Fluorescence imaging method for in vivo pH monitoring during liposomes uptake in rat liver using a pH-sensitive fluorescent dye

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Abstract. Liposomes are known to be taken up by the liver cells after intravenous injection. Among the few techniques available to follow this process *in vivo* are perturbed angular correlation spectroscopy, nuclear magnetic resonance spectroscopy, and scintigraphy. The study of the intracellular pathways and liposomal localization in the different liver cells requires sacrifice of the animals, cells separation, and electronic microscopy. In the acidic intracellular compartments, the *in situ* rate of release of liposomes remains poorly understood. We present a new method to follow the *in situ* and *in vivo* uptake of liposomes using a fluorescent pH-sensitive probe 5,6-carboxyfluorescein (5,6-CF). 5,6-CF is encapsulated in liposomes at high concentration (100 mM) to quench its fluorescence. After laparotomy, liposomes are injected into the penile vein of Wistar rats. Fluorescence images of the liver and the skin are recorded during 90 min and the fluorescence intensity ratio is calculated. Ratio kinetics show different profiles depending on the liposomal formulation. The calculated intracellular liver pH values are, respectively, 4.5 to 5.0 and 6.0 to 6.5 for DSPC/chol and DMPC liposomes. After sacrifice and flush with a cold saline solution, the pH of the intracellular site of the liver (*ex vivo*) is found to be 4.5 to 5.0. This value can be explained by an uptake of liposomes by the liver cells and subsequent localization into the acidic compartment. An intracellular event such as dye release of a drug carrier (liposomes loaded with a fluorescent dye) can be monitored by pH fluorescence imaging and spectroscopy *in vivo* and *in situ*.
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Keywords: liposomes; fluorescence; pH; liver; lysosomes.

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1 Introduction

Liposomes are phospholipid vesicles that have been exploited as carrier systems for cellular delivery of biologically active agents.[1] This carrier system displays several advantages over other methods of encapsulating compounds: (1) it reduces toxicity, (2) it avoids degradation and clearance of labile drug, and (3) it delivers an agent to a target site. Liposomes interact with blood components such as proteins and lipoprotreins[2] and are cleared from the circulation by the mononuclear phagocytic system[3,4] and mainly in the liver.[5,6] Other anatomical sites display reduced clearance capacity; for example, spleen, lung, and bone marrow. The clearance rate of liposomes by this mechanism is highly dependent on several factors: size,[7] dose, charge, lipid composition,[1,8–10] and repeated dosing. Intracellular targeting can be achieved by the use of pH-sensitive liposomes, which are stable at a physiological pH and become leaky in the intracellular acidic compartments.

A few methods are available to evaluate the behavior of liposomes *in vivo* such as nuclear magnetic resonance (NMR) spectroscopy or perturbed angular correlation (PAC) spectroscopy. The PAC technique provides information about the change of microenvironment of an encapsulated gamma-emitting radionuclide and the intactness of liposomes *in vivo*. This technique does not enable the determination of the rate of vesicle destruction in specific organs or tissues.[11] It has been demonstrated by ^{31}P NMR spectroscopy of perfused rat liver that a paramagnetic agent encapsulated in liposomes can be followed in the intracytoplasmic compartment[12] [after intravenous (IV) injection]. At the subcellular level, only *in vitro* microspectrofluorimetric or fluorescence imaging studies have shown the kinetic rate of liposomes uptake by the acidic compartment of liver cells.[13–15] This approach requires the use of a pH-sensitive probe such as pyranine, which is encapsulated in the aqueous phase of liposomes.

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Using a similar method but with different fluorescent probes, we already demonstrated the ability of the dual-wavelength technique to monitor pH in normal and tumor tissues.[16] The use of fluorescent techniques *in vivo* necessitates that the background tissue absorbance be accounted for in the measurements. For example, the measurement of the fluorescence intensity of an exogenous fluorescent dye in a tissue is greatly dependent on the blood content. This parameter can affect the pH measurement if the fluorescence intensities ratio is not corrected for the absorption effect of hemoglobin.[17]

This paper aims to evaluate pH monitoring of the superficial tissue of the liver *in vivo* and *ex vivo* by fluorescence imaging using the pH-sensitive probe 5,6-carboxyfluorescein after IV administration of dye-loaded liposomes. Two different compositions of liposomes[18] are used to evaluate the influence of the formulation on the liposomes behavior in the liver. Carboxyfluorescein is used due to its pH-sensitive property for the study of intracellular acidic compartments and its ability to be quenched at high concentration.

