Eur Biophys J (2006) 35: 155–161
DOI 10.1007/s00249-005-0008-8

ARTICLE

**Nathalie Mignet · Quentin le Masne de Chermont
Tatiana Randrianarivelo · Johanne Seguin
Cyrille Richard · Michel Bessodes · Daniel Scherman**

Liposome biodistribution by time resolved fluorimetry of lipophilic europium complexes

Received: 20 May 2005 / Revised: 24 June 2005 / Accepted: 18 July 2005 / Published online: 14 September 2005

Abstract The use of conventional fluorophores suffers from some limitations in biological fluids due to low signal/background ratio. Today, this sensitivity issue might be reasonably improved thanks to lanthanide chelates, by selective detection of long decay fluorescence. Use of pulsed light source time-resolved fluorimetry takes into account the fluorescence decay time of the lanthanide chelates to gain sensitivity in biological media. Lipid-DTPA: Eu compounds have been prepared and incorporated into liposomes to evaluate europium based detection of liposomes in biological media. Fluorescence emission was not modified by this incorporation. Europium labelled liposomes were used for biodistribution studies and showed their use in this context.

Keywords Lanthanides · Liposomes · Pharmacokinetics

Introduction

Liposomes present a major interest for drug delivery. While their lipidic bilayer helps solubilizing hydrophobic compounds, their internal aqueous center provides a way of encapsulating hydrophilic drugs. For instance, transbilayer pH gradient allows loading doxorubicin very efficiently (Harasym et al. 1997). Today, few drugs formulated into liposomes are available in the pharmaceutical market. However, few methods are available to follow the pharmacokinetics of liposomes. Indeed, lipids might be incorporated into liposomes as markers; however, these lipids must not be exchangeable with membranes. Radioactivity remains the most sensitive method of all. A tracer, such as ^{3}H-cholesteryl hexadecylether, has been integrated in the liposome bilayer to follow the pharmacokinetic and tissue distribution of the liposomes (Parr et al. 1994). Fluorescence is also commonly used, although highly pH and medium dependent. Few lipids labeled with fluorophores are currently available. Among them, dioleoyl-phosphatidylethanolamine–lissamine rhodamine allows the quantification of the liposomes in the tissues. Using this tracer, we optimized the method initially reported by Takeuchi et al. (2001) to follow the fate of liposomes in vivo (Nicolazzi et al. 2003). This method is highly reproducible but requires the extraction of the lipid from the biological media with organic solvent to remove important background and improve sensitivity.

Time resolved fluorimetry of lanthanide chelates has proved to be very sensitive for use with nonisotopic immunoassays. Lanthanides suffer from low molar extinction coefficients, but chelates of lanthanide are being developed to transfer excitation energy to lanthanide ions and reduce this problem. They appear to be a potential alternative to radioisotopic compounds. Lanthanides present numerous advantages, such as large Stoke's shift, narrow emission peaks, and optimal emission wavelengths, for use with biological material. Interference of the natural fluorescence of biological samples, in particular from blood serum, might be highly reduced thanks to selective detection of long-decay fluorescence. Hence, interfering background observed with conventional fluorophores might be avoided (Soini and Kojoia 1983). Lanthanide ions have been previously incorporated into liposomes to increase protein detection (Laukkanen et al. 1995) and to follow the biodistribution of interleukin-2 (Neville et al. 2000). This sensing assay is based on the insertion of DTPA:Eu which is well-suited for protein detection in vitro, but limited for in vivo applications. Sensitization of lanthanide ions by chelidamic based lipids was also recently reported as a good means for protein detection (Roy et al. 2003).

N. Mignet (✉) · Q. le M. de Chermont · T. Randrianarivelo
J. Seguin · C. Richard · M. Bessodes · D. Scherman
CNRS-UMR8151; INSERM U640, Laboratoire Pharmacol. Chim. Genet., Université René Descartes Paris,
4, avenue de l'Observatoire, 75270 Paris cedex 06, France
E-mail: Nathalie.mignet@univ-paris5.fr
Tel.: +33-1-53739-581
Fax: +33-1-43266-918

Our objective was to use the europium ion as a marker to follow the biodistribution of the liposomes. For this purpose, we synthesized amphiphilic ligands composed of a lipidic anchor and a DTPA moiety. The chelatant DTPA was linked to lipidic chains of different nature, octadecyl or cholesterol, in order to favour the anchor of the label into the liposome bilayer. The complexation of lanthanide ions by liposomes containing DTPA lipids or the incorporation of europium labeled lipids into liposomes was evaluated by fluorimetry and dynamic light scattering. The use of these labeled liposomes in biological media as a tool for biodistribution studies was evaluated.

