

## Burst Release of Lipophilic Drugs from Poly (Ethylene Oxide)-B-Polystyrene Micelles is not Caused by Micelle Disassembly

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### ABSTRACT

Burst release is one of the challenges which limit clinical successes of block copolymer micelles for targeted delivery of lipophilic anticancer drugs. It's generally assumed that burst drug release in blood circulation is mainly due to blood dilution and subsequent micelle disassembly after i.v. administration. Hence, investigating the integrity of polymeric micelles is therefore essential to understanding the mechanisms of burst release. In this study, lipophilic cargo was observed to be rapidly released from poly(ethylene oxide)-b-polystyrene (PEG-PS) micelles into PC-3 and MDA-MB-231 cancer cells before the micelles were internalized into cells. Fluorescence resonance energy transfer (FRET) imaging and fluorescence quenching by iron oxide nanoparticles (IONPs) were used to assess *in vitro* and *in vivo* integrity of PEG-PS micelles. The results showed that the disassembly of PEG-PS micelles in cell culture media and fetal bovine serum was very limited over 24 h. When PEG-PS micelles were internalized into cancer cells, a majority of internalized PEG-PS micelles kept integrity. Rapid *in vivo* disassembly of micelles upon blood dilution was not observed and the micelles in mouse body gradually disassembled over 24 h. In summary, burst release of lipophilic drugs from PEG-PS micelles was not caused by micelle disassembly. This finding is useful for developing polymeric micelles with controlled release of lipophilic drugs.

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**Key words:** Burst release; Fluorescence resonance energy transfer; Polymeric micelle; Micelle disassembly; Fluorescence quenching; Anticancer drug delivery

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### INTRODUCTION

Premature release is one of the challenges which limit clinical successes of block copolymer micelles for targeted anticancer drug delivery<sup>[1,2]</sup>. In polymeric micelles, polymer unimers always exist in a dynamic equilibrium with the micelles at concentrations above the critical micelle concentration (CMC). It's generally assumed that burst drug release in blood circulation is mainly due to blood dilution and subsequent micelle disassembly after i.v. administration<sup>[3,4]</sup>. However, rapid cargo release was detected even when the blood concentrations of polymers were much higher than their aqueous CMC<sup>[5,6]</sup>, suggesting burst release may not be caused by blood dilution. In addition, serum proteins alpha- and beta-globulins were found to accelerate lipophilic cargo release from poly(ethylene glycol)-poly(D,L-lactic acid) (PEG-PDLLA) micelles<sup>[5,7]</sup>. It is generally believed that contacts with biological fluids, macromolecules, proteins, lipids and cells might cause disassembly of polymeric micelles<sup>[8]</sup>. However, no direct evidence was provided to support that serum protein could trigger rapid disassembly of micelles. To better predict and reduce burst release, it would be worthwhile to investigate if the burst release is caused by micelle disassembly.

Although the disassembly of polymeric micelles in simple aqueous solutions has been studied using classical microscopy-, spectroscopy-, and chromatography-based methods, intracellular and *in vivo* disassembly is still hard to be monitored and direct evidence for rapid *in vivo* disassembly of micelles is very limited<sup>[8,9]</sup>. The only reported effort to detect intracellular and *in vivo* disassembly of polymeric micelles was the development of a fluorogenic-based approach<sup>[8]</sup>. In this design, a fluorogenic dye fluorescein-5-carboxyl azide diacetate (F-5-CADA) was covalently conjugated to the lipophilic block end of poly(caprolactone)-b-poly(ethyleneoxide) (PEG-PCL). The fluorescence was not detectable until the PEG-PCL micelle was disrupted and the ester group of the dye was cleaved. *In vitro* and *in vivo* micelle disruption was monitored by the increase of fluorescence. However, the limitation of this fluorogenic-based approach is obvious since it requires an additional F-5-CADA

activation step (ester cleavage). The estimation of disassembly was based on two assumptions: F-5-CADA in the core of intact micelles cannot be activated and F-5-CADA is immediately and totally activated upon *in vivo* disassembly. However, ester cleavage might occur even though micelles keep integrity. Furthermore, disassembly of micelles does not guarantee ester cleavage.

Fluorescence resonance energy transfer (FRET) has been employed to investigate lipophilic cargo release from micelles and liposomes<sup>[5-7,10-12]</sup>. The proximity (less than 10 nm) between FRET donor dye and FRET acceptor dye gives rise to FRET effect, which is utilized to measure the distance between the two dyes. The release of donor dye and acceptor dye from nanocarriers causes decrease or disappearance of FRET. Although the reported FRET approaches can detect cargo release from micelles, they are unable to detect micelle disassembly. Covalently conjugating donor and acceptor dyes to lipophilic end of polymers may provide an alternative FRET approach to detect micelle disassembly.