2 Materials and Methods

2.1 *Dye Purification*

The 5,6-carboxyfluorescein (free acid) was purchased from Eastman Kodak. The probe was purified by using a procedure described by Ralston et al.[19] Briefly, the dye was precipitated on active charcoal and then purified on Sephadex LH-20 column (2.5×40 cm) in phosphate buffer solution (PBS: 150 mM, 145 mM NaCl, 145 mM Na_2HPO_4, pH 7.4). The dye purity was assessed by high-performance liquid chromatography. Samples were eluted at room temperature with a linear gradient of methanol in 0.5% acetic acid. The gradient (25 to 100% methanol) was run in 20 min at a flow rate of 2 mL/min on a C 18 reverse phase column (Lichrosphere). A 100-mM solution was prepared by dissolving suitable amount of the purified dye in PBS (pH 7.4).

2.2 *In Vitro pH Calibration*

The excitation spectra of carboxyfluorescein is pH-sensitive. This dye exhibits two major excitation peaks centered at 470 and 490 nm, respectively. The ratio is linked to the pH by this simplified equation according to Whitaker et al.,[20]

$$\mathrm{pH} = \mathrm{pK_a} + (R - R_{\min}/R_{\max} - R),$$

where R is the ratio calculated from intensities measurements, $R_{\min}$ is the minimum limiting value, and $R_{\max}$ is the maximum limiting value. A calibration curve linking ratio (I_{490}/I_{470}) with pH was constructed *in vitro* using a 10^{-6} M 5,6-CF solutions of different pH values (3.5 to 7.5 pH range). The pH of the solutions was measured with a Biotrode electrode (Hamilton, Switzerland). The influence of blood content (proteins and red blood cells) was assessed using the same procedure described in a previous publication.[17] Briefly, blood was collected at the cut tail vein of an anesthetized Wistar rat in an heparinized tube and used immediately for dilution. Dilutions were carried out with isotonic phosphate buffer saline solutions (pH: 7.4) and placed in a dark multiwell. Gentle shaking prevented sedimentation of red blood cells during the experiment. An aliquot was kept to determine hematocrit. Blood was collected in a capillary tube (Blu Tip, Sherwood Medical, Irlande) and centrifugation was carried out in a Bifuge A at 10,000*g* for 7 min. Hematocrits were determined according to the manufacturer recommended procedure (Mikko Haematocrit, Heraus, Separtech, Germany).

2.3 *Preparation of Liposomes*

Liposomes were prepared by the sonication procedure. Two different formulations were used: DMPC (dimyristoylphosphatidylcholine) and DSPC/Chol with a 7:3 molar ratio (DSPC, distearoylphosphatidylcholine; Chol, cholesterol). The lipids were kindly provided by Lipoïd K.G. (Ludwigschaffen, Germany) and the cholesterol was purchased from Sigma (USA). Cholesterol is known to reduce leakage occurring in the serum.[21,22]

The chloroform solution of lipids (DMPC or DSPC/Chol 7:3) was evaporated at 40 °C under reduced pressure in a rotary evaporation flask. After complete removal of the chloroform, a 100mM 5,6-CF solution was added and hydration of lipids was carried out at a temperature above the phase transition temperature of the phospholipid. After an equilibration period, the lipid suspension was sonicated at the same temperature for 10 min (Sonicator Heat-Systems/Misonix, Farmingdale, New York, 500 W, 10% output, 20,000 Hz, 3-mm-diam probe). The suspension of liposomes was centrifuged at 4000 rpm for 20 min (Heraeus Biofuge) to eliminate titanium particles. Liposomes size was determined by quasielastic light scattering at a 90-deg angle (SEMATECH, SM 633/RTG, France) using monomodal analysis. The mean size of liposomes was found as follows. DMPC mean diameter (intensity analysis): 189 nm±30 nm, with dispersion: 95.89 nm±8.7 nm; and for DSPC, mean diameter (intensity analysis) 198 nm±54.2 nm with dispersion: 69.1 nm±24.6 nm. Supernatant was dialyzed over 5-mM phosphate buffer saline (pH=7.4) for 24 h to remove unencapsulated 5,6-CF. Immediately after dialysis, the liposomes were injected into animals to prevent any leakage *in vitro*. The 5,6-CF was encapsulated at high concentration. The quenched encapsulated 5,6-CF has been already used to monitor in real time the leakage of a dye *in vivo* during laser-induced photocoagulation of blood vessels.[16]

