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ORIGINAL PAPER

Microspectroscopic Study of Liposome-to-cell Interaction Revealed by Förster Resonance Energy Transfer

Svetlana L. Yefimova · Irina Yu. Kurilchenko · Tatyana N. Tkacheva · Nataliya S. Kavok · Igor N. Todor · Nataliya Yu. Lukianova · Vasyl F. Chekhun · Yuriy V. Malyukin

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Abstract We report the Förster resonance energy transfer (FRET)-labeling of liposomal vesicles as an effective approach to study in dynamics the interaction of liposomes with living cells of different types (rat hepatocytes, rat bone marrow, mouse fibroblast-like cells and human breast cancer cells) and cell organelles (hepatocyte nuclei). The in vitro experiments were performed using fluorescent microspectroscopic technique. Two fluorescent dyes (DiO as the energy donor and DiI as an acceptor) were preloaded in lipid bilayers of phosphatidylcholine liposomes that ensures the necessary distance between the dyes for effective FRET. The change in time of the donor and acceptor relative fluorescence intensities was used to visualize and trace the liposome-to-cell interaction. We show that FRET-labeling of liposome vesicles allows one to reveal the differences in efficiency and dynamics of these interactions, which are associated with composition, fluidity, and metabolic activity of cell plasma membranes.

Keywords Förster resonance energy transfer · Fluorescent dyes · Liposome · Living cells

S. L. Yefimova (✉) · T. N. Tkacheva · N. S. Kavok · Y. V. Malyukin
Institute for Scintillation Materials, NAS of Ukraine, 60 Lenin ave, 61001 Kharkov, Ukraine
e-mail: ephimova@isma.kharkov.ua

I. Y. Kurilchenko
Slavyansk State Pedagogical University, 19 Batyuka str, Slavyansk 84116, Ukraine

I. N. Todor · N. Y. Lukianova · V. F. Chekhun
R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv 03022, Ukraine

Introduction

The application of nanotechnology for controlling, diagnostics and treatment of biological systems is now referred to as nanomedicine [1, 2]. Nanomedicine is expected to provide dramatic impact on the treatment of atherosclerosis, diabetes, malaria, many infectious, inherited and incurable diseases of today. Development of nano-drugs, which consist of nano-sized carriers and therapeutic, diagnostic or any active compounds, is the most important area of nanomedicine [1–3]. Varieties of nanoparticles, such as different polymeric and metal nanoparticles, liposomal vesicles, micelles, quantum dots, dendrimeres, lipoproteins and different nanoassembles are applied today as nanocarriers for targeted drug delivery into the site of action in living body [1–15]. Nano-sized carriers offer a versatile platform, to which many useful functionalities can be added for improving the specificity and efficiency of drug delivery. Nanocarriers can be designed to overcome many problems related to drug resistance to cancer and can be modified to improve specificity to tumours, thus limiting the drug exposure of healthy tissue and reducing side effects [14].

Liposomal vesicles composed of a bilayer of phospholipids or of any similar amphiphilic lipids were among the first carriers and still remain very efficient for drug delivery [10–15]. Liposomes are biodegradable, biologically inert, weakly immunogenic and they possess limited intrinsic toxicity. Moreover, liposomes afford unique opportunity to deliver the drugs into cells by fusion or endocytosis, i.e. by the way atraumatic for cells [10–15]. Therefore various liposomal formulations are in clinical trials or have been successfully marketed for the treatment of different diseases including cancer (DuanoXome, Myocet, Doxil, Caelyx, onco TCS) [16]. However, for the development of new nano-drugs and

their biomedical and pharmacokinetic research (cellular uptake of nano-drug and active compound release dynamics, etc.), it is necessary to trace nanocarrier's fate during the long-term experiments. Fluorescence-based methods, which imply fluorescent probes application, is a powerful tool to visualize and trace the interaction between carriers and living cells in vivo and in vitro experiments [17–19].

