QUANTIFYING RELEASE FROM LIPID NANOCARRIERS BY FLUORESCENCE CORRELATION SPECTROSCOPY

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ABSTRACT: Understanding the release of drugs and contrast agents from nanocarriers is fundamental in the development of new effective nanomedicines. However, the commonly used method based on dialysis frequently fails to quantify the release of molecules poorly soluble in water, and it is not well-suited for in situ measurements in biological media. Here, we developed a new methodology for quantifying the release of fluorescent molecules from lipid nanocarriers (LNCs) using fluorescence correlation spectroscopy (FCS). LNCs based on nanoemulsion droplets, encapsulating the hydrophobic Nile red derivative NR668 as a model cargo, were used. Our studies revealed that the standard deviation of fluorescence fluctuations in FCS measurements depends linearly on the dye loading in the nanocarriers, and it is insensitive to the presence of less-bright molecular emissive species in solution. In sharp contrast, classical FCS parameters, such as the number and the brightness of emissive species, are strongly influenced by the fluorescence of molecular species in solution. Therefore, we propose to use the standard deviation of fluorescence fluctuations for the quantitative analysis of dye release from nanocarriers, which is unaffected by the “parasite” fluorescence of the released dyes or the auto-fluorescence of the medium. Using this method, we found that LNCs remain intact in water, whereas in serum medium, they release their content in a temperature-dependent manner. At 37 °C, the release was relatively slow reaching 50% only after 6 h of incubation. The results are corroborated by qualitative observations based on Förster resonance energy transfer between two different encapsulated dyes. The developed method is simple because it is only based on the standard deviation of fluorescence fluctuations and, in principle, can be applied to nanocarriers of different types.

INTRODUCTION

Nanocarriers have attracted a lot of interest in the last years because of their possible application as tools for drug delivery, 1,2 in vivo imaging, and image-guided surgery. 3,4 These systems include polymer nanoparticles and lipid-based nanocarriers such as nanoemulsions, liposomes, 7 micelles, and inorganic nanoparticles. Interest in studies of the nanocarrier behavior is increasing, and a lot of research has been devoted to novel ways to characterize their properties. 6 One of the most important properties is their ability to encapsulate hydrophobic or hydrophilic molecules, depending on their composition and morphology.

Optimal application of nanocarriers (NCs) in the drug delivery requires that the drug is maintained inside the nanocarriers until it reaches the target, such as a tumor, followed by controlled release of the drug inside the target. The most common method to study the release of drugs from nanocarriers is dialysis. 7 However, it has limitations when applied to highly hydrophobic drugs poorly soluble in water, 9 and it cannot really be applied to study the release directly in cells and animals. Other methods such as size-exclusion chromatography, continuous flow, and flow cytometry have also been used, 10 but they have limitations similar to those of dialysis. Therefore, understanding the drug release requires simple and effective assays capable to operate directly in situ in complex biological media. Förster resonance energy transfer (FRET) is the method of choice in this case. Several reports have already shown the strong potential of FRET to study cargo release in biological liquids, cells, and even in living animals. 11–15 However, this method requires double labeling of the nanocarriers (i.e., with donor and acceptor). Moreover, it is still challenging to achieve a quantitative characterization of the cargo release using FRET-based methods, although some calibration-based approaches have been suggested recently.12
A promising technique for the characterization of nanobjects in situ is fluorescence correlation spectroscopy (FCS). It is a powerful technique used in biological and biophysical research for investigating fundamental processes such as molecular diffusion, particularly in lipid membranes, and inside the cells, interaction of biomolecules, and (bio)chemical reactions, with sensitivity reaching single-molecule level. FCS is based on measuring the fluorescence intensity fluctuations of emissive species diffusing across a small-excitation focal volume. Autocorrelation analysis of the fluorescence intensity in the focal volume provides information on the concentration, diffusion constant, and brightness of the fluorescent particles. Moreover, analysis of the fluorescence intensity fluctuations by the so-called fluorescence fluctuation spectroscopy enables quantitative analysis of the brightness distribution, which allows characterization of heterogeneous samples containing assembled molecules. FCS serves as a tool for measuring the size and polydispersity of nanoparticles, as well as for evaluating their behavior in complex biological media and their stability. Also in many reports, FCS has been used to characterize the formation of the protein corona on the surface of nanoparticles, or the interaction of human serum albumin with liposomes. However, only few studies have reported the use of FCS to study the release of cargo from nanoparticles. Andresen et al. used FCS for quantification of leakage of dyes from unilamellar lipid vesicles, proposing a mathematical framework for the data analysis. In addition, we used FCS to characterize the leakage of fluorescent dyes from lipid nanoparticles directly into biological media, but these measurements were performed without quantification of release processes.

