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Monitoring drug stability by label-free fluorescence lifetime imaging: a case study on liposomal doxorubicin

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Abstract. In a previous report, we demonstrated that Doxorubicin (DOX) intrinsic fluorescence can be exploited in combination with the phasor approach to fluorescence lifetime imaging microscopy (FLIM) and quantitative absorption/fluorescence spectroscopy to resolve the supramolecular organization of the drug within its FDA-approved nanoformulation, Doxil®. The resulting 'synthetic identity' comprises three co-existing physical states of the drug within Doxil®: a dominating fraction of crystallized DOX (DOX_c >98%), and two minor fractions of free DOX (DOX_f ~1%), and DOX associated with the liposomal membrane (DOX_b <1%). This result serves as a benchmark here to address the time evolution of Doxil® synthetic identity. We probe the effect of temperature for a total duration of 6 months in a non-invasive way by FLIM. We confirm Doxil® stability if stored at 4°C, while we detect marked changes in its synthetic identity at 37°C: crystallized DOX gets progressively disassembled in time, in favor of the other two physical states, free and membrane-associated DOX. Our phasor-FLIM-based approach paves the way to time-resolved biochemical assays on the supramolecular organization of encapsulated fluorescent drugs potentially all the way from the production phase to their state within living matter.

1. Introduction

Cancer is one of the leading causes of death worldwide and Doxorubicin (hereafter referred to as 'DOX') is one of the most effective cytotoxic drugs applied as a chemotherapeutic agent. Nanoformulations of DOX, in particular, proved to reduce a major side effect associated with the use of the isolated drug, i.e. its systemic toxicity, while retaining the overall therapeutic efficacy of the treatment [1]. For instance, Doxil® was approved by the Food and Drug Administration (FDA) agency in 1995 as the first nano-drug and is currently used to treat AIDS-related Kaposi's sarcoma, recurrent ovarian cancer, metastatic breast cancer, and multiple myeloma. Doxil® consists of nanoparticles with a protective lipid bilayer in the "liquid ordered" phase (composed of high-melting-temperature phosphatidylcholine and cholesterol), poly-ethylene glycol segments engrafted into the liposome surface and a high concentration of DOX within the aqueous liposome core [2].

The high drug-encapsulation efficiency is ensured by the use of a specific chemistry of loading (also known as "active remote loading") which induces DOX precipitation into the aqueous liposomal core. The presence of a nanorod-shaped, semi-crystalline phase of precipitated DOX within Doxil® liposomes was demonstrated by X-ray scattering [3] and cryo-EM [4] experiments. However, the semi-quantitative nature of such investigations is not sufficient to distinguish possible coexisting phase-separated drug pools in the formulation (i.e. the drug supramolecular organization), even less to

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quantify the fractional amount of each drug sub-population. These limitations in turn affect our ability to control the performance of encapsulated DOX in delivery applications and to improve by rational design the efficacy of new formulations. Citing verbatim its inventor, Yechezkel Barenholz, “in Doxil® each component matters”[2]: indeed non-monomeric and non-isolated DOX molecules cannot interact with DNA and fulfill their expected cell-killing activity.

In order to monitor the supramolecular organization of the drug within the intact liposomal formulation, we recently proposed a new strategy based on the exploitation of DOX intrinsic fluorescence as a source of signal and fluorescence lifetime imaging microscopy (FLIM) as a tool with exquisite sensitivity to the nanoscale organization of the emitter [5]. Also, the phasor approach was used as a fast, fit-free, fully graphical strategy to extract the quantitative information encrypted within lifetime data [6]. The phasor-FLIM signature of Doxoves® (a research-grade product of PEGylated liposomal DOX whose physical characteristics and pharmacokinetics are comparable to those of Doxil®) was resolved into the contribution of three co-existing fluorescent species, each with its characteristic mono-exponential lifetime, namely: crystallized DOX (DOX_c, 0.2 ns), free DOX (DOX_f, 1.0 ns), and DOX bound to the liposomal membrane (DOX_b, 4.5 ns). Then, the exact molar fractions of the three species are determined by combining phasor-FLIM with quantitative absorption/fluorescence spectroscopy on DOX_c, DOX_f, and DOX_b pure standards. The final picture of the Doxoves® formulation comprises most of the drug in the crystallized form (>98%), with the remaining minor fractions divided between free (~1%) and membrane-bound drug (<1%) [7].

