Liposomes Containing Nickel-Bis(dithiolene) Complexes for Photothermal Theranostics

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Abstract.

New thermosensitive liposomes with a phase transition at 42 °C, containing nickel-bis(dithiolene) complexes as efficient and stable photothermal agents, have been formulated and characterized. These liposomes are highly stable and keep their contents at 37 °C for more than 30 days. On the contrary, the mild hyperthermia generated by the nickel-bis(dithiolene) complex under 940 nm NIR irradiation allows for the fine controlled release of the liposome contents, making such liposomes highly suitable for on-demand drug delivery in human body under NIR laser irradiation. These liposomes can also be directly used, as shown here, as nano-agents for photothermal therapy. In fact, strong cell death can be generated under laser irradiation in presence of these photothermally active nanocargos containing less than 10 %w/w

of metal complex. We also demonstrate, for the first time, that nickel-bis(dithiolene) complexes are good photoacoustic agents, generating easily detectable ultrasonic signals directly proportional to the concentration of complexes and the used laser power.

Introduction.

For decades, the many advantages proposed by the vectorization of active pharmaceutical ingredients (API) have led to the development of a great number of innovative strategies involving advanced nanoscale systems.[1,2] However, achieving drug release at a specific target site with high spatio-temporal control and good biocompatibility is still challenging.[2,3]

Since their introduction in the late 1970s, stimuli-responsive nanocargos seems to show promising perspectives.[4] Many kinds of stimuli have been envisaged in order to trigger drug release from the cargo. Henceforth, various nanosystems have been successfully designed to be sensitive to either endogenous factors such as enzymatic reactions and pH, or exogenous physical stimuli such as ultrasound, magnetic field or light.[5,6] Among them, the thermal stimulus has been deeply investigated as it can initiate a variety of mechanisms leading to drug release.[5] As an example, poly(N-isopropylacrylamide) (PNIPAM) based nanoparticles (NP) had been extensively studied for their ability to shrink because of polymer dehydration occurring when the surrounding temperature goes above its lower critical solution temperature (LCST).[7,8] Lipid vesicles can also be engineered in order to confer them sensitivity to hyperthermia.[9] Hence, the opening of leucine-zipper peptide embed in liposomes membrane can increase the proportion of drug released when submitted to elevated temperature.[10] Membrane permeability can also be improved by using decomposition of sodium bicarbonate at 42 °C to create nanobubbles inside the liposome, creating small depletions nearby the membrane.[11] However, some recent studies demonstrate that repeated exposition to sodium bicarbonate may result in undesired effects due to pH changes in blood.[12]

Owing to its non-invasiveness and high spatio-temporal control, the use of a laser as a light source to irradiate photothermal agents (PTA) in order to induce hyperthermia seems to be a judicious choice.[13] In addition, the induction of localized hyperthermia thanks to photothermal effect can easily be involved in photothermal therapy, by creating a detrimental environment for living cells. Very recently, localized chemotherapy combined with photothermal therapy have given promising results when considering treatment of tumors in mice.[14] As a consequence, several systems have been developed, most of them using graphene oxide or gold nanostructures as PTAs [14,15]. Unfortunately, inorganic structures suffer from major drawbacks such as poor renal clearance and low thermal stability.[15,16,17] Their nanometric size may also prevent high loading in nanoparticles.

Recently, a new class of photothermal agents has been explored to tackle the drawbacks of inorganic nanoparticles. Metal-bis(dithiolene) (MBD) complexes are discrete molecular entities displaying strong photothermal activity under Near Infra-Red (NIR) irradiation in solid–state, in gel and in solution.[18] These complexes, in addition to being small molecules, have already proven their great ability to be used as PTA under NIR irradiation with conversion of light energy into heat as high as 40 % with high stability.[19] In addition, proper functionalization of the complexes has allowed good solubility in water and more importantly, *in cellulo* experiments have demonstrated that NIR-laser irradiation of cultured cells incubated with metal-bis(dithiolene) complexes can induce cell death.[20] More recently, nickel-bis(dithiolene) complexes have been incorporated into biocompatible polymer nanoparticles.[21] It has also been demonstrated that NIR irradiation allows the fine control of fluorophores or drugs release from these nanostructures in solution. In addition, these studies have also shown that nickel-bis(dithiolene) complexes do not produce singlet oxygen under NIR irradiation and that the incorporation of MBD complexes do not increase the cytotoxicity of the organic NPs.

Among others, liposomes have proven being especially efficient and simple vehicles to be used for drug delivery, owing to their good stability and biocompatibility.[22] As showed in Table **1**, those merits led to the development of many types of liposome-based products explaining their major presence on the current pharmaceutical market compared to other types of nanoparticles.

Table 1. Commercially approved nanopharmaceutical for drug delivery. Details on the different products are provided in the given references.

Drug delivery nanosystem category	Number of commercial products	Refs
Liposome/lipid-based NPs	21	23 - 27
Metal-based NPs	6	23, 24
Protein-based NPs	3	23 - 25
Polymer-based NPs	16	24 - 26
Nanoemulsions	5	23, 26
Nanocrystals	11	24, 25, 27

In the field of bioimaging, photoacoustic imaging is a promising medical imaging technique that combines a rich optical contrast and scalable high ultrasonic resolution in a single modality.[28,29,30] The injection of specific photoacoustic/photothermal agents, as dye or gold nanoparticles, has demonstrated the enhanced specificity to image a selected target based on its photoacoustic effect.[31,32,33,34] The combination of photoacoustic imaging and photothermal effect under NIR irradiation for imaging-guided and photocontrolled chemotherapy has only been recently explored but more and more reports on this innovative research topic are emerging today. For example, multifunctional pH- and thermo-sensitive copolymers, exhibiting photothermal and semiconducting properties have been synthetized and nanoprecipitated to encapsulate high quantities of doxorubicin. The polymer nanoparticles proved their ability to

be used for tumor treatment *in vitro* and in mice while allowing for the guidance of precise irradiation through photoacoustic imaging.[35] The quest for synergetic chemo and photothermal therapy also lead to the design of innovative core-shell nanostructures, made of a poly(lactic-co-glycolic acid), PLGA, polymer surrounding liquid perfluorohexane and superparamagnetic oxide. The combination of those elements inside a single nanoplatform allowed for the precise monitoring of their location *in vivo* using bimodal ultrasound/photoacoustic (US/PA) imaging thus helping with the spatiotemporal activation of paclitaxel release and localized hyperthermia leading to severe reduction of mice tumor growth[36]. Nevertheless, those multipotent polymer nanoparticles are still rare and the formulation of a biocompatible lipid version of such cargo, combining anticancer drug delivery, with photothermal therapy and bioimaging, is still challenging.

Herein, we propose to incorporate nickel-bis(dithiolene) complexes, as PTA, into thermosensitive liposomes (TSL) to develop multipotent platforms for combined controlled drug delivery, photothermal therapy and photoacoustic imaging. For this purpose, a new liposomal formulation has been developed for applications adjusted to human body temperature. Indeed, the membrane of the developed liposome can undergo a sol-gel phase transition (T_m) at 42 °C, allowing a rapid release of a model marker or anticancer drug only under NIR laser irradiation. The thermosensitive nanovectors presented here are highly stable over months and they show great ability to keep their content with a weak leakage under storage at 4 °C and under stirring at 37 °C. Nevertheless, fast release of the carboxyfluorescein fluorescent marker, as drug model, can be achieved when the temperature reaches 42 °C. Here, hyperthermia is induced under moderate NIR laser irradiation thanks to the incorporated highly efficient nickel-bis(dithiolene) photothermal agent. Moreover, the hyperthermia generated by the complex inside the liposome under NIR irradiation exhibits a high potential for photothermal therapy. Additionally, nickel-bis(dithiolene) complexes displays ultrasonic

emission when submitted to pulsed laser irradiation, proving that such nanocarriers integrating nickel-bis(dithiolene) complexes can also be used for photoacoustic imaging. The present work highlights the multiple abilities of thermosensitive liposomes containing nickel-bis(dithiolene) combined with NIR laser irradiation for an application in anticancer theranostics.