The common approach in liposome vesicle labeling is based on using organic dye molecules incorporated in liposomes [19]. This approach allows one to visualize liposomes and trace their fate, particularly, their interaction with various biological objects by controlling the change in the probe fluorescence signal (one channel recording methods) [19, 20]. For instance, fluorescence enhancement effect due to the fluorescent probes de-quenching by dilution in appropriate media was used to trace the liposome disruption process [21, 22]. For this purpose, fluorescein or rhodamine dyes were preloaded in liposome interior in high concentration and were nonfluorescent, while their fluorescence appears as a result of the liposome disruption and dye dilution [21, 22]. However, the main disadvantage of the approach based on one channel fluorescence recording is the limited number of parameters to measure fluorescence (fluorescence intensity, anisotropy, fluorescence lifetime) [17–19, 23]. Meantime, the changes of intensity are of low analytical value due to the absence of internal reference. Fluorescence anisotropy and lifetime measurements require complex instrumentation and analysis and are not applied in large-scale practice [19, 23]. Moreover, high concentration of fluorophores preloaded in liposomes in the fluorescence de-quenching method is not often applicable in biological experiments.

The method of λ-ratiometry (recording of fluorescence intensity at two or more wavelengths) allows two-channel fluorescence recording using single or double fluorescence emitters and is based on different mechanisms producing spectroscopic changes, including Förster Resonance Energy Transfer (FRET) [19, 23]. Ratiometric detection of intensities at fixed wavelengths is a simple technique that can use common spectroscopic and microscopic instrumentation and provides the self-referenced ratiometric measurements that are not possible with single-channel recording [23].

FRET is a transfer of electronic excitation energy from one molecule (donor) to other molecule (acceptor) without intermediate photon emission [17–19, 24]. Since FRET efficiency depends drastically on the separation between the donor and acceptor molecules, it is widely used to study a variety of biological processes associated with the intermolecular distance changing [24]. In molecular biology FRET is very often used to analyze the spatial structures of macromolecules (as protein folding, DNA packaging, etc.), supramolecular structures (as ligand receptor interactions, etc.) [17, 19], heterogeneity of plasma membrane [25], study viral membrane fusion [26], probe membrane organization [27, 28], interactions among molecular membrane components, etc. [17, 19, 24].

In the present paper, we report the comparative study of the dynamics and efficiency of the interaction between phosphatidylcholine (PC) liposomes and cells of different type (rat hepatocytes, rat bone marrow, mouse fibroblast-like cells and human breast cancer cells) and cell organelles (hepatocytes nuclei). To study liposome–to cell interaction in dynamics, we used two-channel ratiometric fluorescence recording on the base of FRET effect between two fluorescent probes preloaded in PC liposome bilayers. There are some requirements for FRET donor/acceptor pairs [17–19]: (i) an absorption spectrum of the acceptor must overlap a fluorescence emission spectrum of the donor. At the same time, the acceptor should not be excited directly at the donor excitation wavelength; (ii) the donor should provide optimal lifetime and brightness; (iii) the molar absorbance and fluorescence quantum yield of the acceptor should be sufficient for recording. So, there are some difficulties in optimal choice of the FRET partners.

In our research, hydrophobic dyes 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were used as energy donor and acceptor, respectively (Chart 1, Fig. 1). DiO and DiI dyes and their derivatives are used in various FRET-based applications including the study of nanocarriers (polymeric micelles) stability in various media [29–33]. The dyes possess long hydrocarbon tails $C_{18}H_{37}$ in their structure (Chart 1) which act as "anchors" proving a strong fixation of the dyes within lipid bilayers of liposome vesicles with negligible transbilayer flip-flop [20]. The dyes exhibit high molar extinction coefficients (154,000 M^{-1} cm^{-1} and 148,000 M^{-1} cm^{-1} for DiO and DiI, respectively) and sufficient quantum yields [20]. The encapsulation of the dyes in lipid bilayers of PC liposomes ensures the required distance between the donor DiO and the acceptor DiI to realize FRET [34]. The liposomes loaded with fluorescent dyes in this way

