

# Fast Release of Lipophilic Agents from Circulating PEG-PDLLA Micelles Revealed by *in Vivo* Förster Resonance Energy Transfer Imaging

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Understanding the *in vivo* behavior of nanoparticles is critical for the translation of nanomedicine from laboratory research to clinical trials. In this work, *in vivo* Förster resonance energy transfer (FRET) imaging was employed to monitor the release of hydrophobic molecules from circulating poly(ethylene glycol)-poly(D,L-lactic acid) (PEG-PDLLA) micelles. A lipophilic FRET pair (DiIC<sub>18</sub> and DiOC<sub>18</sub>) was physically entrapped into micelle cores by mimicking the loading of hydrophobic drugs. The FRET efficiency was found significantly reduced within 15 min after intravenous injection, implying that DiIC<sub>18</sub> and DiOC<sub>18</sub> quickly escaped from the circulating micelles. FRET spectroscopy studies further demonstrated that  $\alpha$ - and  $\beta$ -globulins were major factors for the observed fast release, while  $\gamma$ -globulins, albumin, and red blood cells played minor roles. These results provide useful information for developing blood-stable micelles to deliver hydrophobic drugs to the target site via prolonged circulation and extravasation from the vascular system.

## Introduction

Block copolymer micelles<sup>1–5</sup> have been extensively used as vehicles for delivering hydrophobic drugs<sup>6,7</sup> and nucleic acids<sup>8,9</sup> to the target sites. Among the widely studied micelles, PEG-polyester micelles are self-assemblies of amphiphilic copolymers and possess the core-shell architecture with a diameter between 20 and 200 nm.<sup>10</sup> Their hydrophobic cores accommodate drugs with a high loading efficiency, and their brush-like PEG coronas ensure long circulation of micelles *in vivo*. Such a construct not only improves the aqueous solubility of hydrophobic drugs but also circumvents fast elimination induced by host defenses.

As drug delivery vehicles, micelles are expected to alter the pharmacokinetics and biodistribution of drug molecules based on the enhanced permeability and retention effect in tumor therapy.<sup>11</sup> In essence, the pharmacokinetics and biodistribution of drug molecules follow those of micelles only if the drug can

be stably maintained inside micelles. Under such a condition, core-loaded molecules can be released in a controlled manner at target sites over an extended period of time.<sup>12</sup> However, the drug release from micellar systems in blood circulation and the interaction between micelles and blood components remained unclear, and most notably, not much *in vivo* data have been available.<sup>13</sup> In a recent study, Savic et al. showed the loss of integrity of poly( $\epsilon$ -caprolactone)-*b*-poly(ethylene oxide) (PCL-PEO) micelles 1 h after intramuscular or subcutaneous injection.<sup>14</sup> In another study to investigate the biodistribution of paclitaxel delivered by monomethoxy poly(ethylene glycol)-*block*-poly(D,L-lactide) (PEG-PDLLA) micelles,<sup>15</sup> it was found that the distribution of tritium-labeled paclitaxel was different from <sup>14</sup>C-labeled copolymers only 5 min after intravenous administration of paclitaxel-loaded PEG-PDLLA micelles in rats. Tritium radioactivity was observed at a lower level in plasma than <sup>14</sup>C radioactivity, while it was more evenly distributed in the tissues than <sup>14</sup>C radioactivity. These results indicate that paclitaxel was rapidly dissociated from the micelles *in vivo*. However, the mechanism for this fast release of core-loaded molecules from micelles in bloodstream remained elusive.

In this paper, we present an *in vivo* imaging study that explores the release of core-loaded molecules from micelles in circulation. Instead of using a single fluorophore, we loaded a Förster resonance energy transfer (FRET) pair, DiIC<sub>18</sub>(3), a red-orange fluorescent lipophilic probe as an acceptor, and DiOC<sub>18</sub>(3), a green fluorescent lipophilic probe as a donor (referred to as DiI and DiO, respectively, in the rest of the paper) into self-assembled PEG-PDLLA micelles (Figure 1). Because of the close proximity of the dye molecules, an efficient FRET effect was observed.