Experimental

Materials

Solvents were purchased from Merck, egg-phosphatidylcholine, phosphatidylethanolamine-poly(ethyleneglycol)$_{45}$ from avanti polar lipids and cholesterol from Sigma.

Synthesis of lipids

Octadecyl DTPA was prepared in three steps from stearic acid

To a solution of stearic acid (1 g, 3.5 mmol) and *N*-hydroxysuccinimide (402 mg, 3.5 mmol) in THF (10 mL) was added a solution of dicyclohexylcarbodiimide (725 mg, 3.5 mmol) in THF (5 mL). The mixture was stirred for 6 h at room temperature. The solvent was evaporated and the crude diluted in acetone (10 mL). The precipitated dicyclohexylurea was filtered and the octadecyl succinimidyl ester was recovered after evaporation.

Then, a solution of octadecyl succinimidyl ester (42 mg, 110 µmol), amine (Anelli et al. 1999) (80 mg, 110 µmol) and triethylamine (150 µl, 1 mmol) in DMF (5 mL) was stirred for 12 h at room temperature. The solvent was removed and the crude residue was dissolved in ether, washed with saturated $NaHCO_3$, dried, concentrated and purified on silica gel to yield octadecyl-DTPA-ester.

Finally, to a solution of ester (55 mg, 50 µmol) in CH_2Cl_2 (2 mL) cooled to 0°C was added trifluoroacetic acid (250 μL). The mixture was stirred for 24 h at room temperature, concentrated and precipitated in ether. The solid was suspended in water and lyophilized to furnish the desired octadecyl-DTPA. These compounds have been characterized by ^{1}H and ^{13}C NMR.

^{1}H NMR (300 MHz, CD_3OD): δ 0.90 (t, 3H), 1.30 (m, 30H), 1.65 (m, 4H), 2.10 (t, 2H), 2.65 (m, 2H), 2.85 (m, 6H), 3.20 (m, 3H), 3.50 (s, 10H), 5.89 (t, 1H).

^{13}C NMR (75 MHz, CD_3OD): δ 14.1, 22.6, 25.8, 28.2, 28.3, 29.0, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 33.5, 36.8, 39.1, 50.2, 53.6, 55.9, 64.0, 171.1, 176.0.

Cholesteryl-DTPA was prepared as previously described in the literature (Lattuada and Lux 2003).

Europium labeling lipids

To a solution of octadecyl-DTPA (1 mg, 1.5 µmol) in 1 mL H_2O, $EuCl_3$ (2.7 mg, 7.5 µmol) was added. After stirring overnight, a precipitate was formed, which was centrifuged at 2000 rpm, 30 min, 4°C, then washed with H_2O. These steps were repeated until no fluorescence signal was found in the supernatant. The solid was re-suspended in H_2O. The lipid obtained yielded a time-resolved signal of 3215 ± 159 at 5 µM.

To a solution of cholesteryl-DTPA (1 mg, 1.1 µmol) in 1 mL H_2O, $EuCl_3$ (2.7 mg, 5.5 µmol) was added. After stirring overnight, the adduct obtained was dialysed against 1 L H_2O, at 4°C for 24 h. The lipid obtained yielded a time-resolved signal of 5004 ± 169 at 5 µM.

Liposome preparation

Egg-phosphatidylcholine (12 mg, 15 µmol), Cholesterol (3.8 mg, 10 µmol) and phosphatidylethanolamine-poly(ethyleneglycol)$_{45}$ (4 mg, 1.5 µmol) were dissolved in $CHCl_3$. If required, octadecyl-DTPA:Eu (1.5 mg, 1.5 µmol) or cholesteryl-DTPA:Eu (1.7 mg, 1.5 µmol) were incorporated into the mixture. A lipidic film was formed under reduced pressure during 2 h, then hydrated with 1 mL H_2O under slow stirring during 5 h. Liposomes were successively extruded through 0.4 and 0.2 µm filters to obtain 99.79 ± 0.65 nm and 97.49 ± 0.91 nm size liposomes with low polydispersity, 0.247 and 0.241 for the liposomes bearing octadecyl-DTPA:Eu and cholesteryl-DTPA:Eu, respectively.

Phosphorescence spectra

Long-lived fluorescence was recorded with a Varian Cary-Eclipse Fluorescence Spectrophotometer using the phosphorescence mode; $\lambda_{ex} = 396$ nm, $\lambda_{em} = 616$ nm, delay time: 0.1 ms, gate time: 5 ms; for the emission spectra, excitation slit: 20 nm, emission slit: 5 nm. For the excitation spectra, excitation slit: 10 nm (5 nm for the insert), emission slit: 20 nm.

Dynamic light scattering experiments were performed on a Zetasizernano ZS (Malvern Instruments) equipped with a 632.8 nm helium neon laser, 5 mW power, with a detection angle at 173° (noninvasive back scattering).