Due to their high optical absorption, gold nanoparticles and iron oxide nanoparticles (IONPs) are able to quench fluorescent dyes<sup>[13,14]</sup> and quantum dots<sup>[15,16]</sup> in close proximity by attenuating both the excitation beam and the fluorescence signal (inner filter effect). In previous reports, cationic tetramethylrhodamine (TAMRA)<sup>[17]</sup> and sulforhodamine 101<sup>[18]</sup> were conjugated to lipophilic end of block copolymers and the fluorescent dye-labeled polymers were used to encapsulate oleic acid-coated lipophilic IONPs. However, fluorescent dyes could not be self-quenched or quenched by IONPs since the positive charge of the dyes prevented their intermolecular interaction and close interaction with lipophilic IONPs. Furthermore, conjugation with cationic or anionic dyes might change cellular internalization process and intracellular distribution of nonionic block copolymers<sup>[19,20]</sup>. To achieve efficient fluorescence quenching by core-loaded IONPs and monitor the integrity of nonionic micelles, neutral and nonpolar dyes are required.

Bodipy dyes are relatively nonpolar and the chromophore is electrically neutral<sup>[21-23]</sup>. These properties tend to minimize dye-induced perturbation of conjugate functional properties. In current study, a pair of dyes Bodipy-FL (FRET donor) and Bodipy-TMR (FRET acceptor) was conjugated to polystyrene end of PEG-PS. The conjugation with neutral and nonpolar Bodipy dyes allowed the formation of a compact micelle core, which resulted in FRET effect between the two dyes. The FRET effect was utilized to monitor *in vitro* disassembly of PEG-PS micelles. Furthermore, IONPs were encapsulated into the core of PEG-PS micelles to quench the Bodipy dye conjugated to polystyrene end. Fluorescence recovery was utilized to detect *in vivo* disassembly of polymeric micelles.

## MATERIALS AND METHODS

### Materials

Lipophilic IONPs (10 nm) and SuperMag Separator<sup>TM</sup> were supplied by Ocean NanoTech (Springdale, AR). Carboxyl-PEG-PS (9.5 kD-b-18 kD) and PEG-b-PS-NH<sub>2</sub> (5 kD-b-5 kD) were purchased from Polymer Source Inc. (Dorval, Quebec, Canada). Fluorescein-5-carboxamide cadaverine (5-FAM cadaverine, ex/em 490/521 nm) was purchased from AnaSpec (Fremont, CA). Bodipy-FL (ex/em 504/513 nm), Bodipy-TMR (ex/em 535/574 nm), Bodipy-665 (ex/em 650/665 nm) succinimidyl ester and DiI 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 kD) were purchased from Millipore (Billerica, MA). Dialysis tubing (MWCO 3.5-5 kD) was supplied by Spectrum Laboratories, Inc (Rancho Dominguez, CA). Tetrahydrofuran (THF)

and other chemical reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

### Synthesis of fluorescence-labeled PEG-PS

Bodipy dyes were conjugated to polystyrene end of PEG-PS (5 kD-b-5 kD) polymer. PEG-PS-FL polymer: A total of 30 mg of PEG-PS-NH<sub>2</sub> (3 μmol) was dissolved in 2 mL anhydrous THF. 10 mg of Bodipy-FL succinimidyl ester (24 μmol) was added under stirring. To the mixture, 10 μL of triethylamine was added and the reaction continued overnight under stirring. Bodipy-FL labeled PEG-PS (PEG-PS-FL) was purified using Sephadex LH-20 columns in triplicate. The purified PEG-PS-FL in THF was filtered through a 0.45 μm filter and dried under vacuum. 30.6 mg of powder was obtained and stored at -20°C. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 7.39 (s, 0.3 H), 6.25-7.2 (brm, 250 H), 3.57(t, 450 H), 3.28 (t, 1 H) 3.21 (t, 1 H), 2.60 (t, 1 H), 2.51 (s, 1.3 H), 2.26 (s, 1.3 H), 2.16 (t, 1 H), 1.78 (brm, 50 H), 1.36 (brm, 100 H). PEG-PS polymer was labeled with Bodipy-TMR (PEG-PS-TMR polymer) and Bodipy-665 (PEG-PS-665 polymer) following similar procedures. PEG-PS-TMR: <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 7.87 (d, 1 H), 7.27 (d, 1 H), 6.25-7.2 (brm, 250 H), 3.85 (s, 1.4 H), 3.58(t, 450 H), 3.20 (t, 1 H), 2.75(t, 1 H), 2.53 (s, 1.3 H), 2.41 (t, 1 H), 2.17 (t, 1 H), 2.13 (s, 1.3 H), 1.79 (brm, 50 H), 1.36(brm, 100 H). PEG-PS-665: <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 7.56 (m, 1 H), 7.40 (s, 0.4 H), 6.25-7.2 (brm, 250 H), 4.57 (s, 1 H), 3.57 (t, 450 H), 3.29 (t, 1 H), 2.17 (t, 1 H), 1.79 (brm, 50 H), 1.36 (brm, 100 H).

