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Application of Time-Resolved Fluorescence for Direct and Continuous Probing of Release from Polymeric Delivery Vehicles

Mathieu L. Viger^a, Wangzhong Sheng^a, Cathryn L. McFearn^a, Mikhail Y. Berezin^b, and Adah Almutairi^a

^aSchool of Pharmacy and Pharmaceutical Sciences, Departments of NanoEngineering and of Materials Science and Engineering, University of California at San Diego, La Jolla, California 92093

^bDepartment of Radiology, Washington University School of Medicine in St. Louis, Missouri 63110

Abstract

Though accurately evaluating the kinetics of release is critical for validating newly designed therapeutic carriers for *in vivo* applications, few methods yet exist for release measurement in real time and without the need for any sample preparation. Many of the current approaches (e.g. chromatographic methods, absorption spectroscopy, or NMR spectroscopy) rely on isolation of the released material from the loaded vehicles, which require additional sample purification and can lead to loss of accuracy when probing fast kinetics of release. In this study we describe the use of time-resolved fluorescence for *in situ* monitoring of small molecule release kinetics from biodegradable polymeric drug delivery systems. This method relies on the observation that fluorescent reporters being released from polymeric drug delivery systems possess distinct excited-state lifetime components, reflecting their different environments in the particle suspensions, i.e., confined in the polymer matrices or free in the aqueous environment. These distinct lifetimes enable real-time quantitative mapping of the relative concentrations of dye in each population to obtain precise and accurate temporal information on the release profile of particular carrier/payload combinations. We found that fluorescence lifetime better distinguishes subtle differences in release profiles, (e.g. differences associated with dye loading) than conventional steady-state fluorescence measurements, which represent the averaged dye behavior over the entire scan. Given the method's applicability to both hydrophobic and hydrophilic cargo, it could be employed to model the release of any drug-carrier combination.

Keywords

drug delivery systems; kinetics of release; real-time monitoring; time-resolved fluorescence; biodegradable polymers

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Corresponding author: aalmutairi@ucsd.edu.

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Supporting Information Supplementary data including microscopic images of the dye-loaded particles, steady-state fluorescence spectra and release profile, as well as extra lifetime data can be found online at:

INTRODUCTION

Biodegradable particles capable of delivering drugs specifically to diseased tissue have the potential to transform therapeutic delivery, increasing the dose reaching the intended target while lowering the exposure of healthy tissue to toxic side effects [1–3]. For that matter, several biodegradable polymeric carriers offer many of the properties that an ideal particle-based therapeutic should exhibit, including biocompatibility, controlled release, high levels of molecular cargo encapsulation, prolonged systemic circulation, size control and degradation into safe small molecules [4–7]. Part of the challenge of engineering biomaterials for therapeutic delivery lies in accurately evaluating the kinetics of release, which is critical for validating the appropriateness of newly designed drug delivery carriers for *in vivo* applications.

Most analytical strategies currently employed to quantify release kinetics do not allow direct real-time monitoring [8–11]. Instead, they require isolation of the released material from the loaded vehicles and quantification by chromatographic methods, absorption spectroscopy, or NMR spectroscopy. Alternatively, aliquots of particles can be collected and dissolved to quantify the remaining material in the carriers. While allowing precise quantification of the release profile, such measurements require tedious purification of the sample to separate the encapsulated material from the released payload using filtration, dialysis or centrifugation. Due to the labor-intensive sample preparation, time intervals between measurements can be quite long, which greatly limits the number of data points and therefore accuracy when probing fast kinetics associated with rapidly degrading particles. Therefore, direct and continuous real-time monitoring of release from therapeutic carriers is highly desirable for reliable quantification of the dynamics of release.

