Fine and Clean Photothermally Controlled NIR Drug Delivery from Biocompatible Nickel-bis(dithiolene)-Containing Liposomes

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Fine and Clean Photothermally-Controlled NIR Drug Delivery from Biocompatible Nickel-bis(dithiolene) Containing Liposomes

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Keywords: Liposome, Nickel-bis(dithiolene), Photothermal, Drug delivery, Controlled release

Abstract:

This work demonstrates that metal-bis(dithiolene) complexes can be efficiently incorporated inside organic nanocarriers and, that under NIR irradiation, their high photothermal activity can be finely used to release on-demand encapsulated drugs. On the contrary to gold nanoparticles and other organic NIR dyes, nickel-bis(dithiolene) complexes do not produce singlet oxygen under irradiation, a highly desirable characteristic to preserve the chemical integrity and activity of the loaded drug during the NIR triggered release from the nanocarriers. Finally, cytotoxicity experiments performed on various cell lines have shown that the incorporation of such metal complexes do not increase the toxicity of the final liposomal formulation. These results offer great promises for the development of innovative biocompatible drug nanocargos able to safely deliver their content on-demand under NIR laser irradiation. Beside, this work demonstrate that metal-bis(dithiolene) complexes, owing to their versatility of functionalization and metal complexation, are attractive photothermal

agents to develop original NIR responsive materials for application not only in biotechnology but also in material science.

Main text:

In recent decades, owing to the rapid development of nanotechnologies, nanoscale systems for drug delivery and chemotherapy have received tremendous attention to enhance therapeutic efficiency and reduce side effects and inconvenience for the patients.[1] Among them, stimuli-responsive drug delivery systems using "smart" nanocarriers have become a promising modality for enhancing the effects of chemotherapy by controlling the area, time, and dosage.[2] However, the development of such nanocarriers with good targeting selectivity and biocompatibility and on-demand delivery capabilities remains still today a formidable challenge.

To date, various exogenous or endogenous stimuli,[3],[4],[5] such as temperature, pH, enzymatic reactions,[6] magnetic field,[7] ultrasound[8] have been used to control the drug delivery from nanocarriers. Besides, light is also a very versatile stimulus for controlling the on-demand release of entrapped materials from nanocontainers.[9] Indeed, a broad range of parameters, such as frequency, quantity, and duration, can be straightforwardly optimized for a given application. Consequently, this approach confers local control of the nanocontainer permeability, because of the use of light sources such as lasers.

Various strategies have been exploited to date to achieve photocontrolled release from nanovectors. Among these, the use of Near-infrared (NIR) photothermal agents constitutes an emerging approach to induce photocontrolled release of drugs from vesicles. NIR light responsive release is indeed a very important method due to its non-invasive nature, high spatial resolution, and in-depth tissue penetration with minimal damages. Under irradiation, the generated heat stimulates the organic nanocarriers, increases their permeability and allows for the fine control of drug release. Some organic nanosystems for photothermal triggered

release have been developed from liposomes or polymer nanoparticles incorporating for example gold nanoparticles or indocyanine green (ICG).[10],[11],[12] However, gold nanostructures, which are a popular choice in this domain, suffer from several drawbacks such as irreversible morphology changes upon irradiation, slow rates of diffusion and clearance from the body.[13],[14] Organic dyes such as ICG (FDA approved) are also limited due to poor stability, photobeaching and singlet oxygen production.[15],[16]

In that respect, metal-bis(dithiolene) complexes are strong near-IR absorbers with high thermal- and photo-stability. In their neutral state, they display high absorption coefficients (around 30 000 $M^{-1}.cm^{-1}$) in a wide range of NIR absorption maxima that are tunable from 600 to 1600 nm by the judicious combination of metal center and dithiolene substituents. Furthermore, metal-bis(dithiolene) complexes are non-luminescent. This means that all the absorbed energy is released in the environment of heat and, indeed, we have recently demonstrated that metal-bis(dithiolene) complexes displays strong photothermal activities under NIR irradiation in solid–state, as in gel and in solution.[17],[18] In addition, proper functionalization of the complexes has allowed for a very good solubility in water. More importantly, *in cellulo* experiments have demonstrated that NIR-laser irradiation of cellular culture incubated with metal-bis(dithiolene) complexes can induced cell death.[19] These complexes are also highly stable under NIR laser irradiation and no photobleaching has been observed. Thus, all these preliminary results clearly highlight that metal-bis(dithiolene) complexes are strong NIR absorbers with efficient photothermal properties and that they can be good candidates for the photocontrolled release of drugs from organic nanocarriers under NIR irradiation. In addition, Like ICG, the clearance of the molecular nickel complexes should be greatly enhanced compare to metallic nanoparticles.