2.4 *Animals*

Adults Wistar rats weighing 300 to 400 g were anaesthetized (ether for induction and ketamine/ketalar for anesthesia). The rats were surgically prepared to enable fluorescence measurement of the liver surface. A laparotomy was performed to illuminate the liver surface. The liver was kept near the physiological temperature and regular spraying of 37 °C water was performed to avoid drying of the liver surface. For skin measurements, a part of the skin was shaved on the leg. A solution of liposomes (0.8 mL) containing 5,6-CF was slowly injected via the penile vein. For *ex vivo* experiments, the liver was flushed for 90 min after liposomal injection with a cooled saline solution (4 °C) to eliminate any remaining circulating 5,6-CF. This flush was performed through the portal vein, and then the liver was isolated from the main vascular circulation by clamping both inferior and superior cava veins. After the flush, the animals were sacrificed and the liver was surgically removed. Immediately thereafter, the fluorescence was measured *ex vivo* with the fluorescent imaging system.

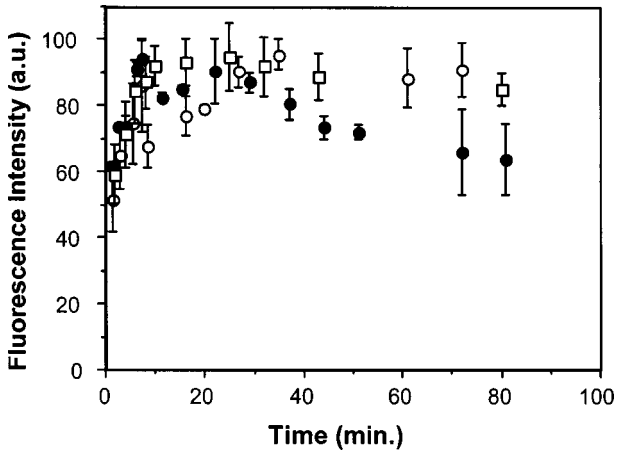

Fig. 1 Kinetic profile of the skin fluorescence intensity as a function of time after injection of (□) free 5,6-CF, liposome-encapsulated 5,6-CF, (○) DMPC liposomes, and (●) DSPC/Chol liposomes ($n=3$).

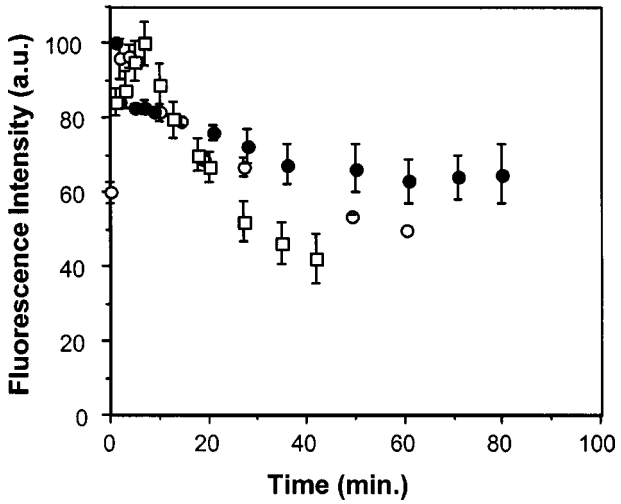

Fig. 2 Kinetic profile of the liver fluorescence intensity as a function of time after injection of (□) free 5,6-CF, liposome-encapsulated 5,6-CF, (○) DMPC liposomes, and (●) DSPC/Chol liposomes ($n=3$) (excitation wavelength is 490 nm).