Direct evaluation of delivery kinetics represents a challenge as it requires sensitive methods capable of differentiating between encapsulated and released materials [12–17]. One of the most common approach to this challenge is to encapsulate solvatochromic fluorophores, whose emission spectra vary depending on the polarity of their environment [18], and measure a steady-state decrease in fluorescent intensity and/or a positive solvatochromic shift in the emission spectrum as they are released into an aqueous environment [19, 20]. While steady-state fluorescence measurements, usually of the hydrophobic dyes Nile Red or Coumarin 153 (C153), are quite straightforward and allow use of readily available conventional instrumentation, they depend strongly on fluorophore concentration, photobleaching, and scattering, the latter being especially problematic given the high turbidity of particle suspensions [21]. In contrast, the excited state decay of fluorophores is generally immune to these parameters and responds to its local environment with the same inherent sensitivity of steady-state fluorescence [22]. Additionally, more information about the molecular environment can be gained from a single experiment exploiting the time-dependent nature of fluorescence, since with fluorescence lifetime one can simultaneously and independently investigate different chemical surroundings of the fluorophore [23–26].

Herein, the release of fluorescent compounds from polymeric carriers was studied in real time by time-resolved fluorescence. This method relies on the observation that the lifetimes of fluorophores being released from polymeric drug delivery systems exhibit multi-exponential decays with distinct excited-state lifetime components, reflecting their different environments in the particle suspensions, i.e., confined in the polymer matrices or free in the aqueous environment. Poly(lactic-co-glycolic acid) (PLGA) polymeric particles were chosen as delivery vehicles since PLGA is a key component of FDA-approved particle formulations for sustained drug delivery due to its desirable mechanical properties, low toxicity (both in polymer and hydrolyzed form), biocompatibility, and predictable biodegradation kinetics [27, 28]. Since drug delivery systems involve delivery of both hydrophilic (e.g. cisplatin,

doxorubicin) and hydrophobic (e.g. paclitaxel, rapamycin) therapeutics, we loaded the PLGA particles with varying concentrations of the hydrophilic model drug fluorescein and hydrophobic model drug C153, which yielded different release profiles associated with the differences in dye polarity and concentration. In addition, steady-state fluorescence was used in parallel to track release of cargo from the drug delivery system and provide additional information to validate the fluorescence lifetime data, as well as to set a useful background for understanding the limits of intensity-based measurements.

MATERIAL AND METHODS

Materials

Poly(lactic-*co*-glycolic acid) (PLGA, 50:50, ester terminated, MW 7–17kDa), Coumarin 153 (C153, 99%, Sigma-Aldrich), fluorescein (99%, Sigma-Aldrich), dichloromethane (DCM, A.C.S. grade), dimethylformamide (DMF, A.C.S. grade), chloroform (CHCl₃, A.C.S. grade), methanol (MeOH, A.C.S. grade), acetone (A.C.S. grade), dimethyl sulfoxide (DMSO, A.C.S. grade) were purchased from commercial sources and used as received. De-ionized water (DI H₂O) was purified from a Millipore system (18.2 M Ω). Phosphate buffered saline (PBS) pH 7.4 was purchased from Life Technologies. Solvents were not degassed.

Particle preparation and characterization

PLGA polymer capsules encapsulating varying amount of fluorophores (C153 or fluorescein) were obtained by electrospray. In this particle formulation method, the applied voltage injects charges into a solution solubilizing the polymer and the payload to produce an aerosol of dense, solid particles collected on a flat surface, typically a standard glass microscope slide [29]. Particle size and morphology can be controlled by the composition of the solvent solution, electrospray distance, voltage, and solution injection rate [30]. Briefly, 100 mg of PLGA was added to 0.75 mL of CHCl₃ and dissolved at 40 °C. Similarly, varying amounts of fluorophores (payload) were added to 0.25 mL of DMF and solubilized at 40 °C. The polymer and payload solutions were combined to create a 3:1 CHCl₃:DMF solvent containing 0.01 %, 0.1 %, and 1.0 % w/w fluorophore (allowing assessment of concentration effects on release kinetics). Particles were initially electrosprayed at 0.5 mL/hr flow rate, 25 kV and 25 cm collection distance. Following microscopy (Nikon Eclipse LV100), parameters were slightly adjusted to produce spherical particles of similar shape and size for both fluorescent delivery systems ($1.6 \pm 0.3 \mu\text{m}$). A 8500 Bench-top FE-SEM (Agilent Technologies) was used to obtain scanning electron micrographs (SEM). Briefly, particles were sprayed onto 1 cm x 1 cm silicon wafers, gold coated using a Sputter Coater 108 Auto (Cresington Sci.) and imaged with a 1000 V beam. Characteristic SEMs of the electrosprayed fluorescein- and C153-loaded PLGA particles are shown in Figure S1A and Figure S1B, respectively. Optical epifluorescence photographs show good encapsulation efficiency with no evidence of dye leaking (fluorescein, Figure S1C; C153, Figure S1D).