The aim of our study is to develop a simple FCS-based technique for the quantification of cargo release from NCs. To this end, we selected lipid NCs (LNCs), the so-called nanoemulsion droplets (Figure 1). These systems are composed of substances generally regarded as safe and therefore, they constitute a promising nontoxic platform for drug delivery. However, the liquid nature of their core raises questions about their stability in vivo and capacity to maintain their cargo until the target. Our recent work has shown that LNCs can retain their contents even in vivo and accumulate in tumors in an almost intact form. However, in this case, the cargo should be sufficiently hydrophobic to remain inside LNCs in a biological environment. Therefore, LNCs can be a suitable platform for encapsulation of hydrophobic drugs and contrast agents. In the present work, we explored a possibility to study FCS the release from LNCs of a model cargo, hydrophobic Nile red derivative NR668 (Figure 1). In addition to classical FCS parameters, such as the number and brightness...
of the emissive species, we also studied the standard deviation (SD) of fluorescence fluctuations as a function of dye loading and the degree of dye release. Remarkably, this SD showed an almost linear dependence on the degree of dye release with minimal perturbation by the fluorescent species outside LNCs. Thus, we propose to use a very simple parameter of FCS measurements to monitor the release of a fluorescent cargo directly in situ in a complex biological context.

■ RESULTS AND DISCUSSION

We first wanted to find conditions under which the content of LNCs is released as a function of time. To this end, we used FRET, which is a well-established technique for studying the stability of nanocarriers. FRET has been used to follow the integrity of different nanoparticles in vivo, such as polymeric micelles, LNCs, and HDL. It acts similar to a molecular ruler to monitor the proximity between the fluorophores inside LNCs, so that FRET is lost as soon as the dyes are released from the nanocarriers and diluted into the medium. In our case, NR668 was selected as an energy acceptor, whereas another highly hydrophobic dye F888 was chosen as an energy donor, as shown in our earlier report.

Nanoemulsion droplets prepared by spontaneous emulsification had a hydrodynamic diameter of 34 nm and a good polydispersity (<0.1) according to dynamic light scattering (DLS). The presence of the dyes did not affect the polydispersity and size of LNCs. An increase in the loading of the FRET pair resulted in the growth of the relative intensity of the acceptor emission, which is an indication of FRET (Figure 2A). The highest acceptor to donor ratio (∼0.75) was obtained for LNCs with 0.5 wt % of F888 and NR668. This formulation was selected for the release studies.

The release experiments were performed in the presence of a 10-time excess of blank LNCs, which was our model of the recipient medium for hydrophobic dye molecules. This mixture was incubated at three different temperatures (20, 37, and 60 °C), and the acceptor to donor intensity ratio was measured after 0.5, 1, 2, 4, and 6 h of incubation. As shown in Figure 2B, at 20 and 37 °C, the acceptor to donor intensity ratio reflecting the FRET efficiency did not change even after 6 h. In contrast, at 60 °C, we observed a decrease in this ratio after the first 30 min, and it continued decreasing during 6 h of the experiment. This decrease is due to an increase in the distance between the donor and acceptor, which implies that dyes are released from our FRET LNCs. These data show that our LNCs can retain the cargo dyes in the release medium even at 37 °C at least for 6 h, which is in line with our earlier work performed in serum. Moreover, we found that 60 °C is a convenient model condition generating dye release on the time scale of hours. However, it is difficult to directly connect FRET efficiency with the degree of dye leakage, unless a dedicated model and corresponding calibrations are realized.

