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Doxorubicin-sensitized Luminescence of NIR-emitting Ytterbium Liposomes : Towards Direct Monitoring of Drug Release

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Abstract: Drug-loaded liposomes are typical examples of nanomedicine. We show here that doxorubicin, the anti-cancer agent in the liposomal drug Doxil[®], can sensitize Ytterbium (Yb³⁺) and generate its near infrared (NIR) emission. When doxorubicin and amphiphilic Yb³⁺ chelates are incorporated into liposomes, the sensitized emission of Yb³⁺ is dependent on the integrity of the particles which can be used to monitor drug release. We also established the first demonstration that the NIR Yb³⁺ emission signal is observable in living mice following intratumoral injection of the Yb³⁺-doxorubicin-liposomes, using a commercial macroscopic setup equipped with a near-infrared camera.

Liposomes represent widely explored nanoplatfroms designed to deliver drugs, diagnostic agents, or both within a single particle. The first clinical nanomedicine approved was a liposome, loaded with doxorubicin (Doxil[®]).^[1] In the body, drugs entrapped into liposomes become bioavailable only upon their release from the nanocarrier, alleviating out-of-target tissue damage. Thus, controlled and localized drug release is crucial.^[2] To this end, various triggers/stimuli have been proposed, including external (heat, ultrasound, light),^[3] as well as endogenous triggers that are intrinsic to the disease site or cellular organelles (enzymes, redox or pH changes).^[1a, 4]

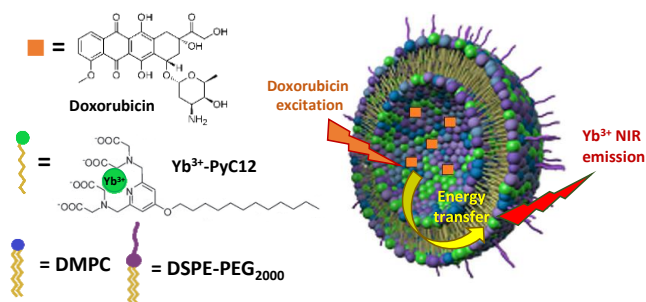
Monitoring drug release is a major field where drug delivery can benefit from molecular imaging. Among the different modalities, magnetic resonance imaging (MRI) has been explored to monitor the *in vivo* localization of drug-loaded liposomes and the release of their cytotoxic cargo with the advantages of high-resolution images and in depth tissue imaging. To achieve this goal, drug-containing liposomes were typically co-loaded with a contrast agent, the release of which is simultaneous with the delivery of the drug from the liposome, resulting in an MR signal increase.^[5] Given the small instrumental footprint and the wide availability of optical detection, most of the approaches for the monitoring of drug delivery are based on fluorescence. For the detection in biological samples, excitation at lower energy and emission in the near infrared (NIR) window are more favorable. Combinations of different types of drugs, delivery platforms, fluorescent probes, and various detection strategies using FRET, fluorescence quenching, aggregation-induced emission, etc. have been explored.^[6] These fluorescence approaches mostly rely on organic dyes or inorganic nanoparticles. Lanthanide luminescence remains largely unexplored in this field despite the versatile and advantageous optical properties of lanthanides, and their emerging success in near infrared biological imaging.^[7] High photostability, large energy difference between excitation and emission bands and sharp emission bands make lanthanide-

based systems attractive for the development of optical detection in biological systems. Importantly, recent results show that lower quantum yields often associated with NIR lanthanide luminescence do not prevent successful microscopic imaging applications in living cells.^[7c, 7g, 7h, 8] An Er³⁺ and Yb³⁺ co-doped NaGdF₄ upconverting nanoparticle has been recently co-encapsulated with doxorubicin (DOX) in the aqueous core of liposomes in order to monitor drug delivery.^[9] Upon DOX release and excitation at 980 nm, the upconverted emission is not quenched by the close proximity of the drug, and the particles emit three characteristic Er³⁺ "green" bands. The emission intensity increases upon release and could be related to the quantity of DOX delivered. To the best of our knowledge, this is the only example of using Ln³⁺ luminescence in the context of drug release monitoring; however, this emission is in the visible region. In comparison to inorganic nanoparticles, like those described in the above example, molecular lanthanide complexes are generally considered to have enhanced biocompatibility. Nevertheless, they have not yet been used for the optical monitoring of drug delivery. In order to emit a sufficient emission signal, luminescent lanthanide cations need to be sensitized by an appropriate highly absorbing chromophore that acts as an antenna and transfers the resulting energy to the metal.^[7f] For an effective energy transfer, antennas and lanthanides have to be in sufficiently close distance. We have previously shown that micelles^[10] or liposomes^[11] allow facile localization in sufficient proximity of the sensitizing antenna and lanthanide cations, without the need of covalent linkage, to generate sensitized NIR emission. Likewise, micellar structures allowed for the joint localization of Ln³⁺ ions and PDT photosensitizer molecules, and *in situ* excitation of the photosensitizer by X-ray induced lanthanide luminescence emission could be performed.^[12]