In this study, we present biocompatible lipid nanoparticles loaded with nickel-bis(dithiolene) complexes for the photothermally controlled released of encapsulated drugs under NIR laser irradiation. EggPC /DSPE-PEG_{2000} liposomes (Lps) were selected as a prototypical model.

They were loaded, depending on the polarity of the nickel-bis(dithiolene) complexes, in the liquid core or in the lipid bilayers. Stable nanocarriers were obtained with diameter ranging between 80 and 100 nm in solution, depending on the nature of the loaded nickel-bis(dithiolene) complex. In addition, cytotoxicity experiments performed on various cell lines have shown that the incorporation of such metal complexes do not increase the toxicity of the final liposomal formulation. Finally, we demonstrate how the high photothermal activity of the nickel-bis(dithiolene) complexes permits the release of carboxyfluorescein, used here as a hydrophilic mimetic drug, with very high levels of temporal control under NIR laser irradiation. Last but not least, we also controlled that nickel-bis(dithiolene) do not produce singlet oxygen under irradiation, on contrary to gold nanoparticles and other organic NIR dyes (cyanine, porphyrin…), an highly desirable characteristic to preserve the chemical integrity and activity of the loaded drug during the NIR triggered release from the nanocarriers.[20]

L-α-phosphatidylcholine/ 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino-poly(ethylene glycol)-2000] (EggPC/DSPE-PEG_{2000}) liposomes are useful biocompatible drug delivery systems for carrying hydrophilic (in the aqueous core) or lipophilic (in the lipid bilayer) drugs, now used in clinical trials (Scheme S1).[21],[22] Introduction of PEG chains enhances the particle stability and *in vivo* circulation time.[23] Liposome with a 9:1 w:w ratio were prepared by the Bangham method involving the hydration of thin film of lipids obtained by evaporation for chloroform solution (10 mg/mL lipid) (see supporting information for details).[24] After hydration and sonication, the liposomes were purified by centrifugation over ultrafiltration units and dispersed in milliQ water. The hydrodynamic diameter of these empty liposomes measured by dynamic light scattering (DLS) was found to be around 87 nm with a polydispersity index (IP) of 0.29 (Figure S1).

For these studies, two nickel-bis(dithiolene) complexes have been carefully selected. One hydrophobic complex carrying eight long C12 alkyl chains ($Ni8C_{12}$), well known for its liquid

crystalline properties,[25] and another hydrophilic one carrying eight 2-methoxy(2-ethoxy(2-ethoxyethyl) PEG chains (Ni8PEG), recently proposed as new agent for photothermal therapy (Figure 1a).[19] Their encapsulation inside the lipid nanocarriers strongly depends on their hydrophilicity. Hydrophilic $Ni8C_{12}$ complex was directly incorporated during the lipid film formation from chloroform solutions. In this way, the hydrophilic complex is localized inside the lipid bilayers of the liposomes. On the contrary, the highly hydrosoluble Ni8PEG was incorporated in the liquid core of the liposomes during the hydration step from its aqueous solution (see Supporting Information for details).

Liposomes incorporating hydrophobic $Ni8C_{12}$ complexes inside the lipid bilayer have been prepared by dissolving directly the nickel complex together with the EggPC and the DSPE-PEG_{2000} in chloroform prior to the thin film formation. The quantity of complex introduced was varied from 5 to 30 w% /lipid. UV-vis-NIR titrations has shown that a maximum of 5 w%/lipid can be incorporated in the lipid bilayer (Figure 1b). UV-vis-NIR titrations performed on the filtrate did not show any absorption band at 990 nm, thus confirming that this $Ni8C_{12}$ complex was completely insoluble in water. The excess of complex was found glued on the wall of the preparation flask (Figure S2). Thus, Lp-$Ni8C_{12}$ with a complex concentration of 5 w%/lipid has been used for the remaining experiments ($[C]_{Lipid}$ = 10 mg/mL and $[C]_{Ni8C12}$ = 500 µg/mL). DLS measurements have shown that these liposomes with 5 w% of complex have a hydrodynamic diameter of ~ 100 nm with a quite narrow dispersity (around 0.3) (Figure S3). The slight increase of the hydrodynamic diameter, compared to that of the empty liposomes, is attributed to the incorporation of the hydrophobic complexes inside the lipid bilayer. Stock Lp-$Ni8C_{12}$ solutions could be stored for more than 30 days with no change in particle size distribution and in $Ni8C_{12}$ content. The Lp-$Ni8C_{12}$ nanoparticles were also found to be stable under NIR irradiation (vide infra) (Figure 1c). In fact, after 5 min irradiation at 940 nm, the mean hydrodynamic diameter of the doped liposomes is not affected. Cryo-transmission electron microscopy (cryo-TEM) was used to