Materials and Methods.

Materials.

Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and lyso-phosphatidylcholine (LPC) were supplied by Avanti Polar Lipids (Alabaster, USA). Surfactant [poly(ethylene glycol)-2000]-amine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (PEG_{2000}-DSPE) was purchased at NOF (USA). Ultrapure water (UP water) was obtained using a Elsa purifying system. Solvents such as chloroform ($CHCl_3$) and methanol (MeOH) were provided by Carlo-Erba (FR) and Sigma-Aldrich (USA), respectively. Other solvents and compounds were purchased from Sigma-Aldrich and used without further purification. The 786-0 were cultured in RPMI-1640 (Life Technologies, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich, USA), 1% penicillin/streptomycin (Sigma-Aldrich, USA). Photothermal agent $Ni8C_{12}$ was synthesized according to the method previously described.[18,37]

Preparation of the liposomes containing nickel-bis(dithiolene) complexes.

Liposomes were prepared by the Bangham method involving the hydration of thin films made of a lipid mixture.[38] First, in volumetric flasks, 10 $mg.mL^{-1}$ solutions of DSPC, LPC and PEG_{2000}-DSPE were prepared in chloroform/methanol (2:1, v/v). In a 10 mL round-bottom flask, 10.9 mg of DPPC were weighted. Then 419 µL of DSPC solution, 250 µL of LPC solution and 295 µL of PEG_{2000}-DSPE solution were added to the round-bottom flask in order to obtain a mixture of DPPC:DSPC:LPC:PEG_{2000}-DSPE (53:21:12:14 %w/w) containing a total of 20.54

mg of lipids dissolved in a $CHCl_3$/MeOH mixture. To prepare liposomes incorporating 8.9 %w/w of $Ni8C_{12}$ complex (mass of $Ni8C_{12}$ = 10 % of the mass of lipids), 200 µL of a 10 $mg.mL^{-1}$ solution of $Ni8C_{12}$ complex in $CHCl_3$ was added to the lipid mixture. The round-bottom flask was then connected to a rotary evaporator (Heidolf, Germany) and left to equilibration at 60 °C for 15 min under vigorous stirring. Solvents were further slowly evaporated under reduced pressure in order to obtain a dry thin film. The newly obtained film was placed under vacuum for 3 hours until traces of residual solvents were evaporated. Hydration of films was performed by placing the round-bottom flasks at 60 °C in a water bath for 2 hours after addition of 2 mL of HEPES buffer (20mM HEPES, 150 mM NaCl, pH = 7.4) containing or not 50 mM of quenched carboxyfluorescein (CF). The resulting hydrated films were sonicated 3 times for 5 min with 5 min resting between two sonications, into a thermally-controlled ultrasonic bath (Transonic T460H, US) set to 60 °C.

In order to remove excess CF and $Ni8C_{12}$, purification was achieved using size exclusion chromatography on prepacked illustra NAP-5 columns (GE Healthcare, Chicago USA). The effective loading of $Ni8C_{12}$ complex was determined by UV-vis spectroscopy and was found to be 6.2 %w/w over the 8.9 %w/w initially added to the formulation. That way, various types of liposomes suspensions with a lipid concentration of 10.27 $mg.mL^{-1}$ (20.54 mg of lipids in 2 mL of HEPES buffer) named after their content could be obtained: liposome with lipids only (Lip(empty)), liposomes incorporating $Ni8C_{12}$ complex (Lip($Ni8C_{12}$)), liposomes containing carboxyfluorescein (Lip(CF)) and liposomes incorporated $Ni8C_{12}$ complex and containing carboxyfluorescein (Lip($Ni8C_{12}$+CF)). Some other complex loadings have been tested with 1.0, 4.8, 13, and 23 %w/w initially (mass of $Ni8C_{12}$ = 1, 5, 15 and 30 % of the mass of lipids) and the effective complex loading of $Ni8C_{12}$ were found to be 1.0, 3.5, 9.6 and 15.4 %w/w.

Characterization.

Dynamic light scattering (DLS) measurements were performed on a Malvern NanoZS system (Malvern, Worcestershire), immediately after the purification step. Solution containing 0.1 $mg.mL^{-1}$ of lipids in HEPES buffer were analyzed in triplicate, using backscattering angle (173 °). Liposome size and morphology were also observed using transmission electron cryo-microscopy (cryo-TEM) to keep the samples hydrated. Samples of Lip(empty), Lip($Ni8C_{12}$), and Lip($Ni8C_{12}$+CF) have been observed using a 200 kV electron microscope (Tecnai G2 T20 Sphera, FEI) equipped with a LaB6 filament and a 4k x 4k CCD camera (USC4000, Gatan). Samples were frozen by quick immersion in liquid ethane using an automatic plunge freezer (EM GP, Leica) regulated at 20 °C and 95 % humidity. Four microliters of the solutions were deposited at the surface of holey carbon coated grids (Quantifoil R 2/2), blotted for 0.8 to 1.4 sec and quickly plunged into liquid ethane. Grids were transferred to a cryo-holder (Gatan 626) and were observed at -176 °C. Lip($Ni8C_{12}$) suspensions were also studied after 5 min irradiation at 940 nm, with a power density of 5 $W.cm^{-2}$ and after been heated at 50 °C before instant freezing in liquid ethane. UV-Vis-NIR absorption spectra in solution were recorded on a Shimadzu UV3600 Plus spectrophotometer. Samples were placed in 1 cm path length quartz cuvettes. For the photothermal studies, 1 mL of liposome suspensions were irradiated through a quartz cuvette with a 940 nm-wavelength semiconductor laser (BWT Beijing LTD) for 10 min. The laser power density could be adjusted externally (0-10 W). The output power was independently calibrated using an optical power meter. A thermocouple with an accuracy of ± 0.1 °C connected to an Agilent U1253B multimeter was immersed into the solution. The thermocouple was inserted at such a position that direct irradiation from the laser was avoided. The temperature was measured every 1s. Differential scanning calorimetry (DSC) analyses have been performed using a NETZSCH DSC 200 F3 instrument equipped with an intracooler. To do so, 200 µL of liposome suspension have been concentrated by simple evaporation under air flow for 1 hour. Then, 40 µL of the concentrated solution were introduced into a sealed

aluminum crucible and submitted to 3 cycles of temperature ramps ranging from 10 °C to 70 °C at 10 K.min^{-1}. 40 µL of HEPES buffer were added in the reference crucible. The pure phospholipids were analyzed by submitting a crucible containing 40 µL of a 50 mg.mL^{-1} solution of each phospholipid in HEPES buffer to the same temperature program.

Evaluation of stability of the liposomes at 4 °C and 37 °C.

Purified suspensions of Lip(empty), Lip(Ni8C$_{12}$), and Lip(Ni8C$_{12}$+CF) have been stored either in the fridge at 4 °C without stirring or in an incubator at 37 °C for 3 months under gentle stirring at a concentration of 1 mg.mL^{-1}. At selected time points, aliquots of each sample were submitted to triplicate DLS measurements after proper dilution to 0.1 mg.mL^{-1}.