2.5 *Fluorescence Imaging System*

Fluorescence measurements were performed with a fluorescence imaging system developed in our laboratory. The instrumental device was described in a previous paper.[23] Schematically, it includes a filtered 150-W xenon lamp with two narrowband interference filters (FWHM=10 nm) centered at 490 and 470 nm. The image field was 40×40 mm (1600 mm^2) to visualize the entire liver. The peak fluorescence emission was selected using a narrowband interference filter (515 nm, FWHM=1 nm, Corion, USA) and imaging was performed with an ultra-high-sensitivity intensified camera (model C2400-20, Hamamatsu Photonics, Japan) connected to an image processor (Argus-50, Hamamatsu, Japan) and equipped with a a Nikon 60-mm macro (NIKOR 60-mm Macro $F/2.8$). Fluorescence images were obtained every 40 ms. The video signal was fed to the image processor, which performs integration of 512×512 pixels images with 14-bit digital resolution. Fluorescence intensities and ratios were determined within a fixed area corresponding to 4000 pixels. The background intensity (intensity correlated to the imaging system recorded without any animal) was taken into account by *in vitro* measurements of background plus autofluorescence (fluorescence intensity recorded on the tissue before dye injection) for the *in vivo* experiments. Ratio images were calculated on the same area using fluorescence images obtained at 515 nm for each excitation wavelength (490/470 nm) with subtraction of the background and the autofluorescence for *in vivo* measurements. At these excitation wavelengths, background and autofluorescence were negligible. Fluorescence imaging was performed between 0 and 80 min, except for free 5,6-CF (45 min) due to its short circulating half-life.

3 Results

The fluorescence kinetic profiles recorded on the skin with a 490-nm wavelength excitation are shown in Fig. 1. At this wavelength, skin autofluorescence was very low compared to the skin spectra after dye injection wheter the formulation was free or encapsulated within the liposomes. Comparison of the free 5,6-CF fluorescence kinetics to the liposomal encapsulated dye kinetics were expressed in percentage of fluorescence (100% of fluorescence corresponding to the tissue fluorescence peak). The free 5,6-CF fluorescence intensity increased and reached a plateau phase 10 min after the dye injection. When 5,6-CF filled liposomes were administered, the fluorescence kinetic profiles were slightly different depending on the liposomal formulation. Within the 0 to 30-min time interval, there was no significant difference between DMPC and DSPC/Chol liposomes. After 30 min and until the end of the experiment, the DMPC fluorescence intensities were constant but always higher than DSPC/Chol. The fluorescence kinetic profile of the liver after free 5,6-CF injection (Fig. 2) showed a maximum peak of fluorescence 10 min after injection, followed by a rapid decrease. Liver autofluorescence was also very low compared to the liver intensities recorded after dye injection independant of the formulation (free or encapsulated in liposomes). Note that 5,6-CF fluorescence intensities were very low compared to either liposomal formulation. No fluorescence could be measured 40 min after free dye injection. Both liposomal formulations exhibited maximum fluorescence 5 min after injection. The fluorescence kinetic of DSPC/Chol liposomes, reached a plateau between 30 and 80 min postinjection. For the DMPC liposomes, the fluorescence intensity decreased slowly until the end of the experiment and no fluorescence could be measured after 60 min.

In reference to the ratio (I_{490}/I_{470}) kinetic profiles measured on the skin (Fig. 3), for all the formulations (free dye, DMPC, and DSPC/Chol liposomes), a plateau phase was observed 15 min after injection with an average ratio value of 2.4. Figures 4(a) and 4(b) show typical fluorescence images recorded on the liver of an anaesthetized rat 10 min after DSPC/Chol liposomes injection and the I_{490}/I_{470} ratio image [Fig. 4(c)]. The image recorded with at a 490-nm excitation wavelength [Fig. 4(a)] presents a high level of fluorescence compared to the image recorded at the 470 nm excitation wavelength [Fig. 4(b)]. The calculated ratio on the resulting ratio image [Fig. 4(c)], was 1.75, but a corrected ratio was calculated on the basis of the calibration previously described