visualize the morphology of Lp-Ni8C_{12} directly in water. The cryo-TEM images confirm the formation of unilamellar liposomes, having a spherical shape with diameters ranging from 15 nm to 110 nm (Figure 1d). It should be noticed that hydrodynamic diameters measured by DLS are always overestimated due to the hydration layer and the dispersity of the size distribution.

Lp-Ni8PEG nanocarriers were prepared by hydrating the lipid bilayer with an aqueous solution of the complex at 40 weight% relative to the lipid content (4 mg/mL). UV-vis-NIR titrations of the liposome solution and of the filtrate have revealed that 13 w%/lipid of nickel complex is encapsulated in the liquid core of the liposomes and 27 w%/lipid remains in the water solution (Figure S4). Only 1/3 of the complex initially introduced in the aqueous hydrating solution have been encapsulated in this way ($[C]_{Lipid}$ = 10 mg/mL et $[C]_{Ni8PEG}$ = 1.3 mg/mL). Higher concentrations have not been tested yet. The hydrodynamic diameter determined by DLS measurements was found to be 90 nm with a polydispersity of 0.3 (Figure S5). The introduction of nickel complex in the liquid core do not significantly affects the diameter of the EggPC/DSPE-PEG_{2000} liposomes. Time-dependent DLS measurements have also shown here that the Lp-Ni8PEG remain stable after ~30 days and laser irradiation (Figure 1c). However, leaching of hydrophilic Ni8PEG complexes into the exterior aqueous solution cannot be excluded.

Figure 1

The photothermal activity and the light conversion efficiencies (η) of aqueous suspensions (10 mg/mL) of Lp-Ni8C_{12} containing 5 w%/lipid in the lipid bilayer or Lp-Ni8PEG containing 13 w%/lipid in the liquid core were evaluated by monitoring the temperature profile during 10 min under continuous laser irradiation at 940 nm for 10 min after turning off the laser (Figure

S6 and S7). The η values were calculated according to the equation reported by Roper et al (Table S1).[15] The temperature increase under laser irradiation is strongly dependent on the nature and concentration of the complex. The photothermal efficiency of $Ni8C_{12}$ complex incorporated inside the lipid bilayer was found to be 25% whereas the η value of the free Ni8PEG in aqueous media is only 16 %. This difference on the conversion efficiency of both encapsulated complexes is directly reflected by the maximum temperature change observed. In fact, with Ni8PEG, a maximum temperature increase ($\Delta T_{max} = T_{max}-T_0$) of 55.6 °C was measured, whereas with $Ni8C_{12}$ complex, which 2.6 time less concentrated, a ΔT_{max} of 66.6 °C is easily reached. Nevertheless, the temperature increase measured in both cases under irradiation at 940 nm at 5 w/cm^2 was sufficient to induce a melting of the lipid bilayer. In addition, these two suspensions were found to be highly photothermally stable. No fatigue was observed after four heating and cooling cycles performed on suspensions of Lp-$Ni8C_{12}$ and Lp-Ni8PEG (Figure S8). In addition, the size of the liposomes was not affected by the laser irradiation (Figure 1c). Such complexes with high temporal and photothermal stability can be promising candidates for long term photo-controlled drug deliveries. This marked difference in the photothermal conversion efficiency is likely attributed to the confinement of the $Ni8C_{12}$ complex inside the pseudo solid-like environment of the lipid bilayer, contrary to the Ni8PEG complex, which is free in solution and can undergo faster deactivation and heat dissipation with the solvent.