Evaluation of CF leakage in storage conditions at 4 °C and at 37 °C.

The amount of carboxyfluorescein passively released from the liposomes stored in the fridge at 4 °C has been quantified over months (CFR4°C). To proceed, a freshly purified liposome suspension in HEPES buffer with a lipid concentration of 1 mg.mL^{-1} was stored at 4 °C for 3 months. At selected time points (x), 50 µL of this solution was diluted in 2 mL HEPES buffer and the fluorescence intensity was measured at 25 °C (Ix). Then, 5 µL of a 10% triton X100 in water were added to the cell in order to destroy the liposomes and the fluorescence intensity was measured again (Ixmax). At t=0, the amount of detected fluorescence which is attributable to the fraction of unquenched fluorescence was calculated as follow (1):

$$CFR0 = (I_0/I_0max)x100 \qquad (1)$$

For every other time point, the same process was repeated in triplicate. The CF release percentage was then normalized compared to values at t = 0 and calculated as being:

$$CFR4°Cx = Ix - (I0*Ixmax/I0max)/Ixmax - (I0*Ixmax/I0max) \qquad (2)$$

Similar experiments have been conducted on liposome suspensions stored at 37 °C under gentle stirring (CFR37°C). The sample preparation and calculation methods were identical to that at 4 °C.

NIR light-controlled release of CF.

Release profiles under different irradiation conditions were evaluated using continuous laser at 940 nm plugged into a fluorimeter (HORIBA Jobin Yvon) bearing a temperature-controlled cell-holder set to 37 °C. For kinetic studies, excitation wavelength was set to 450 nm and emission detected at 518 nm. Temperature was recorded using a thermocouple placed at the upper part in the cell content and connected to a multimeter (Agilent, Santa Clara). To ensure the homogeneity in temperature and fluorescence inside the cuvette, a magnetic bar was added for vigorous stirring. Release experiments have been performed in HEPES buffer and in DMEM+10 % FBS (Fetal Bovine Serum).

Cell culture.

The 786-O renal cells were routinely cultured in RPMI supplemented with 10 % FBS, 100 $\mu g.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin at 37 °C in a humidified atmosphere under 5 % CO_2.

In vitro cytotoxicity of the liposomes.

The 786-O renal cells were incubated for 24h in presence of various amounts of liposome suspensions in sterile 96 wells plates (MicortestTM-96-Becton Dickinson). The cytotoxicity of liposomes was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The absorbance at 570 nm was measured using a BMG Labtech FLUOstar Optima plate reader. To evaluate the potential of the nickel-bis(dithiolene) containing

liposomes for photothermal therapy, cell cultures incubated with 75 and 150 µg.mL^{-1} of Lip(empty) and Lip(Ni8C$_{12}$) suspensions were irradiated, after 24 h incubation, at 940 nm for 5 min with a laser power of 5 W.cm^{-2} with or without washing of cells with phosphate buffer saline (PBS) before addition of fresh biological media. Experiments were performed in triplicate.

In vitro confocal fluorescence microscopy

Images of 786-O renal cells were acquired on SP5 confocal microscope with a 40× objective (40x/1.25-0.75 HC PL APO; Leica). Images were acquired with the LAS AF software and assembled for illustration using ImageJ software. Cells were incubated with Lip(CF) and Lip(Ni8C$_{12}$+CF) for 24 h, at a concentration of 150 µg.mL^{-1}. Each culture was then observed before being irradiated 5 min at 5 W.cm^{-2}, with or without washing of the surrounding media with PBS. Quantifications of particle number, areas and fluorescence intensities were performed using ImageJ software.

Photoacoustic experiments.

To perform the photoacoustic imaging, a Nd:YAG pulsed laser (Quanta-Ray, INDI Series, Spectra-Physics, USA) with a pulse duration of 6 ns, a repetition rate of 10 Hz and a wavelength of 1064 nm has been used. The acoustic detection was conducted using the 256-channel open research scanner ULA-OP 256 (University of Florence) and a 7 MHz linear ultrasound array (LA523E, Esaote, Florence, Italy). Measurements have been performed by placing 500 µL of each appropriate suspension of purified liposomes in a size-calibrated inclusion of 4 % agar gel. Each measurement was performed in ultra-pure water, HEPES buffer at pH 7.4 (HEPES 20 mM + NaCl 150 mM) and in DMEM + 10% FBS. The agar phantoms have been irradiated with an increasing laser energy in the range 0-200 mJ/pulse. For each energy level, 20

consecutive photoacoustic acquisitions have been averaged. An acquisition without any laser excitation was recorded to evaluate the residual noise of the acquisition system.

Results and discussion

The liposomes with or without incorporated nickel-bis(dithiolene) complexes were prepared by the Bangham method involving the hydration of thin film of lipids obtained by evaporation for chloroform solution of dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC).[38] When needed, the nickel-bis(dithiolene) PTA agent carrying eight C_{12} carbon chains ($Ni8C_{12}$) was directly introduced inside the lipid films and these lipid films were then hydrated with pure water or aqueous solutions of carboxyfluorescein (CF). This way, various liposomal formulations were obtained: liposome with only lipids (Lip(empty)), liposomes incorporated $Ni8C_{12}$ complex (Lip($Ni8C_{12}$)), liposomes containing carboxyfluorescein (Lip(CF)) and liposomes incorporated $Ni8C_{12}$ complex and containing carboxyfluorescein (Lip($Ni8C_{12}$+CF)). After hydration and sonication, the liposomes were purified by using size exclusion chromatography.

The hydrodynamic diameter and polydispersity index (PDI) for each formulation are listed in Table 2.

Table 2. Comparison of the sizes of each type of liposomes. Measurements have been performed by DLS, on 3 different as-prepared batches for each type of liposomes to assess the reproducibility of the formulation method.

Type of liposome	Hydrodynamic diameter (nm)	PDI
Lip(empty)	79 ± 2.6	0.299 ± 0.023
Lip($Ni8C_{12}$)	128 ± 9.4	0.321 ± 0.056
Lip(CF)	91 ± 1.2	0.399 ± 0.026

Lip(Ni8C_{12}+CF)	151 ± 7.9	0.396 ± 0.035

The intercalation of the nickel-bis(dithiolene) complex inside the lipid bilayer strongly increases the size of the liposomes from 80 to 130 nm. The liposome size increase with the amount of incorporated Ni8C_{12} complexes. For an effective loading of 3.5 %w/w, the liposome size is around 126 nm, 128 nm for 6.2 %w/w, 185 nm for 9.6 %w/w and 199 nm for 15.4 %w/w. The incorporation of CF in the liquid core of the liposome only leads to a slight size increase by 10-20 nm in diameter. The use of ultrasonic bath often leads to high polydispersity as highlighted by elevated PDI.[39, 40] These observations are probably due to the formation of lipid disc-like micelles in parallel of liposomes, which is common when using saturated phospholipids, especially after temperature cycles above the transition temperature of crystalline phase.[41,42] However, it is well known that DLS is poorly adapted for size measurements on non-spherical nanoparticles. The formation of such structures was observed by cryo-TEM (*vide infra*). However, using such sonication method, compared to sonic probes allows for the production of suspensions free of titanium particles.[43] Filtration steps could be added at the end of the process to reduce PDI and ensure sterilization of the formulation, but would lead to the loss of a large part of the lipid material. Anyway, this method allows to obtain reproducible formulations of liposomes with sizes suitable for taking advantage of the enhanced permeability and retention (EPR) effect while allowing long circulating time after administration.[44,45]