Having set up our model release system, we then explored the possibility to quantify the release of dyes using FCS measurements. Because dye release should affect the nanodroplet brightness and the quantity of emissive species in solution, FCS appears as a promising method to quantify this release. Following this logic, the release of hydrophobic dye into the recipient medium should result in an increase in the number of emissive species and a decrease in the particle brightness, as we showed in our earlier work. However, the problem comes when we do not know much about the biological medium, where the dye is released. In this case, the fluorescence of the released dyes as well as the autofluor-
of light. LNCs, being much brighter than calcein, were the main source remaining unchanged in the broad concentration range of increase in the number of the less bright species decreased the brightness per particle at 10 mW, 830 nm illumination), an times brighter according to the FCS measurement of NR668, are much brighter than single calcein molecules (concentration increased the observed dilution, estimated based on FCS focal volume (0.34 fL) and studied calcein concentrations (0.1 μM) were much larger so that an increase in the NR668 loading would mimic the changes in the dye content in LNCs because of dye leakage. For FCS measurements, a two-photon excitation at 830 nm was used and the emission was collected over the entire visible spectrum, so that fluorescence signal from both NR668 and calcein could be recorded. The following FCS parameters were studied: (i) brightness (B), (ii) number of emissive species (N), and (iii) SD of signal fluctuations.

As shown in Figure 3A,B, an increase in the calcein concentration increased the N value but decreased the B value. This was expected because higher concentrations of calcein should increase the number of emissive species. Indeed, the studied calcein concentrations (0.1–3.6 μM) were much larger than that of LNCs. The concentration of LNCs at 10 000-fold dilution, estimated based on FCS focal volume (0.34 fL) and the observed N value (0.58, without calcein), was ~2.8 nM. Moreover, because LNCs, containing a large number of NR668, are much brighter than single calcein molecules (~74 times brighter according to the FCS measurement of brightness per particle at 10 mW, 830 nm illumination), an increase in the number of the less bright species decreased the overall brightness per particle. Remarkably, the SD parameter remained unchanged in the broad concentration range of calcein. This parameter did not change probably because LNCs, being much brighter than calcein, were the main source of light fluctuations, totally controlling the SD value. On the other hand, the SD value showed an almost linear dependence on the NR668 dye loading (Figure 3C). The brightness also increased with NR668 loading, although the dependence was clearly not linear with a moderate increase at low NR668 loadings, followed by a stronger increase at higher loadings (Figure 3D). Remarkably, the number of emissive species showed a complicated behavior: it rapidly decreased at low dye loadings and then decreased much slower at higher loadings.

The behavior of B and N parameters is complex because at very low dye loading, the contribution of the calcein emission to the autocorrelation curve is important, which increases the overall N values and decreases B. At higher loading, LNCs become much brighter than calcein, so that they start dominating the fluctuation analysis. Nevertheless, even at the highest loading the N and B parameters in the presence of 0.2 μM calcein were quite different from those for LNCs in Milli-Q water, whereas SD showed practically the same value for these two conditions (Figure 3).

These two experiments, which model the release of dyes from LNCs, suggest that B and N are not suitable parameters for quantification of the release because they are nonlinearly affected by the molecules present in the medium. The only parameter, which appears useful for the leakage quantification is SD. Indeed, it is only affected by the change in the dye content inside bright LNCs but is practically independent from free single molecules of calcein in solution, which are much less bright than single LNCs. Thus, SD can directly indicate the dye content in LNCs without being influenced by the fluorescence of the released dye.

However, SD is not an absolute parameter, being strongly dependent on instrument settings, such as laser power and instrumental alignment as well as on LNC concentration. Therefore, we introduced a normalized parameter SD/SD0, where SD0 represents the SD of the fluorescence fluctuations for 1% NR668 LNCs in the absence of leakage. The dependence of the SD/SD0 parameter on the NR668 loading was verified for different laser powers and concentrations of LNCs.

LNCs loaded with different NR668 concentrations were measured by FCS at two laser powers (7 and 10 mW) and at different LNC concentrations. We found that the SD/SD0 parameter was not affected by a change in the laser power (Figure 4A) or LNC concentration (Figure 4B), showing that it is a robust parameter. The linear dependence of SD/SD0 on dye loading was then used as a calibration curve (Figure 4A). It should be mentioned that the data point corresponding to 1% NR668 LNCs in the absence of leakage. The dependence of the SD/SD0 parameter on the NR668 loading was verified for different laser powers and concentrations of LNCs.