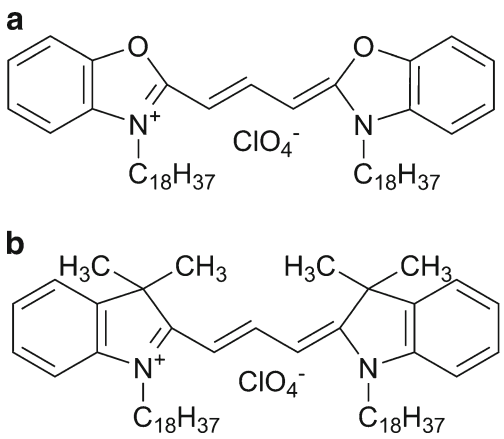

Chart 1 Structural formulas of the dyes used: (**a**) DiO; (**b**) DiI

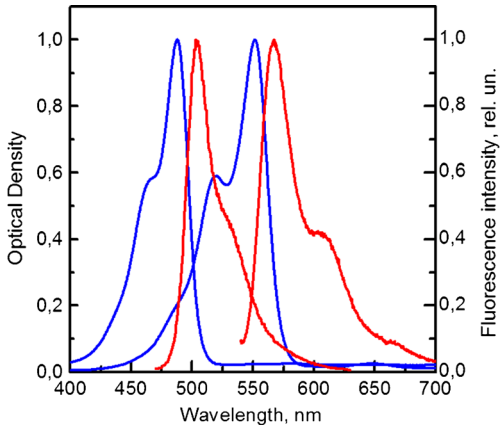

Fig. 1 Absorption (*blue*) and fluorescence (*red*) spectra of the DiO and DiI dyes in ethanol

we call "FRET liposomes". The release of the dyes from liposomes into the cell membrane causes a loss of the FRET effect and redistribution of the relative donor and acceptor fluorescence intensities due to an increase of the donor-acceptor distance that is used as a "signal system" to monitor the liposome-to-cell interaction [17, 19, 23].

Experimental Section

Chemicals L-α-Phosphatidylcholine from egg yolk and dyes DiO and DiI were purchased from Sigma–Aldrich and used without purification. The excitation spectra of the dyes match their absorption ones that indicates dyes purity (spectra not presented). Chloroform (Sigma–Aldrich) used to prepare lipid and dye stock solutions was a spectroscopic grade product.

Preparation of Lipid Vesicles with DiO and DiI Probes Unilamellar PC lipid vesicles containing DiO and DiI dyes were prepared by the extrusion method [35]. Appropriate amount of PC (40 mg/ml) and dyes (10^{-3} M) stock solutions in chloroform were mixed in a flask and dried until complete chloroform evaporation. The thin lipid-dyes film was then hydrated with 2 ml of Eagle's medium +10 % fetal calf serum (pH=7.4). Final concentration of PC was 1×10^{-3} M. The obtained lipid-dyes suspension was finally extruded through 100 nm pore size polycarbonate filter using a mini-extruder (Avanti Polar Lipids, Inc.). The concentrations of each dye in the liposomal suspension were 2×10^{-5} M.

Cells and Cell Lines Freshly isolated hepatocytes were derived from rat's liver by the nonenzymatic method [36] with following washing-out with Eagle's medium with 10 % fetal calf serum.

Isolated hepatocyte nuclei were obtained as described by Kaufmann [37]. For separate nuclei, a liver was homogenized using Downs's homogenizer in ice-cold 0.25 M sucrose solution containing 5 mM $MgSO_4$, 50 mM Tris, pH 7.4. The homogenate was filtered through Muslin cloth and spun at low speed 800 g for 5 min. The pellet was rinsed and spun twice as it was described above.