Thermal properties of the various liposomes were investigated by Differential Scanning Calorimetry (DSC). The transition temperature (Tm) reported for DPPC in water is 41 °C.[46] This reversible transition can easily be observed using polarized optical microscopy (POM) (see Figure S1) and was also confirmed by DSC measurements (Figure S2). The presence of

saturated fatty acids carried by DPPC results into the formation of organized and rigid crystalline domains at ambient temperature.[47] When reaching the sol-gel transition temperature, fluidity between chains increases explaining the apparition of disorganized amorphous material. Surprisingly, based on our release experiments and on the literature,[48] it is found that releases occur at lower temperature around 38 °C with pure DPPC liposomes. Thus, to increase the phase transition temperature, DPPC was mixed with DSPC having a thermal transition at 55 °C.[46] This way, liposomes with a transition temperature starting at 42 °C (Figure 1, Lip(empty)) in HEPES buffer have been designed. Moreover, the introduction of the CF in the liquid core of the liposome does not affect the reversible phase transition of the lipid bilayer which remains at 42 °C (Figure S2, Lip(CF)). Introduction of up to 10 %w/w of nickel complex and incorporation of CF in the liquid core of the liposomes weakly affect the phase transition of the lipid bilayer which shifted to 42.7 and 42.8 °C for Lip(Ni8C$_{12}$) and Lip(Ni8C$_{12}$+CF), respectively (Figure 1).

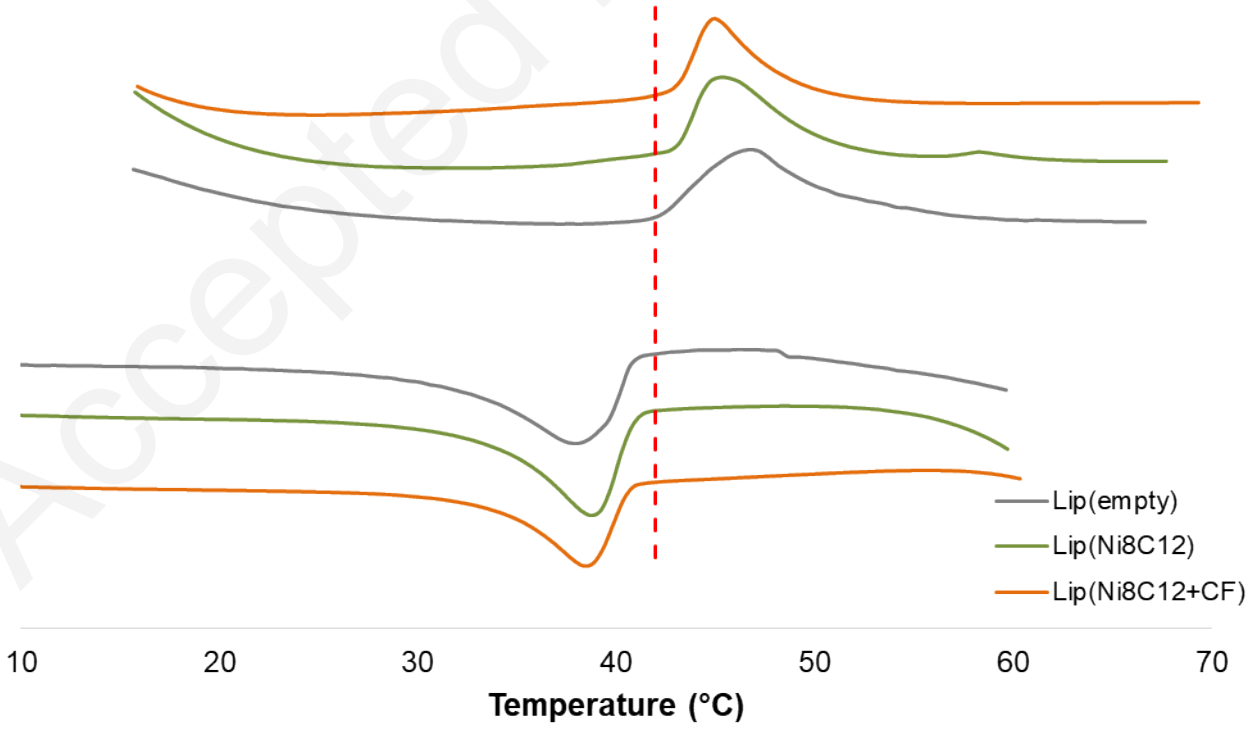

Figure 1. DSC traces for Lip(empty), Lip(Ni8C_{12}), Lip(Ni8C_{12}+CF) in HEPES buffer (top: heating curves, bottom: cooling curves, the vertical dotted line is placed at 42 °C).

The morphology of the liposomes containing the PTA agent in HEPES buffer below and above the phase transition at 42 °C was explored thanks to cryo-TEM experiments. As showed in Figure 2a, small unilamellar vesicles (SUV) with a mean diameter around 100 nm have been successfully designed in HEPES buffer at room temperature. The lipid bilayer, 4 nm wide, is clearly visible. Interestingly, Lip(Ni8C_{12}) liposomes samples which have been cryogenized from the sample prepared at a temperature of 25 °C are not spherical but display a polygon-shape. This specific morphology is likely attributed to the tendency of saturated phospholipids to form rigid lamellar homeodomains into the lipid bilayer.[49] Interestingly, the size and the morphology of the Lip(Ni8C_{12}) is not affected after 5 min irradiation at 940 nm with a laser power of 5 W.cm^{-2} (Figure 2b). Upon introduction of CF, the liposomes keep the polygon-shape but larger sizes are observed, confirming the DLS results (Figure 2c). It should be noted, as already mentioned, that some non-spherical nanoparticles are also formed (Figure S3). Above the transition temperature of 42 °C, the liposomes adopt a spherical shape, attesting the increase of mobility of the lipids constituting the bilayer (Figure 2d). As commonly described, the observed size is slightly lower than the hydrodynamic diameter obtained with DLS measurement, as the PEG crown and the hydration layer around the membrane are not visible by cryo-TEM analysis.[50,51]

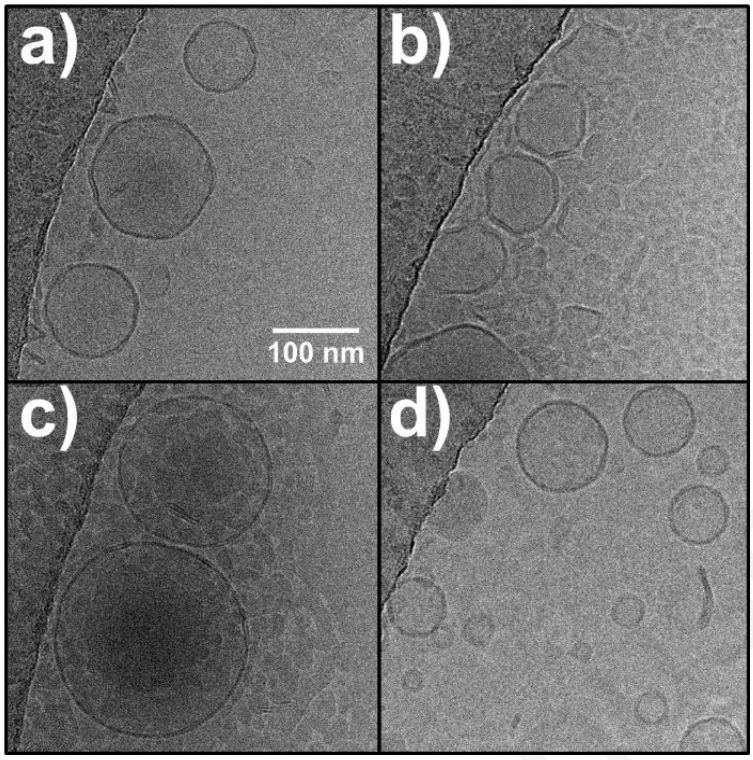

Figure 2. a) Lip(Ni8C_{12}) cryogenized in liquid ethane from sample prepared at 25 °C b) Lip(Ni8C_{12}) after 5 min irradiation, 5 W.cm^{-2}, 940 nm c) Lip(Ni8C_{12} + CF) cryogenized in liquid ethane from sample prepared at 25 °C and d) Lip(Ni8C_{12}) cryogenized in liquid ethane from sample prepared at 50 °C