Particles were collected and purified as follows. A glass slide containing microparticles was placed in 50 mL centrifuge tubes, immersed in PBS, and sonicated (Branson 2510) for approximately one minute to free the particles from the glass slides. Residual particles were scraped from the glass slide using a flat spatula. The solution was centrifuged at 4500 RPM (Beckman-Coulter, Allegra X-15R) for 10 min at 20 °C to obtain a particle pellet. The supernatant was removed and the pellet (~2 mg/slide) was re-suspended in 5 mL of fresh PBS (0.4 mg/mL). This process was repeated three times. The glass slide was weighed before and after removal of particles to calculate the mass of particles collected. Dye-loading was determined from UV-Vis spectroscopy using a dual beam UV-Vis-NIR Spectrophotometer (UV-3600, Shimadzu). All spectra were acquired at room temperature. Standard curves were obtained by dissolving different concentrations of fluorescein in DI

H₂O and C153 in CHCl₃. Concentration versus peak absorbance was plotted to produce a linear calibration curve. C153-loaded PLGA particles (5 mL, 0.4 mg/mL) were completely solubilized in 50 mL CHCl₃, whereas fluorescein-loaded PLGA particles were solubilized in 0.5 mL of CHCl₃ first and then diluted in DI H₂O to a volume of 50 mL. The UV-Vis spectra of the sample were acquired and the absorption peak intensity was fitted to the standard curve to determine the amount of payload encapsulated.

Steady-state and time-resolved fluorescence measurements

Fluorescence spectra were recorded using a Fluorolog-3 (Horiba Jobin Yvon) equipped with a photomultiplier tube (PMT, Hamamatsu R928) for detection and a xenon arc lamp (Ushio Inc.) for excitation. Fluorescence lifetime was measured using a time-correlated single photon counting (TCSPC) system (Horiba) equipped with a NanoLED excitation source (488 nm, 1 MHz impulse repetition rate, Horiba) and a R928P detector (Hamamatsu Photonics, Japan). The detector was set to 520 and 540 nm for detection of fluorescein and Coumarin 153 (C153), respectively. The slit width was varied between 2 and 20 nm to achieve an appropriate count rate. The instrument response function (IRF) was obtained by using a scattering solution of Ludox-40 (Sigma-Aldrich) in water (prompt) at 480 nm emission. Both samples and the prompt were measured in a semi-micro quartz cuvette. The lifetime was recorded on a 450 ns scale. A total of 4094 channels were used with a time calibration of 0.110 channel/ns. Except when taken in pure solvent (one exponential), all decay curves were fit to a sum of three exponentials. The fluorescence lifetimes were extracted using DAS6 v6.6 decay analysis software (Horiba). The goodness of fit was judged by χ^2 values, Durbin-Watson parameters, and visual observation of the fit line and residuals, which should be distributed randomly about zero. The average lifetime (τ_{AVE}) was calculated according to $\tau_{AVE} = \sum f_i \tau_i$, where f_i is the fractional contribution of the individual lifetime of each component (τ_i). In all cases, lifetime components with insignificant fractional contributions ($f_i < 3\%$) were discarded and decays were considered to be mono- or two-exponential if the sum of the fractional contributions (f_1 or $f_1 + f_2$) was higher than 90 %.