Time-resolved Fluorimetry (TRF) was performed on a Victor, Perkin Elmer_Wallac multiplate reader. TRF program for europium performs 1000 pulses/s with an excitation light at 320 nm. In the period between flashes, 615 nm fluorescence of the sample is measured for 400 µs that allows the short-time fluorescence to decay. The photons counted during 1 s were recorded and expressed as counts per second (cps).

In vivo distribution

Lewis lung carcinoma tumors were implanted subcutaneously in the right flank of 5-week-old female C57Bl/6 mice (Janvier) and 10–14 days after, biodistribution studies were undertaken. Mice were anesthetized by intraperitoneal injection of a mix of Ketamine (85.8 mg/kg; Centravet) and Xylazine (3.1 mg/kg; Bayer) diluted in 150 mM NaCl. Europium-labeled liposomes (2 μmol, 200 μl) were injected into the mouse tail vein. Blood was collected by cardiac puncture, mice were euthanized at 1 h after injection and the liver, spleen, lungs and tumor were collected, weighed and homogenized in pH 7.4 PBS using an Ultra Thurax (Diax 600, Heidolph, Fisher). Experiments were conducted following the NIH recommendations and in agreement with a regional ethic committee for animal experimentation (no. P2.PB003.04). The fluorescence intensity of europium-labeled lipids was detected directly on the plasma by TRF with a spectrofluorometer (Victor, Perkin Elmer). Tissue homogenates at 2.5 mL/g were incubated with lysis buffer (Roche) 1× (1/1, v/v), vigorously mixed overnight, then centrifuged (1,000 rpm, 5 min). The fluorescence intensity was assayed on the supernatant by TRF. The amount of liposome in the plasma or tissue homogenates was evaluated with a calibration curve performed in the tissues of interest (Fig. 4) and expressed as the remaining percentage of injected dose (Fig. 5).

Results

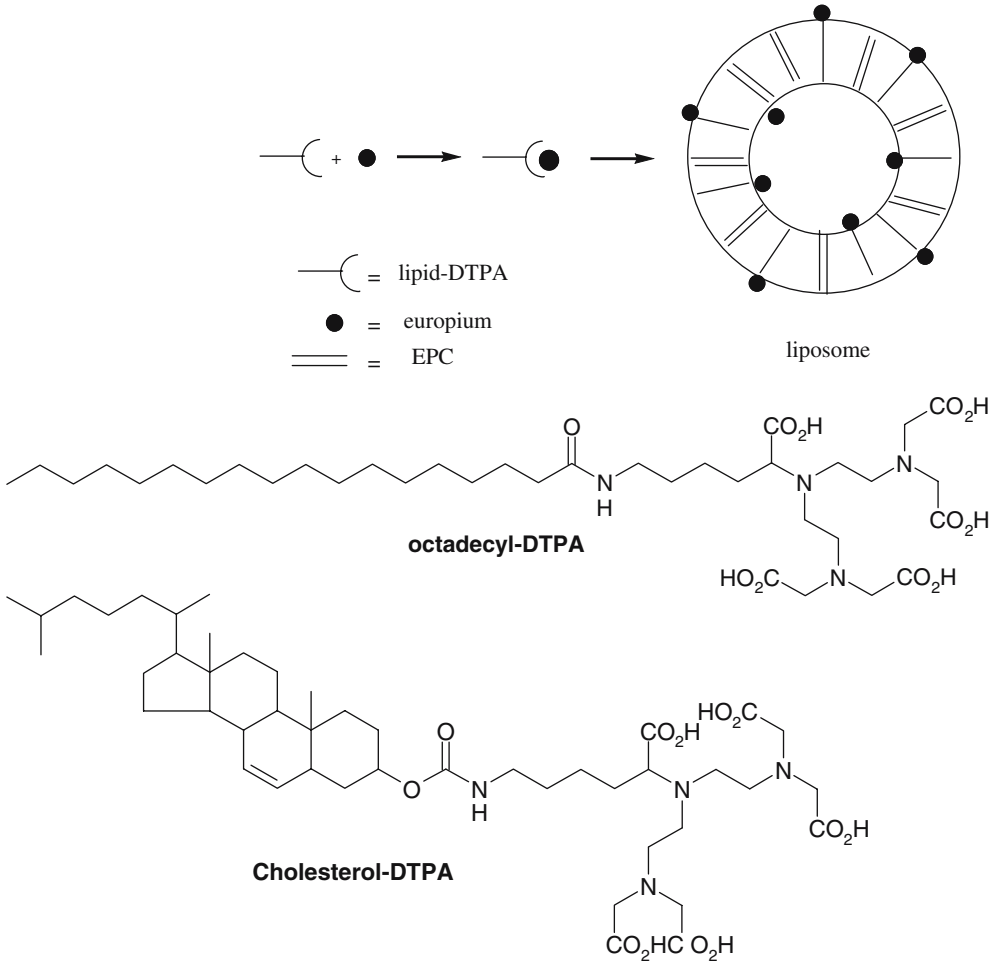
Labeling liposomes with europium-lipids

The way we proceeded is schematically represented in the following scheme. First, the DTPA-lipid was reacted with europium chloride, then the labeled lipid was inserted into liposomes.