For drug delivery purposes, the light induced stimuli should not lead to the production of singlet oxygen in order to preserve the chemical integrity of the encapsulated drugs. To verify that the nickel-bis(dithiolene) complexes do not produce singlet oxygen under laser irradiation, toluene solutions of $Ni8C_{12}$ and Ni8PEG complexes have been irradiated in presence of 1,3-diphenylisobenzofuran (DPBF) compound. DPBF compound is well-known to react quickly with $^1O_2$ to form a product with a decreased absorption maxima and associated fluorescence (Figure S9).[26] For this purpose, toluene solutions of DPBF (2×10^{-5} M) and nickel complexes

(1.6×10^{-4} M, ~ 10 eq.) were irradiated for 15 min at 940 nm with a laser power of 5W/cm^2. The fluorescence spectrum recorded before and after irradiation show almost the same emission intensities without any change of the emission band profile (Figure S10). This simple experiment clearly indicates that the nickel-bis(dithiolene) complexes do not produce singlet oxygen under laser irradiation and that they can safely be used in photo-controlled drug release experiments.

To further demonstrate the potential of these nickel-bis(dithiolene)-doped liposomes for the photothermal control release of encapsulated drugs, carboxyfluorescein (CF) was encapsulated in the aqueous interior of the liposomes (Lp-Ni8C$_{12}$-CF). Lp-Ni8C$_{12}$ were selected to avoid leaching of the metal complex in surrounding aqueous medium and to leave more space inside the liquid core of the liposomes for the encapsulated drugs. To study the photothermal control release ability of these novel nanocarriers, EggPC/DSPE-PEG$_{2000}$ lipid films containing 5 w% of Ni8C$_{12}$ complex were directly hydrated with a saturated aqueous solution of CF. After sonication and purification, the liposomes (150 µL of the solution at 10mg/mL of lipids) were directly poured in a home-made dialysis bag (3500 Da) placed directly in a quartz cell (see SI). The release of CF from the liposomes was monitored by fluorescence spectroscopy, in dark, with or without laser irradiation. The percentage of CF released was calculated according the following equation: %CF released = $((F_m-F_i)/F_t)\times100$ where F_m is the measured fluorescence, F_i is the initial fluorescence and F_t is the fluorescence measured after addition of 50 µL of Triton X-100. In the absence of laser irradiation, the CF is slowly released from the liposomes and only 30 % of the CF was liberated in the aqueous medium after 90 min and 80 % after 15 h (Figure 2 and Figure S11). On the contrary, 15 min of laser irradiation at the beginning of the process leads to a boost of the CF release and 80 % of the CF is released in 60 min. The on-demand drug delivery ability of this photothermally stable system was also evaluated by applying short intermittent laser irradiation pulses.

During the first 30 min without irradiation, the release kinetics are identical to that found initially on non-irradiated Lp-Ni8C_{12}-CF. Thereafter, the suspension was irradiated for different time intervals. The results indicate that the CF release is proportional to the irradiation time. Indeed, higher percentage of CF is released under longer the irradiation times. This means that, during irradiation, the porosity of the liposome increases, resulting in the release of CF which results in a sudden rise in fluorescence. Then, as soon as the laser is switched off, the temperature of the medium decreases and the liposome recovers its initial permeability and therefore its original release kinetics. In this way, for example, 80 % of the CF could have been released in 80 min after four irradiations. It should be noticed that UV-vis-NIR titration performed on the dialysate after irradiation did not shown absorption in the NIR region, meaning that this hydrophobic Ni8C_{12} nickel-bis(dithiolene) complex remains within the liposomes. The release kinetic will depend as illustrated here, on the number of irradiations, the duration of the irradiation, the duration of the resting plateaux and likely on the laser power used. Thus, as clearly demonstrated here, Lp-Ni8C_{12} can be efficiently used to release a selected molecule in a controlled manner with various kinetics which are directly related to the duration of the NIR laser irradiation. This result is particularly interesting since nickel-bis(dithiolene) complexes owing to their versatility of functionalization, which is more difficult to access with cyanine derivatives, can be properly incorporated inside the lipid nanoparticles with a high level of control in term of localization and concentration. In addition, such small molecular photothermal centers should be more easily eliminated by the human body compared to inorganic nanostructures (gold, CuS_x…).