Studies of the evolution in size and in dispersity have also been carried out while incubating the samples at 37 °C for one month in HEPES buffer. As shown in Figure 3a and Figure S4a, a slight increase in size and in PDI is observed for Lip(CF) and Lip(Ni8C_{12}+CF) during the first 24 h of incubation. In addition for Lip(CF), a gel-like phase at the bottom of the tube starts to appear after 1 week. As reported, long-time storage, particularly at elevated temperature, may lead to a loss of liposome stability translated by a structure modification from polygon-shape to disk-like shape, consequence of phospholipid hydrolysis.[52] Moreover, hydrolysis of the DSPE unit coupled to PEG_{2000} used in the formulation is known to occur quickly at elevated temperature, resulting in a loss of its efficiency as a surfactant, accelerating Ostwald ripening phenomena.[53,54] On the contrary, Lip(Ni8C_{12}+CF) remains completely stable over 1 month and

no formation of a gel-like phase has been detected. Thus, interestingly, the incorporation of the PTA agent seems to stabilize the liposomes at 37 °C. This is in good agreement with the observation made by Bothun for liposomes containing Ag nanoparticles inside the bilayer.[40] It appears that the presence of nickel complex inside the bilayer participates in the stabilization of the membrane when the temperature approaches Tm.

When stored at 4 °C, good stability of Lip(CF) suspensions was observed over 3 months, in terms of size (Figure 3 a) and dispersity (Figure S2a) and no gel-like precipitate was observed at the bottom of the tube. On contrary, for Lip(Ni8C_{12}+CF) suspensions, a slight decrease of both parameters (Figure 3b and Figure S4b) was observed together with the appearance of a small amount of precipitate of Ni8C_{12} at the bottom of the tube after 3 weeks. We assume that this is due to the slow desorption of dithiolene complex embed inside the lipid bilayer after long-time storage at low temperature, allowing for compaction of the crystalline structure of lipids in a more favorable state, thus reducing the size of the liposomes.

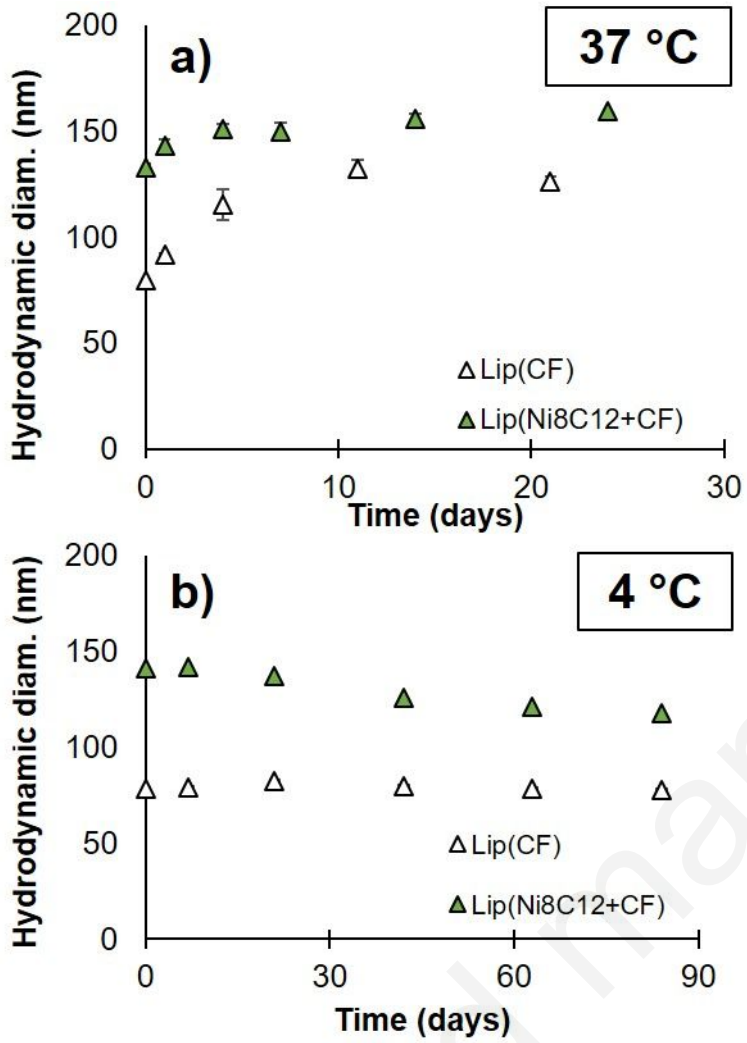

Figure 3. Evolution of the hydrodynamic diameter of Lip(CF), Lip(Ni8C_{12}), Lip(Ni8C_{12}+CF) incubated a) at 37 °C for 1 month or b) at 4 °C for 3 months (standard deviations have been calculated from three measurements performed on three dilution) (if not visible, the standard deviation is lower than the size of the symbol).

The passive leakage of the encapsulated CF from the liposomes stored at 4 °C and at 37 °C has also been evaluated. CF was used in order to mimic the behavior of an active pharmaceutical ingredient (API). When highly concentrated inside the liposome, the CF tends to form non-fluorescent dimers able to trap the energy of the free molecules upon collisions.[55] This results in poor fluorescence detection when the dye is highly concentrated in the internal media of

liposomes. However, when released from the liposomes, CF flows out to the diluted external media, leading to an increase of the fluorescence.

Minimal leakage over time in storage conditions is a pre-requisite to ensure long-term conservation of the pharmaceutical preparation. The amount of CF released from Lip(Ni8C_{12}+CF) formulation stored in the fridge at 4 °C have been evaluated over 3 months, with a diluted suspension of liposomes (Figure 4). Even after 3 months at 4 °C, it appears that less than 7% of the encapsulated fluorophore was released in the external media, highlighting the good stability of the formulation when kept in the fridge for long-term storage. When incubated for a month at 37 °C, sample of Lip(CF) also exhibit good ability to keep their content as only 10 % of CF were passively released from the vesicles over a month. (Figure 4). It is known that the crystalline structures forming the phospholipid bilayer are permeable to water but weakly permeable to charged or larger molecules, resulting in good retention of the liposome inner content.[22,38,43,56] With Lip(Ni8C_{12}+CF), when nickel complex is added to the formula, 30 % of CF is passively released after 24 days incubation. This higher release rate is probably due to the complex entrapped in the bilayer which prevents from a perfect organization of lipids into a crystalline hermetical structure, allowing for the diffusion of encapsulated molecules through the membrane. This passive release is though still acceptable for target *in vivo* application as only approximately 15 % of loaded molecule is released during the first week of incubation at 37 °C from Lip(Ni8C_{12}+CF).