To obtain rat bone marrow cells, rats were sacrificed by cervical dislocation and then placed in 70 % alcohol for 10 min. Both femurs from one rat were taken and stripped of adherent muscles of the knee end. A needle was inserted into the bone and cells were aspirated followed by several flushes through the bone using a 1 ml syringe filled with culture medium, until all the bone marrow was flushed out of the bone. A similar procedure was performed from the other end of the bone as close to the tip as possible. The marrow thus obtained was suspended by pipetting the large marrow cores through a 1 ml pipette. The suspension was then centrifuged at 200 g for 5 min. The pellet thus obtained was dissolved in 3 ml of Erylyse buffer for 10 min, than final volume 14 ml, centrifuged at 200 g for 5 min. The treatment pellet of cells in Erylyse buffer (3 min) repeated twice, cells filtered from debris and were dissolved in Phosphate buffered saline (PBS); the cell count was done in a Neubauer chamber and tested for viability by the Trypan Blue dye exclusion test.

L929 cells (ATCC CCL 1) are fibroblast-like cells cloned from strain L. The parent strain was derived from normal subartaneous areolar and adipose tissue of a male C3H/An mouse.

The human breast cancer cells of the MCF-7 line were cultivated in modified Dulbecco's medium ISCOV ("Sigma", Germany) with addition of 10 % of fetal calf serum ("Sangva", Ukraine) at the temperature of 37 °C and CO_2 concentration of 5 %. Cells were reseeded twice a week at the density $2–4\times 10^4$ cells/cm^2, when cell layer covered about half of the flask surface.

Cell Labeling Procedure Isolated hepatocytes pellet (50 μl 10^7 cells/ml), hepatocytes nuclei and rat bone marrow cells (50 μl 10^7 cells/ml) were incubated with liposomal suspension (50 μL) in 1 mL of Eagle's medium with 10 % fetal calf serum at 37 °C for required time intervals. Afterwards non-bound liposomes were removed by centrifugation at 500 g and washing-out by adding HBSS (HEPES buffered saline solution) buffer (pH=7.4) with 0,1 % BSA.

Cell lines MCF-7 and L929 in growing medium were cultured with 5 μl liposomal suspension at 37 °C for the desired lengths of time. Afterwards non-bound liposomes were also removed by centrifugation at 500 g and washing-out by adding HBSS.

Spectroscopy and Microspectroscopy Fluorescence spectra of DiO-DiI-and DiO/DiI-loaded liposomes in water and DMF:water binary solutions were recorded by Lumina spectrofluorimeter (Thermo Scientific, USA). Cell imaging was

performed using inverted fluorescent microscope Olympus IX71 with digital camera Olympus C-5060. BP 460–490 and BP 510–550 filters were used to excite DiO and DiI, respectively. To study FRET modulation in cells, microspectroscopic technique was used. For this purpose, fluorescent microscope Olympus IX71 was supplied with a fiber optic spectrometer USB4000 (Ocean Optics, USA) for the fluorescence detection from selected cells. For FRET experiments BP 460–490 filter was used.

Results and Discussion

Manifestation of FRET Modulation in Liposomal Vesicles

DiO and DiI dyes are highly hydrophobic and exhibit extremely low water solubility [20]. In the PC liposomes with overage diameter of 100 nm they are located in lipid bilayers and the close proximity of the dye molecules ensures an efficient FRET between the donor and acceptor molecules that was confirmed by the fluorescence spectra (Fig. 2, curve 1). The DiO fluorescence ratio in the total fluorescence signal $I_{DiO}/(I_{DiO}+I_{DiI})$ was calculated to be 0.1, where I_{DiO} and I_{DiI} are the fluorescence intensities of DiO at 510 nm and DiI at 565 nm, respectively. It should be noted that direct excitation of DiI at the wavelength of 460 nm is small in comparison with the total DiI signal of FRET liposomes (Figs. 1 and 2, curve 2). The contribution of DiO signal to the DiI emission is negligible, because of low DiO signal in the presence of FRET. The addition of DMF into the water liposomal suspension causes the liposome distortion and the dyes release. As a result, the DiO fluorescence ratio increases remarkably (from 0.1 to 0.9), because the distance between donor and acceptor molecules cannot be kept within the range of FRET efficiency (Fig. 2, curve 3).

