Published in final edited form as:

Mol Pharm. 2013 November 4; 10(11): 4185–4194. doi:10.1021/mp4002393.

Noninvasive Fluorescence Resonance Energy Transfer Imaging of *in vivo* Premature Drug Release from Polymeric Nanoparticles

Peng Zou, Hongwei Chen, Hayley J. Paholak, and Duxin Sun*

Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109

Abstract

Understanding *in vivo* drug release kinetics is critical for the development of nanoparticle-based delivery systems. In this study, we developed a fluorescence resonance energy transfer (FRET) imaging approach to noninvasively monitor *in vitro* and *in vivo* cargo release from polymeric nanoparticles. The FRET donor dye (DiO or DiD) and acceptor dye (DiI or DiR) were individually encapsulated into poly(ethylene oxide)-*b*-polystyrene (PEO-PS) nanoparticles. When DiO (donor) nanoparticles and DiI (acceptor) nanoparticles were co-incubated with cancer cells for 2 h, increased FRET signals were observed from cell membranes, suggesting rapid release of DiO and DiI to cell membranes. Similarly, increased FRET ratios were detected in nude mice after intravenous co-administration of DiD (donor) nanoparticles and DiR (acceptor) nanoparticles. In contrast, another group of nude mice *i.v.* administrated with DiD/DiR co-loaded nanoparticles showed decreased FRET ratios. Based on the difference in FRET ratios between the two groups, *in vivo* DiD/DiR release half-life from PEO-PS nanoparticles was determined to be 9.2 min. In addition, it was observed that the presence of cell membranes facilitated burst release of lipophilic cargos while incorporation of oleic acid-coated iron oxide into PEO-PS nanoparticles slowed the release of DiD/DiR to cell membranes. The developed *in vitro* and *in vivo* FRET imaging techniques can be used to screening stable nano-formulations for lipophilic drug delivery.

Keywords

burst release; fluorescence resonance energy transfer; imaging; polymeric nanoparticle

Polymeric nanoparticles have emerged as attractive tools for targeted delivery of anticancer drugs to minimize toxic side effects¹. However, many challenges remain for their clinical application. Premature release of encapsulated drugs from polymeric nanoparticles is one of the main obstacles for site-specific drug delivery to tumor tissues²⁻⁵. Various *in vitro* drug release assays have been developed to examine the potential of *in vivo* premature release⁶. However, the *in vitro* assays might not exactly mimic *in vivo* conditions due to the lack of sink conditions, dynamic blood flow, and reliable approaches to separate released lipophilic drug and nanocarriers².

Currently, the most widely used approach to detect *in vivo* premature drug release is comparison of pharmacokinetic profiles after *i.v.* administration of nano-formulations and free drug. There are some limitations for this approach: 1). Most current sample preparation methods (liquid-liquid extraction and protein precipitation) cannot accurately distinguish

*To whom correspondence may be addressed: Duxin Sun, Ph.D., Department of Pharmaceutical Sciences, College of Pharmacy, The University of Michigan, Room 2020, 428 Church Street, Ann Arbor, MI 48109, Tel: 1-734-615-8740, Fax: 1-734-615-6162, duxins@umich.edu.

Supporting Information This material is available free of charge via the Internet at <http://pubs.acs.org>

encapsulated drug and free drug in blood and tissue matrices. 2). Solid phase extraction^{7, 8}, size exclusion column⁹ and ultrafiltration^{7, 9} can distinguish released free drug and unreleased drug but they cannot distinguish released protein-bound drug and unreleased drug. Furthermore, the sample preparation procedures may trigger drug release. 3) Individual radiolabeling of drug and vehicle may detect premature release¹⁰ but the experiments are labor-intensive and time-consuming. Intratumor microdialysis is another useful approach for detecting *in vivo* drug release from nano-formulations^{11, 12}. However, this microdialysis technique cannot detect released tissue-bound drug, which lead to underestimation of drug release from nano-formulations. Hence, it is critical to develop a simple and reliable method to visualize *in vivo* drug release from nanocarriers in real-time.

Fluorescence resonance energy transfer (FRET) has been employed to investigate cargo release from micelles and liposomes^{3, 4, 13-16}. Two lipophilic dyes DiO (donor) and DiI (acceptor) are loaded into polymeric nanoparticles and the proximity (typically less than 10 nm) between the two dyes gives rise to FRET effect¹⁷. Typically, the decrease of FRET effect is used to monitor cargo release from nanocarriers in test tubes^{4, 16, 18}, cell culture³ and blood stream^{14, 19}. However, the presence of nanoscale biological acceptors in the body complicates the changes of FRET effect. For example, rapid transfer of lipophilic dyes from polymeric nanoparticles (60-100 nm in diameter) to cell membranes (3-4 nm in thickness)²⁰ resulted in a recovery of FRET in endosomes/lysosomes³, which compensates decreased FRET effect in polymeric nanoparticles and hence makes quantitative measurement of *in vivo* cargo release difficult. Furthermore, the low tissue penetration of DiO and DiI fluorescence has limited their *in vivo* imaging applications.

One objective of this study is to develop a FRET approach to noninvasively assess *in vivo* lipophilic cargo release in real-time. Previous studies revealed rapid release of DiO and DiI from poly(ethylene oxide)-*b*-poly(D,L-lactide) (PEO-PDLLA)³, poly(ethylene oxide)-*b*-poly(ϵ -caprolactone) (PEO-PCL)³, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol) (DSPE-PEG)⁴, and poly(lactic-co-glycolic acid) PLGA²¹ nanoparticles. The cargo release from the biodegradable nanoparticles was too fast to accurately determine *in vivo* release kinetics. Hence, in this study, we selected poly(ethylene oxide)-*b*-polystyrene (PEO-PS) nanoparticle as a model drug carrier because of its high lipophilic core, leading to a longer cargo release half-life than biodegradable nanoparticles. It was found that the release of FRET donor and acceptor dyes from polymeric nanoparticles to cell membranes resulted in increased FRET ratios in both cell membranes and intracellular organelles. To overcome the limitations of current FRET methods, we utilized both FRET ratio increase and decrease to quantitatively measure release kinetics. In our new FRET method, donor dye and acceptor dye were individually loaded into PEO-PCL or PEO-PS nanoparticles for *in vitro* FRET imaging, respectively. While two near-infrared lipophilic dyes DiD and DiR were used as donor and acceptor dyes for *in vivo* FRET imaging, respectively. When mixed donor nanoparticles and acceptor nanoparticles were co-incubated with cancer cells and co-administered into nude mice through tail vein, both *in vitro* and *in vivo* FRET imaging showed increased FRET effect, indicating rapid transfer of dyes from polymeric nanoparticles to cell membranes. Donor dye and acceptor dye individually loaded nanoparticles showed advantages over donor/acceptor co-loaded nanoparticles for *in vivo* FRET imaging in terms of specificity and accuracy. Furthermore, incorporation of oleic acid-coated iron oxide nanoparticles (IONPs) into PEO-PS nanoparticles was found to be able to slow the transfer of dyes from polymeric nanoparticles to cell membranes. Hence, premature release could be reduced by increasing lipophilicity of polymeric nanoparticle core. To our knowledge, this is the first application of quantitative whole-body FRET imaging to measure *in vivo* cargo release kinetics in real-time.