Release Experiments

Fluorescein release was induced by incubation of the particles in PBS (1X, pH=7.4) at 37 °C using a thermostatted multicell holder (Horiba) connected to a heated bath circulator (RTE 7, Thermo Scientific). Because of its more hydrophobic nature and higher affinity to the PLGA matrix, release of C153 from the PLGA particles was achieved through dissolution of the PLGA network in basic conditions (NaOH, pH = 14) at 65 °C. Aliquots were collected at precise time intervals and *in situ* steady-state fluorescence spectra and fluorescence decay curves were acquired. The aliquots containing C153 were adjusted to pH 7.4 using KH₂PO₄ prior to any measurements. For all release experiments, readings were done on 500 μ L aliquots of dye-doped PLGA polymer particle suspensions (0.2 mg/mL) in micro quartz cuvettes.

Release kinetics modelling

Two phase decay modelling, implemented in data analysis software Prism 5 (GraphPad Software Inc., La Jolla, CA) was applied to the steady-state fluorescence dataset (C153, fluorescein) obtained at different time points. In this model the outcome is assumed to be the result of the sum of a fast and slow exponential decay according to the set of following equations:

$$Y = \tau_{\infty} + \left(\text{SpanFast} \times e^{-K_{\text{fast}}t} \right) + \left(\text{SpanSlow} \times e^{-K_{\text{slow}}t} \right) \quad (1)$$

$$\text{SpanFast} = (\tau_0 - \tau_\infty) \times (\text{PercentFast}) / 100 \quad (2)$$

$$\text{SpanSlow} = (\tau_0 - \tau_\infty) \times (100 - \text{PercentFast}) / 100 \quad (3)$$

where τ_0 is the value of the fluorescence when time is zero, τ_∞ is the fluorescence at infinite time, K_{fast} and K_{slow} are the two rate constants, all expressed in min^{-1} , and PercentFast, which is the fraction of the span (from τ_0 to τ_∞) accounted for by the faster of the two components. The result of this model provides two time constants t_{fast} and t_{slow} where:

$$t_{\text{fast}} = 1/K_{\text{fast}} \quad (4)$$

and

$$t_{\text{slow}} = 1/K_{\text{slow}} \quad (5)$$

Half-life (fast) and Half-life (slow) are computed as $\ln(2)/K_{\text{fast}}$ and $\ln(2)/K_{\text{slow}}$, respectively.

RESULTS

Steady-state fluorescence does not reflect the state of the cargo

The release of an encapsulated material from PLGA carriers may involve either or both of two processes: diffusion out of the polymer matrix and degradation of the matrix through hydrolysis of its ester linkages [31]. The contribution of these two components to the release from PLGA carriers depends strongly on the polarity of the cargo. In an aqueous environment, hydrophilic compounds with high water solubility, such as fluorescein, will diffuse rapidly out of the matrix, while hydrophobic compounds with higher affinity to the hydrophobic PLGA carrier, such as C153, will show limited diffusion, as well as a release profile that matches closely with the degradation rate of the polymer matrix [32].