This FLIM-based quantitative picture of Doxoves® synthetic identity served as a benchmark for the measurements reported here on the time evolution of such identity upon exposure of Doxoves® to 37°C.

2. Materials and methods

2.1. Materials

Doxoves® (F30204B-D2) was purchased from FormuMax Scientific (Sunnyvale, CA, USA). Liposomes were made of HSPC/CHOL/mPEG2000-DSPE, loaded with 2 mg/mL doxorubicin, and dissolved in the manufacturer’s buffer containing 10 mM Histidine and 10% sucrose at pH=6,5. Doxoves® was divided into aliquots held at different temperatures: half batch at 4°C (storage temperature suggested by the pharmaceutical manufacturer) and the other half at 37°C. Each aliquot (50 µL) was measured at least four times after a different period of storage/incubation at the given temperature. The sample stored at a constant temperature was analyzed for the first three months every week and then once a month for up to 6 months.

2.2. Lifetime Measurements

Fluorescence Lifetime Imaging Microscopy was performed using Leica TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany). The samples were observed by a pulsed diode laser operating at 40 MHz with an excitation wavelength of 470 nm. The emission was collected in the wavelength range between 510 and 660 nm by a photomultiplier tube interfaced with a time-correlated single photon counting (TCSPC) card (PicoHarp 300, PicoQuant, Berlin). For each sample and timing, measurements were performed at multiple distances from the bottom glass to have an average value independent of any sample stratification.

2.3. Phasor Analysis

Phasor-FLIM analysis was performed using a dedicated routine of SimFCS software (Laboratory for Fluorescence Dynamics, University of California, Irvine). With this approach, each pixel can be mapped onto a so-called “phasor” plot whose polar coordinates are derived by the Fourier transform of the fluorescence decay in time at the angular repetition frequency of the measurement. The Cartesian coordinates g and s are respectively the real and the imaginary parts of the Fourier transform of the fluorescence impulse response and are positive. Therefore a mono-exponential decay stays on the

Universal Circle, that is a half-disk centered at (0.5,0) with a radius of 0.5, where the zero lifetime is located at (1,0) and the infinite lifetime at (0,0). If a pixel contains a combination of two (or more) distinct lifetime decays, its position in the phasor plot is obtained by the weighted linear combination of these contributions [8]. Consequently, the intensity contribution of each mono-exponential species can be estimated graphically [6] or by simple linear algebra:

$$I_x = \frac{distance_{point \to intercept}}{distance_{x \to intercept}} \tag{1}$$

This procedure requires the knowledge of the lifetimes of the contributing species and does not account for differences in brightness (given by the product of quantum yield Q_x and molar absorption coefficient ε_x). Indeed the fractional-intensity contributions of two or more species coincide with their actual molar fractions only if the pure species have the same brightness under the experimental conditions. Knowing the spectroscopic parameters (such as quantum yield and molar absorption coefficient) for each species it's possible to convert the fractional-intensity contribution I_x into the actual molar fraction M_x using the following equation:

$$M_x = \frac{I_x}{\varepsilon_x * Q_x * \sum_x \frac{I_x}{\varepsilon_x * Q_x}} \tag{2}$$

3. Results and discussion

3.1. Molar composition of Doxoves®

The phasor plot in Figure 1A shows the experimental lifetime cluster derived from a FLIM measurement on Doxoves® in solution, at 40-MHz frequency of the excitation laser. The excitation wavelength was set to 470 nm in order to selectively excite the intrinsic fluorescence of DOX. Based on our previous study [7], we know that three pure species concur to generate the measured lifetime of Doxoves®, each with its own mono-exponential characteristic lifetime and relative abundance: crystallized DOX (DOX_c), free DOX (DOX_f), and DOX associated with the liposomal membrane (DOX_b) (Figure 1B).