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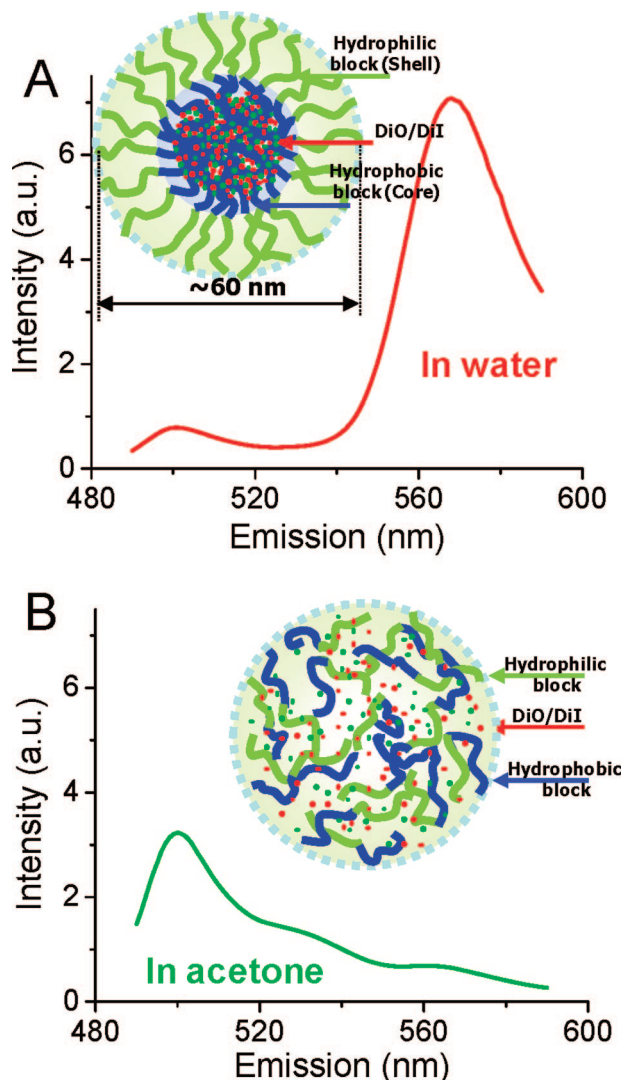
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**Figure 1.** Fluorescence spectra of PEG-PDLLA FRET micelles diluted by  $10\times$  water (A) and  $10\times$  acetone (B). Diagram of a FRET micelle prepared with 0.75% DiO and 0.75% DiI at 2 mg/mL polymer concentration is shown as the inset in A.

Release of core-loaded molecules led to an increase of the distance between the fluorescent molecules, and thus a reduction in FRET efficiency. This design allowed us to directly monitor the release of DiI and DiO from micelles under different conditions, as detailed below.

### Materials and Methods

**Materials.** Bovine serum albumin,  $\alpha$ - and  $\beta$ -predominant globulins, and  $\gamma$ -globulins were purchased from Sigma-Aldrich (St. Louis, MO). DiI and DiO were purchased from Invitrogen (Carlsbad, CA).