In this work, we have hypothesized that DOX, a widely used anti-cancer molecule and therapeutic agent in the liposomal drug Doxil[®], could act as sensitizer for the NIR-emitting Yb³⁺. DOX is a derivative of anthraquinone which has already been reported to sensitize NIR-emitting lanthanides.^[13] Amphiphilic lanthanide complexes can be easily incorporated in a liposome bilayer to create the proximity of Yb³⁺ ions and encapsulated DOX. As long as the liposome nanoparticle remains intact, lanthanide luminescence can be observed. Upon drug release, the lanthanide luminescence signal is expected to decrease. As a major advantage of this approach, the drug is directly implicated in the generation of the lanthanide NIR emission, which will be thus an unequivocal and instantaneous indicator of the presence of the drug inside the liposome, ultimately enabling a direct and real-time follow-up of drug release. Conversely, the impossibility

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to distinguish low liposome concentration from dissolved liposome/released drug represents a drawback of this strategy. To demonstrate the validity of our approach, we have created and characterized PEGylated DMPC / DSPE-PEG(2000) (1,2-dimyristoyl-sn-glycero-3-phosphocholine / 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]) liposomes incorporating amphiphilic chelates of the NIR emitting Yb³⁺ (Yb-pyC12) in their bilayer.^[11] The lanthanide-containing liposomes were further loaded with doxorubicin via a pH-gradient methodology^[14] (Scheme 1).



Scheme 1. Schematic representation of the liposomes studied and their NIR emission generation mechanism.

The liposomes were ~50 nm in diameter. Their Yb³⁺ content was determined by ICP and the doxorubicin loading efficiency was assessed by absorption and fluorescence studies (see ESI). The luminescence properties of the liposomes have been studied to evaluate if the DOX operates as an antenna for the sensitization of Yb³⁺. Figure 1 shows the emission and excitation spectra of Yb³⁺-liposomes incorporating DOX. The excitation on the DOX electronic levels at 500 nm allows the observation of the characteristic Yb³⁺ emission band ranging from 940 to 1075 nm, which is assigned to the ²F_{5/2} → ²F_{7/2} electronic transition (spectra A and B). As Yb³⁺ does not possess any electronic transition located in an energy domain corresponding to the visible range of wavelength, the only way to populate the excited states of Yb³⁺ is by taking advantage of the electronic structure of the DOX to absorb the excitation energy. In absence of DOX, it was not possible to observe the emission band of Yb³⁺ upon excitation at 500 nm. In addition to the NIR Yb³⁺ emission, a broad band in the range 510-850 nm could also be observed (Figure 1, Table 1) and is assigned to the DOX emission.

Luminescence lifetimes of Yb³⁺ compounds are sensitive to the surrounding -OH, -NH and -CH vibrations and reflect the lanthanide coordination environment. Luminescence lifetimes of the Yb³⁺-DOX liposomes were measured upon excitation of the doxorubicin ($\lambda_{exc} = 532$ nm). We identified two distinct values, a longer one at 1.4 μ s and a shorter one at 0.44 μ s (Table 1). This observation is in agreement with previous results, where we showed that liposomes provide two distinct environments to Ln³⁺, with 1.77 and 0.46 μ s obtained for Yb³⁺-Anthracene liposomes.^[11] Yb³⁺ ions pointing inside the hydrophilic core are more 'protected' against quenching effects, while Ln³⁺ ions on the external surface are more prone to non-radiative deactivation due to a more important exposure to water, and hence have shorter lifetime.

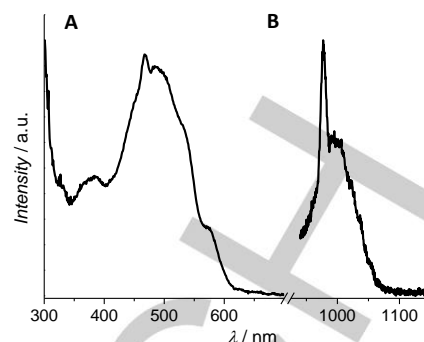


Figure 1. (A) Excitation spectrum ($\lambda_{em} = 980$ nm) and (B) emission spectrum after subtraction of residual doxorubicin emission in the range 940-1150 nm ($\lambda_{ex} = 500$ nm) of Yb³⁺-DOX liposomes. All spectra have been corrected and normalized. The Yb³⁺ concentration was 0.5 mM in the liposome samples which were prepared in PBS. All data were acquired at room temperature.