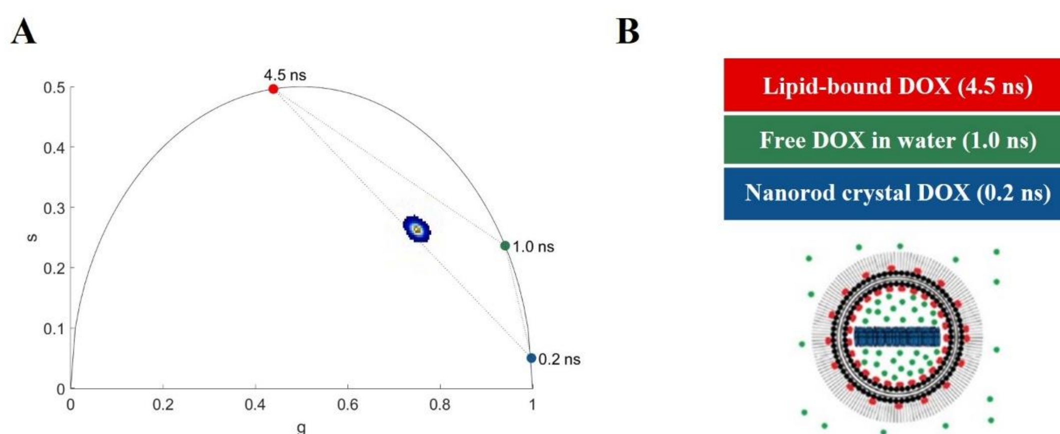

Figure 1: Phasor plot of standard liposomal doxorubicin. A) Experimental phasor plot of Doxoves®. B) Schematic representation of liposome with the three pure species derived by phasor-FLIM analysis. Blue depicts the nanorod-shaped crystal of ammonium-sulfate DOX, while green and red mean respectively DOX free and bound to the lipid membrane.

As a result, the FLIM signature of Doxoves® falls within the triangle having the lifetimes of the three pure species as vertices, as expected from the well-known algebraic rules of phasor composition [6].

Using these latter, the fractional intensity contribution of each species can be easily calculated (a schematic representation of the procedure is reported in Figure 2A). Please note that, at this level, the procedure can already be used to quantitatively compare different datasets (e.g. distinct drug preparations). Still, the fractional-intensity contribution of a species will coincide with its actual molar fraction only if the distinct pure species have the same brightness (given by the product of their quantum yield Q_x and their molar absorption coefficient ε_x) under the experimental conditions used. These latter parameters were experimentally derived or estimated in a previous report [7] and integrated into Equation 2 to obtain the molar fraction of each species in the Doxoves® batch measured here (Figure 2B): crystallized DOX is 98.419±0.005, free DOX 1.036±0.005 and lipid-associated DOX is 0.545±0.001.