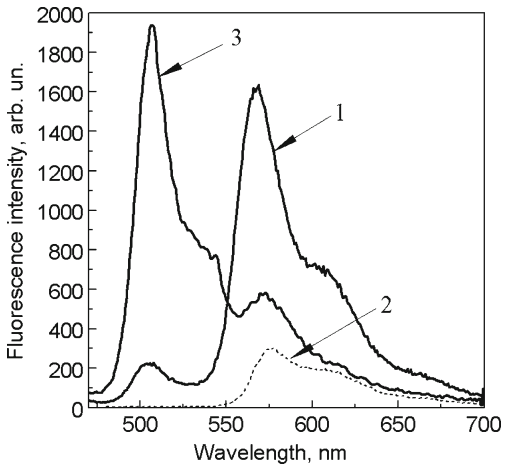

Fig. 2 Fluorescence spectra of liposome suspensions: 1–FRET-liposomes; 2–DiI-loaded liposomes; 3–FRET-liposomes in a binary DMF:water solution (1:1). Fluorescence was excited at 460 nm (the donor absorption band)

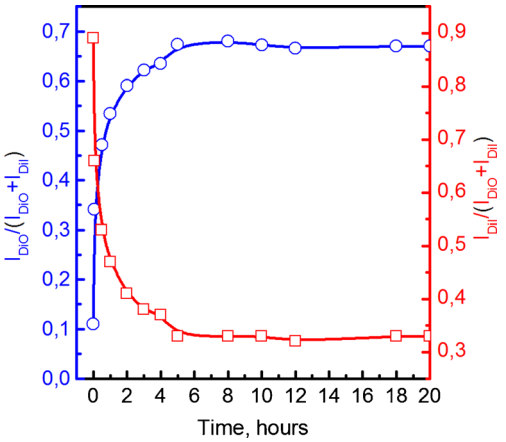

Fig. 3 The changes of DiO $I_{DiO}/(I_{DiO}+I_{DiI})$ and DiI $I_{DiI}/(I_{DiO}+I_{DiI})$ fluorescence ratio in the total fluorescence signal (I_{DiO} and I_{DiI} were recorded at 510 and 565 nm, respectively) as a function of FRET-liposome–to–empty liposome interaction time

The same process is observed when FRET-liposomes interact with other lipid bilayers, for instance, other liposomes (Fig. 3). Liposome vesicles are known to be flexible and highly dynamic supramolecular structures. Aggregation (or clustering), fusion and fission are the most common processes which can lead to the liposome mixing or leaking out of its content [38]. So the addition of "empty" liposomes to the FRET-liposomes suspension causes the dye molecules redistribution due to the membrane fusion process. As a consequence, the FRET signal diminishes, so that the value of $I_{DiO}/(I_{DiO}+I_{DiI})$ increases, while the DiI fluorescence ratio $I_{DiI}/(I_{DiO}+I_{DiI})$ decreases in time (Fig. 3). Since liposome vesicles serve as useful mimics of biological membranes, the interaction of liposomes with cells is supposed to be a similar dynamic process. Analyzing the dynamic changes of the dyes fluorescence ratio, we can monitor the interaction of liposomes with cells in real time and reveal the features of this process for cells of different types.

The application of this simple approach based on a comparison of FRET donor/acceptor emission ratios allows us to avoid the involvement of common methods of FRET measurements as "netFRET" and "three-cube FRET" (FRET microscopy technique) resulting in cross talk and bleed-thought effects that complicates data analysis [39]. Moreover, microspectroscopic technique allows us to deal with a single cell or group of cells and avoid ensemble averaging. Examining the cells under the fluorescence microscope, we can eliminate cells with low functional state or damaged ones which emission contaminates the FRET signal. The application of microspectroscopy is also very prospective way in case of cell culture growing in a Petri dish or special flasks, because allows the stress related to live cell removal from the growing surfaces to be avoided.

