 nm liposomes administration (Figure 7), there was a bright luminescence observed in the reticuloendothelial cells similar to that in the MLV group.

50 nm liposomes administration provided the histology picture and luminescent characteristics similar to those observed in 100 nm liposomes group (Figure 8-10).

4. DISCUSSION

Matching the data on liposomal dye accumulation in the tissues and the images showing the distribution of the dye among the liver cells according to the size of vesicular particles allows to see certain consistent patterns.

After the MLV are administered they are rapidly absorbed from the blood. Within 1 hour after the administration the liver had up to 80% of the dye. Histology samples showed two types of cells according to the liposomal distribution features. By form and localization they can be identified as liver parenchymal cells and reticuloendothelial cells.

Liver parenchymal cells mostly had peripheral luminescence. This can prove that the MLV absorption is, in this case, due to the particle adhesion and fusion with the external cell membrane. Then the localization and the intensity of the luminescence did not differ significantly during the whole study period.

Reticuloendothelial cells had a diffuse localization in the tissue and demonstrated another type of luminescence. It was localized in the entire cell area with the brightness increased in the centre of the cell and depending on the size of the lipid particles and the duration of observation. The highest intensity of the luminescence and its further changes in this type of cells was observed after the MLV administration. 1 hour after the MLV administration reticuloendothelial cells had very bright luminescence unlike parenchymal cells. The luminescence in the entire cell area and its maximum intensity in the centre allows to suggest that reticuloendothelial cells absorbed MLV by endocytosis, which is characteristic of macrophages. MLV appear to be identified and absorbed by reticuloendothelial cells as foreign particles. The intensive luminescence inside the cells can suggest that after endocytosis MLV migrate into the lysosomes. A high intensity of the dye luminescence and a large number of those cells allows to suggest that they contribute a lot to the MLV absorption process.

2 hours after the MLV administration the picture obtained by fluorescence microscopy changed. The luminescence in reticuloendothelial cells significantly decreased. The luminescence intensity in reticuloendothelial cells did not differ greatly from that in parenchymal cells; however, unlike in

parenchymal cells the luminescence was intensive inside the cells. Simultaneous two-fold decrease in the total dye concentration in the same liver samples can probably be explained by the dye release from the reticuloendothelial cells. At this observation stage the released dye is not fully absorbed.

24 hours after the MLV administration a significant increase of the dye concentration in the liver tissues was observed. Histology studies of the sections found the increase in reticuloendothelial cells luminescence. This demonstrates that at that stage of observation the dye is reabsorbed by those cells. Since lipoproteins play an important role in the absorption and distribution of lipids and other lipophilic substances in the blood we can suggest that after lipophilic PKH-26 is released into the blood it is partly absorbed by lipoproteins and then absorbed by reticuloendothelial cells inside the lipoproteins. This migration cycle was described for liposomal radioactively labeled lecithin (Scherphof et. al.,1983).

When 100 nm liposomes were administered their total absorption was the least and made up 46.2% of the total liposomal concentration 1 hour after the administration. 2 hours after the administration there was 1.6 times decrease in the dye concentration. Liver parenchymal cells luminescence was different in differently localized cells, some liver trabeculars had higher luminescence intensity compared with the adjacent ones. Thus, 100 nm liposomes are minimally absorbed both by liver parenchymal cells and reticuloendothelial cells.

1 hour after the administration of 50 nm liposomes their absorption was 52.9% of the total liposomal concentration and there was 1.3 times decrease by the 2-hour timepoint. Light luminescence of reticuloendothelial cells and relatively little decrease in the dye concentration by the 2-hour endpoint suggest that 50 nm liposomes are minimally absorbed by reticuloendothelial cells. The most of 50 nm liposomes are absorbed by parenchymal cells.

From a therapeutical point of view the essential structural targets for hepatoprotection are not only parenchyma but also vascular endothelium (Kamp et. al.,1997), so the absorption of the dye by the endothelium in different liposomal compositions was examined. The luminescence of liver lobe central veins was very light in all the liposomal compositions. The endothelium of large vessels demonstrated visible luminescence after the administration of the liposomes. The maximum luminescence was observed for 100 nm liposomes and the minimum luminescence for the MLV.

Liposomal biologically active substances have a marked hepatoprotective effect in the experiments (Korb et. al., 2008, Mukhamadiyarov et. al., 2011). Therefore, a relevant today's pharmacology issue is searching for specific substances and forming liposomal compositions based on those substances, which would have a therapeutic effect on various pathologic liver conditions. This will allow to formulate medications based on those liposomal compositions with their pharmacokinetics and pharmacodynamics being different from those in other drugs (see for example Adrian et. al., 2005, Erion et. al., 2005, Korb et. al., 2008, Mukhamadiyarov et. al., 2011, Torchilin, 2005).

The use of liposomes with incorporated luminescent dye can be an effective tool for monitoring the dynamic changes of their absorption and distribution in the target organs.

CONCLUSIONS

1. Incorporating PKH-26 luminescent dye into the liposomal membrane allows to assess the dynamics of their absorption and distribution in the tissues and organs. This does not require complex or expensive equipment and is safe unlike radionuclide techniques.

2. The absorption of the liposomes by the liver tissues depends on the size of the liposomal particles. The maximum absorption was observed for multilamellar vesicles, then, in decreasing order, for 50 nm liposomes and for 100 nm liposomes.

3. The use of liposomes containing PKH-26 allows to differentiate the localization of those liposomes by the type and the intensity of luminescence both inside the cells and when distributed around the tissue.

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