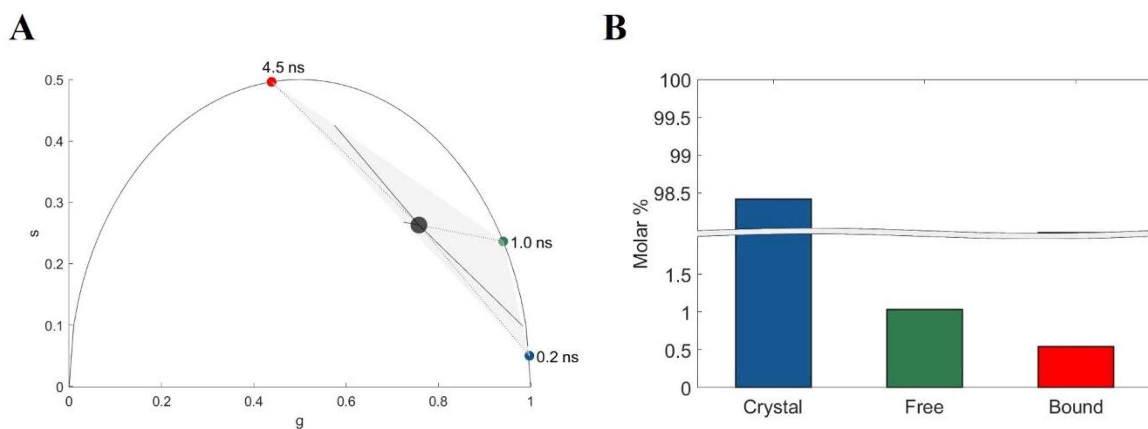

Figure 2: Phasor analysis of standard liposomal doxorubicin. A) Graphical calculation of fractional-intensity contributions. Dotted lines connect each pure species to the experimental phasor and are extended as a continuous line to the edge joining the other two pure species. Equation 1 determines the fractional intensity. B) Resulting molar fractions in Doxoves® after the application of Equation 2. Crystal DOX is 98.419±0.005, free DOX 1.036±0.005 and lipid-bound DOX is 0.545±0.001.

3.2. Time-resolved FLIM-based analysis of Doxoves® stability at 37°C

At this point, the molar composition of Doxoves® can be non-invasively monitored in time by the phasor-FLIM approach. As shown in Figure 3, we performed consecutive FLIM measurements on Doxoves® stored at 37°C for a total duration of the experiment of about 6 months. A marked shift of the phasor-center position (dark to white dots in Figure 3) was detected over time. In particular, the phasor moved progressively towards the segment connecting two pure species out of three, namely free DOX and DOX associated with lipids (green and red cursor, respectively).

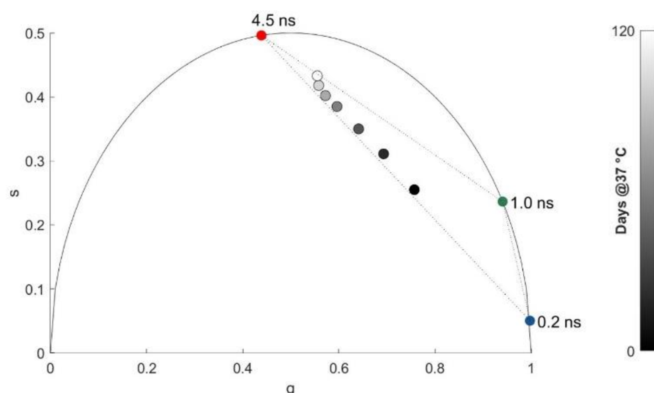

Figure 3: Phasor shift of Doxoves® at 37 °C over time. Each point corresponds to the center of the cluster given by the measures at a fixed time indicated by the grayscale. The distance of each point from a pure species (located on the Universal Circle) is inversely proportional to the fraction of that species present in the sample.

As explained above (i.e. algebraic rules and Equation 2), the phasor characteristic position can be used to calculate the corresponding molar fractions as a function of time. For this conversion, we assumed that no additional species contribute to the measured lifetimes throughout the experiment, although we cannot exclude the emergence of new (unknown) species with different lifetimes over time. Under these assumptions, data are displayed in Figures 4A and 4B, corresponding to the experiment performed at 37°C and 4°C, respectively. Of note, storage of the drug at 37°C progressively results in the almost complete dissolution of the crystallized DOX fraction into the remaining two pure species, free and membrane-associated DOX, which after 6 months reach a stationary state with a relatively more abundant contribution from free DOX. By contrast, storage of the drug at 4°C for the same amount of time does not result in a change in the molar fraction of the contributing species, a clear indication that Doxoves® maintains its synthetic identity.