Experiments with Living Cells

The cells and hepatocytes nuclei under study were incubated with FRET-liposomes for different time periods (1, 2, 4, 8, 10, 18, 24 h). After each time interval their fluorescent images and fluorescent spectra taken from the cells by microspectroscopy technique were analyzed. Figure 4 represents the redistribution of relative donor and acceptor fluorescence intensities in time. Figure 4 shows different dynamics and time-scale of the redistribution process for different cells.

Rat hepatocytes exhibit gradual accumulation of the dye-loaded liposomes. As one can see in Fig. 4a and f, curve1, there is a gradual increase of the donor DiO fluorescence ratio in total fluorescence signal $I_{DiO}/(I_{DiO}+I_{DiI})$ from 0.1 up to 0.7 within about 4 h. The value of $I_{DiI}/(I_{DiO}+I_{DiI})$ reveals the opposite behavior, i.e. decreasing DiI fluorescence signal from 0.9 down to 0.3 (not presented). After 4 h incubation, the fluorescent image of hepatocytes represent bright uniformly stained cells, green at blue and orange at green excitation that indicates the accumulation of both dyes in cells. The long-

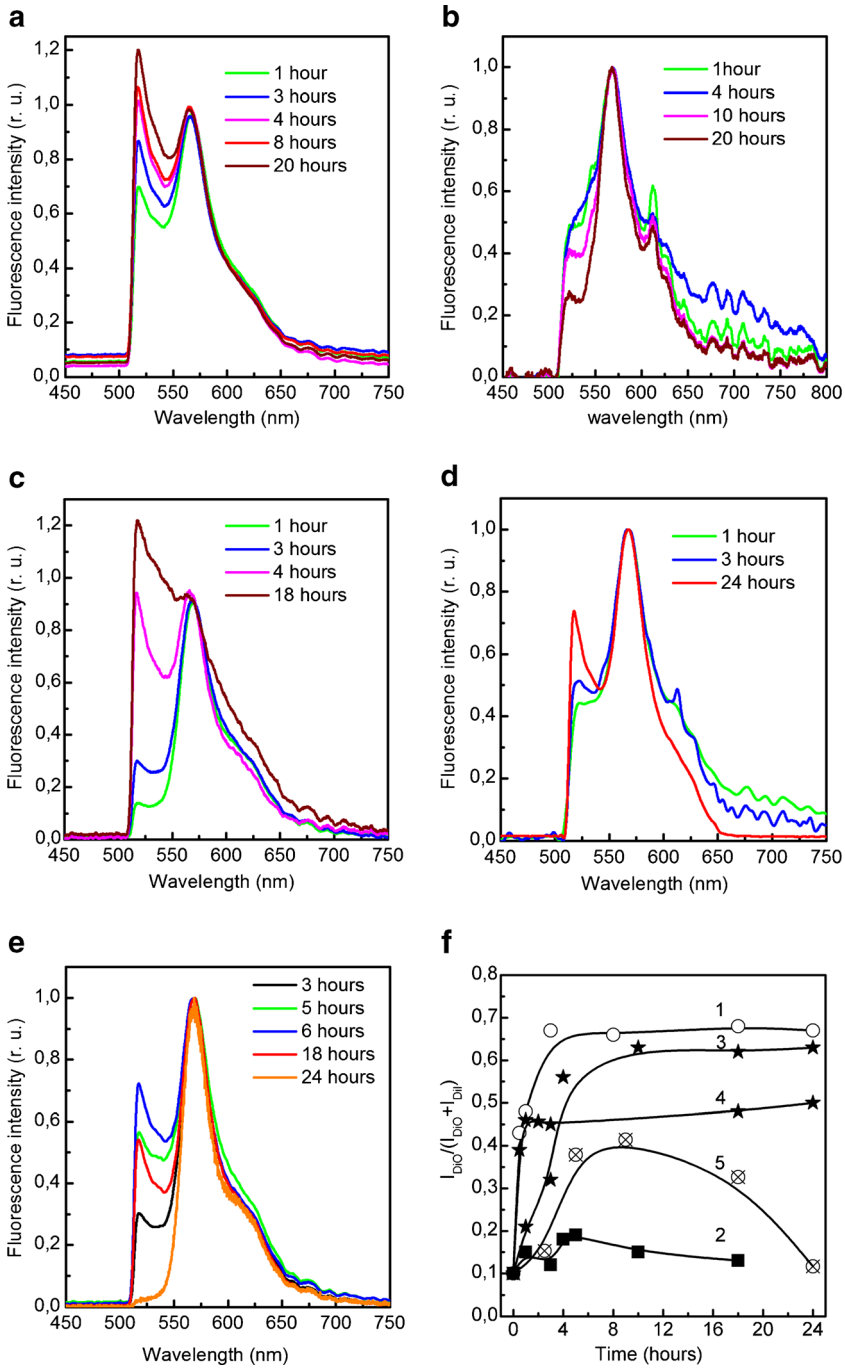

Fig. 4 Fluorescence spectra taken after different time periods of cells incubation with FRET-liposomes using spectromicroscopic technique (excitation filter BP 460–480): (**a**) rat hepatocytes, (**b**) hepatocyte nuclei, (**c**) mouse fibroblast-like cells, (**d**) rat bone marrow, (**e**) human breast cancer cells; (**f**) changes of the DiO fluorescence ratio in the total fluorescence signal in time: 1–rat hepatocytes, 2–hepatocyte nuclei, 3–mouse fibroblast-like cells, 4–rat bone marrow, 5–human breast cancer cells. Spectra (**a**–**e**) were normalized relative to the acceptor band maxima