Materials & methods

Materials

Oleic acid-coated lipophilic iron oxide nanoparticles (IONPs) and SuperMag Separator™ were supplied by Ocean NanoTech (Springdale, AR). PEO-PCL (5.8 kD-b-22.5 kD) and carboxyl-PEO-PS (9.5 kD-b-18kD) were purchased from Polymer Source Inc. (Dorval, Quebec, Canada). DiO, DiI, DiD and DiR were purchased from Invitrogen (Carlsbad, CA). Sephadex LH-20 and PD-10 desalting columns were purchased from GE Healthcare (Piscataway, NJ). Centrifugal filter units (MWCO 10 kDa) were purchased from Millipore (Billerica, MA). Dialysis tubing (MWCO 3.5-5 kDa) was supplied by Spectrum Laboratories, Inc (Rancho Dominguez, CA). CytoVu® imaging slides (Part number C1000-MP8NP50) were obtained from SiMPore (West Henrietta, NY). CD-1 mouse plasma in sodium citrate, human plasma, THF and all other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Polymeric nanoparticle preparation

IONP-loaded PEO-PS nanoparticles were prepared by a precipitation method. 10 mg of PEO-PS, 0.075 mg of DiO or DiI and 1 mg of oleic acid-coated IONPs were dissolved in 0.5 ml of THF. 2 mL of deionized water were added to the solution at a speed of 6 ml/h using a syringe pump (Fisher Scientific, Pittsburgh, PA) under vigorous stirring. The solution was then dialyzed against 2 liters of deionized water using Spectra/Por (MWCO 6-8 kD) (Spectrum OR, Irving, TX) for 2 days. Water was changed each day. The nanoparticles passed through a PD-10 desalting column (GE Healthcare, Piscataway, NJ) to remove unencapsulated dyes. Then, IONP-loaded DiO or DiI nanoparticles were isolated and concentrated using a magnetic separator (Ocean NanoTech, Springdale, AR). Finally, the IONP-loaded nanoparticles were suspended in PBS and filtered through a 0.45 µm filter, and stored at 4°C. Following the similar procedures, PEO-PCL nanoparticles and PEO-PS nanoparticles were prepared by individually loading or co-loading 0.75% of DiO and 0.75% of DiI in the absence of IONPs. After purification using PD-10 columns, the nanoparticles were concentrated using 10 kDa MWCO centrifugal filter units and suspended in PBS. For *in vivo* imaging, 6% of DiD and 6% of DiR were individually loaded or co-loaded into PEO-PS nanoparticles in the absence or presence of 10% of IONPs. The concentrations were calculated according to the amount of polymers, dyes and deionized water used.

Characterization of polymeric nanoparticles

The average hydrodynamic sizes were measured by dynamic light scattering (DLS) technique on a Zetasizer Nano ZS particle sizer (Malvern Instruments Ltd, Westborough, MA). The hydrodynamic sizes of nanoparticles were reported as the intensity-weighted average with standard deviation. Fluorescence spectra of nanoparticles were measured on an LS55 PerkinElmer luminescence spectrometer (Waltham, MA) with an excitation at 480 nm (DiO) or 610 nm (DiD). To monitor the possible cargo release or exchange, time-resolved fluorescence study of nanoparticles individually loaded or co-loaded with FRET donor and acceptor dyes was performed in cell culture media (10% FBS), human plasma, and mouse plasma. Fluorescence spectra were recorded every 5 min over a 2 h period. The rate of energy transfer depends on the extent of spectral overlap between the donor emission and acceptor absorption spectra, the quantum yield of the donor dye, the relative orientation of the donor and acceptor transition dipole moments, and the distance separating the donor and acceptor dyes. Hence, previously reported spectra properties of donor and acceptor dyes were summarized in Supporting Information (Table S1).

Cell culture and xenograft mice

A breast cancer cell line MDA-MB-231 obtained from American Type Culture Collection (ATCC, Rockville, MD) was cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA). The cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C, with the medium changed every other day. Cells were cultured on 8-well Lab-Tek glass chamber slides (Thermo Fisher Scientific, Rochester, NY) for FRET confocal imaging. 1×10⁴ cells per well were incubated for 2 days to allow cell adherence. 20 μL of nanoparticles (2 mg/mL) were added to cells in 0.18 ml of culture medium and incubated at 37°C for the desired lengths of time before imaging.

Eight week old female athymic nude mice (nu/nu), obtained from the National Cancer Institute (Bethesda, MD), were subcutaneously inoculated in the back with 5×10⁶ MDA-MB-231 cells suspended in a mixture of 50 μL of PBS and 50 μL of matrigel basement membrane (BD Biosciences, San Jose, CA). When the tumor implants reached 0.4 cm in diameter, the tumor-bearing mice were subjected to the *in vivo* imaging.

FRET confocal microscopy

FRET images were acquired using a digital camera (C9100, Hamamatsu Photonics, Japan) mounted on a Visitech VT Infinity 3 array-scanning confocal system (VisiTech International Ltd., United Kingdom) attached to a Nikon TE-2000U microscope with a 60X Nikon Plan Apo water-immersion objective. Images were recorded in the DiO channel (488 nm excitation, 535 ± 20 nm emission), FRET channel (488 nm excitation, 620 ± 20 nm emission) and DiI channel (543 nm excitation, 620 ± 20 nm emission). The exposure time was 200 ms for the DiO and FRET channels and 100 ms for the DiI channel. Cells were incubated with 0.2 mg/mL of PEO-PS (or PEO-PCL) nanoparticles co-loaded with 0.75% DiO and 0.75% DiI. Additionally, cells were incubated with a mixture of 0.2 mg/mL of PEO-PS (or PEO-PCL) nanoparticles with 0.75% DiO and 0.2 mg/mL of PEO-PS (or PEO-PCL) nanoparticles with 0.75% DiI. Similarly, PEO-PS nanoparticles loaded with FRET dyes and 10% IONP were incubated with cells. After 2 h incubation, images were obtained before or after PBS washing by using MetaMorph v6.5.3 (Universal Imaging, Malvern, PA).

The images obtained after PBS washing were background-subtracted using the “Background Correction” tool in MetaMorph. Due to the presence of nanoparticles in the media, the images obtained before PBS washing were not subjected to background subtraction. Crosstalk correction and FRET ratios calculation were carried out using an in-house FRET Calculator program and Matlab. To correct crosstalk, correction coefficients α and β were determined from cells incubated with DiI nanoparticles only ($\alpha = I_{\text{FRET}}/I_{\text{DiI}}$) and DiO nanoparticles only ($\beta = I_{\text{FRET}}/I_{\text{DiO}}$)²²⁻²⁴. I_{FRET} , I_{DiI} and I_{DiO} represent the intensities in each region of interest (ROI) under FRET, DiI, and DiO filter sets, respectively. Net FRET was calculated as follows $I_{\text{FRET}}' = I_{\text{FRET}} - \alpha \times I_{\text{DiI}} - \beta \times I_{\text{DiO}}$ and FRET ratio = $I_{\text{FRET}}' / (I_{\text{FRET}}' + I_{\text{DiO}}')$ ³, where I_{FRET}' and I_{DiO}' are the corrected values.