**Micelle Preparation.** Synthesis of block copolymers is described in the Supporting Information. FRET micelles were prepared by the precipitation and membrane dialysis method.<sup>16</sup> 100 mg PEG-PDLLA, 0.75 mg DiO, and 0.75 mg DiI were dissolved in 5 mL acetone. After stirring for 30 min, the solution was dropped into 50 mL deionized water at the speed of 2 mL/min and followed by 3 h stirring to vaporize acetone. The aqueous solution was then dialyzed against 2 L deionized water (Spectra/Por MWCO 3500) for 48 h. The water was changed after 24 h. Finally, the solution was filtered

through a  $0.45\ \mu\text{m}$  filter to remove undesirable aggregates and stored at  $4\ ^\circ\text{C}$ . The polymer concentration in the filtered solution was measured after freeze-drying the solution. The final micelle concentration was found to be  $0.63\ \text{mg/mL}$ . The size of the micelles was measured by dynamic light scattering at  $23\ ^\circ\text{C}$ , using a DynaPro99 molecular-sizing instrument equipped with a microsampler (Protein Solutions, Inc.). The CORAN software was used for data analysis. The critical micelle concentration (cmc) was measured to be  $1.37\ \mu\text{g/mL}$  by monitoring the fluorescence emission spectrum of pyrene.<sup>17</sup>

**Animal Study.** All animal studies were approved by Purdue Animal Care and Use Committee (PACUC). Three 4- to 6-week-old Balb/C mice ( $\sim 17\ \text{g}$ ) were anesthetized by intraperitoneal injection of avertin ( $500\ \text{mg/kg}$ ) before administering  $100\ \mu\text{L}$  of FRET micelles via tail vein injection. According to the body weight, the blood volume of a mouse is  $\sim 1.7\ \text{mL}$ , and thus the micelle concentration in blood was estimated to be  $38\ \mu\text{g/mL}$ . For *in vivo* imaging of FRET micelles, a mouse ear was placed onto a glass bottom culture dish using glycerol as an interstitial medium to reduce refractive index mismatch.

**Red Blood Cells (RBCs).** Blood was extracted from an anesthetized mouse in a heparinized tube by paraorbital bleeding (PACUC protocol # 96-069-04). RBCs were isolated, washed, and resuspended at a 0.3% hematocrit in PBS. The RBCs were then incubated with  $63\ \mu\text{g/mL}$  FRET micelles at  $37\ ^\circ\text{C}$  for different time periods before imaging as indicated.