term incubation of hepatocytes with FRET-liposomes does not revealed any sufficient changes in cell brightness or dyes relative fluorescence intensities (Fig. 4f, curve 1).

The pattern of the relative donor and acceptor fluorescence intensities redistribution in time is similar to that observed in the experiment with the cell membrane model ("empty" liposomes, Fig. 3) and indicates the liposome incorporation into cell membrane and dye dilution with the membrane that takes about 4 h.

Isolated Hepatocyte Nuclei In contrast to hepatocytes, fluorescence images of isolated hepatocytes nuclei taken from different time intervals show a slight accumulation of dyes on the surface of the nuclei. The fluorescence signals detected in both channels (DiO and DiI) are weak for all time intervals (Fig. 4b and f, curve 2). The DiO fluorescence ratio does not change practically (Fig. 4f, curve 2). Therefore, the data described point to the low efficiency of liposome–to–nuclei interaction.

Mouse Fibroblast-like Cells L929 This cell line reveals the dynamics of liposome accumulation, which is similar to that for hepatocytes cells, but a bit slower at the first 3 h of the experiment duration. Figure 4c and f, curve 3 show a gradual increase in the DiO fluorescence ratio $I_{DiO}/(I_{DiO}+I_{DiI})$from 0.1 upto 0.65 within about 5 h that indicates the increase of the donor–acceptor distance due to dye release. Further increase of the cell incubation time does not lead to the remarkable changes in DiO/DiI fluorescence redistribution.

Rat bone marrow cells reveal the increase of the DiO fluorescence ratio $I_{DiO}/(I_{DiO}+I_{DiI})$ from 0.1 upto 0.46 in the first hour of cell incubation with FRET-liposomes that points to the effective liposome–to–cell interaction, whereas experiment duration up to 24 h reveals just modest DiO/DiI fluorescence redistribution (Fig. 4d and f, curve4).