5-FAM was conjugated to PEG end of PEG-PS (9.5 kD-b-18 kD) polymer. A total of 110 mg of carboxyl-PEG-b-PS (4 μmol) was dissolved in 2.5 mL anhydrous THF. 82.5 mg of DCC (0.4 mmol) and 69 mg of NHS (0.6 mmol) were added to the polymer solution and stirred for 20 min. Following the addition of 92.1 mg of 5-FAM cadaverine (200 μmol), 10 μL of triethylamine was added to the mixture and the reaction continued overnight under stirring at room temperature. 5-FAM labeled PEG-b-PS (5FAM-PSO-PS) was purified using Sephadex LH-20 columns. The purified 5FAM-PEG-PS was dried under vacuum and stored at -20°C. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 10.44 (s, 0.44 H), 8.41 (d, 0.69 H), 8.02 (dd, 0.64 H), 7.64 (d, 0.72 H), 7.47 (d, 0.70 H), 6.25-7.20 (brm, 880 H), 3.58 (t, 906 H), 3.24 (t, 1.6 H), 2.98 (t, 1.5 H), 2.55 (t, 1.5 H), 1.67-1.84 (brm, 180 H), 1.20-1.36 (brm, 350 H).

### Fabrication of polymeric micelles

IONP-loaded PEG-PS-665 micelles were prepared by a precipitation method. 10 mg of PEG-PS-665 was dissolved in 0.5 mL of THF and 1 mg of IONPs was added to the solution. 2 mL of deionized water was dropped at a speed of 6 mL/min using a syringe pump (Fisher Scientific, Pittsburgh, PA) under vigorous stirring and followed by 20 min of stirring. The solution was then dialyzed against 2 liters of deionized water for 2 days. Water was changed every day. IONP-loaded nanoparticles were isolated and concentrated using a magnetic separator (Ocean NanoTech, Springdale, AR). Finally, the IONP-loaded nanoparticles were resuspended in PBS (equivalent to 0.5 mM polymer) and filtered through a 0.45 μm filter, and stored at 4°C. By following this procedure, IONP-free PEG-PS-FL, PEG-PS-TMR, and PEG-PS-665 micelles were prepared. 10 mg of PEG-PS-FL and 10 mg of PEG-PS-TMR were dissolved in 0.5 mL of THF to prepare FRET micelles. The micelles were concentrated using a 10 kD MWCO centrifugal filter unit and suspended in PBS (equivalent to 1 mM of polymers). 5-FAM labeled and DiI-loaded PEG-PS micelles were prepared by following similar procedures. Briefly, 10 mg of 5FAM-PEG-PS and 0.1 mg of DiI were dissolved in 0.5 mL of THF. 2 mL of deionized water were added under vigorous stirring.

After dialysis, the micelle solution passed through a PD-10 desalting column (GE Healthcare, Piscataway, NJ) to remove unencapsulated DiI. The micelles were suspended in PBS (equivalent to 0.5 mM polymer).

#### Characterization of polymeric micelles

Average hydrodynamic sizes of micelles were measured using a Zetasizer Nano ZS particle sizer (Malvern Instruments Ltd, Westborough, MA). The morphology of IONP-loaded polymeric micelles was obtained on a Philips CM-100 transmission electron microscope (TEM). Fluorescence spectra of various micelles were measured on an LS55 PerkinElmer luminescence spectrometer (Waltham, MA) with an excitation at 488 nm for Bodipy-FL and FRET effect, 520 nm for Bodipy-TMR and 610 nm for Bodipy-665. To evaluate quenching effect of IONPs on fluorescence of Bodipy-665, IONP-loaded micelles were suspended in PBS or THF to record the fluorescent spectra. To measure FRET ratios, FL/TMR FRET micelles and mixed FL micelles and TMR micelles were excited at 488 nm and the emission scan was 500-620 nm. The FRET ratios were calculated as  $\text{FRET ratio} = \text{IFRET} / (\text{IFRET} + \text{IFL})^{[6]}$ . IFRET and IFL are the fluorescence intensity at 570 nm and 513 nm respectively.

#### Integrity of micelles in PBS, cell culture media and fetal bovine serum

Aliquots of FL-TMR FRET micelles were incubated in phosphate-buffered saline, RPMI 1640 with 10% fetal bovine serum (FBS), or 100% FBS in triplicate in a 96-well plate at 37°C for 24 h. The final incubation concentrations of polymers were 50  $\mu\text{M}$  and 5  $\mu\text{M}$ . Fluorescence of Bodipy-FL (ex/em 488/513 nm) and FRET (ex/em 488/570 nm) were measured using a Synergy plate reader (BioTek, Winooski, VT) at 0.5 h, 6 h and 24 h. The FRET ratios were calculated as  $\text{FRET ratio} = \text{IFRET} / (\text{IFRET} + \text{IFL})$ . Meanwhile, the FRET ratio of FRET micelles in THF/water (50%/50%) was measured as a control. The FRET ratio of mixed PEG-PS-FL micelles and PEG-PS-TMR micelles (molar ratio 1:1) in PBS was also measured as a negative control.

#### Fluorescence microscopy and FRET confocal microscopy

A prostate cancer cell line PC-3 and a breast cancer cell line MDA-MB-231 obtained from American Type Culture Collection (ATCC, Rockville, MD) were cultured on 8-well Lab-Tek glass chamber slides (Thermo Fisher Scientific, Rochester, NY) for FRET and fluorescent imaging. 104 cells per well were incubated for 2 days to allow cell adherence. To visualize DiI release and micelle internalization, 5-FAM labeled and DiI-loaded PEG-PS micelles (equivalent to 10  $\mu\text{M}$  polymer) were incubated with cancer cells at 37°C for the desired lengths of time before imaging. Nuclei were stained with Hoechst. Images were obtained with TRITC (DiI), FITC (5-FAM) and DAPI channels on a Nikon TE2000S epifluorescence microscope coupled with a standard mercury bulb illumination, a CCD camera (Roper Scientific, Tucson, AZ). Images were overlaid using MetaMorph<sup>®</sup> software (Molecular Devices Corporation, Sunnyvale, CA).