Incorporation of lipid-DTPA:Eu into liposomes

Synthesis of the europium labeled DTPA lipids

Two different lipids linking the DTPA moiety were investigated. An octadecyl and a cholesterol were linked through a butylamine linker to the DTPA as described in the experimental section. The synthesis of DTPA



Scheme 1 Representation of the labelled liposomes. Structure of the octadecyl-DTPA and cholesteryl-DTPA used in the study

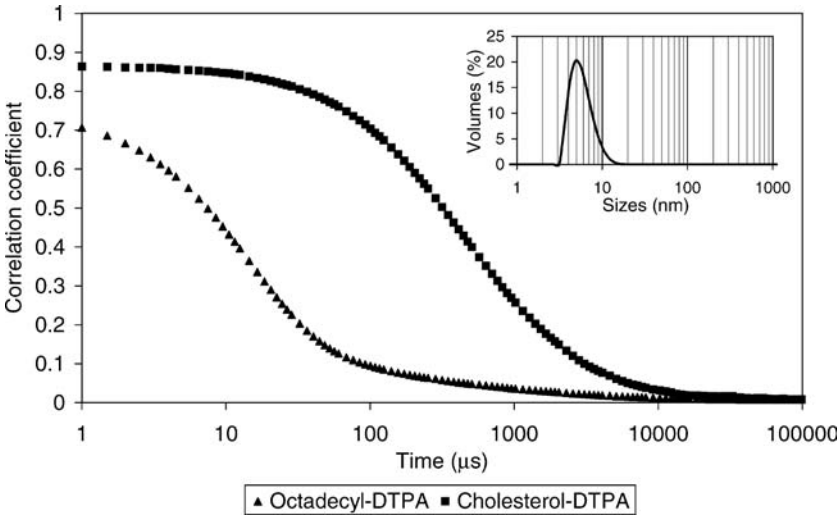


Fig. 1 Raw data correlation function of the octadecyl-DTPA and cholesteryl-DTPA obtained by dynamic light scattering. The insert corresponds to the volume distribution of the octadecyl-DTPA compound

cholesterol was previously described for gadolinium chelation (Lattuada and Lux 2003). Addition of $EuCl_3$ was performed in H_2O at a concentration of 1 mM. We observed that micelles composed of the octadecyl lipid did aggregate after europium chloride chelation while cholesteryl-DTPA did not as much. After centrifugation of the reaction, the supernatant for the monocatenar lipid gave a low signal in time resolved fluorimetry while the whole cholesteryl-DTPA:Eu was mostly found in the supernatant. By dynamic light scattering, a peak exhibiting a hydrodynamic diameter of 8 nm was measured corresponding to the micellar organisation of the octadecyl-DTPA compound (Fig. 1), as reported for a related compound (Accardo et al. 2004). On the contrary, high size heterogeneity of the cholesteryl-DTPA sample did not allow obtaining a reliable size distribution (Polydispersity index was 0.305 and 0.780 for the octadecyl-DTPA and the cholesteryl-DTPA, respectively). The raw data correlation function represented in Fig. 1 indicates the presence of diffusing species with coarser particle size in the cholesteryl-DTPA sample. After complexation of the lipids with Eu^{3+}, formation of clusters with high heterogeneity has been observed by dynamic light scattering (unshown data). A different complexation DTPA:Eu due to the conformation strength of the cholesterol over the monocatenar lipid might occur.

Stoechiometry between the lipid-DTPA and Eu(III)

The ability of the two DTPA lipids to chelate europium has been monitored by fluorescence spectroscopy. The stoichiometry of each lipid:Eu(III) complexes has been determined by the Job method in H_2O. The mixture lipid:Eu revealed a difference of interaction between the DTPA moiety and the europium depending on the nature of the lipid linked to the DTPA (Fig. 2). The highest TRF signal was obtained for a mixture L_3Eu_2 and L_3Eu for the octadecyl-DTPA and the cholesteryl-DTPA, respectively. The micellar aggregation of the lipids might induce the formation of bridges between the DTPA of different lipids and europium, leading to different possible stoechiometry between the lipid and the ion, probably allowing different amount of H_2O molecules to interact with the complex.