In vivo FRET imaging

In vivo fluorescence imaging was performed with an IVIS Spectrum imaging system (Xenogen, Alameda, CA). Images were recorded in DiD channel (640 nm excitation, 680 nm emission), FRET channel (640 nm excitation, 780 nm emission) and DiR channel (710 nm excitation, 780 nm emission). The exposure time was 1s for all of the channels. Identical illumination settings were used for acquiring all images. Images were acquired and analyzed using Living Image 2.5 software (Xenogen, Alameda, CA).

To determine FRET ratios of nanoparticles under the IVIS Spectrum imaging system, 1 mL of nanoparticles (0.01 mg/mL in PBS) in an eppendorf tube was imaged using DiD (Ex/Em 640/680 nm) and FRET (Ex/Em 640/780 nm) filter sets. The average fluorescence intensities were calculated using the automatic ROI (Region of Interest) tool of Living Image 2.5 software. FRET ratio was calculated as follows: $\text{FRET ratio} = I_{\text{FRET}} / (I_{\text{FRET}} + I_{\text{DiD}})$, where I_{FRET} and I_{DiD} are the average intensities of nanoparticles under FRET and DiD filter sets, respectively.

The animal procedures were performed according to a protocol approved by the by the University Committee for the Use and Care of Animals (UCUCA) at the University of Michigan. Five groups of mice with MDA-MB-231 xenograft tumors ($n=3$) were i.v. administered with one of the following treatments: PBS, DiD nanoparticles (1 mg), DiR nanoparticles (1 mg), mixed DiD nanoparticles (1 mg) and DiR nanoparticles (1 mg), or DiD/DiR co-loaded nanoparticles (1 mg). The mice anesthetized with isoflurane were imaged at 10 min and 2 h after injection. Similarly, five groups of mice with xenograft tumors ($n=3$) were i.v. administered with one of the following treatments: PBS, IONP (10%)/DiD (6%) nanoparticles, IONP (10%)/DiR (6%) nanoparticles, mixed IONP/DiD nanoparticles and IONP/DiR nanoparticles, or IONP (10%)/DiD (6%)/DiR (6%) co-loaded nanoparticles. The mice were imaged at 10 min, 2 h and 6 h after injection. The average I_{FRET} and I_{DiD} from whole mouse body were calculated using the ROI tool. The FRET ratio was calculated as: $\text{FRET ratio} = I_{\text{FRET}} / (I_{\text{FRET}} + I_{\text{DiD}})$.

Calculation of in vivo release half-life

The differences in average FRET ratios between the mice treated with DiD/DiR co-loaded nanoparticles and the mice treated with mixed DiD nanoparticles and DiR nanoparticles were plotted against time post injection. *In vivo* DiD/DiR release half-life was calculated by fitting the first-order decay curve.

Results

Characterization of polymeric nanoparticles

As shown in Table 1, PEO-PCL nanoparticles loaded with 0.75% of DiO or DiD showed hydrodynamic sizes of 106-110 nm. The hydrodynamic sizes of PEO-PS nanoparticles loaded with one or two FRET dyes ranged from 45 – 54 nm. The incorporation of 10% IONPs increased the size of PEO-PS nanoparticles to 156 – 177 nm.

Fluorescence spectra of polymeric nanoparticles containing donor dye, acceptor dye or both dyes were obtained. Fig S1 showed the changes in FRET ratio with the increased DiI and DiO concentration ratio. With the increase of encapsulated DiI and DiO ratio, the FRET ratio of nanoparticles increased. Spectral crosstalk was observed when DiI nanoparticles are excited at wavelength 480 nm. Fluorescence spectra of DiD nanoparticles, DiD/DiR co-loaded nanoparticles and DiR nanoparticles were presented in Fig S2. The data showed that spectral crosstalk of DiR nanoparticles was very low when DiR was excited at 610 nm.

Stability on cell culture media and plasma

When DiO nanoparticles and DiI nanoparticles were mixed with a molar ratio 1:1 in cell culture medium and incubated at 37°C for 2 h, time-resolved spectra did not detect changes in FRET signals, suggesting no significant inter-nanoparticle DiO/DiI exchange (Fig 1a and 1b). DiO and DiI were not released from PEO-PCL nanoparticles and PEO-PS nanoparticles. To increase lipophilicity of polymeric nanoparticles, 10% oleic acid-coated IONPs and 0.75% DiO (DiO/IONP nanoparticles) or 0.75% DiI (DiI/IONP nanoparticles) were encapsulated into PEO-PS nanoparticles. Time-resolved spectra did not detect changes

of FRET signals when DiO/IONP nanoparticles and DiI/IONP nanoparticles were co-incubated in medium for 2 h, suggesting no significant inter-nanoparticle DiO/DiI exchange (Fig 1c).

On the other hand, both DiO and DiI were loaded in to PEO-PS nanoparticles (Fig S3a), PEO-PCL nanoparticles (Fig S3b) and IONP-loaded PEO-PS nanoparticles (Fig S3c). Intensive FRET signals were detected at 580 nm when the nanoparticles were excited at 480 nm. Similarly, the three types of nanoparticles were incubated in cell culture media (Fig S3) and human plasma (Fig S4) for 2 h at 37°C and no remarkable change in FRET signals was observed, indicating no release of DiO and DiI.

To visualize *in vivo* cargo release, we used two near-infrared dyes DiD (donor) and DiR (acceptor) for FRET imaging. Six types of polymeric nanoparticles were prepared: PEO-PS with 6% DiD (DiD nanoparticles), PEO-PS with 6% DiR (DiR nanoparticles), and PEO-PS with both 6% DiD and 6% DiR (DiD/DiR nanoparticles), PEO-PS with 10% IONPs and 6% DiD (IONP/DiD nanoparticles), PEO-PS with 10% IONPs and 6% DiR (IONP/DiR nanoparticles), and PEO-PS with 10% IONPs, 6% DiD and 6% DiR (IONP/DiD/DiR nanoparticles). When DiD nanoparticles and DiR nanoparticles were mixed (molar ratio 1:1) in mouse plasma, time-resolved spectra did not show inter-nanoparticle exchange of DiD and DiR during 2 h incubation (Fig 1d). Similarly, no DiD and DiR exchange was observed between IONP/DiD nanoparticles and IONP/DiR nanoparticles in mouse plasma (Fig 1e).