To track intracellular disassembly of micelles, cancer cells were incubated with 50  $\mu\text{M}$  of FL/TMR FRET micelles for 8 h. In parallel, cells were incubated with mixed PEG-PS-FL micelles (25  $\mu\text{M}$ ) and PEG-PS-TMR micelles (25  $\mu\text{M}$ ) for 8 h as a control. The FRET confocal images were acquired by 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 at room temperature. Images were recorded under Bodipy-FL channel (488 nm excitation, 535 $\pm$ 20 nm emission), FRET channel (488 nm excitation, 580 $\pm$ 20 nm emission) and Bodipy-TMR channel (543 nm excitation, 580 $\pm$ 20 nm emission). The exposure time was 200 ms. Images were obtained using MetaMorph v6.5.3 (Universal Imaging, Malvern, PA). The images were background-subtracted using the "Background Correction" tool in MetaMorph. Crosstalk correction and FRET ratios calculation were carried out using an in-house FRETcalculator program and Matlab. To correct crosstalk, correction coefficients  $\alpha$  and  $\beta$  were determined from cells incubated with PEG-PS-TMR micelles only ( $\alpha = \text{IFRET}/\text{ITMR}$ ) and PEG-PS-FL micelles only ( $\beta = \text{IFRET}/\text{IFL}$ )<sup>[24-26]</sup>. IFRET, ITMR, and IFL were intensities in each region of interest (ROI) under FRET, Bodipy-TMR, and Bodipy-FL filter sets, respectively. FRET ratios were calculated as:  $\text{FRET ratio} = \text{IFRET} / (\text{IFRET} + \text{IFL})$ .

#### Xenograft mice, in vivo and ex vivo fluorescent imaging

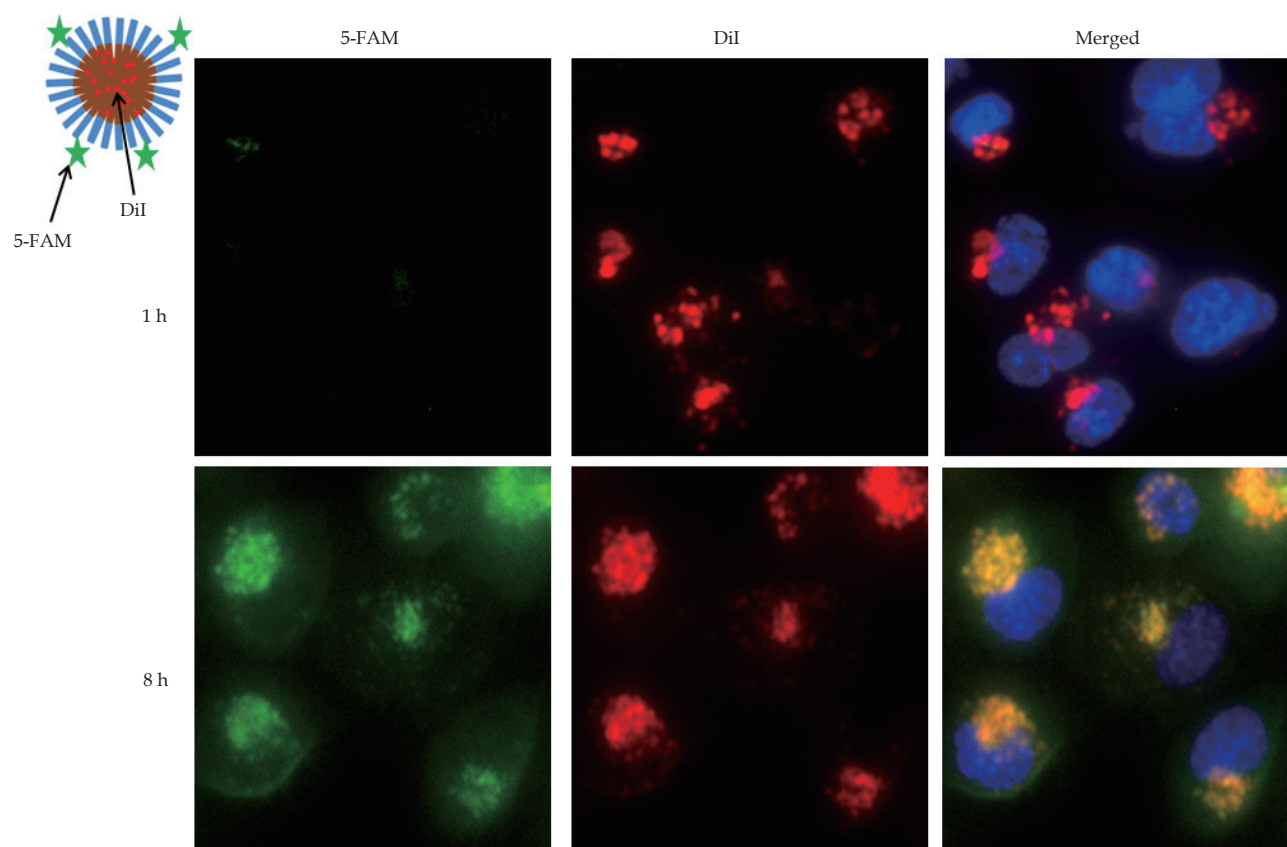
The animal procedures were performed according to a protocol approved by the University Committee for the Use and Care of Animals (UCUCA) at University of Michigan. Female athymic nude mice (nu/nu), obtained from National Cancer Institute (Bethesda, MD) at 8 weeks of age, were subcutaneously inoculated in the back with  $5 \times 10^6$  PC-3 cells suspended in a mixture of 50  $\mu\text{L}$  of PBS and 50  $\mu\text{L}$  of matrixgel basement membrane (BD Biosciences, San Jose, CA). When the tumor implants reached 0.8 cm in diameter, the tumor-bearing mice were subjected to the imaging studies.