Incorporation of the labeled DTPA lipid into liposomes

Different amounts of labeled DTPA lipid were incorporated into the preparation of conventional liposomes constituted of egg phosphatidylcholine, cholesterol and phosphatidylethanolamine-poly(ethyleneglycol)$_{75}$ (EPC/Chol/PE-PEG$_{45}$). The octadecyl-DTPA:Eu and the cholesteryl-DTPA:Eu were incorporated during the film formation in the initial mixed lipid solution in $CHCl_3$. After hydration, the liposomes were extruded to lead to multilamellar vesicles.

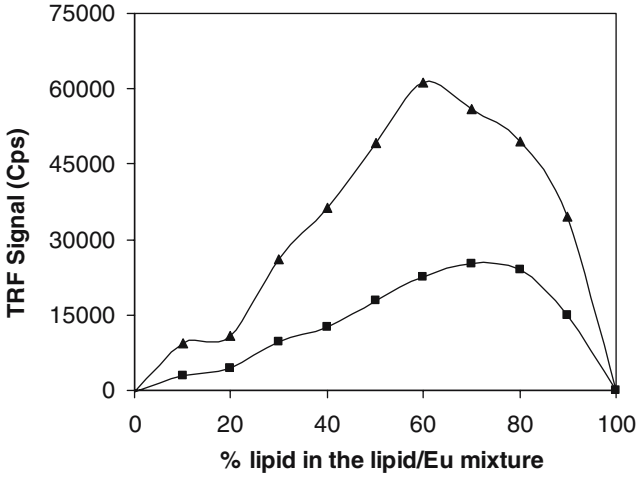


Fig. 2 A job diagram obtained by time resolved fluorimetry. *Triangles* relate to the octadecyl-DTPA:Eu lipid at a concentration of 500 µM, *squares* represent the cholesteryl-DTPA:Eu lipid at a concentration of 400 µM. Signal from the $EuCl_3$ was removed