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Finally, we applied our new FCS-based methodology to study the release of the model cargo NR668 from LNCs. To this end, a release experiment was performed by incubating LNCs loaded with 1 wt % NR668 at 20, 37, and 60 °C in three different media: (i) pure water, (ii) water with blank LNCs,
and (iii) water with 10% fetal bovine serum (FBS). During incubation in pure water, the NR668 LNCs were very stable in water, showing no dye release even at 60 °C (Figure 5A), probably because NR668 is very hydrophobic and cannot leak into neat water. In the presence of blank LNCs or FBS, capable to solubilize NR668, we observed dye release at 60 °C. Approximately 50 and 75% release was observed after 6 h incubation for blank LNCs and FBS, respectively (Figure 5B,C). At 37 °C, the dye release was observed only for FBS medium with nearly 50% release after 6 h. The results observed with blank LNCs are perfectly in line with our FRET data showing dye release exclusively at 60 °C (Figure 2B). However, in contrast to the FRET approach, our methodology based on FCS enabled a quantitative description of the dye release.

**CONCLUSIONS**

In summary, we have developed a new methodology for quantifying the release of fluorescent dyes from LNCs. This methodology is based on the SD of fluorescence fluctuations in FCS measurements. The proof of concept was validated using the hydrophobic Nile red dye, NR668, encapsulated in lipid nanoemulsions. The results suggest that LNCs remain intact in water, whereas in FBS medium, they release their content in a temperature-dependent manner. At 37 °C, the release is relatively slow reaching 50% after 6 h of incubation. This method of quantification is simple because it requires only measurement of the SD of the fluorescence signal. This means that the recording can be done in principle without any analysis of the autocorrelation curve. Therefore, we expect that this method could be further extended to basic confocal microscopy, where the release kinetics could be directly followed by recording signal fluctuations in the focal volume.

**MATERIALS AND METHODS**

**Materials.** All chemicals and solvents for synthesis were from Sigma-Aldrich. Labrafac WL (medium chain triglycerides) by Gattefossé (Saint-Priest, France). Kolliphor HS15 (non-ionic surfactant) was obtained from BASF (Ludwigshafen, Germany). Ultrapure water was obtained using a Milli-Q filtration system (Millipore, Saint-Quentin-en-Yvelines, France). FBS was acquired from Lonza (Verviers, Belgium) and Gibco-Invitrogen (Grand Island, USA). Dihexylamino-2-(2-ethyl-hexyloxy)-benzo[a]phenoxazin-5-one (NR668) and 4’-dioctylamino-3-octyloxyflavone (F888) were synthesized as described before.**

**Preparation and Characterization of Lipid Nano-carrier LNCs.** Dye-loaded nanoemulsions were prepared by spontaneous nanoemulsification.** We dissolved the dyes (NR668 or F888) in Labrafac WL (35 mg) at concentrations of 1% by weight (unless indicated). Then, Kolliphor HS15 was added (65 mg), and the mixture was homogenized under magnetic stirring at 90 °C for 5 min. Finally, nanoemulsions were generated by the addition of ultrapure (Milli-Q) water (230 mg) under stirring. Size distribution was determined by DLS using a Zetasizer Nano series DTS 1060 (Malvern Instruments SA). In DLS, measurement statistics by volume was used.

**Fluorescence Spectroscopy.** Fluorescence spectra were recorded on a FluoroMax 4 (Jobin Yvon, HORIBA) spectrofluorometer. Fluorescence emission spectra were performed at room temperature with 520 nm excitation wavelength for NR668 dye and 390 nm for F888 dye. Emission spectra were corrected for lamp fluctuations and for wavelength-dependent response of the detector. All fluorescence measurements were carried out using solutions with absorbance ≤0.1 at the wavelength of excitation.

**FRET-Based Stability Test.** The stability of the dye-loaded LNCs was estimated at three different temperatures 20, 37, 60 °C, by FRET between two encapsulated dyes, 0.5 wt % of F888 (with respect to Labrafac WL) used as energy donor and 0.5 wt % of NR668 used as energy acceptor. The LNCs were diluted 1000 times from the original formulation and incubated in water and 10-fold excess (100-fold dilution from the original formulation) of blank nanocarriers (without encapsulated dyes). The donor in the nanocarriers was excited at 390 nm, and the emission spectra were recorded for both donor and acceptor.