*In vivo* fluorescence imaging was performed with an IVIS Spectrum imaging system (Xenogen, Alameda, CA). The exposure time was 1 s. Images were acquired and analyzed using Living Image 2.5 software (Xenogen, Alameda, CA). Images were recorded under an excitation of 640 nm and an emission of 680 nm at 2 h and 24 h after i.v. administration of PEG-PS-665 micelles (equivalent to 0.1  $\mu\text{mole}$  of polymer) or IONP-loaded PEG-PS-665 micelles (equivalent to 0.1  $\mu\text{mole}$  of polymer). Simultaneously, 0.2 mL of PEG-PS-665 micelles or IONP-loaded PEG-PS-665 micelles in an eppendorf tube (10  $\mu\text{M}$  in THF, PBS or mouse plasma) were imaged with mice. At the end of experiment, the mice were sacrificed using CO<sub>2</sub>. Blood and tissues (tumor, heart, lung, liver, spleen, and kidneys) were collected and tissues were rinsed with PBS. Blood and tissue samples were imaged using the identical settings as *in vivo* imaging.

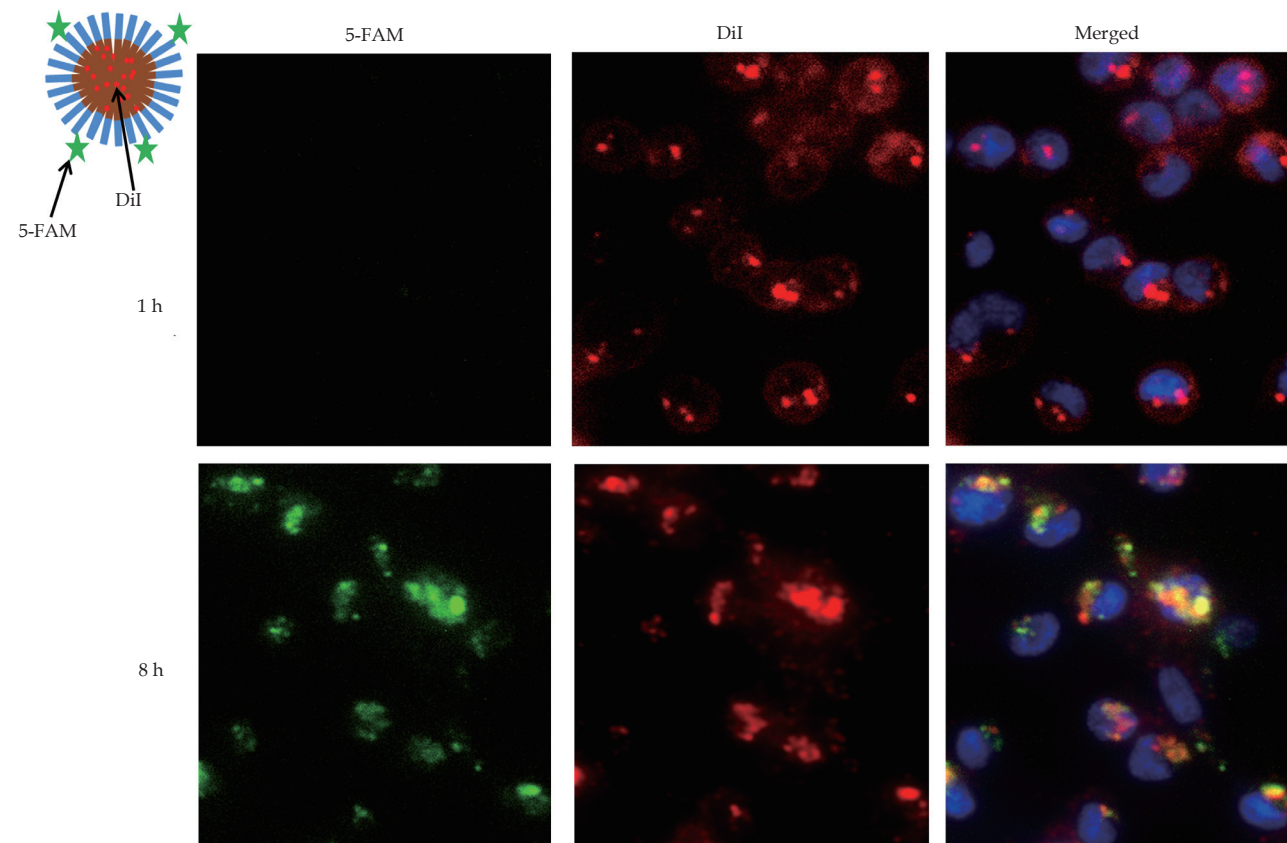
## RESULTS

#### Rapid release of DiI from 5FAM-PEG-PS micelles

Rapid release of lipophilic cargos from PEG-PDLLA, PEG-PCL, PEG-b-poly 4-(vinylpyridine) (PEG-PVPy) and PEG-b-distearoylphosphatidylethanolamine (PEG-DSPE) micelles has been detected *in vitro*<sup>[6,7,12,27]</sup> and *in vivo*<sup>[5]</sup>. To investigate cargo release from PEG-PS micelles, PC-3 cells were incubated with 5-FAM and DiI dual-labeled PEG-PS micelles (Figure 1). After incubation for 1 h, encapsulated DiI was released into cells and accumulated in endosomes and lysosomes. No significant uptake of 5-FAM labeled micelles was observed (Figure 1). In contrast, at 8 h, both 5-FAM labeled micelles and DiI were internalized into cells and co-localized in endosomes and lysosomes. Similar results were observed in MDA-MB-231 cells (Figure 2). The results suggested that lipophilic cargo and PEG-PS micelles were not internalized into cells simultaneously. This is consistent with previous reports which revealed that DiI dye was rapidly released from PEG-PDLLA<sup>[6]</sup>, PEG-PCL<sup>[6]</sup>, PEG-PVPy<sup>[12]</sup> and PEG-DSPE<sup>[7]</sup> micelles prior to cellular uptake of the micelles.



**Figure 1** Separate uptake of 5-FAM labeled PEG-PS micelles and DiI by PC-3 cells. PC-3 cells were incubated with 0.275 mg/mL 5-FAM-labeled micelles containing 1% DiI for 1h and 8 h. The green color represents fluorescence from 5-FAM labeled micelles and the red color represents DiI fluorescence.



**Figure 2** Separate uptake of 5-FAM labeled PEG-PS micelles and DiI by MDA-MB-231 cells. MDA-MB-231 cells were incubated with 0.275 mg/mL 5-FAM-labeled micelles containing 1% DiI for 1 h and 8 h. The green color represents fluorescence from 5-FAM labeled micelles and the red color represents DiI fluorescence.

**Characterization of FRET micelles and PEG-PS-665 micelles**

To elucidate the mechanisms of rapid release of DiI from PEG-PS micelles, it is worthwhile to test *in vitro* and *in vivo* integrity of PEG-PS micelles. Bodipy dyes (FL, TMR and 665) were conjugated to the amine group at the end of polystyrene block. Lipophilic IONPs were entrapped in the core of PEG-PS-665 micelles to quench Bodipy-665. The average hydrodynamic sizes of Bodipy dye labeled micelles ranged from 35 nm to 46 nm (Table 1). Incorporation of 10% IONPs increased the average size of PEG-PS-665 micelles from 40 nm to 167 nm.

**Table 1** Hydrodynamic size of polymeric micelles.

	Average hydrodynamic sizes (nm)
PEG-PS-FL	35 ± 8
PEG-PS-TMR	46 ± 15
PEG-PS-FL/TMR	39 ± 11
PEG-PS-665	40 ± 9
PEG-PS-665+IONPs	167 ± 43