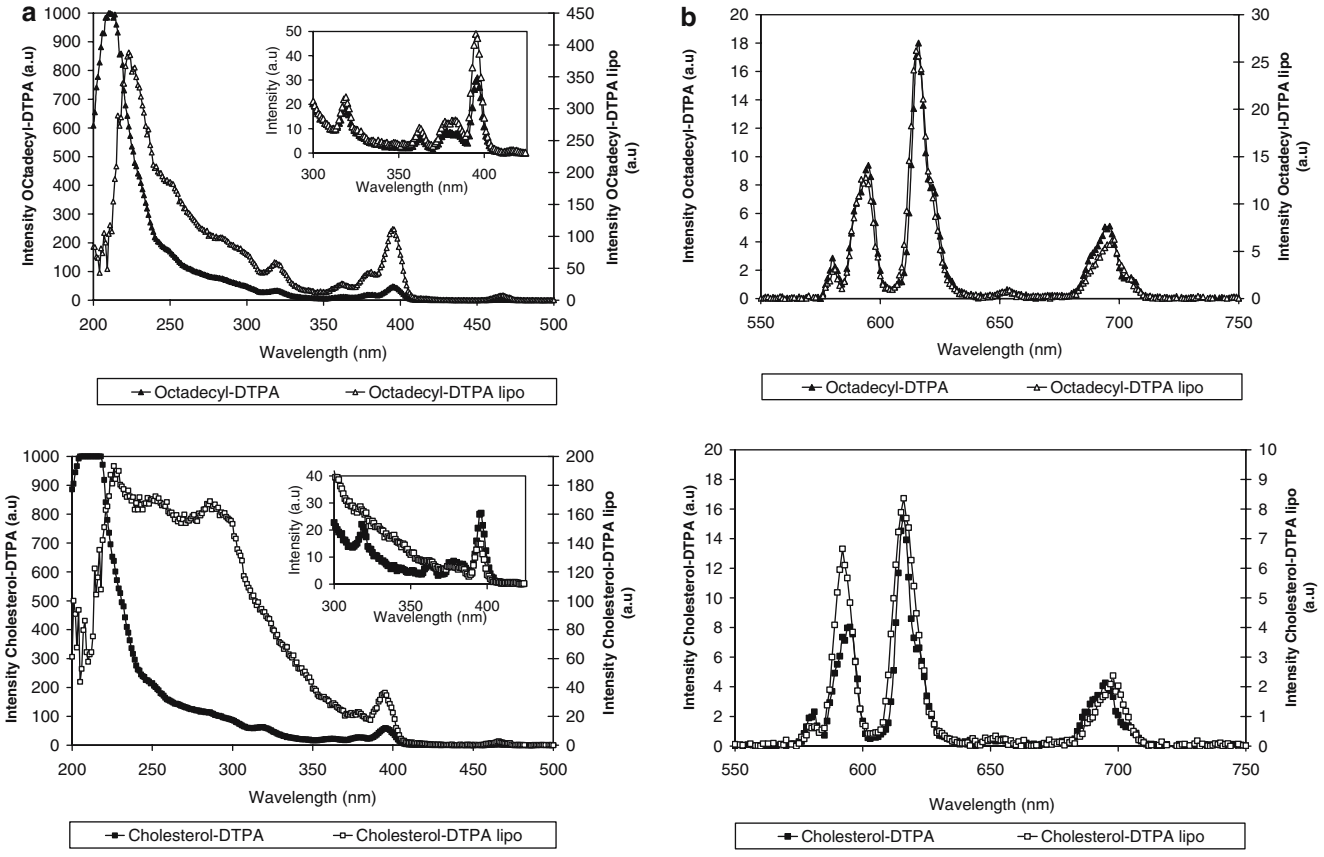


Fig. 3 Excitation (**a**) and emission (**b**) spectra of the octadecyl-DTPA:Eu (*triangles*) and cholesteryl-DTPA:Eu (*squares*) incorporated or not incorporated into liposomes. *Open Symbols* represent free lipids, and *solid symbols* represent lipids incorporated into liposomes. The *left axis* represents the free lipids intensity, the *right axis* represents the liposome intensity

Similar excitation and absorption profiles were previously reported for polymerizable chelidamic lipids (Roy et al. 2003). In our case (Fig. 3a), the main peak at 210 nm was slightly shifted and became broader when the lipids were included into the liposomes (open triangles and squares, right axis), when compared with the lipids themselves (black triangles and squares, left axis) (Fig. 3). Octadecyl-DTPA:Eu and cholesteryl-DTPA:Eu gave a similar excitation profile with a peak around 210 nm, which was diminished in both cases when inserted into liposomes. This decrease was more important in the case of the cholesteryl-DTPA:Eu (More than a factor 5 when compared to a factor of 2.5 for the octadecyl-DTPA:Eu). This nonradiative desexcitation, which was more intense in the case of the cholesteryl-DTPA, might be caused by the formation of less structured liposomes in this case. Less organized lipid bilayers are probably formed by insertion of the cholesteryl-DTPA as compared to the octadecyl-DTPA insertion. This different lipid organisation might lead to different environment of the DTPA:Eu moiety, inducing a much broader excitation spectrum between 220 and 350 nm in the case of the cholesteryl-DTPA.

The fluorescence emission spectra of the labeled lipids prior to and after insertion in liposomes are presented in Fig. 3b. The luminescent bands obtained are characteristic of Eu^{3+} and correspond to the different transition from $^5D_0$ to ^7F (Parker et al. 2002). The main peaks result from the transitions $^5D_0 \rightarrow {}^7F_1$(594 nm) and $^5D_0 \rightarrow {}^7F_2$ (616 nm). As expected, no changes in the characteristic bands were noted. On the contrary, the emission intensity level relates to the environment change of the DTPA:Eu moiety prior to and after liposome insertion. A signal increase was measured after octadecyl-DTPA insertion, while a signal decrease was measured after cholesteryl-DTPA insertion. These changes evidence the different stoichiometry changes due to lipid insertion into liposome bilayers.

TRF Signals in biological media

Labeled liposomes were submitted to TRF measurements in biological samples, in particular tissue homogenates, to evaluate their potential use in these systems. Experiments in organs were straightforward since the tissues were homogenated in detergent and submitted to measurements as described in the Experimental section. The difference obtained for the octadecyl-DTPA:Eu labeled liposome when compared with cholesteryl-DTPA:Eu labeled liposome might come from the fact that excitation wavelength used for these TRF mea-