In vitro imaging of cells incubated with DiO/DiI co-loaded nanoparticles

DiO/DiI co-loaded PEO-PS nanoparticles were incubated with MDA-MB-231 cells for 2 h and the cells were imaged prior to PBS washing. As shown in Fig 2c, the accumulation of DiO and DiI resulted in FRET signals from cell membranes and subcellular organelles. The pseudo colors in Fig 2d show higher FRET ratios ($\text{FRET ratio} = I_{\text{FRET}}/(I_{\text{FRET}} + I_{\text{DiO}})$) from cell membranes and subcellular organelles than those from nanoparticles in the medium, suggesting a closer proximity between DiO and DiI on cell membranes. This is due to the small cell surface area and high accumulation of DiO and DiI on cell surface (Fig 2e). Similarly, FRET effect was observed in cell membranes in a previous study²⁵ where cells were stained with 5-dodecanoylamino fluorescein (donor) and Nile red (acceptor).

Subsequent endocytic internalization of cell membranes stained with DiO and DiI resulted in high FRET ratios on subcellular organelles. *In vitro* cargo release was clearly demonstrated by decreased FRET ratios in medium and increased FRET ratios on cell membranes and subcellular organelles. However, the recovery of FRET ratios on cell membranes and subcellular organelles complicates *in vivo* FRET imaging, which makes it difficult to quantitatively measure *in vivo* release.

In vitro imaging of cells incubated with mixed DiO nanoparticles and DiI nanoparticles

To overcome the limitation of donor/acceptor co-loaded nanoparticles, donor and acceptor individually loaded nanoparticles were used for FRET imaging. When MDA-MB-231 cells were incubated with DiO loaded PEO-PS nanoparticles and DiI loaded PEO-PS nanoparticles, FRET imaging detected increased FRET ratios on both cell membranes and intracellular organelles (Fig 3-a4). Consistently, high FRET ratios were detected on cell membranes and intracellular organelles when cells were incubated with DiO and DiI individually loaded PEO-PCL nanoparticles (Fig 3-b4). The cells incubated with PEO-PCL nanoparticles showed higher FRET ratios than the cells incubated with PEO-PS nanoparticles, indicating faster release of DiO and DiI from PEO-PCL nanoparticles.

In vivo imaging of cargo release from PEO-PS nanoparticles

Mixed DiD loaded PEO-PS nanoparticles and DiR loaded PEO-PS nanoparticles in PBS (molar ratio 1:1) exhibited an average background FRET ratio of 0.23 ± 0.01 ($\text{FRET ratio} = I_{\text{FRET}}/(I_{\text{FRET}} + I_{\text{DiD}})$). However, when DiD nanoparticles and DiR nanoparticles were co-injected into mice through tail vein, average FRET ratio ($n = 3$) increased to 0.29 ± 0.04 at 10 min (Fig 4a and c) and 0.48 ± 0.02 at 2 h (Fig 4b and c). FRET signals were not detected from the mice treated with PBS, DiD nanoparticles alone, or DiR nanoparticles alone (Fig 4a and 4b). The data suggest that DiD and DiR were rapidly released into cell membranes, thereby increasing FRET ratios *in vivo*.

In contrast, DiD/DiR co-loaded PEO-PS nanoparticles in PBS exhibited an average FRET ratio of 0.89 ± 0.02 . When the DiD/DiR nanoparticles were administered (i.v.) to mice, average FRET ratios decreased to 0.60 ± 0.03 at 10 min ($n = 3$) (Fig 4 a and c) and 0.48 ± 0.03 at 2 h ($n = 3$) (Fig 4b and c), respectively. This indicates that DiD and DiR are diluted when released to cell membranes due to the much larger volume of phospholipid bilayers in mice than the total polystyrene volume in nanoparticles. In addition, it is worthy to note that, due to the large amount of phospholipid bilayers in mice, the concentrations of DiD and DiR on the surface of mouse cell membranes were much lower than that of DiO and DiI on MDA-MB-231 cell surface. Hence, the FRET ratios observed in mice were lower than that observed on MDA-MB-231 cell membranes.

Since the mice had comparable total volumes of lipid bilayers and were given the same dose of FRET dyes, for the group treated with mixed nanoparticles and the group treated with co-loaded nanoparticles, the final concentrations of dyes on cell membranes were expected to be equal at the end of cargo release. The equal average FRET ratio between the two groups at 2 h (0.48) indicated the end of release (Fig 4c).

In vitro imaging of cells incubated with mixed IONP/DiO nanoparticles and IONP/DiI nanoparticles

The sink effect of cell membranes is likely due to the high lipophilicity ($\log P$ 7-8) of fatty acids in lipid bilayers. Hence, we hypothesized that incorporating oleic acid ($\log P$ 7.7) into PEO-PS nanoparticles could increase lipophilicity of polystyrene cores and thus slow the release of lipophilic dyes. To achieve this, we encapsulated oleic acid-coated iron oxide nanoparticles (IONPs) into the PEO-PS nanoparticles (Fig S5). Fig 3-c3 shows that incorporation of 10% IONPs into PEO-PS nanoparticles (with DiI or DiO) eliminated FRET signals on cell membranes, suggesting that the encapsulated oleic acid layers slowed DiO and DiI release.

In vivo imaging of cargo release from IONP-loaded PEO-PS nanoparticles

Five groups of mice ($n = 3$) were administered with one of the following treatments: PBS, IONP/DiD nanoparticles, IONP/DiR nanoparticles, mixed IONP/DiD nanoparticles and IONP/DiR nanoparticles, or IONP/DiD/DiR nanoparticles. FRET images were obtained at 10 min (Fig 5a), 2 h (Fig 5b) and 6 h (Fig 5c). The mixed IONP/DiD nanoparticles and IONP/DiR nanoparticles (molar ratio 1:1) showed a background FRET ratio of 0.19 ± 0.01 in PBS. When they were co-administered to mice, the average FRET ratios ($n = 3$) increased to 0.22 ± 0.01 (10 min), 0.44 ± 0.01 (2 h), and 0.47 ± 0.02 (6 h), respectively (Fig 5d). These data suggested that DiD and DiR were released into cell membranes, resulting in increased FRET ratios. When mice were administered with IONP/DiD/DiR nanoparticles, the average FRET ratios ($n = 3$) were 0.65 ± 0.01 (10 min), 0.58 ± 0.03 (2 h), and 0.56 ± 0.05 (6 h), respectively (Fig 5d). Even at 6 h the average FRET ratios between the two groups were not equal, indicating an incomplete release of DiD/DiR. These data suggest that although

encapsulated oleic acid layers were unable to completely abolish DiD/DiR release due to the ubiquitous cell membranes in the body, they could slow the release.

In vivo release half-life

As shown in Fig 6, the release of DiD and DiR from PEO-PS nanoparticles followed first-order kinetics and the release half-life was 9.2 min in nude mice. Encapsulation of 10% oleic acid coated IONPs slowed DiD/DiR release and extended the release half-life to 50.8 min. Due to the rapid release of DiD and DiR from nanoparticles, tumor accumulation of DiD and DiR was not observed for the mice treated with PEO-PS nanoparticles (Fig 4) or IONP-loaded PEO-PS nanoparticles (Fig 5).