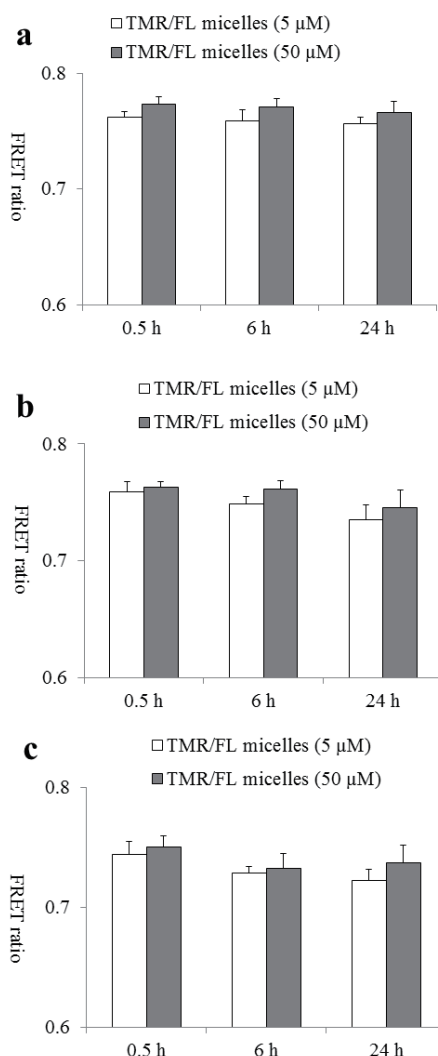
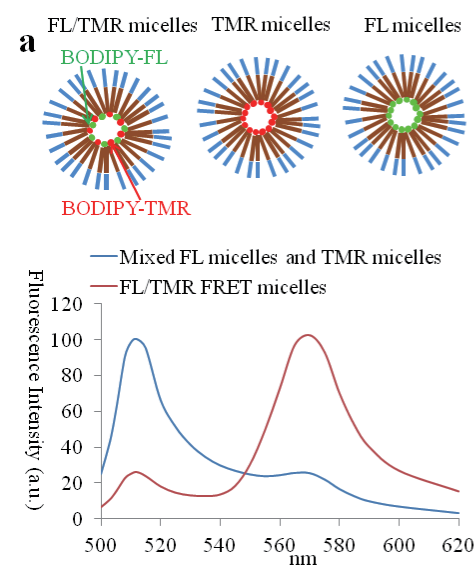
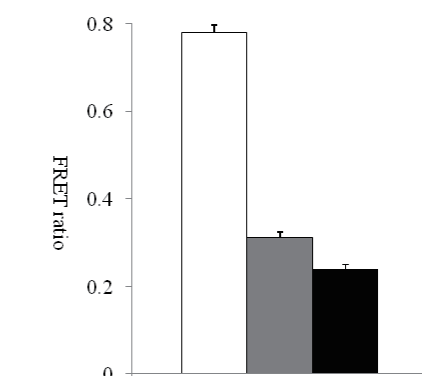
Figure 3a shows the diagrams and fluorescence spectra of FL micelles, TMR micelles and FL/TMR FRET micelles. FL/TMR micelles in PBS were excited at 488 nm. The formation of a compact lipophilic core of FL/TMR FRET micelles gave rise to the FRET effect between FL and TMR with 480 nm excitation (Figure 3a, red line). FRET ratio was determined to be  $0.78 \pm 0.02$  (Figure 3b). When mixed