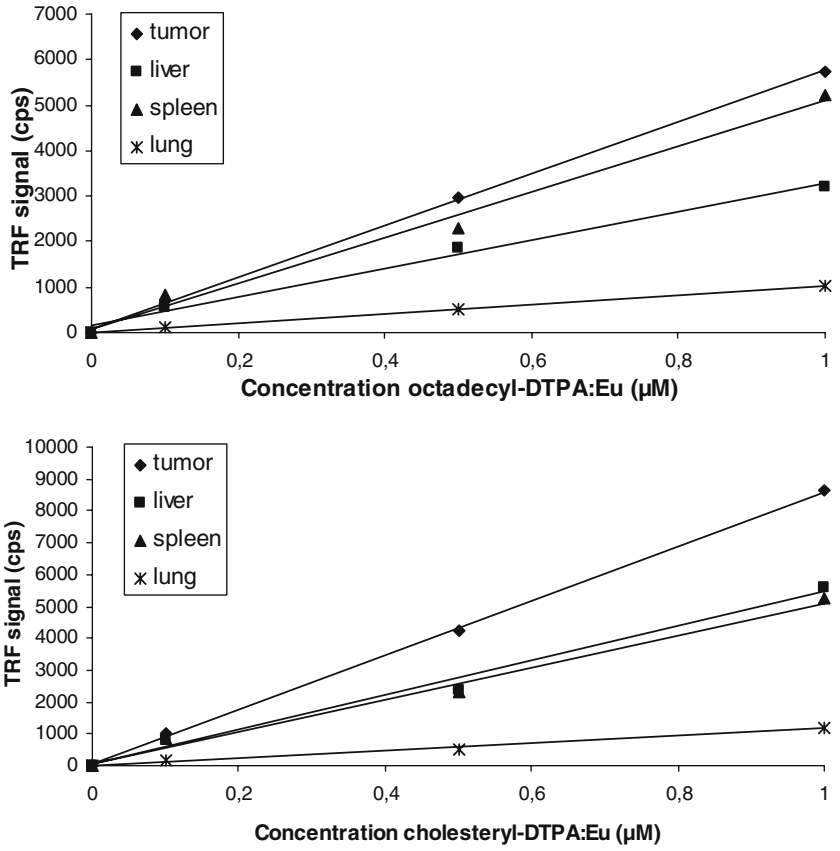


Fig. 4 Calibration curves of the europium labeled liposomes in different tissue homogenates

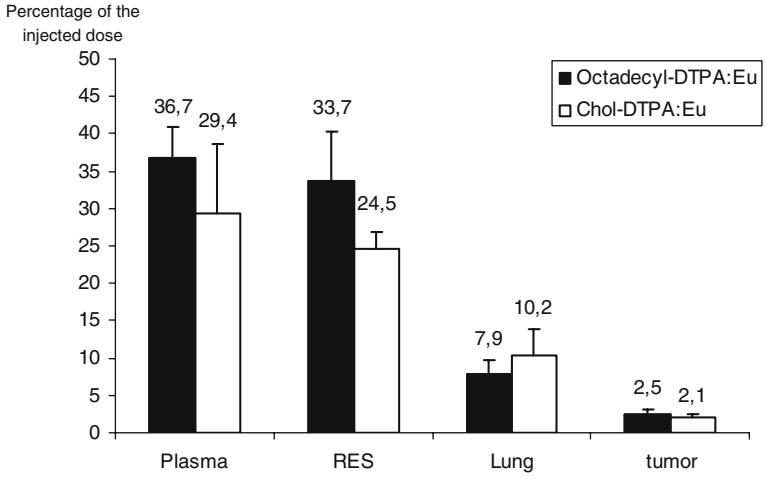


Fig. 5 Biodistribution of the europium labeled liposomes in mice. The results shown represent a calculated mean from three animals

surements (320 nm) was not optimized for both lipids. However, the calibration curves obtained show a good linearity in the concentration range needed for biodistribution studies (Fig. 4).

In vivo application

Liposomes labeled with the octadecyl-DTPA:Eu and cholesteryl-DTPA:Eu were injected into C57Bl6/6J mice, bearing 3LL tumors. One hour following injection, we found 36.7 and 29.4% of the injected dose in the plasma for the octadecyl-DTPA bearing liposomes and the cholesteryl-DTPA bearing liposomes, respectively (Fig. 5). These data correlate with what was previously reported in the literature for a similar liposome isotopically labeled (40% recovery in the plasma) (Parr et al. 1994). Indeed, as the excitation band used was 320 nm in this experiment, which does not correspond to a maximum in the excitation spectrum, values might be under estimated. From both type of liposomes, the octadecyl-DTPA:Eu labeled one allowed the highest recovery, which tends to suggest that a part of cholesteryl label might be lost. It is quite surprising since the commonly

used non exchangeable radioactive tracer ^{3}H label is cholesteryl based. The difference obtained between both europium labeled liposomes could also relate to a weaker stability of the liposome due to more cholesterol insertion. It is noteworthy that the protocol was relatively simple since plasma was obtained by centrifugation of the collected blood and submitted immediately to measurements without further treatment.

Discussion

In our quest of a new lipid that would not be exchangeable for the in vivo detection of liposome distribution, we evaluated octadecyl-DTPA:Eu and cholesteryl-DTPA:Eu as new possible liposome tracers. Despite their structural organization difference in aqueous media, we could efficiently label the DTPA bearing lipids with europium and insert these adducts into liposomes as expected (Marchi-Artzner et al. 2003; Moulin et al. 2003; Roy et al. 2003). Interestingly enough, the differences in TRF signal obtained for the two lipids changed when incorporated into liposomes. Despite a similar DTPA chelating moiety, the nature and positioning of the lipid anchor into the bilayer leads to different energy transfer to Eu^{3+}.