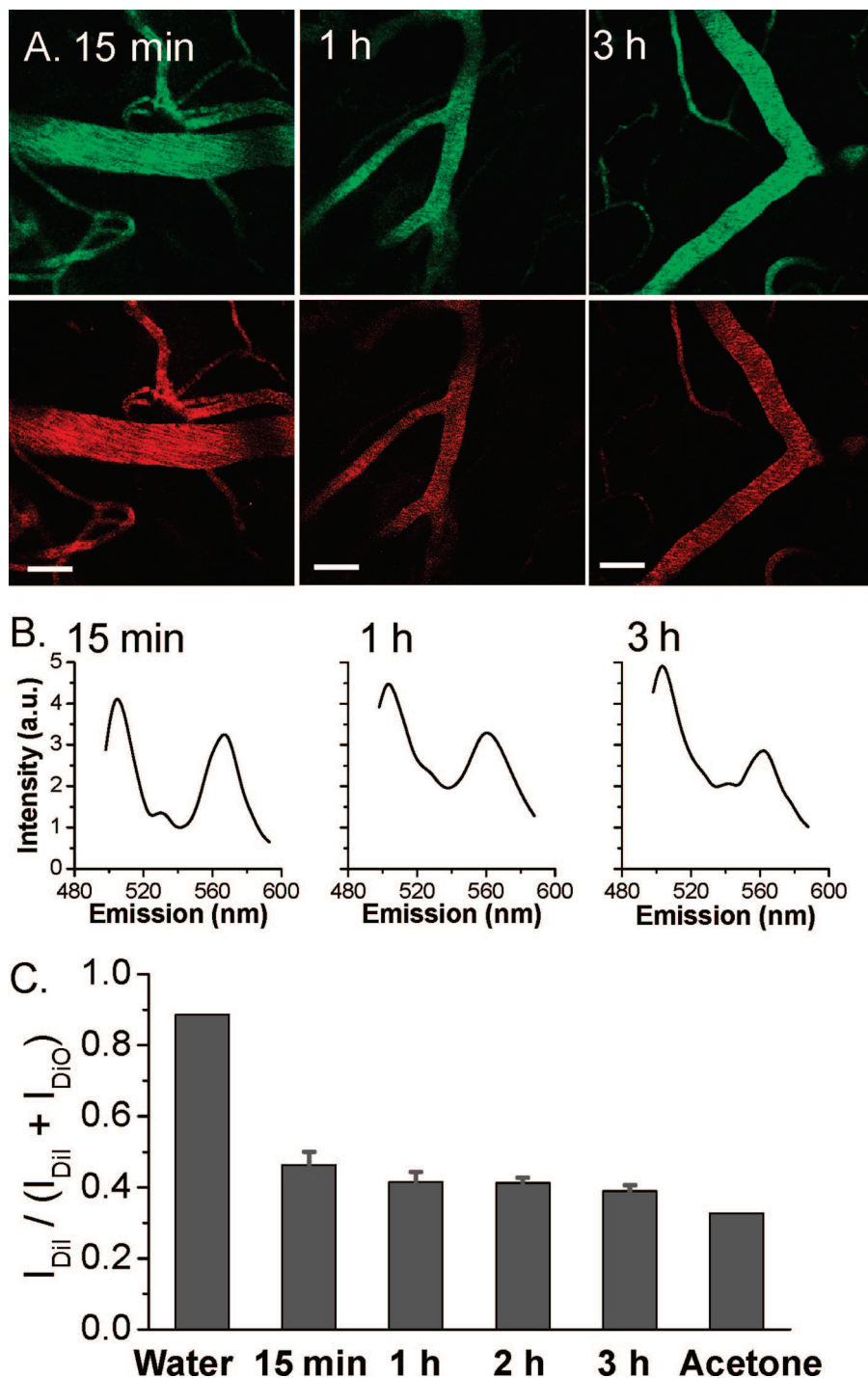
**FRET Imaging and Spectroscopy.** Both *in vitro* and *in vivo* imaging were performed on a FV1000 confocal system (Olympus, Tokyo, Japan). A  $40\times$  water-immersion objective with a working distance of 3.3 mm was used to image the ear vasculature. A 488 nm  $\text{Ar}^+$  laser was used to excite DiO. The spectral filters were tuned to 500–530 nm for DiO detection and 555–655 nm for DiI detection, respectively. Microspectroscopy at pixels of interest was carried out using a spectral detector with emission scan from 495 to 595 nm. To remove signal variations caused by different vessel depths, the *in vivo* fluorescence intensities of FRET micelles were rescaled into the same range for easy comparison of spectral profile. Fluorescence spectra ranging from 490 to 590 nm of FRET micelles in different solutions were obtained on an AMINCO-Bowman series 2 Luminescence spectrometer (SLM Aminco Bowman, Urbana, IL) with 484 nm excitation at room temperature.

### Results and Discussion

FRET micelles (the inset of Figure 1A) formulated by loading 0.75% DiI and 0.75% DiO (see Materials and Methods) were used to study the release of core-loaded molecules from micelles *in vitro* and *in vivo*. These micelles with an average diameter of 60 nm were stable for more than 3 months. The occurrence of FRET was confirmed by fluorescence spectra of  $63\ \mu\text{g/mL}$  micelles in deionized water or acetone measured with 484 nm excitation. An efficient FRET was observed (Figure 1A) from micelles in deionized water due to the distance proximity of DiI and DiO inside micelle cores. The FRET ratio  $I_R/(I_G + I_R)$  was measured to be 0.90, where  $I_R$  and  $I_G$  were fluorescence intensities of DiI at 565 nm and DiO at 501 nm, respectively. When micelles were decomposed in acetone, energy transfer disappeared because the distance between DiI and DiO cannot be retained within the FRET range (Figure 1B). Accordingly, the FRET ratio  $I_R/(I_G + I_R)$  decreased to 0.17. To estimate the cross-talk of donor emission and direct excitation of acceptor probes, we have measured the spectra of FRET micelles with 0.75% DiI and DiO, micelles with 0.75% DiI alone, and micelles with 0.75% DiO alone (Figure S1). The cross-talk of DiO to DiI channel is negligible because of low DiO signal in the presence of FRET. The direct excitation of DiI by the 484 nm laser was small in comparison with the total DiI signal of FRET micelles. Therefore, by monitoring the