FL micelles and TMR micelles (molar ratio 1:1) in PBS was excited at 480 nm, no FRET effect was observed (Figure 3a, blue line). The fluorescence at 570 nm was due to spectral crosstalk. The FRET ratio was  $0.24 \pm 0.02$ . The disassembly of FL/TMR FRET micelles in 50% THF decreased FRET ratio to 0.31, indicating that the decrease of FRET ratio can be utilized to detect the disassembly of micelle.

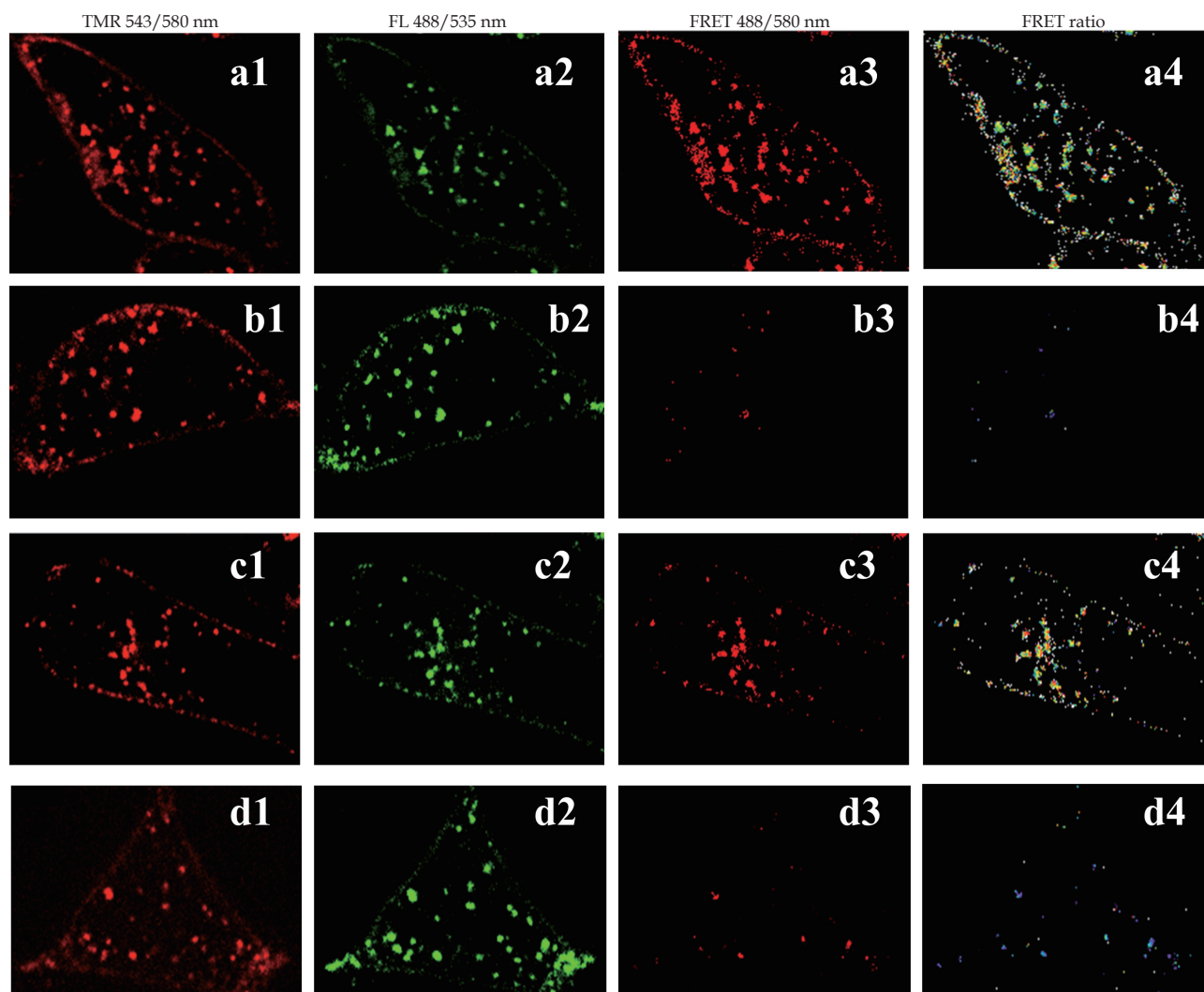
**Integrity of PEG-PS micelles in PBS, cell culture media and FBS**