Discussion

To our knowledge, this is the first application of near-infrared FRET imaging in noninvasively measuring *in vivo* cargo release kinetics in whole animal body. For the mice administered with DiD/DiR co-loaded nanoparticles, the release of DiD and DiR from PEO-PS nanoparticles caused decreased FRET ratios. Meanwhile, the accumulation of DiD and DiR on the surface of cell membranes and intracellular organelles resulted in increased FRET ratios, which compensated the FRET ratio decrease caused by cargo release. In contrast, for mice co-administered with DiD nanoparticles and DiR nanoparticles, there were no initial baseline FRET signals from polymeric nanoparticles and all the FRET signals were from DiD/DiR on the surface of cell membranes and intracellular organelles. Hence, increased FRET ratio among mice co-administered with DiD nanoparticles and DiR nanoparticles is a more sensitive indicator of cargo release.

In theory, if two mice are given the same amount of FRET dyes and the total amount of phospholipid bilayer in each mouse is similar, the densities of dyes on membrane surface should be comparable at the end of dye release, resulting in comparable FRET ratios between the two mice. Hence, the difference between the FRET ratios of the mice co-administered with DiD nanoparticles and DiR nanoparticles and the mice administered with DiD/DiR co-loaded nanoparticles can be used to determine the half-life of DiD/DiR release in real-time. The release half-life can be used as an indicator of *in vivo* stability of nano-formulations. The longer the release half-life, the less potential of burst release. Although the developed FRET imaging method cannot determine release kinetics of real drugs, it is an effective tool for screening stable nano-formulations.

In this study, polymeric nanoparticles composed of PEO-PS were selected for FRET studies to noninvasively measure *in vivo* cargo release kinetics. Due the high glass transition temperature of polystyrene (107°C)²⁶, PS cores were kinetically frozen with low liquidity at 37°C. Thus, PEO-PS nanoparticles showed very limited cargo release in cell culture medium and mouse plasma in the absence of cells. However, in the presence of cells, dyes were rapidly released into cell membranes and generated FRET signals. Compared with polystyrene (logP = 2.95)²⁷, fatty acids (such as oleic acid, logP = 7.7)²⁸ in lipid bilayers are more lipophilic. Due to its Brownian motion, each polymeric nanoparticle in the medium has chances to collide with cell membranes in two hours. A previous study revealed that instant interactions between polymeric nanoparticles and cell membranes during the collision were sufficient to trigger drug release from polymeric nanoparticles although the nanoparticles did not bind to cells². The lipophilic cargos were rapidly extracted from polymeric nanoparticles when they were in contact with cell membranes.

The FRET effect observed on cell membranes was due to the accumulation of dialkylcarbocyanine dyes on membrane surface and parallel orientation of donor and acceptor dyes. Dialkylcarbocyanine dyes are composed of two long alkyl tails and a charged

fluorophore group. The alkyl tails of are inserted into membrane lipid bilayers while fluorophore head is located at cell membrane surface and parallel to each other. Once the alkyl tails are inserted into cell membrane, the dyes are rapidly and homogeneously distributed to the whole membrane surface through lateral diffusion²⁹. It was also reported the flip-flop of dialkylcarbocyanines from membrane surface to inner leaflet was limited³⁰. A quantitative analysis is conducted to demonstrate the high densities of DiO and DiI on cell membrane surface when the cells are incubated with 20 μL of DiO and DiI co-loaded nanoparticles. Typically, a human cell has cell surface area of $155.9 \mu\text{m}^2$ ³¹. The density of MDA-MB-231 cells for imaging is 4×10^4 per well. Hence, the total cell surface area is approximately $6.23 \times 10^{12} \text{ nm}^2$. The total number of DiO molecules in the incubation is $0.02 \text{ mL} \times 2 \text{ mg/mL} \times 0.75\% \div 881(\text{g} \cdot \text{mole}^{-1}) \times 6.02 \times 10^{23} = 2.05 \times 10^{14}$ and the total number of DiI molecules in the incubation is $0.02 \text{ mL} \times 2 \text{ mg/mL} \times 0.75\% \div 934(\text{g} \cdot \text{mole}^{-1}) \times 6.02 \times 10^{23} = 1.93 \times 10^{14}$. If we assume that only 1% of DiO and DiI molecules are transferred to cell membranes, there would be 33 DiO molecules ($2.05 \times 10^{14} \times 1\% \div (6.23 \times 10^{10}) = 33$) and 31 DiI molecules ($1.93 \times 10^{14} \times 1\% \div (6.23 \times 10^{10}) = 31$) per 100 nm^2 of cell surface. In contrast, the total volume of polystyrene cores in the cell culture medium is 0.025 mm^3 or $2.5 \times 10^{16} \text{ nm}^3$ ($0.02 \text{ mL} \times 2 \text{ mg/mL} = 0.04 \text{ mg PEO-PS}$, equal to 0.0262 mg of PS or 0.025 mm^3). The number of DiO and DiI in every 1000 nm^3 of polystyrene are 8.2 ($2.05 \times 10^{14} \div (2.5 \times 10^{13})$) and 7.7 molecules ($1.93 \times 10^{14} \div (2.5 \times 10^{13})$), respectively. DiO and DiI exhibit a two-dimensional distribution on cell membrane surface and the densities are 31 and 33 molecules per 100 nm^2 of cell surface. In contrast, DiO and DiI exhibit three-dimensional distribution in polystyrene cores and the density is 8 molecules per 1000 nm^3 . For both scenarios, the proximity between DiO and DiI is sufficiently close to generate FRET effect but the FRET ratios from cell membranes are expected to be higher than that from polymeric nanoparticles. In addition, the relative orientation of the DiO and DiI transition dipole moments can affect the FRET ratios. Fluorophore head groups of DiO and DiI on cell membrane surface are parallel to each other. It is known that a parallel orientation of donor and acceptor leads to robust energy transfer³². In contrast, DiO and DiI in polystyrene cores show random orientation, which decreases the rate of energy transfer.

It is generally believed that premature drug release is due to disassembly of polymeric nanoparticles in blood circulation by polymer concentration dilution and interactions with plasma protein and lipids³³. However, in this study, the *in vitro* incubation concentration of nanoparticles (0.2 mg/mL) was much higher than the reported critical micelle concentrations (CMC) of PEO-PS ($< 1 \mu\text{g/mL}$) in water³⁴ to ensure the nanoparticles were not disrupted. Since the total plasma volume in mice is 1 mL ³⁵, the plasma concentration of PEO-PS is approximately 1 mg/mL which is much higher than its CMC. Furthermore, polymeric nanoparticles loaded with dyes showed reasonable stability in mouse and human plasma (Fig 1d and Fig S4). Thus, *in vivo* premature cargo release was not likely caused by the disassembly of nanoparticles or polymer exchange.