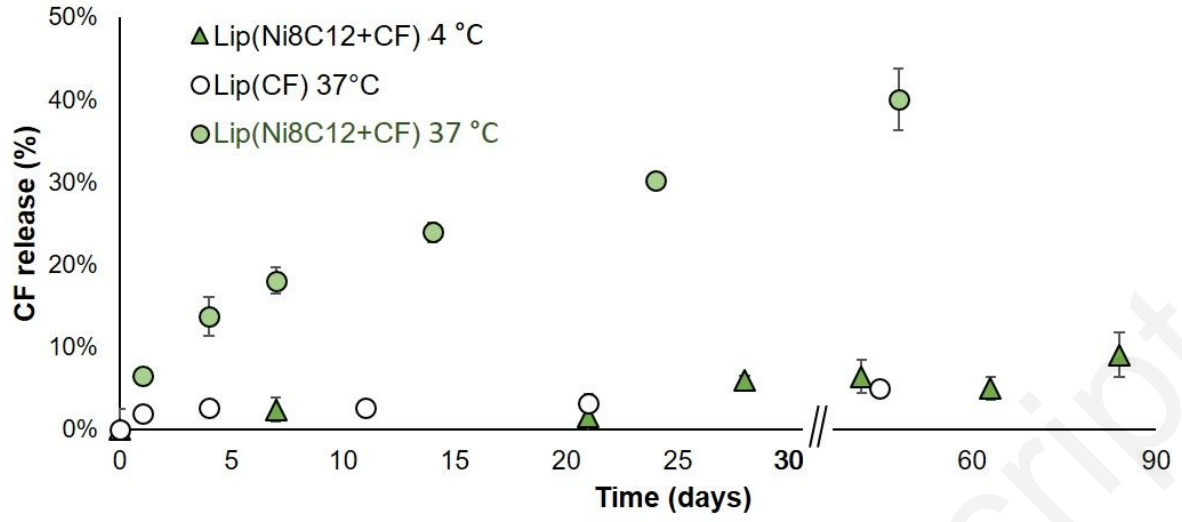

Figure 4. CF release over 3 months from a 1 mg.mL^{-1} suspension from Lip(Ni8C$_{12}$+CF) in HEPES (20 mM + 150 mM NaCl) stored at 4 °C. CF release from Lip(CF) and Lip(Ni8C$_{12}$+CF) suspensions at 2 mg.mL^{-1} stored in HEPES (20 mM + 150 mM NaCl) at 37 °C over 42-45 days (standard deviations have been calculated from measurements performed on three dilutions) (if not visible, the standard deviation is lower than the size of the symbol).

Photothermally controlled release of CF from these newly developed nickel-bis(dithiolene) containing liposomes has also been evaluated under laser irradiation. Ni8C$_{12}$ complex strongly absorbs at 940 nm, thus a 940 nm laser was used for these experiments.[21] Release kinetics have been measured on solutions containing 100 µg.mL^{-1} of Lip(CF) or Lip(Ni8C$_{12}$+CF) dispersed in DMEM + 10% FBS and then placed in a thermostated fluorimeter set to 37 °C. After 1 min equilibration, solutions were submitted to moderate power densities of 1, 2 or 3 W.cm^{-2} for 90 seconds. As showed on Figure 5, liposomes which do not encapsulate nickel-bis(dithiolene) complex Ni8C$_{12}$ display no release for 1 and 2 W.cm^{-2}. Only for the highest studied power density (3 W.cm^{-2}), weak and slow release is observed, reaching 11 % of release after 5 min. However, when complex is added, 85 % of the encapsulated CF is released in less than a minute with a laser power of 3 W.cm^{-2}, testifying of the ability of Ni8C$_{12}$ to induce a mild hyperthermia necessary to trigger drug release. Indeed, as showed by Figure S5, the time delay between laser

activation and CF release can be explained by the duration necessary for the solution to reach the transition temperature of 42 °C. After 1 min irradiation at 3 W.cm^{-2} at 37 °C, 85 % of the encapsulated CF is released and no further release was observed for longer irradiation times. The rest of the fluorophore remains stacked and quenched inside the nanoparticles.

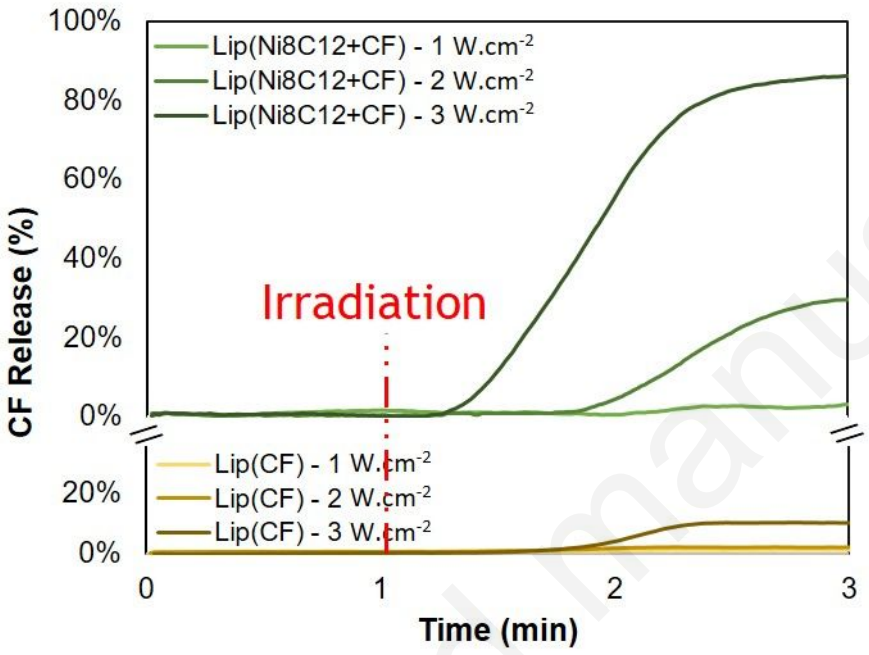

Figure 5. Release kinetics of CF dye from liposomes without complex (Lip(CF)) or containing nickel-bis(dithiolene) complex (Lip(Ni8C$_{12}$+CF)), as a function of the laser power. The liposome concentration inside the cuvette was 100 µg.mL^{-1}.

Additional experiments have also shown that release kinetics as well as the amount of active molecule released can be finely tuned by controlling parameters such as complex loading or power and duration of laser irradiation (Figure S6). The temperature profiles measured during the irradiation experiments of Lip(Ni8C$_{12}$+CF) containing different amounts of Ni8C$_{12}$ and under various laser power (Figure S7) show that the liposome content is effectively released only when the temperature is above 42 °C.