Table 1. Photophysical properties of PEGylated Yb³⁺-DOX containing liposomes.^[a]

Q(DOX) / % ^[b]	Q(Yb ³⁺) / % ^[c]	τ_1 / μ s ^[d]	τ_2 / μ s ^[d]
1.75(4)	1.8(1)·10 ⁻³	1.4(1): 51(5) %	0.44(2): 49(5) %

[a] 0.5 mM Yb³⁺ in PBS, room temperature, 2 σ values between parentheses. [b] DOX-centered emission (510-850 nm) under excitation at 500 nm. [c] Yb³⁺-centered emission (940-1150 nm) under excitation at 500 nm. [d] Under excitation at 532 nm, Yb³⁺ emission was monitored at 980 nm; population parameters $P_i = \frac{\beta_i \tau_i}{\sum_{i=1}^n \beta_i \tau_i}$ in % are given after the colon.

The efficacy of DOX as antenna for Yb³⁺ ions was assessed by the determination of the absolute quantum yield of Yb³⁺-DOX-liposomes (Table 1). The value of Q(Yb³⁺) is comparable to those reported for polystyrene beads incorporating Yb³⁺ trifluoromethanesulfonates and 1,4-dihydroxyanthraquinone chromophores (1.22·10⁻³ %)^[13d] but lower than those reported for similar pyridine based systems: Φ pyOMe = 1.7·10⁻⁴ ^[15], Φ Yb/Ant liposomes = 2.4·10⁻⁴ ^[11], or other ligands.^[7d-f, 16]

In vitro drug release was modeled by adding increasing amounts of *N*-octyl-glucoside to Yb³⁺-DOX liposomes as they induce their disruption. Upon addition of 40% (v/v) of *N*-octyl-glucoside, a ~35% decrease of the Yb³⁺ luminescence (985 nm) was observed (Figure S2). Upon liposome lysis, the energy absorbed by the doxorubicin is no longer transferred to the lanthanide, leading to reduced lanthanide emission. Liposome rupture was confirmed by DLS measurements (Figure S3). These results evidence that the liposome integrity is a prerequisite of Yb³⁺ sensitization by DOX.

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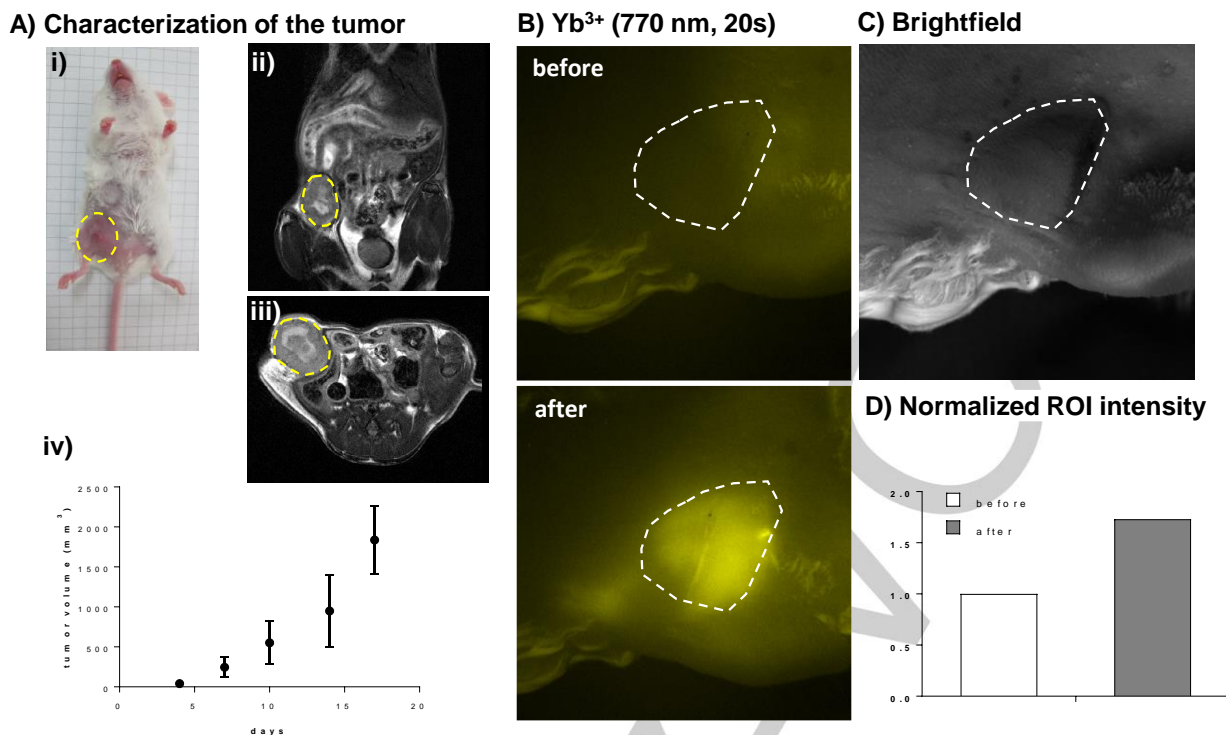