**FCS and Data Analysis.** FCS measurements were performed on a two-photon platform based on an Olympus IX70 inverted microscope, as described previously. Two-photon excitation at 830 nm (10 or 7 mW laser output power) was provided by a mode-locked femtosecond Ti:Sa laser (Tsunami, Spectra Physics) pumped by a solid-state laser (Milennia V, Spectra Physics). The measurements were performed in a 96-well plate (Krystal, Porvair Science) using a 200 mL volume per well at 25 °C. The focal spot was set...
about 20 μm above the bottom of the well. This condition was found to provide an optimal signal/noise ratio and to minimize the probe photobleaching. Emitted fluorescence was filtered using a short-pass filter with a cutoff wavelength of 680 nm (F75-680; AHF). The fluorescence was directed to a fiber-coupled APD (SPCM-AQR-14-FC; PerkinElmer), and the fluorescence fluctuation signals \( F(t) \) were collected directly by a Multiple Tau correlation ALV-5000E correlator (ALV GmbH). The autocorrelation curves were obtained in real time by the correlator with the autocorrelation function \( G(τ) \) (eq 1)

\[
G(τ) = \frac{⟨\delta F(t) × δF(t + τ)⟩}{⟨F(t)⟩^2}
\]

where \( ⟨F(t)⟩ \) is the mean of the fluorescence signal \( F(t) \) and \( τ \) is the lag time. Assuming that LNCs diffuse freely in the excitation volume, each correlation function, \( G(τ) \), was analyzed independently on a homemade MATLAB (v.2010, MathWorks) program 16 using the standard 3D diffusion model with one diffusing species (eq 2), according to Thompson 49

\[
G(τ) = \left( \frac{1}{N} \right) × \left[ \frac{1}{\left( 1 + \frac{τ}{τ_0} \right)} \right] × \left[ \frac{1}{\sqrt{\left( 1 + \frac{τ}{τ_0} × s \right)}} \right]
\]

where \( τ_0 \) is the diffusion time, \( N \) is the mean number of fluorescent species within the two-photon excitation volume, and s is the ratio between the axial and lateral radius of the excitation volume. The excitation volume was calculated with a solution of 6-carboxy-tetramethylrhodamine (TMR, Sigma-Aldrich) at 50 nM in phosphate buffer. The focal volume obtained in these conditions was about 0.34 fL and \( s \) was between 3 and 4 (calibration before each acquisition series).

In parallel, the SD of the fluorescence signal was calculated by eq 3 and the brightness per molecule \( (B) \) with eq 4

\[
SD = \sqrt{\frac{1}{n - 1} \sum_{i=1}^{n} [F(i) - F(τ)]^2}
\]

\[
B = \frac{F(τ)}{N}
\]

with \( n \) scalar observations.

The dye-loaded LNCs used for FCS measurements were diluted 10 000-fold from the originally prepared LNCs. For each measurement, 20 acquisitions of 10 s duration for LNCs (30 s for TMR) were performed, and the results correspond to the median value obtained from these 20 repeats. For each condition, the measurement was made in triplicate.

**Samples for FCS.** The FCS parameters for in situ release quantification were evaluated by measuring SD, \( N \), and \( B \) in two different series of samples. The first one consisted 1 wt % NR668 LNCs and different concentrations of free calcein dyes (0.1, 0.2, 0.5, 1, 2, and 3.6 μM). The second one consisted a fixed concentration of calcein (0.2 μM) and variable concentrations of NR668 in LNCs (0.1, 0.2, 0.4, 0.6, 0.8, and 1 wt %). The SD parameter was calibrated using LNCs in Milli-Q water (without calcein) with different concentrations of encapsulated NR668. The obtained SD values were normalized by dividing by SDo, the SD of control LNCs without leakage (1 wt % NR668 LNCs in Milli-Q water). The SD/SDo ratio was plotted versus the wt % of NR668 in LNCs, and the data were fitted with a linear function \( y = A + B × x \), where \( a = 0.116; b = 0.994; y \) is SD/SDo, and \( x \) is the dye loading in LNCs (wt %).

**Dye-Release Studies by FCS.** Dye-loaded LNCs were diluted 10 000-fold (~2.8 nM) in three different media: pure water, water with blank nanocarriers at 1000-fold dilution (10-fold excess with respect to dye-loaded droplets), and water with 10% of FBS. Then, samples were incubated at three different temperatures 20, 37, and 60 °C, for 0.5, 1, 2, 4, and 6 h. SD was measured as a function of time and then converted into SD/SDo to obtain quantitative data on the dye release.

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**Notes**

The authors declare no competing financial interest.

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