A steady-state fluorescence profile was first obtained for the release of C153. As stated in the methods section, because of the high affinity of the hydrophobic fluorescent guest molecule to the hydrophobic host matrix and the slow degradation of PLGA, simply incubating the particles at 37 °C did not allow measurement of release within hours. To achieve faster degradation of the PLGA particles and, hence, faster release, C153-loaded particles were incubated in a pH 14 environment at 65 °C. Under these conditions, complete particle degradation could be obtained in 2–3 hours, which could be assessed qualitatively as the solution changed from cloudy to clear. Fluorescence intensity of the suspension was recorded over time and revealed a fast quenching of C153 fluorescence (Figure 1A). This decrease in intensity can be explained by the hydrophobic nature of C153. In a non-polar environment, e.g. inside the hydrophobic carriers, C153's fluorescent intensity is much greater than when it is in a polar solvent, where it has a very low fluorescence intensity (see also Figure 1A) [25]. Interestingly, no trend in kinetics of release related to differences in dye loading could be observed as all the release profiles seemed to display very similar rate constants. A concentration-dependence would have been expected since hydrophobic compounds have been shown to hinder diffusion of water molecules in PLGA matrices, a rate-limiting step for hydrolysis [32, 33]. The degradation of PLGA polymer is the collective process of bulk erosion and surface erosion. In systems where the diffusivity of water into the matrix is high, faster bulk erosion dominates, whereas in systems where the diffusion of water into the particles is low, slower surface erosion prevails. Different concentration of encapsulated hydrophobic materials should have a corresponding impact on the amount of water associated with the polymer particles. One must note that the release kinetics for C153

could not be fitted adequately with the two phase decay modeling process, therefore no quantification of the rate of release could be provided.

Incubation of the fluorescein-loaded particle suspensions at 37 °C was sufficient to activate fast diffusion of the payload out of the particles. The rate of release of fluorescein from the PLGA particles was monitored by steady-state fluorescence. Fluorescence intensity, for all three concentrations, followed a first-order kinetic, also known as a burst release profile [34], indicating a release process that starts fast and progressively slows down as the amount of material available for release is reduced (Figure 1B). Fluorescein's emission intensity increased upon release because of its hydrophilic nature, causing higher fluorescence intensity in polar environments (see also Figure 1B) [21]. Since hydrolytic degradation of PLGA polymers progresses very slowly, the process of release in this case relies entirely on diffusion out of the polymer matrix, with degradation-induced release being almost non-existent in this short time frame. Also, the recorded fluorescence (Figure 1B) showed that the relative emission intensity at any given time point reaches a maximum value for the fluorescein-loaded particles with the highest initial dye loading (0.3 % w/w), while lower encapsulated dye concentrations (0.05 and 0.008 % w/w) led to lower fluorescein fluorescence intensity; a typical manifestation of the concentration dependence of the fluorescence signal, meaning that a higher cargo loading leads to a higher amount of released material. Interestingly, one could hypothetically appreciate differences in release kinetics. In the opposite way of C153, hydrophilic content facilitates water penetration in polymeric carriers and leads to the creation of more porous and swelled polymer networks [35]. Since diffusion is a function of the water concentration, so that the release behavior is intimately related to the swelling, the rate of release and the importance of the burst release should correlate with the amount of encapsulated fluorescein. When modeling the release kinetics, both a fast and a slow kinetic could be detected, with the fast kinetic being 10 times that of the slow kinetic. The goodness of the fit was impeccable for all three concentrations with R^2 values very close to 1. However, as seen on Figure S2, the rate constants were found to be independent of the dye-loading concentration since no statistically significant differences were found.

Time-resolved fluorescence provides valuable information on cargo environment

C153, a member of the 7-aminocoumarin dye family, displays useful solvatochromism properties due to its large positive change in dipole moment [26]. This can be first appreciated by examining fluorescence emission spectra for C153 in solvents of varying polarities (Figure S3), where the fluorescence emission at the wavelength of maximum intensity shifts progressively to longer wavelengths as the surrounding solvent goes from relatively non-polar (DCM) to polar (PBS). Fluorescence decay curves for C153 in the different solvents also reflect the sensitivity of the dye to the environment; the lifetime of C153 decreased from 5.78 to 1.65 ns as the polarity of the solvent was increased (Table S1). Our observation of shorter lifetimes in more polar environments is in good agreement with previously published results [25, 26]. In contrast, fluorescein did not show a measureable sensitivity to its surroundings and no conclusive trend could be extracted from the same type of experiments (see Figure S4 for the emission spectra and Table S2 for emission maxima and lifetime values).