Figure 2. *In vivo* proof-of-concept of optical detection in 4T1-tumor bearing mice. A) Characterization of the tumor: (i) photograph; coronal (ii) and axial (iii) MRI images of the tumor, providing tumor volume evolution measurement over time (iv). Luminescence monitoring of Yb³⁺-DOX containing liposomes: B-D) Yb³⁺ (long path 770 nm filter) based emission upon excitation with light selected using 482 nm band path 35 nm filter, before and after injection (B), brightfield (C) and normalized ROI intensity quantification (D).

In order to demonstrate the potential *in vivo* detectability of the Yb³⁺ optical signal generated *via* DOX sensitization, a proof-of-concept study was performed in 4T1-tumor bearing mice (n=5). Tumors were induced by injection of 4T1 cells in the 4th mammary gland of Balb/C female mice (Figure 2 shows a representative mouse). Tumor growth, position and volume were visualized by MRI (Fig. 2A). Optical imaging was performed using a microscope equipped with an EMCCD camera with sensitivity in the NIR range. To maximize the *in vivo* detection of Yb³⁺ emission, mice were injected intratumorally with Yb³⁺-DOX liposomes (20 μ L; 3.16 mM concentration of Yb³⁺). Yb³⁺ emission (long path 770 nm filter, 20 s acquisition time per image) images upon excitation light selected using a 482 nm band path 35 nm filter were acquired before and 10 min after intratumoral injection. These images (Fig. 2B) evidence a clear signal enhancement after injection. Normalized ROI intensities were further quantified before and after injection (Fig. 2D). Intravenous injections of Yb³⁺-DOX liposomes (100 μ L; 3.16 mM Yb³⁺ concentration) into 4T1-tumor bearing mice did not allow to detect signal increase in the tumor. A biodistribution study realized in mice by ICP-OES following intravenous injection in order to determine the Yb³⁺ content in different tissues indicate a promising tumor uptake of 3.0 %ID/g (ESI). However, such tumor accumulation is not sufficient to provide a detectable optical signal in our macroscopic setup. To address this limitation, active targeting of the lanthanide-containing liposomes to the tumor will be necessary.

These results show for the first time in an *in vivo* model that it is possible to observe the NIR signal arising from Yb³⁺ sensitized by DOX. Liposomes co-loaded with Yb³⁺ complexes and a drug can provide a specific luminescence signal allowing the detection of the delivery of nanomedicine to a tissue of interest. It is also possible to monitor the release of DOX by following the time-dependent variation of Yb³⁺-emission.

In summary, we report on a novel concept towards direct monitoring of doxorubicin release from liposomes containing NIR-emitting lanthanide complexes. We evidence that the widely used anti-cancer agent doxorubicin is capable of sensitizing the NIR luminescence of Yb³⁺, if both partners are encapsulated in liposomes. The NIR luminescence emission generated in this way is dependent on the integrity of the liposomes allowing for a direct and real-time monitoring of drug release. This luminescence is detectable in living mice with a commercial macroscopic setup equipped with a near-infrared sensitive camera. This observation of the NIR emission was possible despite the small quantum yields of Yb³⁺ upon sensitization by doxorubicin which can be explained by the high density of lanthanide emitter and sensitizer per unit volume in these liposomes.

While this remains a proof-of-concept study, we believe that the same strategy can be extended to other liposome compositions more adapted to specific applications (e.g. triggered drug release), optimized to biological targeting or eventually containing other chemotherapeutic drugs with extended aromatic structure.

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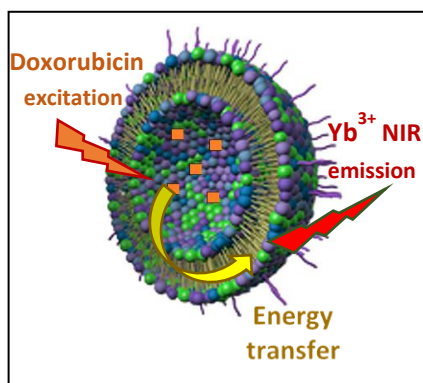
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Keywords: Lanthanide sensitization • Liposomes • Doxorubicin • Drug delivery • In vivo imaging

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Doxorubicin-sensitized near infrared emission of Yb^{3+} is observable in solutions of liposomes encapsulating the drug and amphiphilic Yb^{3+} complexes. This emission vanishes when the liposome is destroyed and doxorubicin is released. The NIR Yb^{3+} luminescence could be observed in living mice with a commercial macroscopic setup.

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