In parallel, the cytotoxicity of fresh formulations of Lip(empty), Lip(CF), Lip($Ni8C_{12}$) and Lip($Ni8C_{12}$+CF) have been evaluated *in cellulo* on human renal carcinoma 786-O cancer cells with and without laser irradiation at 940 nm (Figure 6 and Figure S8). The cells were incubated for 24 h with different amount of each liposome prior to irradiation. Results are represented in Figure 6. It appears that the intrinsic toxicity of the lipid vesicles Lip(empty) is negligible, as 90 % of the cell survived, even when exposed to concentrations as high as 150 $\mu g.mL^{-1}$ (Figure 6, grey bars). Similar observations can be made with Lip(CF) (Figure 6, yellow bars), highlighting the weak toxicity of the fluorophore encapsulated inside the liposomes and also of the small part which has leached from the liposomes. A slight decrease in cell viability can be observed when photothermal agent $Ni8C_{12}$ is added into the formulation. More than 70 % of cells survived after 24 h exposure to culture medium containing up to 150 $\mu g.mL^{-1}$ of Lip($Ni8C_{12}$), equivalent to ~15 $\mu g.mL^{-1}$ of complex (Figure 6, green bars), while Lip($Ni8C_{12}$+CF) exhibit a slightly more important toxicity (60 % survival) (Figure 6, orange bars),. The slight decrease of the cell viability suggests that some complex is accessible from the outer part of the bilayer, thus being available for interactions with the surrounding media. More interestingly, irradiation of cell cultures incubated with Lip(empty) did not show any additional cytotoxic effect. These experiments clearly shows that laser irradiation in itself does not affect cell viability, which is in good agreement with previous observations.[21] On the contrary, for the liposome formulations incorporating $Ni8C_{12}$ complex, the obtained results clearly show that the combination of laser irradiation and the presence of the Ni-bis(dithiolene) photothermal agent leads to a drastic drop in cell viability by more than 85 % for the highest concentrations (Figure 6), thus underlying the promising perspectives of using such liposomes incorporating nickel-bis(dithiolene) complex for photothermal therapy.[57] Confocal microscopy investigations performed after incubation of 786-O renal cells with Lip($Ni8C_{12}$+CF) with or without laser irradiation have revealed that the liposomes are not internalized inside the cells.

Washing the cell cultures after 24 h incubation in presence of nickel-bis(dithiolene) containing liposomes and before 5 min laser irradiation suggest that the liposomes do not interact with the cells (Figure S9), since no luminescence has been detected in the cell interior. The non-internalization of the liposomes into the cells was also confirmed by irradiation experiments performed after washing of the cell cultures (Figure S10 and S11). These results highlight that there is no need to internalize the liposomes inside the malignant cells to induce strong cell death by photothermal effect, i.e. a close proximity is sufficient.

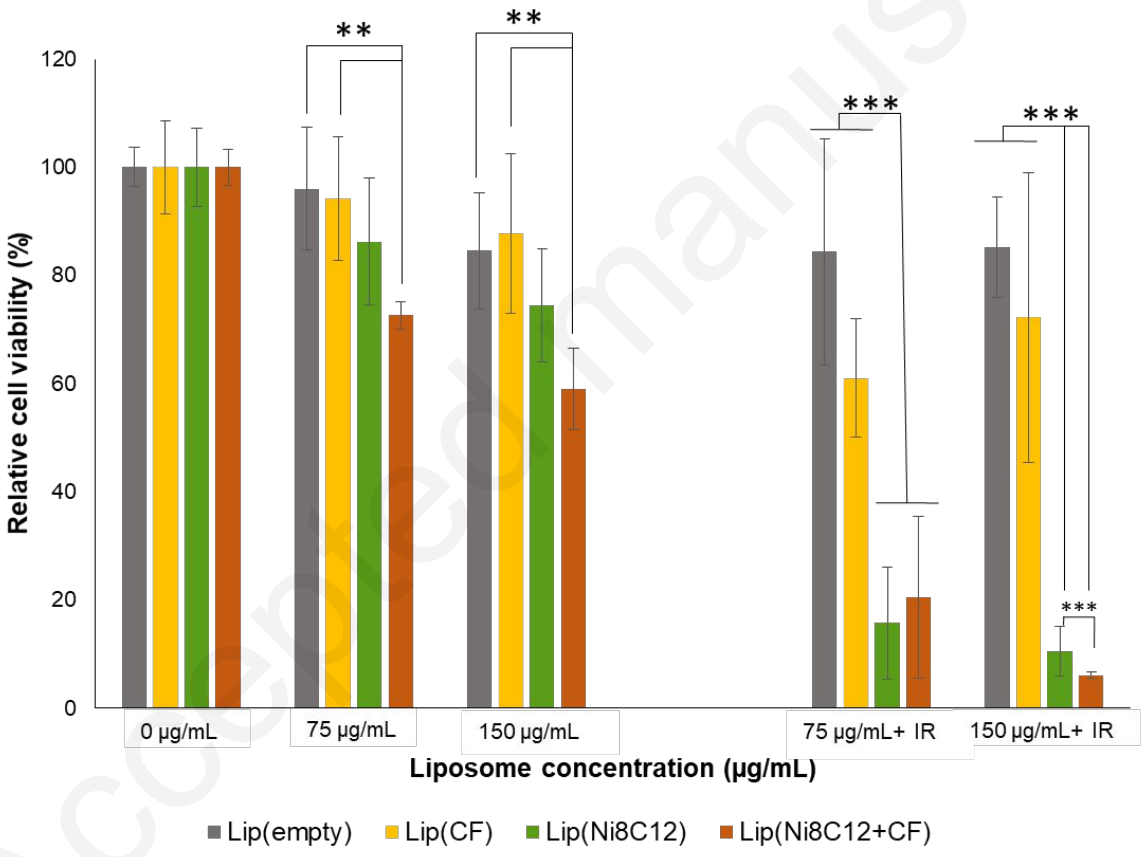

Figure 6. Relative viability of 786-O cancer cells after 24 hours of incubation with various liposome formulations, with or without irradiation (5 min, 5 W.cm^{-2}). Statistical significance was assessed using appropriate tests (Kruskal-Wallis, Mann-Whitney and ANOVA). Significant differences are marked with ** or *** for $p<0.01$ and $p<0.001$ respectively.

Statistical analysis has been performed on two independent experiments involving triplicates each group of treated cells (n = 6).

Finally, the photoacoustic properties of these liposomes containing a nickel-bis(dithiolene) photothermal agent were also explored. In photoacoustic imaging, a short (nanosecond) non-ionising laser pulse is absorbed by the biological tissue, leading to a transient thermal expansion of the optical absorbers in the tissue and subsequent ultrasound emission. The generated ultrasound waves are acquired by an ultrasound transducer to form a photoacoustic image. However, many diseases or tissues do not manifest an endogenous photoacoustic contrast and it is necessary to introduce exogenous photoacoustic contrast agents with significant photothermal effect to convert light into heat for acoustic imaging. Thus, new thermally- and photo-stable exogenous photoacoustic contrast agents with high photothermal activity are highly desirable for photoacoustic imaging.

As expected, a weak photoacoustic signal is detected in pure water and in biological media under laser irradiation (Figure 7). The same observation has been made for liposome suspension (Lip(CF) and Lip(empty) which do not contain nickel-bis(dithiolene) complex, even at high lipid concentrations (Figure S12). Meanwhile, when $Ni8C_{12}$ is added to the formula, strong photoacoustic signals were detected. As expected, the intensity of the photoacoustic signal is proportional to the concentration of liposomes, i.e. nickel-bis(dithiolene) complexes, and to the power of the laser pulses. Therefore, photoacoustic signals have been easily detected from suspensions of Lip($Ni8C_{12}$) and Lip($Ni8C_{12}$+CF) with concentrations as low as 10 $\mu g.mL^{-1}$ in lipids, corresponding to concentrations in photothermal agent $Ni8C_{12}$ as low as 1 $\mu g.mL^{-1}$ (Figure 7). The same observations were made on liposomes containing $Ni8C_{12}$ co-encapsulated with either CF proving that the co-encapsulation of an API is compatible with the utilization of these nanoparticles for an application in bioimaging (Figure S12). It can be anticipated that this

feature can be useful for the tracking and the quantification of the liposomes at target site before triggering drug release.