Several DSC experiments have been performed on concentrated aqueous suspensions of Lp-Ni8C_{12} (100 mg/mL). No phase transition has been detected between 5 °C and 80 °C, meaning that the lipid bilayer is already in a fluid state in this temperature range (Figure S12). The use of EggPC, which is a mixture of lipids, do not favor the crystallization of the bilayer and in addition DSPE-PEG_{2000} lipids exert a lateral expansion which favor the fluid membrane

phase.[27] The fluid nature of the lipid bilayer is also in line with the natural CF release observed at room temperature without laser irradiation. In the present case, the heat release generated by the nickel complexes under laser irradiation simply increases the fluidity of the membrane, allowing for higher release rates.

Figure 2

Finally, the cytotoxicity of the liposomes containing nickel-bis(dithiolene) complexes has been evaluated by thiazolyl-blue-tetrazolium-bromide (MTT) assays on two cell lines (HeLa and MDA) after 24h incubation (see SI for details). The first results obtained have shown that the EggPC/DSPE-PEG_{2000} liposome have a marked cytotoxicity on both cell lines for concentrations above 50 µg/mL. The cytotoxicity of the liposomes on both cell lines is comparable but with a cell viability of the MDA cells slightly lower compared to the HeLa cells. It should be noticed that in order to detect any effect of the presence of the metal-complex, high concentrations of liposomes have been used. The IC50 of Ni8PEG is around 500 µg/mL.[19] The IC50 of $Ni8C_{12}$ could not have been determined due to its strong hydrophobicity. In the case of Lp-$Ni8C_{12}$, cytotoxicity is only due to the liposome and not to the encapsulated complex, as the cytotoxicity is the same whether the complex is present or not (Figure 3a and 3b). On the other hand, metal complex–containing Lp-Ni8PEG are more toxic than the liposomes alone. The hypothesis is that the Ni8PEG complex is released from the liposome and disseminates into the culture medium, due to its hydrophilic character, thus inducing additional cytotoxicity. However, these cytotoxicity experiments clearly show that the trapping of an hydrophobic nickel-bis(dithiolene) complex inside the lipid bilayer do not import any additional toxicity to the organic nanocarriers.

Additional experiments have been performed on HeLa and MDA cell cultures in presence of empty liposomes or Lp-$Ni8C_{12}$ liposomes with and without laser irradiation at 940 nm (Figure

S13). Cell cultures without any liposomes have also been irradiated at 940 nm as control test. These experiments clearly shows that laser irradiation in itself does not affect cell viability. Moreover, laser irradiation of cells in presence of empty liposomes or Lp-Ni8C_{12} liposomes does not induce any additional cell death. This confirm that the presence of Ni8C_{12} complexes do not import any additional toxicity to the liposomes. The concentration of complexes is also not sufficient here to induce a significant photothermal effect.

Figure 3

In conclusion, it has been demonstrated here for the first time that NIR irradiation of liposomes containing nickel-bis(dithiolene) complexes allows for the fine control release of fluorophores or drugs from these nanostructures in solution. Additional studies have also shown that nickel-bis(dithiolene) complexes do not produce singlet oxygen under NIR irradiation and that the incorporation of metal-bis(dithiolene) complexes do not increase the cytotoxicity of the organic nanoparticles. This original combination of the photothermal effect of the nickel-bis(dithiolene) complex with the photo-controlled delivery of drugs into a single nanocarrier allows now for the development of innovative therapeutic agents for a dual chemo- and photothermal-therapy of great interest for the treatment of cancers. These results have recently been patented.[28] Future works will be devoted to the optimisation of these novel photoresponsive drugs delivery nanocarriers to reach a rapid and controlled response with minimal metal complex content and even low laser power. Future works will also be devoted to the delivery of active drugs in biological media and to the introduction of additional functionalities on the outside of the liposomes for local and targeted treatments of diverse diseases. Biodistribution, biotransformation and clearance of the metal-complexes as well as their pharmacological specificities will also have to be considered and studied. The