The cell membrane-triggered cargo release was further confirmed by demonstrating decreased cargo release after incorporating oleic acid layer into PEO-PS particles. In our previous report³⁶, we found that doxorubicin encapsulated into oleic acid layer of IONPs could be internalized into cancer cells together with IONPs and released in endosomes/lysosomes. The fatty acids in cell membrane (phospholipid tails are composed of fatty acids) have comparable hydrophobicity with oleic acid, which might explain the extended intracellular release of doxorubicin. Hence, we hypothesized that incorporation of oleic acid coated IONPs could decrease the burst release. Our FRET results showed that incorporation of oleic acid-coated IONPs into PEO-PS nanoparticles could extend the half-life of DiD/DiR release from 9.2 min to 50.8 min, suggesting that premature release of lipophilic drugs can be slowed by incorporating lipids. This strategy has been successfully applied to geldanamycin³⁷, paclitaxel³⁸ and docetaxel³⁹ delivery. Specifically, lipophilicity of

geldanamycin, paclitaxel and docetaxel was increased by conjugating lipid anchors to the drug. The lipid-taxane prodrugs and phospholipids were coencapsulated into PEO-PS nanoparticles. Prolonged blood circulation of taxanes in mice was achieved using the lipophilicity-enhanced nanoformulations.

Consistently, a recent *in vivo* pharmacokinetic study on paclitaxel-loaded PEO-PCL micelles supported *in vivo* burst release of real drug⁴⁰. By dual radiolabeling PEO-PCL copolymers and paclitaxel with ³H and ¹⁴C, the authors compared *in vitro* and *in vivo* stability of paclitaxel loaded PEO₁₁₄-b-PCL₁₉ and PEO₁₁₄-b-PCL₁₀₄ micelles. It was found that a longer hydrophobic PCL block decreased CMC of PEO₁₁₄-b-PCL₁₀₄ micelles and retains paclitaxel payload in human plasma incubation. Upon injection into mice, PEO₁₁₄-b-PCL₁₀₄ micelles circulated for longer than PEO₁₁₄-b-PCL₁₉. However, paclitaxel was rapidly eliminated from the blood with less than 1% of the injected dose per ml of plasma remaining 15 min postinjection regardless of the formulation. The micelles with different *in vitro* release kinetics exhibited similar burst release rates, indicating that the *in vitro* drug release assay is a poor predictor of the *in vivo* stability. The burst release is probably caused by the lipophilic gradient between endogenous lipids and PCL core instead of micelle disassembly.

The identification of cell membranes as a sink which triggers premature drug release has some implications for the design and development of nano-formulation for lipophilic drug delivery. First, although some polymeric nanoparticles such as PEO-PS and PEO-PCL nanoparticles are able to improve solubility, absorption and systemic exposure (AUC) of water-insoluble drugs, they cannot efficiently deliver drugs to tumor sites due to burst release. Second, premature release cannot be eliminated by only decreasing CMC. Third, increasing lipophilicity of both the core of nanocarrier and delivered drugs might be a feasible strategy to reduce burst release. Fourth, the sink effect of cell membranes suggests that traditional *in vitro* drug release assays conducted in the absence of phospholipid bilayers do not reflect the real *in vivo* release conditions. Therefore, new *in vitro* drug release assays need to be developed.

Conclusions and perspective

In summary, we have developed a noninvasive FRET imaging method to quantitatively measure *in vivo* release of lipophilic cargos, which can be used to examine various nanoparticles for lipophilic drug delivery. Utilizing the FRET imaging method, we demonstrated rapid *in vitro* and *in vivo* cargo release from PEO-PS and PEO-PC nanoparticles. In addition, this sink effect of cell membranes poses a substantial barrier for targeted delivery of lipophilic drugs, and necessitates the design of a new generation of polymeric nanoparticle-based delivery vehicles.

Driven by the vast demand in the field of targeted drug delivery, various *in vitro* methods have been developed to measure drug release from nanoparticles but most of them have been found to be poor predictors of *in vivo* release. To identify promising candidates for clinical investigation, it is urgent to develop rapid and simple approaches which can quantitatively compare *in vivo* release kinetics of various drug delivery systems. The noninvasive FRET imaging reported here provides a useful tool for screening stable lipophilic drug delivery systems. However, noninvasively measuring *in vivo* release of hydrophilic drugs from nanocarriers such as doxorubicin liposomes is still a challenge. Further research aiming at imaging hydrophilic drug release is warranted. Furthermore, due to the *in vivo* sink effect of cell membranes and lipoproteins, burst release is a concern for targeted delivery of lipophilic drugs. More research is needed to reduce burst release of lipophilic drugs by increasing lipophilicity of both the core of nanocarriers and encapsulated drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We sincerely thank Dr. Andrew Wang (Ocean NanoTech, Springdale, Arkansas) for generously providing iron oxide nanoparticles. This work was partially supported by the National Institutes of Health (RO1 CA120023 and R21 CA143474); University of Michigan Cancer Center Research Grant (Munn); and University of Michigan Cancer Center Core Grant to DS.

References

1. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol.* 2007; 2(12):751–60. [PubMed: 18654426]
2. Wang H, Xu J, Wang J, Chen T, Wang Y, Tan YW, Su H, Chan KL, Chen H. Probing the kinetics of short-distance drug release from nanocarriers to nanoacceptors. *Angewandte Chemie.* 2010; 49(45):8426–30. [PubMed: 20718010]
3. Chen H, Kim S, Li L, Wang S, Park K, Cheng JX. Release of hydrophobic molecules from polymer micelles into cell membranes revealed by Forster resonance energy transfer imaging. *Proc Natl Acad Sci U S A.* 2008; 105(18):6596–601. [PubMed: 18445654]
4. Diezi TA, Bae Y, Kwon GS. Enhanced stability of PEG-block-poly(N-hexyl stearate l-aspartamide) micelles in the presence of serum proteins. *Mol Pharm.* 2010; 7(4):1355–60. [PubMed: 20575526]
5. Danson S, Ferry D, Alakhov V, Margison J, Kerr D, Jowle D, Brampton M, Halbert G, Ranson M. Phase I dose escalation and pharmacokinetic study of pluronic polymer-bound doxorubicin (SP1049C) in patients with advanced cancer. *Br J Cancer.* 2004; 90(11):2085–91. [PubMed: 15150584]
6. D'Souza SS, DeLuca PP. Methods to assess in vitro drug release from injectable polymeric particulate systems. *Pharm Res.* 2006; 23(3):460–74. [PubMed: 16400516]
7. Mayer LD, St-Onge G. Determination of free and liposome-associated doxorubicin and vincristine levels in plasma under equilibrium conditions employing ultrafiltration techniques. *Anal Biochem.* 1995; 232(2):149–57. [PubMed: 8747469]
8. Deshpande NM, Gangrade MG, Kekare MB, Vaidya VV. Determination of free and liposomal amphotericin B in human plasma by liquid chromatography-mass spectroscopy with solid phase extraction and protein precipitation techniques. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2010; 878(3-4):315–26.
9. Krauze MT, Noble CO, Kawaguchi T, Drummond D, Kirpotin DB, Yamashita Y, Kullberg E, Forsayeth J, Park JW, Bankiewicz KS. Convection-enhanced delivery of nanoliposomal CPT-11 (irinotecan) and PEGylated liposomal doxorubicin (Doxil) in rodent intracranial brain tumor xenografts. *Neuro Oncol.* 2007; 9(4):393–403. [PubMed: 17652269]
10. Zolnik BS, Stern ST, Kaiser JM, Heakal Y, Clogston JD, Kester M, McNeil SE. Rapid distribution of liposomal short-chain ceramide in vitro and in vivo. *Drug Metab Dispos.* 2008; 36(8):1709–15. [PubMed: 18490436]
11. Blakeley J, Portnow J. Microdialysis for assessing intratumoral drug disposition in brain cancers: a tool for rational drug development. *Expert Opin Drug Metab Toxicol.* 2010; 6(12):1477–91. [PubMed: 20969450]
12. Zamboni WC, Gervais AC, Egorin MJ, Schellens JH, Hamburger DR, Delauter BJ, Grim A, Zuhowski EG, Joseph E, Pluim D, Potter DM, Eiseman JL. Inter- and intratumoral disposition of platinum in solid tumors after administration of cisplatin. *Clin Cancer Res.* 2002; 8(9):2992–9. [PubMed: 12231546]
13. Lu J, Owen SC, Shoichet MS. Stability of Self-Assembled Polymeric Micelles in Serum. *Macromolecules.* 2011; 44(15):6002–6008. [PubMed: 21818161]
14. Chen H, Kim S, He W, Wang H, Low PS, Park K, Cheng JX. Fast release of lipophilic agents from circulating PEG-PDLLA micelles revealed by in vivo forster resonance energy transfer imaging. *Langmuir.* 2008; 24(10):5213–7. [PubMed: 18257595]