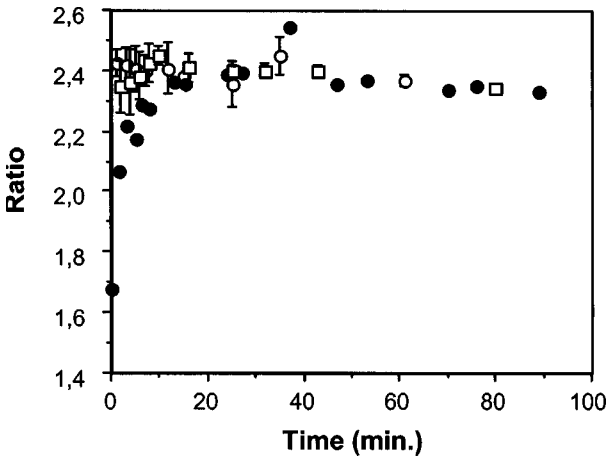

Fig. 3 Evolution of the ratio of the skin as a function of time after injection of (□) free 5,6-CF and liposome-encapsulated 5,6-CF: (○) DMPC liposomes and (●) DSPC/Chol liposomes ($n=3$) (excitation wavelength is 490 nm).

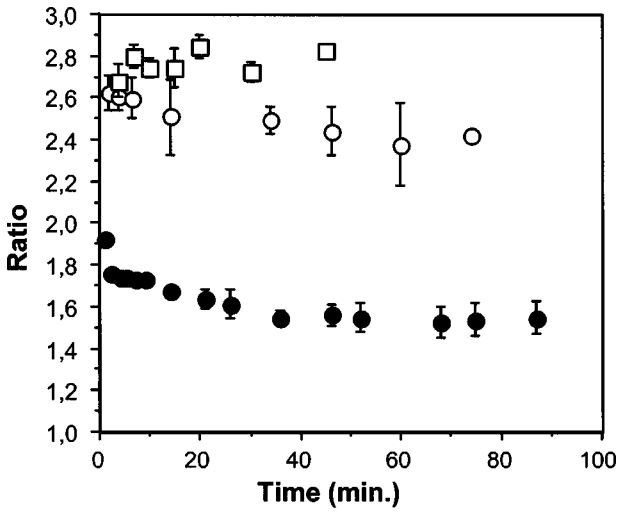

Fig. 5 Evolution of the ratio of the liver as a function of time injection of free 5,6-CF (□), liposome-encapsulated 5,6-CF: (○) DMPC liposomes and (●) DSPC/Chol liposomes.

to take into account the effect of the red blood cells.[17] Briefly, a calibration curve of 5,6-CF ratio was realized as a function of the hematocrit. *In vitro*, dilutions of 5,6-CF (5,6-CF concentration is constant) were done in the presence of an increasing rate of hematocrit from 5 to 44% (whole blood). An increased ratio between 5 and 20% hematocrit followed by a plateau from 20 to 44% was observed. The variation of the ratio between solutions containing 5 and 44% hematocrit was 0.55. Because of the importance of the increase in the presence of a large amount of hemoglobin, the ratio must be corrected for vascularized tissue (such as liver).

The ratio kinetic profiles in the skin (Fig. 5) were different as a function of the formulation. When free 5,6-CF was injected, the ratio remained constant and high (2.8) but could only be calculated within the 0- to 40-min time interval because of the low fluorescence intensities. After DMPC injection, the ratio was also high but decreased slowly from 2.6 to 2.4. After DSPC/Chol liposomes injection, the ratio was lower (1.7) and decreased over the course of the experiment (1.5).