Fluorescence lifetime of C153 during release from PLGA particles was monitored by recording fluorescence decays over time under the same conditions (pH, temperature) used for the steady-state fluorescence experiments. From the fluorescence decays, two major time components, whose values remain stable over time, could be extracted. As C153 is a solvatochromic dye, these components may be attributed to interactions with environments of distinct polarity. The first component from the fit, τ_1 , has a long lifetime with a value around 5.4 ns, suggesting a hydrophobic environment surrounding C153 (Table S1).

Therefore, τ_1 was assigned as the dye-PLGA interaction. The shorter time constant, $\tau_2 = 1.6$ ns, could be attributed to the interaction between the dye molecules and water, as this value matches that for C153 in PBS (Table S1). The evolution of the corresponding fractional contributions of these constants through the degradation process is plotted in Figure 2. The fractional contribution of τ_1 decreased quickly in the first 30 min from 91 % to 35 %, followed by a slower decrease over the remaining time period, reaching 28 % after 125 min (Figure 2, black squares). This decrease was related to the progressive loss of hydrophobic interactions as the carriers were degrading. Not surprisingly, a matching increase in fractional contribution of τ_2 was observed (Figure 2, red circles), from 9 % to 63 % in the first 30 min and from 65 % to 68 % in the next 95 min. This behavior reinforces the hypothesis that more C153 resides in a hydrophilic environment as the degradation of the polymer matrix progresses. The amount of probe released from the particles or remaining in the particles at different time points can be easily obtained by multiplying the varying C153-water contribution (τ_1) or the C153-polymer contribution (τ_2), respectively, by the initial dye concentration found in the particles, which was previously quantified by steady-state fluorescence (see Materials and Methods for experimental details). Changes in concentration of encapsulated (μg of fluorescein/mg of PLGA) and released (mol of fluorescein/L of solution) payload, derived from Figure 2, can be found in Figure S5. Interestingly, since lifetime is an absolute measure, it is not necessary to compare its value with those of a set of samples of known concentrations to quantify the amount of payload released, as would be the case with steady-state fluorescence. Also, because of the drastic and rapid quenching of C153 fluorescence due to hydrophobic effects (Figure 1A), direct quantification of the amount of released materials using steady-state measurements would not be possible.

Examination of the fractional contribution of the dye-water component (τ_2) at time zero reveals useful information about dye distribution within particles and particle hydration. If the C153 dye was perfectly shielded from the surrounding aqueous environment when encapsulated in the particles, one could expect the initial fractional contribution of the dye-water component (τ_2) to be zero, but the contribution we consistently observed was ~10%. This indicates that a portion of the C153 dye is in contact with the aqueous environment immediately upon addition of the buffer solution, prior to elevating the pH to 14 to initiate degradation of the polymer matrix. Increasing the number of washes of the particles did not cause the contribution to decrease, so it likely did not result from non-encapsulated C153 dye. We hypothesized that the origin of this observation is twofold. First, since C153 molecules are located throughout the whole polymer carrier, some C153 molecules located on the surface of the particles with a direct interaction with the aqueous environment are likely to be present. Second, physicochemical characterizations of PLGA have shown that the first stage of the hydrolytic degradation process involves swelling, water uptake, and formation of domains of water in the hydrophobic structure as soon as dry particles are dispersed in water, which could equally contribute to a non-zero fractional contribution of τ_2 at $t = 0$ (Figure 2, red circles) [27, 36]. Since only averaged behavior can be extracted from steady-state fluorescence spectra, such fine information is generally lost as its contribution is buried under the overall readout signal.