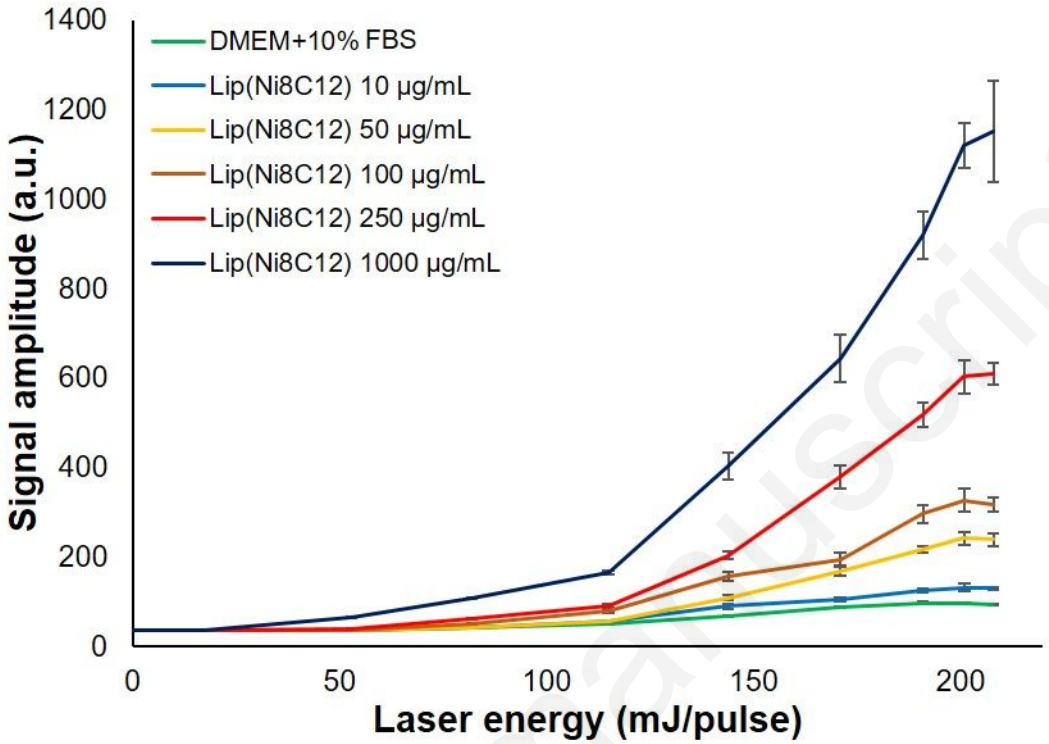

Figure 7. Photoacoustic signal detected depending on the power of laser pulses and on the liposome concentration (standard deviations have been calculated on 20 images).

Conclusion.

New thermosensitive liposomes have been developed by a judicious mixing of dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC) and nickel-bis(dithiolene) complexes as photothermal agent. Small unilamellar vesicles (SUV) with a mean diameter around 100 nm, suitable for the EPR effect, displaying a thermal phase transition at 42 °C have been obtained. The mild hyperthermia generated under NIR laser irradiation at 940 nm by nickel-bis(dithiolene) complexes allows for the fine controlled release of the liposome contents whereas these liposomes are highly stable and keep their contents at 37 °C

for several days. The liposomes are able to deliver almost all their content only when the temperature reaches 42 °C. Drug delivery can be achieved by adjusting the nickel-bis(dithiolene) concentration, or by tuning the laser power and the irradiation time. Such liposomes are highly suitable for on-demand drugs delivery in human body under NIR laser. Moreover, the hyperthermia generated by the complex inside the liposome under NIR irradiation exhibits a high potential for photothermal therapy. In fact, in presence of these nickel-bis(dithiolene) containing liposomes and under NIR laser irradiation, strong cells death can be induced. Finally, it has been demonstrated, for the first time, that nickel-bis(dithiolene) complexes also exhibit photoacoustic properties so ultrasonic waves can be generated under laser stimulation. The photoacoustic signal coming from the nickel-bis(dithiolene) complex is easily detected and its intensity is directly proportional to the concentration of complex and to the laser energy. These last results show that this class of complexes can also be used as exogenous contrast agents for photoacoustic bioimaging. Compared to the current alternatives, metal-bis(dithiolene) complexes are highly stable photothermal agent, contrary to indocyanine green, they will offer better clearance than inorganic nanoparticules such as gold nanorods and their photoacoustic signal can be detected at very low concentrations. In conclusion, the newly developed liposomes integrating nickel-bis(dithiolene) complexes as photothermal agent can be of great interest for imaging-guided photocontrolled drugs delivery and photothermal therapy (dual therapy) in human body.

Keywords. Theranostic nanoparticles, Liposome, Photocontrolled Drug Release, Nickel-bis(dithiolene) complex, Photothermal therapy, Photoacoustic.

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Supporting information.

POM images of DPPC and DSPC; DSC diagrams of DSPC, DPPC and Lip(CF) in water; Cryo-TEM images of Lip($Ni8C_{12}$); Evolution of the PDI of Lip (CF), Lip($Ni8C_{12}$), Lip($Ni8C_{12}$+CF) for 3 months at 37°C and 4°C; Release kinetics of CF from Lip($Ni8C_{12}$+CF) in HEPES; CF release kinetics in HEPES from formulations of Lip($Ni8C_{12}$+CF) containing 1, 3.5, 6.2 or 9.6 %w/w of $Ni8C_{12}$; CF release and temperature profile observed when irradiating liposomes suspensions containing 1 %w/w, 3.5 %w/w, 6.2 %w/w or 9.6 %w/w of $Ni8C_{12}$; Relative viability of 786-O cancer cells cultures with various liposomes formulations; Confocal images of 786-O cells incubated with lip(CF) or Lip($Ni8C_{12}$+CF); Confocal images of 786-O cells with

or without laser irradiation after washing; Relative viability of 786-O cancer cells cultures incubated with various liposomes formulations with or without laser irradiation; Photoacoustic signal detected in HEPES from suspensions of Lip(empty), Lip(CF) and Lip(Ni8C_{12}+CF).

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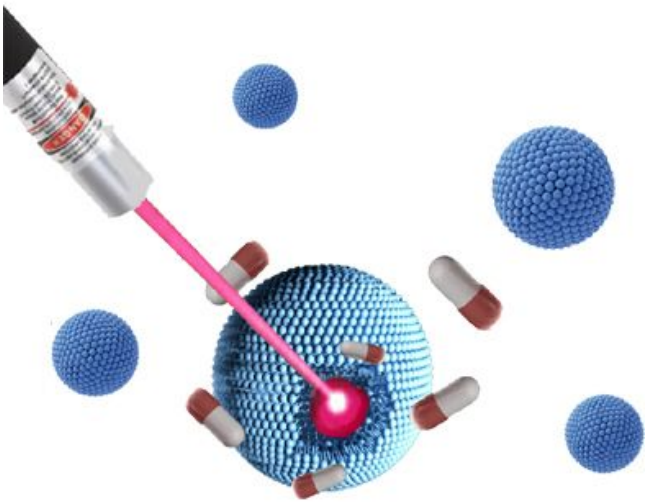

Original thermosensitive liposomes containing a nickel-bis(dithiolene) complex, as photoactive component, are able to deliver their contents on demand under NIR laser irradiation at 42 °C. In addition, the newly developed nanocarriers can be used as exogenous photoacoustic agents and as biocompatible photothermal agents.