The pattern of the FRET-liposomes interaction with human breast cancer cells MCF-7 differs sufficiently from the ones above described (Fig. 4e and f, curve 5). Figure 4f, curve 5 shows the gradual increase of the $I_{DiO}/(I_{DiO}+I_{DiI})$ value from 0.1 up to 0.4 during about 9 h of the cells incubation with FRET-liposomes. However, contrary to other cells under study, further long-term incubation of MCF-7 cells with the dye-loaded liposomes leads to the recovery of FRET effect, the DiO fluorescence ratio $I_{DiO}/(I_{DiO}+I_{DiI})$ decreases from 0.4 down to 0.1 after 24 h incubation. The fluorescence image of these cells shows very bright yellow-green emission that indicates that both dyes are accumulated in a cell membrane in a great amount. The same effect was described by *Chen* and *co-workers* after a long-time KB cell incubation with dye-preloaded polymeric micelles and explained by the dyes molecules concentration in cell endocytic vesicles after dyes internalization that causes the donor-acceptor distance decrease and a partial recovery of FRET signal [32].

Thus, taking all experimental facts together, we can conclude that FRET-liposomes interact with the cells of all types, but the dynamics, efficiency and probably mechanisms of this process differ remarkably. The common effect observed in the experiment with all cells is the decrease in time of FRET signal and increase of DiO fluorescence intensity. Explaining these results we have to exclude the release of dyes from the liposomes due to liposome instability or liposome bilayers fusion. Our study has reveled that during the analyzed time period (24 h) PC liposomes are stable, the leakage of hydrophobic dyes DiO and DiI is not observed, and there are no changes in the fluorescence spectra. So, we can conclude that the observed redistribution of the relative DiO and DiI fluorescence intensities (Fig. 4) is due to the liposome-to-cells interaction. The observed differences in the dynamics of these interactions can be explained by several reasons.

There are a number of mechanisms of the liposome-to-cell interaction: (i) stable absorption, i.e. association of intact liposomes with the cell surface without their internalization; (ii) endocytosis, i.e. uptake of intact liposomes into endocytotic vesicles; (iii) fusion or merging of the liposome lipid bilayers with the cell plasma membrane; (iv) lipid transfer, i.e. the transfer of lipid molecules between liposomes and cell without cell association of aqueous liposome contents [40]. The relative values of these processes are different for different cells and even for different experimental conditions.

For cells of different types, there are certain differences in cell plasma membrane composition (lipid content, cholesterol, protein and other component ratio, etc.) [41] that can affect the membrane fluidity/rigidity and, consequently, the ability of dyes to diffuse within a cell membrane and their intracellular uptake. This factor is one of the possible reasons of the different degree of DiO/DiI relative fluorescence intensities redistribution observed for different cells (Fig. 4). Significant differences in lipid composition of tumour cells [42] can be responsible for the observed FRET recovering effect as a result of dyes accumulation in a great amount in a cell membrane after a long-term incubation of MCF-7 cells with the dye-loaded liposomes.

Moreover, it is known that living cells or cell organelles exhibit different metabolic activity. Cells with high metabolic activity, such as rat hepatocytes or mouse fibroblast cells, demonstrate the most efficient dyes-loaded liposomes uptake, while isolated hepatocyte nuclei with their passive metabolic process, have revealed extremely low efficiency of liposome uptake.

Conclusion

The changes with time in the FRET signal between two fluorescent dyes preloaded in PC liposomes were used to

visualize and study the liposomes interaction with living cells of different types and cell organelles. It has been shown that FRET-labeling of liposome vesicles allows one to reveal the differences in dynamics and efficiency of liposome-to-cell interactions which are associated with the cell plasma membrane composition, fluidity, cell metabolic activity and mechanisms of liposome-to-cell interactions. The generation of wavelength-ratiometric signal due to redistribution in time of fluorescence relative intensities of FRET donor and acceptor dyes has the prospect to be applied as a test system to trace the delivery of active compound inside living cells using nanocarriers.

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