Our objective was to validate the use of europium adducts for liposomes or drug pharmacokinetics studies. We showed that these adducts might be very useful in this context. Comparison with the fluorescence measurements that we commonly used for liposome detection (Nicolazzi et al. 2003) is in favor of europium adducts in terms of protocol simplification. Indeed, measurements were directly performed in biological media, or in the tissue homogenates, and no extraction with organic solvents was required.

Sensitivity was not issued in this preliminary study but ways of improvement are open to increase the sensitivity as numerous studies aim at increasing the detection threshold (Carlos et al. 2003). Energy transfer to europium with more appropriate chelating systems might be coupled to an octadecyl lipid to apply more efficiently our technique. Small chelating moieties should be more appropriate to perfectly fit in the membrane without altering the structure of the liposome.

It also opens ways to investigate simultaneously drug and carrier pharmacokinetic studies thanks to other available lanthanides. One can imagine labeling the liposomes and the drug of interest with two different lanthanides (for instance europium and terbium), to be able to follow both products directly in biological media. The organic solvent extraction allows solely the detection of hydrophobic compounds, the hydrophilic ones being lost in the tissue homogenates. Direct observation of lanthanide labeled entities eliminates this gap. Today, only radioactive detection allows working directly in biological media with high sensitivity. The drawbacks involved, such as safety cautions and waste processing, certainly show the need for alternative solutions. Again, lanthanide labeling appears to be such an alternative.

References

Accardo A, Tesauro D, Rocsigno P, Gianolio E, Paduano L, D'Errico G, Pedone C, Morelli G (2004) Physicochemical properties of mixed micellar aggregates containing CCK peptides and Gd complexes designed as tumor specific contrast agents in MRI. J Am Chem Soc 126:3097–3107

Anelli P, Fedeli F, Gazzotti O, Lattuada L, Lux G, Rebasti F (1999) L-glutamic acid and L -lysine as useful building blocks for the preparation of bifunctional DTPA-like ligands. Bioconjugate Chem 10:137–140

Carlos L, De mello donega C, Albuqerque R, Alvers S, Menezes J, Malta O (2003) Highly luminescent europium(III) complexes with naphtoyltrifluoroacetone and dimethyl sulphoxide. Mol Phys 101:1037–1045

Harasym T, Cullis P, Bally M (1997) Intratumor distribution of doxorubicin following i.v. administration of drug encapsulated in egg phosphatidylcholine/cholesterol liposomes. Cancer Chem Pharmacol 40:309–317

Lattuada L, Lux G (2003) Synthesis of Gd-DTPA-cholesterol: a new lipophilic gadolinium complex as a potential MRI contrast agent. Tetrahedron Lett 44:3893–3895

Laukkanen M-L, Orellana A, Keinänen K (1995) Use of genetically engineereed lipid-tagged antibody to generate functional europium chelate-loaded liposomes. Application in fluoroimmunoassay. J Immunol Methods 185:95–102

Marchi-Artzner V, Brienne MJ, Gulik-Krzywicki T, Dedieu JC, Lehn JM (2003) Selective complexation and transport of europium ions at the interface of vesicles. Chem Eur J 10:2342–2350

Moulin C, Amekraz B, Steiner V, Plancque G, Ansoborlo E (2003) Speciation Studies on DTPA Using the complementary nature of electrospray ionization mass spectrometry and time-resolved laser-induced fluorescence. Appl Spectrosc 57:1151–1161

Neville M, Richau K, Boni L, Pflug L, Robb R, Popescu M (2000) A comparison of biodistribution of liposomal and soluble IL-2 by a new method based on time-resolved fluorimetry of europium. Cytokine 12:1702–1711

Nicolazzi C, Mignet N, de la Figuera N, Cadet M, Torero Ibad R, Seguin J, Scherman D, Bessodes M (2003) Anionic polyethyleneglycol lipids added to cationic lipoplexes increase their plasmatic circulation time. J Control Release 88:429–443

Parker D, Dickins R, Puschmann H, Crossland C, Howard J (2002) Being excited by lanthanide coordination complexes: aqua species, chirality, excited-state chemistry, and exchange dynamics. Chem Rev 102:2389–2403

Parr M, Ansell SM, Choi LS, Cullis PR (1994) Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles. Biochim Biophys Acta 1195:21–30

Roy B, Santos M, Mallik S, Campiglia A (2003) Synthesis of metal-chelating lipids to sensitize lanthanide Ions. J Org Chem 68:3999–4007

Soini E, Kojoia H (1983) Time-resolved fluorimeter for lanthanide chelates—a new generation of nonisotopic assays. Clin Chem 39:65–68

Takeuchi H, Kojima H, Yamamoto H, Kawashima Y (2001) Evaluation of circulation profiles of liposomes coated with hydrophilic polymers having different molecular weights in rats. J Contr Rel 75:83–91