15. Jiwanich S, Ryu JH, Bickerton S, Thayumanavan S. Noncovalent encapsulation stabilities in supramolecular nanoassemblies. *J Am Chem Soc.* 132(31):10683–5. [PubMed: 20681699]
16. Miller T, Rachel R, Besheer A, Uezguen S, Weigandt M, Goepferich A. Comparative investigations on in vitro serum stability of polymeric micelle formulations. *Pharm Res.* 2012; 29(2):448–59. [PubMed: 21879388]
17. Gordon GW, Berry G, Liang XH, Levine B, Herman B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys J.* 1998; 74(5):2702–13. [PubMed: 9591694]
18. Jee JP, McCoy A, Mecozzi S. Encapsulation and release of Amphotericin B from an ABC triblock fluoruous copolymer. *Pharm Res.* 2012; 29(1):69–82. [PubMed: 21739321]
19. Li Y, Xiao W, Xiao K, Berti L, Luo J, Tseng HP, Fung G, Lam KS. Well-defined, reversible boronate crosslinked nanocarriers for targeted drug delivery in response to acidic pH values and cis-diols. *Angew Chem Int Ed Engl.* 2012; 51(12):2864–9. [PubMed: 22253091]
20. Lewis BA, Engelman DM. Lipid bilayer thickness varies linearly with acyl chain length in fluid phosphatidylcholine vesicles. *J Mol Biol.* 1983; 166(2):211–7. [PubMed: 6854644]
21. Xu P, Gullotti E, Tong L, Highley CB, Errabelli DR, Hasan T, Cheng JX, Kohane DS, Yeo Y. Intracellular drug delivery by poly(lactic-co-glycolic acid) nanoparticles, revisited. *Mol Pharm.* 2009; 6(1):190–201. [PubMed: 19035785]
22. Xia Z, Liu Y. Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. *Biophys J.* 2001; 81(4):2395–402. [PubMed: 11566809]
23. Hoppe AD, Swanson JA. Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis. *Mol Biol Cell.* 2004; 15(8):3509–19. [PubMed: 15169870]
24. Hoppe A, Christensen K, Swanson JA. Fluorescence resonance energy transfer-based stoichiometry in living cells. *Biophys J.* 2002; 83(6):3652–64. [PubMed: 12496132]
25. Xiao L, Xiong X, Sun X, Zhu Y, Yang H, Chen H, Gan L, Xu H, Yang X. Role of cellular uptake in the reversal of multidrug resistance by PEG-b-PLA polymeric micelles. *Biomaterials.* 2011; 32(22):5148–57. [PubMed: 21546083]
26. Rharbi Y. Reduction of the glass transition temperature of confined polystyrene nanoparticles in nanoblends. *Phys Rev E Stat Nonlin Soft Matter Phys.* 2008; 77(3 Pt 1):031806. [PubMed: 18517413]
27. Morata TC, Campo P. Ototoxic effects of styrene alone or in concert with other agents: A review. *Noise Health.* 2002; 4(14):15–24. [PubMed: 12678924]
28. Khan TR, Daugulis AJ. Application of solid-liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. *Biotechnol Bioeng.* 107(4):633–41. [PubMed: 20552670]
29. Loura LM, Fedorov A, Prieto M. Partition of membrane probes in a gel/fluid two-component lipid system: a fluorescence resonance energy transfer study. *Biochimica et biophysica acta.* 2000; 1467(1):101–12. [PubMed: 10930513]
30. Wolf DE. Determination of the sidedness of carbocyanine dye labeling of membranes. *Biochemistry.* 1985; 24(3):582–6. [PubMed: 3994976]
31. Jones DA. The important of surface area/volume ratio to the rate of oxygen uptake by red cells. *The Journal of general physiology.* 1979; 74(5):643–6. [PubMed: 512634]
32. Meng F, Sachs F. Orientation-based FRET sensor for real-time imaging of cellular forces. *Journal of cell science.* 2012; 125(Pt 3):743–50. [PubMed: 22389408]
33. Bae YH, Yin H. Stability issues of polymeric micelles. *Journal of controlled release : official journal of the Controlled Release Society.* 2008; 131(1):2–4. [PubMed: 18625275]
34. Wilhelm M, Zhao CL, Wang Y, Xu R, Winnik MA, Mura JL, Riess G, Croucher MD. Poly(styrene-ethylene oxide) Block copolymer micelle formation in water: a fluorescence probe study. *Macromolecules.* 1991; 24(5):1033–1040.
35. Davies B, Morris T. Physiological parameters in laboratory animals and humans. *Pharm Res.* 1993; 10(7):1093–5. [PubMed: 8378254]
36. Zou P, Yu Y, Wang YA, Zhong Y, Welton A, Galban C, Wang S, Sun D. Superparamagnetic iron oxide nanotheranostics for targeted cancer cell imaging and pH-dependent intracellular drug release. *Mol Pharm.* 2010; 7(6):1974–84. [PubMed: 20845930]