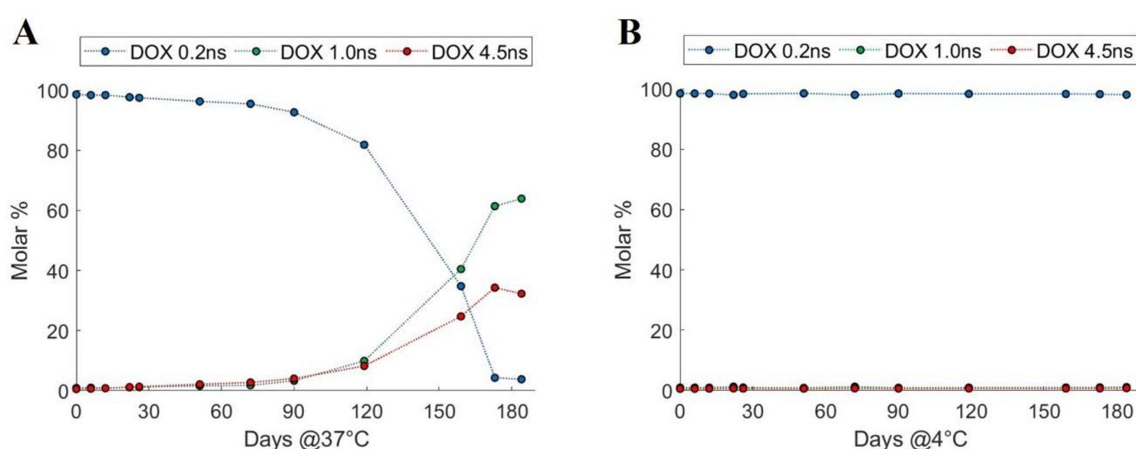

Figure 4: Temporal evolution of the molar fractions in Doxoves® at different temperatures.
A) Doxoves®, if stored at 37 °C, shows a significant change of its synthetic identity in terms of molar fractions of the three pure species. B) By contrast, if Doxoves® is stored at 4 °C, it remains stable (in terms of molar fractions of the contributing species) for the total duration of the experiment.

4. Conclusions

We were able to investigate Doxoves® stability for a total duration of 6 months by exploiting a label-free technique with nanoscale sensitivity to the supramolecular organization of fluorescent encapsulated molecules. Phasor-FLIM is a fast and fit-free data-analysis procedure and does not require chemical modification of the sample. It is compatible with standard optical setups and allows a time resolution of a few seconds. In addition, the use of visible light makes phasor-FLIM promising for cellular studies, in particular, to highlight drug interactions with living matter that can change its supramolecular organization. The platform used in this work can provide fast readouts along the production line of formulations not only in a pharmaceutical company but also in the chemical industry (for controlled-release pesticides, paints, adhesives, inks, anti-counterfeiting inks, cosmetics, nutraceutical/dietary supplements).

References

[1] Borišev I, Mrdanovic J, Petrovic D, Seke M, Jović D, Srdenović B, Latinovic N and Djordjevic A 2018 Nanoformulations of doxorubicin: How far have we come and where do we go from here? *Nanotechnology* **29** 332002

[2] Barenholz Y 2012 Doxil® - The first FDA-approved nano-drug: Lessons learned *Journal of Controlled Release* **160** 117–34

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[3] Schilt Y, Berman T, Wei X, Barenholz Y and Raviv U 2016 Using solution X-ray scattering to determine the high-resolution structure and morphology of PEGylated liposomal doxorubicin nanodrugs *Biochim Biophys Acta* Gen Subj. **1860** 108–19

[4] Wei X, Shamrakov D, Nudelman S, Peretz-Damari S, Nativ-Roth E, Regev O and Barenholz Y 2018 Cardinal Role of Intraliposome Doxorubicin-Sulfate Nanorod Crystal in Doxil Properties and Performance *ACS Omega* **3** 2508–17

[5] Digman MA, Caiolfa VR, Zamai M and Gratton E 2008 The phasor approach to fluorescence lifetime imaging analysis *Biophys J* **94** L14-L16

[6] Ranjit S, Malacrida L, Jameson DM and Gratton E 2018 Fit-free analysis of fluorescence lifetime imaging data using the phasor approach *Nat Protoc* **13** 1979–2004

[7] Tentori P et al 2022 Fluorescence lifetime microscopy unveils the supramolecular organization of liposomal Doxorubicin *Nanoscale* **14** 8901–5

[8] Weber G 1981 Resolution of the Fluorescence Lifetimes in a Heterogeneous System by Phase and Modulation Measurements Introduction The determination of fluorescence lifetimes by phase *J. Phys. Chem.* **85**