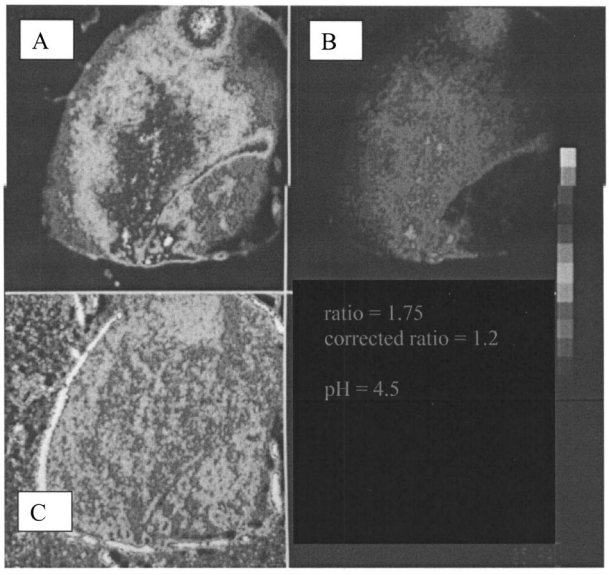

Fig. 4 Fluorescence and ratio images of the liver after injection of the DSPC/Chol 5,6-CF loaded liposomes: (a) fluorescence image at a 490-nm excitation wavelength, (b) fluorescence image at a 470-nm excitation wavelength, and (c) I_{490}/I_{470} ratio image ($n=3$).

4 Discussion

This paper aimed to evaluate pH monitoring of the superficial tissue of the liver *in vivo* and *ex vivo* by fluorescence imaging using the pH-sensitive probe: 5,6-CF encapsulated in liposomes. This fluorescent dye has two main advantages: (1) efficient encapsulation into liposomes (1,4,15) and (2) to measure pH because of its pH-sensitivity[23] between 5.5 and 8.0. Furthermore, Straubinger et al.[14] demonstrated that the molecular structure of 5,6-CF leads to different permeability properties in the acidic range. If 5,6-CF is a trivalent anion at physiological pH, it has no charge at acidic pH. This probe underwent fluorescence quenching at high concentration (100 mM). At the time of injection, in intact liposomes, the charged form of the probe was quenched[24,25] (nonfluorescent). In this study, we used two different compositions of liposomes to evaluate the influence of the formulation on the breakdown of liposomes in lysosomes. These liposomes differed by their phospholipid composition (DMPC or DSPC) and their ratio of cholesterol (0 and 7:3) in the transcutaneous measurements (Fig. 1), the fluorescence kinetic profile of free 5,6-CF in the skin demonstrated a plateau phase 10 min after injection, thereafter remaining constant until the end of the experiment. This plateau phase indicated the diffusion of the probe throughout the capillaries leading to the perfusion of the skin. The probe localization can be extravascular and/or intravascular in very small capillaries.[26] This phenomenon of endothelium lining is well known during fluorescein angiography.[27] In the case of liposomal formulations, the fluorescence intensities variation may be attributed to two parameters: (1) the leakage of 5,6-CF in blood after liposomes interactions with blood components (proteins and lipoproteins) and (2) the diffusion of free 5,6-CF throughout the capillaries. In particular,

DSPC/Chol liposome fluorescence intensity decreased more rapidly than that of DMPC. This difference could be attributed to individual bilayer properties: the DMPC liposomes with a transition phase at 23 °C are more leaky at the physiological temperature (37 °C) than the DSPC/Chol liposomes with a transition phase at 41 °C (*in vitro* studies, data not shown). The leakage of the dye therefore occurred more rapidly across the phospholipid bilayer of the DMPC liposomes, which was in a fluid state. It is well known[2,28,29] that fluid liposomes (like DMPC) are rapidly degraded and destroyed in the blood stream due to the interaction of these liposomes with high-density lipoprotein (HDL).