37. Forrest ML, Zhao A, Won CY, Malick AW, Kwon GS. Lipophilic prodrugs of Hsp90 inhibitor geldanamycin for nanoencapsulation in poly(ethylene glycol)-b-poly(epsilon-caprolactone) micelles. *J Control Release*. 2006; 116(2):139–49. [PubMed: 16926059]
38. Ansell SM, Johnstone SA, Tardi PG, Lo L, Xie S, Shu Y, Harasym TO, Harasym NL, Williams L, Bermudes D, Liboiron BD, Saad W, Prud'homme RK, Mayer LD. Modulating the therapeutic activity of nanoparticle delivered paclitaxel by manipulating the hydrophobicity of prodrug conjugates. *J Med Chem*. 2008; 51(11):3288–96. [PubMed: 18465845]
39. Johnstone, SA.; A, SM.; Xie, S.; Harasym, TO.; Mayer, LD.; Tardi, PG. Development of hydrophobic taxane prodrug nanoparticles with enhanced plasma circulation lifetime and improved efficacy. The Annual Meeting of the American Association for Cancer Research; Washington, DC. 2010.
40. Letchford K, Burt HM. Copolymer micelles and nanospheres with different in vitro stability demonstrate similar paclitaxel pharmacokinetics. *Mol Pharm*. 2012; 9(2):248–60. [PubMed: 22204437]

Figure 1. Time-resolved spectra of PEO-PS nanoparticles incubated in RPMI 1640 media and mouse plasma

(a) mixed (molar ratio 1:1) PEO-PS nanoparticles loaded with either 0.75% DiO or 0.75% DiI in RPMI 1640 media containing 10% FBS; (b) mixed PEO-PCL nanoparticles loaded with either 0.75% DiO or 0.75% DiI in RPMI 1640 media containing 10% FBS; (c), mixed PEO-PS nanoparticles loaded with 10% IONPs, 0.75% DiO and PEO-PS nanoparticles loaded with 10% IONPs, 0.75% DiI in RPMI 1640 media containing 10% FBS; (d), mixed PEO-PS nanoparticles loaded with either 6% DiD or 0.75% DiR in mouse plasma; (e), mixed PEO-PS nanoparticles loaded with 10% IONPs, 6% DiD and PEO-PS nanoparticles loaded with 10% IONPs, 6% DiI in mouse plasma. Fluorescence spectra were measured with an excitation at 480 nm or 610 nm and an emission scan 490-600 nm or 650-850 nm every 5 min over a 2 h period. No significant inter-nanoparticle cargo exchange was observed.

Figure 2. MDA-MB-231 cancer cells incubated with DiO/DiI co-loaded PEO-PS nanoparticles (a) Fluorescence of DiI; (b) Fluorescence of DiO; (c) FRET signals; (d) FRET ratios, FRET ratio = $I_{\text{FRET}}/(I_{\text{FRET}} + I_{\text{DiO}})$. FRET imaging showed increased FRET ratios from cell membranes and decreased FRET ratios of nanoparticles in the media.

Figure 3. MDA-MB-231 cancer cells incubated with mixed DiO and DiI individually loaded nanoparticles

(a) Mixed PEO-PS nanoparticles loaded with either 0.75% DiO or 0.75% DiI; (b) Mixed PEO-PCL nanoparticles loaded with either 0.75% DiO or 0.75% DiI; (c) mixed PEO-PS nanoparticles loaded with 10% IONPs, 0.75% DiO and PEO-PS nanoparticles loaded with 10% IONPs, 0.75% DiI. Release of DiO/DiI from polymeric nanoparticles resulted in increased FRET ratios in cell membranes. Incorporation of oleic acid-coated IONPs into polymeric nanoparticles reduced DiO/DiI release.

Figure 4. *In vivo* FRET imaging DiD and DiR release from PEO-PS nanoparticles

Five groups of mice ($n = 3$) were i.v. administered with one of the following treatments: PBS, DiD nanoparticles, DiR nanoparticles, mixed DiD nanoparticles and DiR nanoparticles, or DiD/DiR co-loaded nanoparticles. Images were obtained at 10 min (**a**) and 2 h (**b**) post injection. **a**, The detection of FRET signals from the mice co-administered with mixed DiD nanoparticles and DiR nanoparticles at 10 min suggested the rapid premature cargo release. **b**, Increased FRET signals from the mice co-administered with mixed DiD nanoparticles and DiR nanoparticles at 2 h suggested the release of DiD and DiR from nanoparticles into cell membranes. **c**, Average FRET ratios measured on the whole mouse body, $\text{FRET ratio} = I_{\text{FRET}} / (I_{\text{FRET}} + I_{\text{DiD}})$. The comparable average FRET ratios of the two groups at 2 h indicated that *in vivo* cargo release completed within two hours. *, $P < 0.05$; **, $P < 0.01$

Figure 5. *In vivo* FRET imaging DiD and DiR release from IONP-loaded PEO-PS nanoparticles
 Five groups of mice ($n = 3$) were i.v. administered with one of the following treatments: PBS, IONP/DiD nanoparticles, IONP/DiR nanoparticles, mixed IONP/DiD nanoparticles and IONP/DiR nanoparticles, or IONP/DiD/DiR co-loaded nanoparticles. Images were obtained at 10 min (a), 2 h (b) and 6 h (c) post injection. d, Average FRET ratios measured on the whole mouse body, $\text{FRET ratio} = I_{\text{FRET}} / (I_{\text{FRET}} + I_{\text{DiD}})$. The FRET ratios of mice co-administered with mixed IONP/DiD nanoparticles and IONP/DiR nanoparticles increased, while the FRET ratios of mice administered with IONP/DiD/DiR co-loaded nanoparticles decreased, suggesting that the incorporated oleic acid could not abolish the release. However, even at 6 h, the average FRET ratios between the two groups were not equal, indicating that the *in vivo* release was slowed by the oleic acid. *, $P < 0.05$; **, $P < 0.01$

Figure 6. *In vivo* release half-lives of DiD and DiR from PEO-PS nanoparticles in the absence and presence of IONPs

Table 1

Hydrodynamic sizes of PEO-PS nanoparticles and PEO-PCL nanoparticles

Nanoparticles	Average hydrodynamic sizes (nm)
PEO-PS with 0.75% DiO	45 ± 9
PEO-PS with 0.75% DiI	46 ± 8
PEO-PS with 0.75% DiO and 0.75% DiI	49 ± 14
PEO-PCL with 0.75% DiO	106 ± 30
PEO-PCL with 0.75% DiI	110 ± 24
PEO-PS with 10% IONPs and 0.75% DiO	156 ± 45
PEO-PS with 10% IONPs and 0.75% DiI	162 ± 50
PEO-PS with 6% DiD	50 ± 9
PEO-PS with 6% DiR	49 ± 11
PEO-PS with 6% DiD and 6% DiR	54 ± 13
PEO-PS with 10% IONPs and 6% DiD	177 ± 58
PEO-PS with 10% IONPs and 6% DiR	169 ± 52
PEO-PS with 10% IONPs, 6% DiD and 6% DiR	175 ± 61