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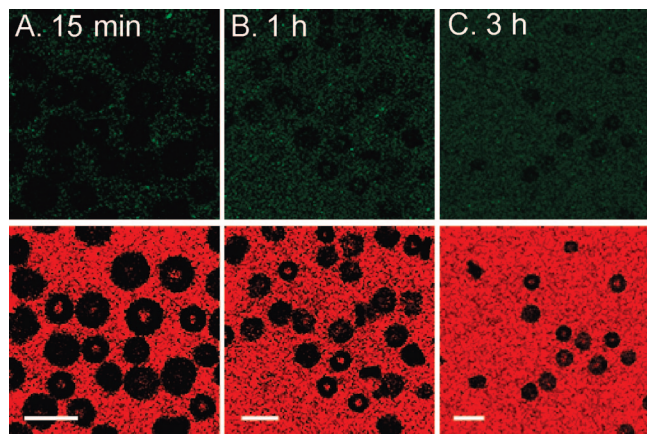
**Figure 2.** *In vivo* imaging of FRET micelles intravenously injected into a mouse through the tail vein. The fluorescence signals were measured during a 3 h period. (A) DiO (green) and DiI (red) signals observed in blood vessels. Bar = 50  $\mu\text{m}$ . (B) Fluorescence spectra of FRET micelles in blood vessels. (C) FRET ratio,  $I_{DiI}/(I_{DiI} + I_{DiO})$ , measured over time and compared with those in water and acetone.

dynamic change of FRET ratio, we were able to monitor the release of DiI and DiO from micelles in real time.

To study the release of core-loaded molecules from PEG-PDLLA micelles in blood circulation, we intravenously injected 100  $\mu\text{L}$  of FRET micelles into three mice through the tail veins. The blood vessels in the ear lobes were imaged in real time with 488 nm excitation to monitor the release of DiI and DiO from micelles. DiO (green) and DiI (red) signals in the blood vessels were measured at 15 min, 1 h, and 3 h (Figure 2A). The spectra (Figure 2B) recorded at the same time showed a strong peak around 501 nm. In comparison with the spectrum of intact micelles (Figure 1A), the appearance of this DiO fluorescence peak

indicates a decrease of FRET. The ratio,  $I_{DiI}/(I_{DiO} + I_{DiI})$ , was calculated to quantify the FRET change, where  $I_{DiI}$  and  $I_{DiO}$  are the average intensities of interested blood vessels in the images with DiI and DiO detections as we showed in Figure 2A. It was found that  $I_{DiI}/(I_{DiO} + I_{DiI})$  rapidly decreased to 0.463 ( $\pm 0.037$ ) at 15 min postinjection and gradually decreased to 0.415 ( $\pm 0.029$ ) at 1 h and 0.390 ( $\pm 0.017$ ) at 3 h (Figure 2C). Comparing with the ratios in water (0.886) or acetone (0.327), our observation suggests a quick and nearly complete release of core-loaded molecules from circulating micelles in an uncontrolled fashion.