Free 5,6-CF exhibited a rapid clearance in blood, resulting the rapid decrease of fluorescence intensity in the liver (Fig. 2). No fluorescence was measured 40 min after injection because the intensity recorded is too low. The rapid increase of liver fluorescence intensity measured during the first 2 min can be attributed to two different kinds of leakage (Fig. 2). First, the leakage of 5,6-CF in blood after interaction of liposomes with blood components, particularly proteins and lipoproteins[2] was observed. Second, opsonization occurred once the opsonins adsorbed onto the liposomal surface allowing their recognition and capture primarily by the phagocytic cells of liver (Kupffer cells), leading to the degradation of liposomal membrane and release of the dye. In this study, the liposomes injected into the Wistar rats were of a mean size of 189±30 nm for DMPC and 198±54.2 nm for DSPC/Chol. Liposomes in this size range get taken up by the liver in a very short time (40 s) as compared to the whole process leading to an intracellular localization of the liposomes that transpires[30] over a period of approximately 2 h. After the hepatic uptake of the labeled liposomes in the acidic compartments (lysosomes), the probe has a neutral charge. In this form, it can diffuse through the membrane of the lysosomes to the intracytoplasmic compartment (physiological pH) where it becomes highly fluorescent because of the dilution. Below pH 5.5, the leakage of 5,6-CF out of liposomes was 17 times greater than at neutrality.[1,22] The probe could diffuse through the cytoplasmic membrane into the vascular compartment (physiological pH) where it was eliminated. This diffusion explained the rapid increase of fluorescence intensity irregardless of the liposomal composition (DMPC or DSPC/Chol 7:3). The 5,6-CF present in both acidic and normal pH compartments contributed to the increase of fluorescence intensity.

When free dye was injected, the measured ratio of the skin was constant during the whole experiment (Fig. 3). The ratio value (2.4) corresponded to the physiological pH. The dye localized in the blood stream due to its ability to diffuse across biological membrane toward the interstitial tissue. For liposomal formulations, the ratio can be calculated when fluorescence intensities at both excitation wavelength (490 and 470 nm) were measurable. In the case of DSPC/Chol, the ratio value (2.4) was reached more slowly than DMPC (20 min after injection). This could be explained by the higher stability of the DSPC/Chol in the blood limiting its release rate.

The image obtained after DSPC/Chol liposomes injection [Fig. 4(c)] corresponded to a mean pH of 4.5. Such a low pH value indicated that the pH-sensitive dye (5,6-CF) was mainly localized in the acidic intracellular compartment of the Kupffer cells. Note that 5,6-CF at acidic pH has no charge and can diffuse through the lysosomal membrane. Since 5,6-CF can escape from these acidic compartments and become highly fluorescent, this low pH value demonstrated that a high amount of the dye was localized in the acidic compartment. Moreover, after flushing the liver with cold saline solution to eliminate blood cells and circulating free dye, a similar pH value was obtained, confirming that the dye is mainly localized in the intracellular acidic compartments.

When free 5,6-CF was injected, the measured ratio in the liver was very high (2.8). Taking into account the high blood content in the liver hematocrit (44%), a correction factor of 0.55 was applied. The corrected ratio value of 2.25 corresponded to a pH of 7.2. After DMPC liposomes injection, the ratio values were lower and decreased slowly, consistent with the liposomes releasing their content rapidly in the liver. As free 5,6-CF was cleared from the liver, an equilibrium was reached between the amount of dye in the intracellular acidic compartment and the amount of dye localized in physiological pH compartments as evidenced by the pH measured at the plateau phase (6.0). In the case of DSPC/Chol liposomes, the ratio values were very low and reached a plateau phase (1.6) 20 min after injection. The increase of the ratio showed that the efflux of 5,6-CF from the DSPC/Chol in the acidic cells was greater than the clearance. The ratio increased to a plateau where the leakage and the efflux were equilibrated. The high stability of this liposomal formulation evidenced by a very low release rate leading to a major localization of the dye in the intracellular acidic compartment is an explanation for a low pH value of 4.5. The plateau phase (20 to 90 min) suggested a continuous leakage of the dye in the acidic compartment of Kupffer cells. The calculated pH of the liver was about 4.5 close to the pH of lysosomes demonstrating the ability of the method to detect fluorescence in an intracellular compartment *in vivo* and *in situ*.

In conclusion, an intracellular event such as dye release of a drug carrier (liposomes loaded with a fluorescent dye) can be monitored by pH fluorescence imaging *in vivo* and *in situ*. Currently, only microspectrofluorometry has been applied *in vitro* to the study liposomal uptake by macrophages. The liposomal formulation has an important contribution on the leakage properties of liposomes in lysosomes; there was a clear relationship between formulation and stability *in vivo*. Further studies are necessary to determine the therapeutic potential of IV-administered liposomes in terms of their behavior, their kinetics of lysosomal uptake, their subsequent leakage, and finally the dye elimination by the liver.

Acknowledgments

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