liposomes should rapidly be removed from the circulation by the cells and the organs comprising the reticuloendothelial system after opsonization.[29] The absorbed metal ions should then be concentrated into the kidney tissue and eliminated from the body via the urinary tract.[30] The versatility of metal-bis(dithiolene) complexes combined to their ease of functionalization makes this class of metal complexes highly attractive to develop original NIR responsive materials with tuneable photothermal properties for dedicated applications in biotechnology but also in material science.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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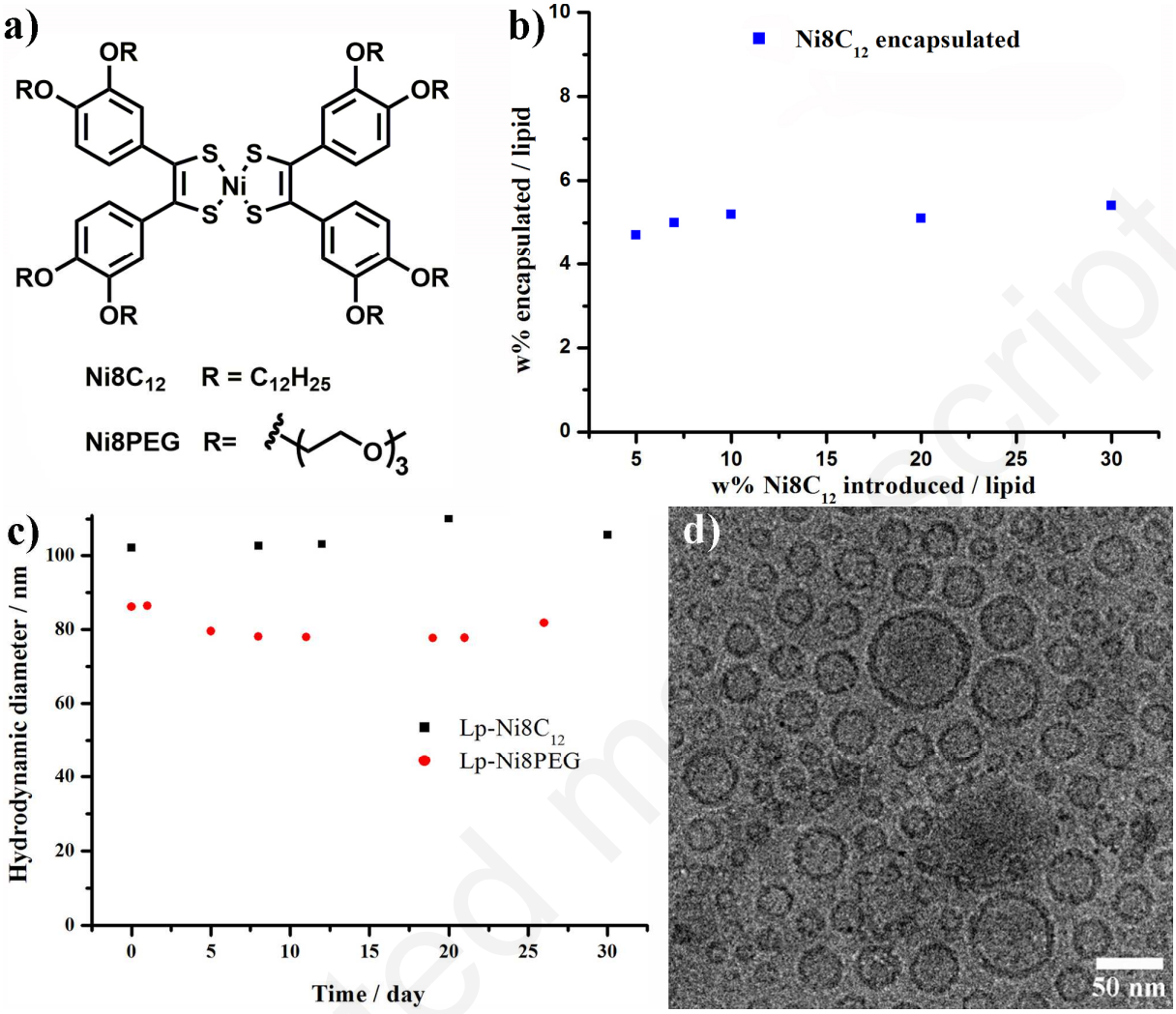

Figure 1. a) Chemical structures of the two nickel-bis(dithiolene) complexes which have been incorporated in EggPC/DSPE-PEG$_{2000}$ liposomes; b) Mass fraction of Ni8C$_{12}$ incorporated as a function of the mass fraction of Ni8C$_{12}$ introduced in the formulation; c) Evolution of the hydrodynamic diameter determined by DLS measurements as a function of the time. After 30 days, identical hydrodynamic diameters has been measured before and after laser irradiation (940 nm, 10 min, 5 W.cm^{-2}) for both liposomal formulations with Ni8C$_{12}$ and Ni8PEG; d) Cryo-TEM image of the Lp-Ni8C$_{12}$ (10 mg/mL of lipids).