In order to generate a single curve characterizing the kinetics of release, the average lifetime values may be calculated at each time point from the components of each decay curves (Figure 3). The overall lifetime for the three samples of different C153 concentrations decreases over time, with a faster decrease in the first 30 min, often referred to as burst release, and a more sustained decrease afterwards (see also Figure S6 for decay curves). Burst release has been observed in previous studies and is attributed, among other things, to a dramatic initial molecular weight loss due to rapid cleavage of particularly accessible surface bonds.[36] Figure 3 also reveals that the kinetics of release depends on loading density: the rate of release decreased as the hydrophobic cargo concentration was increased.

In other words, the degradation rate was highest for the lowest C153-loading and vice versa. As discussed above, hydrophobic payloads have been shown to impede water diffusion in PLGA matrices and influence the rate of hydrolysis. Thus, as the concentration of hydrophobic C153 in the carriers increases, the availability of water molecules decreases and degradation slows down (Figure 3).

Similarly to C153, two major time components could be extracted from the fluorescence decay curves recorded for the release experiments involving fluorescein (Figure 4; see also Figure S7 decay curves). For fluorescein, the first time constant, τ_1 , is very short and increases in value from 0.33 to 0.87 ns and decreases in fractional contribution from 23 to 2 % (Figure 4A) as fluorescein is progressively released from the PLGA particles. This short lifetime component is a clear manifestation of homotransfer, energy transfer between close pairs of identical fluorophores (generally < 10 nm apart) [21, 37]. Typically, such transfer is ubiquitous for fluorophores like fluorescein that feature small Stokes shifts and hence high spectral overlap between their excitation and emission spectra. The amplitude of this phenomenon is concentration-dependent, so the increase in lifetime of the fast component likely occurs because the fluorescein concentration inside the polymeric vehicles decreases over time due to diffusion into the aqueous environment. This results in an increase in the intermolecular distance, which weakens the interactions between the dye molecules and lowers the probability of homotransfer. The second time component, τ_2 , has a much longer excited-state lifetime which decreases very rapidly from 6.0 to 4.0 ns, with its fractional contribution increasing from 49 % to 98 % (Figure 4B). The initial high lifetime value measured prior to degradation at $t = 0$ ($\tau_2 = 6.0$ ns) may be attributed to an apparent lengthening of the lifetime resulting from reabsorption of photons which depends on the overlap between the emission and absorption spectra, as well as the concentration of the sample [22]. This effect of the release on the lifetime was found to be lost in the first 15 min of the fluorescein release experiment as the dye molecules escaped from the matrix. This is corroborated by a rapid equilibration of the fluorescence lifetime to a value of 4.0, the well-established value for diluted fluorescein in aqueous solutions (see Figure 4B and Table S2).

Changes in the average lifetime of PLGA particles containing three different concentrations of fluorescein are compared in Figure 5. The variation between the average lifetime profiles clearly demonstrates concentration dependence. The importance of the burst release could be evaluated by analysing the speed at which the average excited-state lifetime increases. While all profiles rapidly equilibrate to a value close to what corresponds to free fluorescein molecules in water, i.e., ~ 4.0 ns, as fluorescein diffuses out of PLGA, this occurs over varying periods of time. For a concentration of 0.008 and 0.05 % w/w, the average fluorescein lifetime value in water was found to reach a 100 % water interaction value after 15 and 5 min, respectively (Figure 5, black squares; Figure 5, red circles). For the highest fluorescein loading (0.3 % w/w), the magnitude of the burst release, and the resulting high concentration of fluorescein instantaneously discharged in the aqueous environment upon dispersion of the particles in water, was so significant that the average lifetime value was entirely dominated by the fractional contribution of fluorescein in water even at $t = 0$ (Figure 5, blue triangles). While valuable insights on the mechanism of release could be extracted from these experiments, the overwhelming contribution of the burst release at $t = 0$ resulted in non-reliable release kinetics for fluorescein. This also clearly limits the direct quantification of the amount of released/encapsulated payload using the fractional contributions of the lifetime components. Hence, the search for a probe with more linear fluorescence lifetime response covering a larger range of concentrations presents an exciting direction and logical continuation of this work.