There are several possible reasons for the fast release. First, micelles are often diluted to a concentration below their cmc

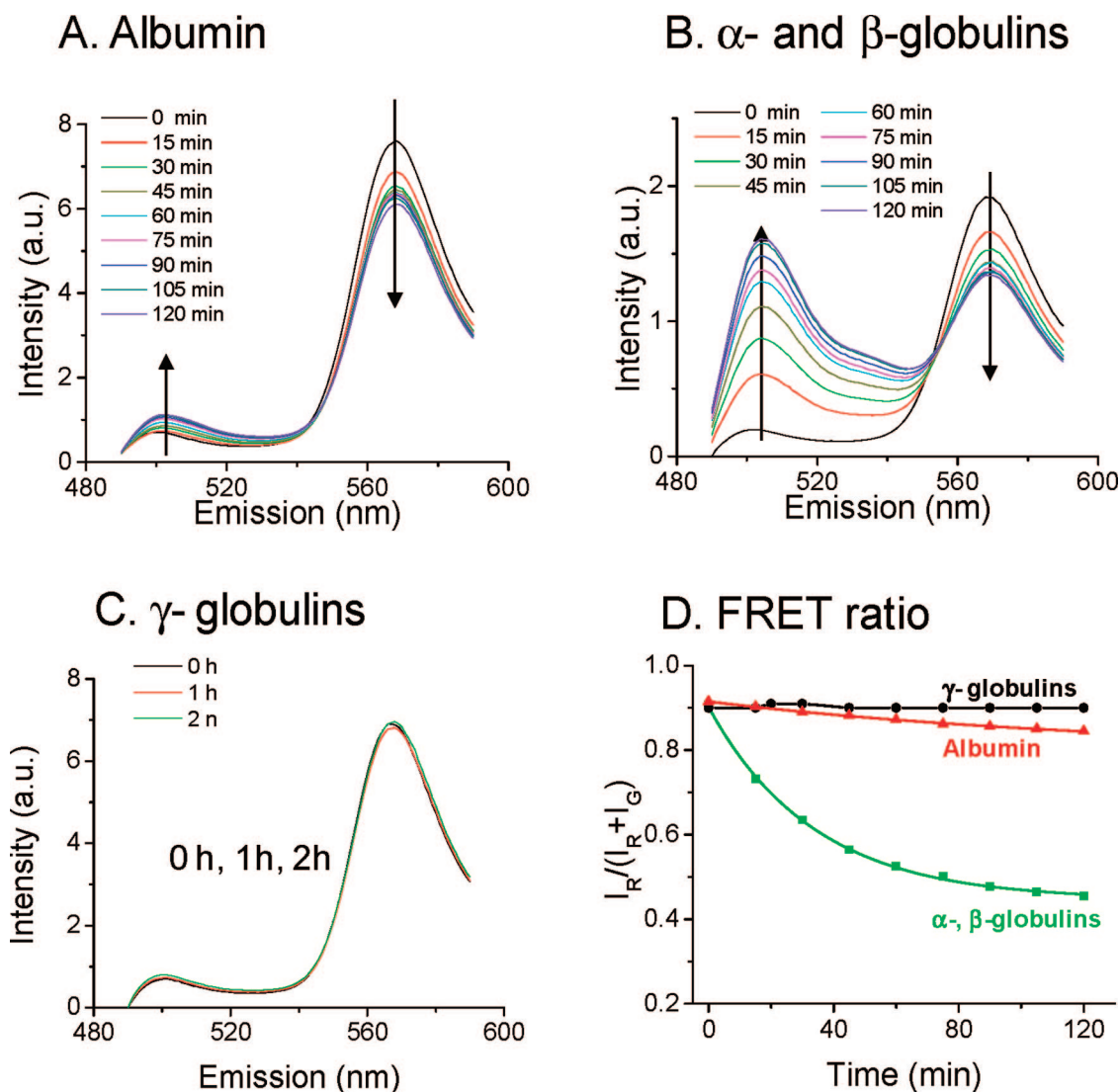


**Figure 3.** Confocal fluorescence images of DiO (green) and DiI (red) signals from FRET micelles incubated with red blood cells for (A) 15 min, (B) 1 h, and (C) 3 h. The micelle concentration was 63  $\mu\text{g/mL}$ . Bar = 10  $\mu\text{m}$ .