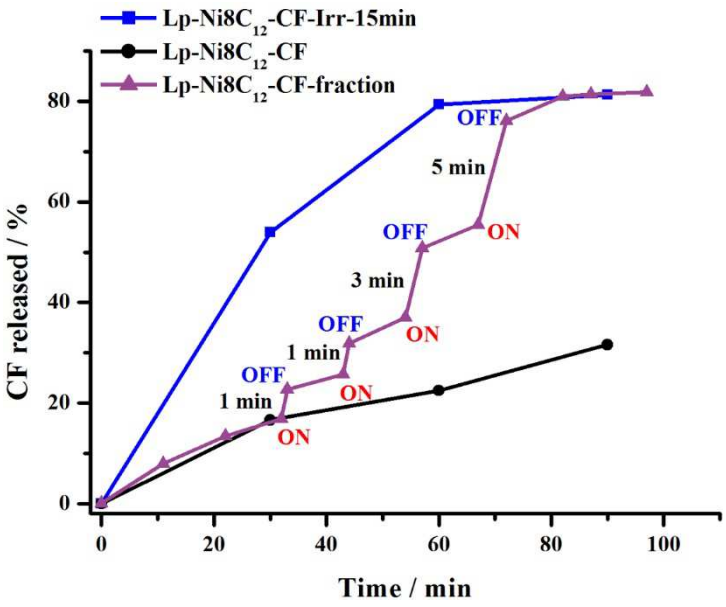

Figure 2. Release of CF from photothermosensitive EggPC/DSPE-PEG_{2000} liposomes containing 5 w% of $Ni8C_{12}$ complex in water under dark (Black curve (circle): without laser irradiation, Blue curve (square): with 15 min initial laser irradiation and purple curve (triangle): with various laser irradiation duration at different time intervals) (940 nm, 5 W/cm^2).

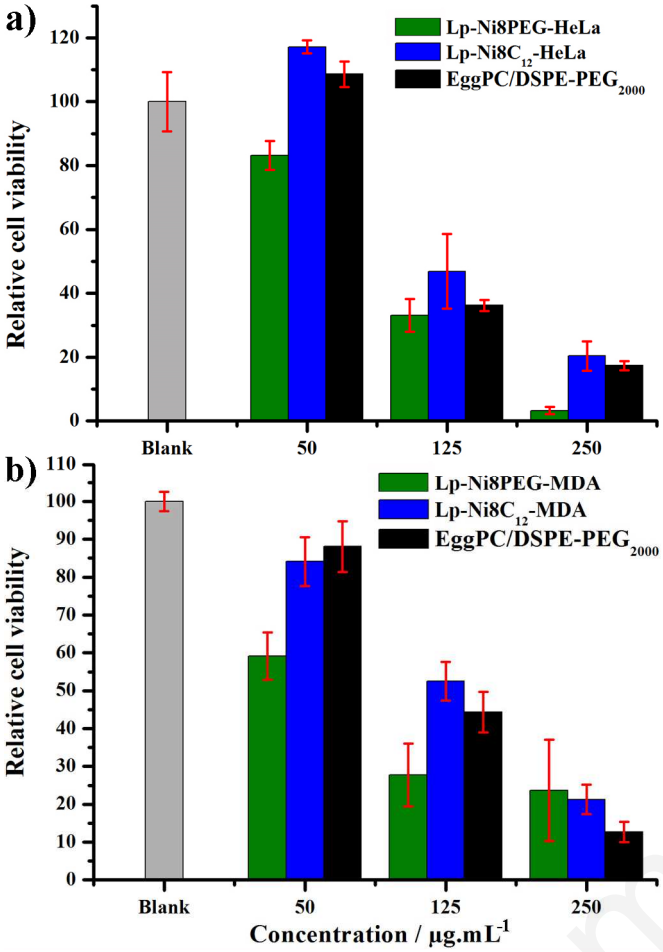

Figure 3. Relative Cell viability of HeLa (a) and MDA (b) cells incubated with different concentrations of EggPC/DSPE-PEG$_{2000}$ liposomes, Lp-Ni8C12 (5w%/lipid) and Lp-Ni8PEG (13w%/lipid).

TOC

Incorporation of nickel-bis(dithiolene) complexes inside biocompatible liposomes allows for the fine photothermal control of drug release without any increase of the cytotoxicity or any production of singlet oxygen.

Keyword: Liposome, Nickel-bis(dithiolene), Photothermal, Drug delivery, Controlled release

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