Time-resolved fluorescence provides additional information that cannot be gathered from the standard intensity-based release profiles (Figure 1), simultaneously revealing details

regarding multiple processes on a molecular level. Steady-state fluorescence experiments provide more limited information since a plot of fluorescence intensity versus wavelength represents the averaged behavior of what occurs during the entire scan, whereas the measure of the time a molecule spend in the excited before relaxing back to the ground state gives an absolute measure and provides a dynamic picture of the fluorescence, regardless whether the dye used is solvatochromic or not. For C153, steady-state fluorescence measurements showed a very drastic (~80 %) fluorescence quenching in 5 min, which could be interpreted as if 80 % of the payload was released in the first 5 min. However, we have observed much more progressive changes in lifetime suggesting longer lasting hydrophobic interactions between C153 and the polymer matrix, with a 30 % fractional contribution still remaining after 2 hours of degradation. Furthermore, only when using fluorescence lifetime were C153-loading dependent differences in the kinetics of release and degradation observed. Using time-resolved fluorescence, we could bring to light dynamic spectrophysical phenomena before and during the diffusion process (i.e., homotransfer and reabsorption effects), as well as variability in the burst release related to dye loading. However, the overwhelming contribution of the burst release at $t = 0$ limited the reliability of the release kinetics for high concentrations of fluorescein obtained using lifetime measurements.

CONCLUSION

With increasing interest in the development and application of polymeric particles as delivery vehicles for therapeutic and diagnostic purposes, direct and real-time methods to characterize release and gather information on the encapsulated molecules' environment will become increasingly important. We have shown that with the appropriate probe fluorescence lifetime measurements are suitable for *in situ* determination of small molecule release kinetics from polymeric drug delivery systems. By simultaneously following specific lifetime parameters indicative of dye-carrier, dye-water, or dye-dye interactions over time, we could distinguish subtle differences in kinetic profiles associated with differences in dye loading, which was impossible using conventional steady-state fluorescence experiments. Lifetime measurements were taken directly from the particle suspensions without any additional treatment (e.g. dialysis, centrifugation). The ease of data acquisition and analysis using such an accessible method provides a simple route for lifetime measurements to become commonly used in the drug delivery field. Given the abundance of fluorophores available with a variety of spectral properties and polarities, release kinetics for a specific polymer-drug combination can be extrapolated from fluorescence lifetime measurements using a dye of equivalent polarity and size. Fluorescence lifetime measurements could also be extended beyond small molecules to release of fluorescently labeled proteins to examine delivery systems for protein therapeutics as well. Furthermore, advances in fluorescence lifetime imaging microscopy (FLIM) will extend release studies to cellular *in vitro* experiments.[38] With the availability of whole animal fluorescence lifetime imagers, release kinetics may eventually be measurable *in vivo*.[39]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Steady-state fluorescence release kinetics for (A) Coumarin 153- and (B) fluorescein-loaded PLGA particles.

Figure 2.

Fractional contribution of each lifetime component for C153 (C153-PLGA interaction, black squares; C153-water interaction, red circles) over time as it is released from PLGA particles.

C153-PLGA = 5.4 ns, C153-water = 1.6 ns. [C153]_{PLGA} = 0.2 % w/w.

Figure 3.

Change of the average lifetime of C153 encapsulated as function of incubation time reflects the release of the dye from PLGA particles. Dye loading: 0.001 % w/w (black squares), 0.03 % w/w (red circles), and 0.2 % w/w (blue triangles).

Figure 4. Evolution of the (A) τ_1 and (B) τ_2 lifetime component of fluorescein as it is released from PLGA particles. $[\text{Fluorescein}]_{\text{PLGA}} = 0.008\% \text{ w/w}$.

Figure 5. Changes in average lifetime of fluorescein, encapsulated at 0.008 % w/w (black squares), 0.05 % w/w (red circles) and 0.3 % w/w (blue triangles), as it is released from PLGA.