leading to the liberation of core-loaded molecules.<sup>18</sup> However, in our experiment, the estimated concentration of micelles in

blood ( $\sim 38 \mu\text{g/mL}$ ) was much higher than their aqueous cmc (1.37  $\mu\text{g/mL}$ ). Furthermore, it was reported previously that, even when the aqueous concentration of micelles falls below the cmc, micelles could remain intact for hours.<sup>19</sup> Therefore, the dilution effect induced by intravenous injection could not result in the rapid release in blood. Second, core-loaded molecules could be translocated from intact micelles to abundant blood components via dynamic instability of micelles. However, such translocation was found to be relatively slow by a FRET recovery experiment (Figure S2), in which two populations of micelles, one with only DiI and the other with only DiO, were mixed in aqueous solution at room temperature. It was found that  $I_R/(I_G + I_R)$  gradually increased from 0.28 at 0 h to 0.68 at 19 h. This recovery of FRET demonstrated a slow translocation of core-loaded molecules between two different micelles, while the integrity of micelles was sustained. The gradual recovery was much slower than the burst release observed *in vivo* (Figure 2). Therefore, we hypothesized that the stability of the micelles was compromised in blood circulation, leading to the fast release of core-loaded molecules.

To find out the dominant factor in blood that accounts for the fast release of DiI and DiO, FRET micelles were incubated with



**Figure 4.** Fluorescence spectral measurement of FRET micelles in solutions containing different blood proteins. (A) Time-resolved spectra of FRET micelles in albumin solution (40 mg/mL). (B) Time-resolved spectra of FRET micelles in  $\alpha$ -,  $\beta$ -globulins solution (14 mg/mL). (C) Time-resolved spectra of FRET micelles in  $\gamma$ -globulins solution (10 mg/mL). (D) Time traces of FRET ratio,  $I_R/(I_R + I_G)$ , in solutions of albumin (red curve),  $\alpha$ -,  $\beta$ -globulins (green curve), and  $\gamma$ -globulins (black curve).

several components isolated from blood. RBCs were first selected for being the major component (~45% in volume) of whole blood. FRET micelles were mixed with isolated RBCs and imaged (Figure 3A–C) at the same time points as Figure 2A, i.e., 15 min, 1 h, and 3 h. The final concentration of micelles was 63  $\mu\text{g}/\text{mL}$ . Release of DiI and DiO from micelles to RBCs was not observed, although DiI and DiO are known to be able to label RBC membrane readily.<sup>20</sup> This result indicates that DiI and DiO were retained by micelles in the presence of RBCs.

Second, albumin and globulins ( $\alpha$ -,  $\beta$ -,  $\gamma$ -) were studied due to their prevalent existence in plasma. A previous study by Chowdhary et al. has suggested that a hydrophobic drug, benzoporphyrin derivative, can be transferred from the lipid phase to albumin and lipoproteins.<sup>21</sup> Therefore, an albumin solution at 40 mg/mL close to its plasma concentration (35–50 mg/mL) was prepared and then mixed with FRET micelles at the concentration of 63  $\mu\text{g}/\text{mL}$ . The sample was gently vortexed to ensure sufficient interaction between micelles and albumin. To determine the decrease of FRET efficiency, time-resolved spectra were measured during a 2 h period with 15 min interval. As shown in Figure 4A, DiI fluorescence intensity decreased, while DiO fluorescence intensity increased over time, as indicated by arrows. However, the FRET ratio  $I_R/(I_G + I_R)$  decreased slightly from 0.91 to 0.84 in the first 2 h period as shown in Figure 4D (the red curve), implying that the release occurred slowly and the integrity of micelles was considerably sustained in the presence of albumin. A similar observation was documented by Kwon et al., in which 80% of doxorubicin was still in micelles after 100 h incubation with serum albumin.<sup>22</sup>

The roles of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins were investigated similarly. The solution of  $\alpha$ - and  $\beta$ -globulin mixture was prepared at a concentration of 14 mg/mL in water based on their blood concentration (14–25 mg/mL). FRET micelles were spiked into 2 mL  $\alpha$ - and  $\beta$ -globulins solution at the final concentration of 63  $\mu\text{g}/\text{mL}$ , followed by gentle vortexing. Time-resolved spectra were measured during a 2 h period with 15 min interval. A remarkable decrease of DiI fluorescence intensity and a rapid increase of DiO fluorescence intensity were observed and shown in Figure 4B, indicating a fast release of DiI and DiO from micelles. The FRET ratio,  $I_R/(I_G + I_R)$ , decreased from 0.91 to 0.45 in the first 2 h period (Figure 4D, the green curve), which was close to the *in vivo* result (Figure 2B). In an independent experiment, after addition of FRET micelles into a solution of  $\gamma$ -globulins (10 mg/mL), FRET ratio did not change during the 2 h period (Figure 4C,D), indicating that the effect of  $\gamma$ -globulins is negligible. The above results indicate that  $\alpha$ - and  $\beta$ -globulins are major factors responsible for the fast release of DiI and DiO by compromising the integrity of micelles, while the involvement

of  $\gamma$ -globulins is insignificant. Meanwhile, it was noticed that the release caused by  $\alpha$ - and  $\beta$ -globulins was not at the same time scale as our *in vivo* experiments. Several factors may account for this difference. First, the ratio of blood proteins to micelles used in our *in vitro* experiments could be lower than that in blood, resulting in slower action toward the micelles. Such a concentration effect was demonstrated in Figure S3 in which a faster decrease of FRET ratio was observed when the micelle concentration was 6.3  $\mu\text{g}/\text{mL}$  (10 times lower than the one used in Figure 4). Therefore, the release could be faster with a lower ratio of micelles to  $\alpha$ - and  $\beta$ -globulins. Second, the fast blood flow with a strong shear stress could mix the micelles with blood proteins more thoroughly than *in vitro* vortexing, leading to a faster release accordingly. Third, a higher temperature can increase the activity of molecules and lead to the fast disassembly of micelles in a blood stream. Fourth, other molecules in blood which are not tested in our experiments could also contribute to the release process. In addition, different factors may cooperate to accelerate the disassembly of micelles *in vivo*. Nevertheless, our *in vitro* results demonstrate that  $\alpha$ - and  $\beta$ -globulins are prime factors responsible for the release of hydrophobic molecules from PEG-PDLLA micelles in blood.

## Conclusions

*In vivo* FRET imaging was used to study the behavior of PEG-PDLLA micelles injected into the blood stream of a live animal. A fast release of core-loaded molecules from micelles was observed in blood circulation due to the loss of micelle integrity. FRET analysis with individual blood components showed that  $\alpha$ - and  $\beta$ -globulins are the major factors for the fast disassembly of micelles *in vivo*. Our studies provide an explanation to the earlier observation that paclitaxel was rapidly disassociated from micelles after injection into blood stream.<sup>15</sup> Such dissociation is possibly due to the decomposition of micelles and translocation of paclitaxel to the abundant lipid components and carriers in blood. Therefore, it is critical to formulate micelles resistant to disassembly in circulation for drug delivery. To address this technical issue, one possible strategy is to design shell-cross-linked biodegradable PEG-polyester micelles to offset the drug loss.<sup>23</sup> In such micelles, physically incorporated drug molecules can be shielded tightly by the cross-linked corona. Moreover, a biodegradable cross-linking can allow the release of the drug from micelles in a controlled manner.

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**Supporting Information Available:** Details of polymer synthesis and Figures S1–S3. This material is available free of charge via the Internet at <http://pubs.acs.org>

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