FL/TMR FRET micelles were incubated in PBS, RPMI 1640 with 10% of FBS and 100% FBS at concentrations of 50  $\mu$ M and 5  $\mu$ M. Fluorescence of Bodipy-FL and FRET were measured at 0.5 h, 6 h and 24 h. The decrease of FRET ratio was used to detect the disassembly of FRET micelles. As shown in Figure 4a, FRET ratios of FRET micelles were 0.75 at 5  $\mu$ M and 0.77 at 50  $\mu$ M. At concentrations of 50  $\mu$ M and 5  $\mu$ M, the FRET ratios of micelles in PBS were constant over 24 h, indicating that PEG-PS micelles were not disrupted in PBS within 24 h.

In RPMI 1640 media with 10% of FBS (Figure 4b), the FRET ratio of FL/TMR micelles (5  $\mu$ M) decreased from 0.76 at 0.5 h to

0.73 at 24 h, indicating a slight increase of the mean particle size of micelles or the disassembly of a small fraction of micelles. Similarly, when FL/TMR micelles (5  $\mu$ M) were incubated in 100% of FBS, FRET ratios decreased from 0.74 at 0.5 h to 0.72 at 24 h (Figure 4c). The results suggested that a majority of micelles kept their integrity in PBS, RPMI 1640 and FBS within 24 h.

**Figure 4** Integrity of micelles and IONP-loaded micelles in (a) PBS, (b) RPMI 1640 medium, and (c) FBS. Open bar: concentration of micelle polymer 5  $\mu$ M; solid bar: concentration of micelle polymer 50  $\mu$ M.**Figure 3** Fluorescence spectra and FRET ratios of FL/TMR FRET micelles. (a) Diagrams and fluorescence spectra of FL/TMR micelles (red) and mixed FL micelles and TMR micelles (blue), excitation: 480 nm; (b) FRET ratios of FL/TMR FRET micelles in PBS and THF and FRET ratios of mixed FL micelles and TMR micelles in PBS. FRET ratio =  $IFRET / (IFRET + IFL)$ , IFRET and IFL are the fluorescence intensity at 570 nm and 513 nm respectively (Excitation 488 nm).



**Figure 5** FRET images of PC-3 cells and MDA-MB-231 cells. PC-3 cells were incubated with FL/TMR FRET micelles (a1-a4), and mixed PEG-PS-PL and PEG-PS-TMR micelles (b1-b4) for 8 h. MDA-MB-231 cells were incubated with FL/TMR FRET micelles (c1-c4), and mixed PEG-PS-PL and PEG-PS-TMR micelles (d1-d4) for 8 hours. FRET ratio =  $\text{IFRET} / (\text{IFRET} + \text{IFL})$ ; FL channel: excitation 488 nm, emission  $535 \pm 20$  nm; FRET channel: excitation 488 nm, emission  $580 \pm 20$  nm; and TMR channel: excitation 543 nm, emission  $580 \pm 20$  nm.

#### FRET confocal imaging

To detect intracellular disassembly of micelles, PC-3 cells were incubated with FL/TMR FRET micelles (Figure 5 a1-a4) and mixed FL micelles and TMR micelles (Figure 5 b1-b4) in parallel. FRET signals were detected on both cell membranes and intracellular organelle membranes after incubation with FL/TMR FRET micelles for 8 h (Figure 5 a4), indicating the integrity of micelles during cellular internalization process. The FRET ratios detected from endosomes/lysosomes were slightly lower than that from cell membranes, suggesting a gradual disassembly of micelles in endosomes/lysosomes. In contrast, no FRET was observed when the cells were incubated with mixed FL micelles and TMR micelles (Figure 5 b4). Similar results were observed when MDA-MB-231 cells were incubated with FL/TMR FRET micelles (Figure 5 c1-c4) and mixed FL micelles and TMR micelles (Figure 5 d1-d4).

#### Fluorescence quenching by the formation of micelles and IONP-loaded micelles

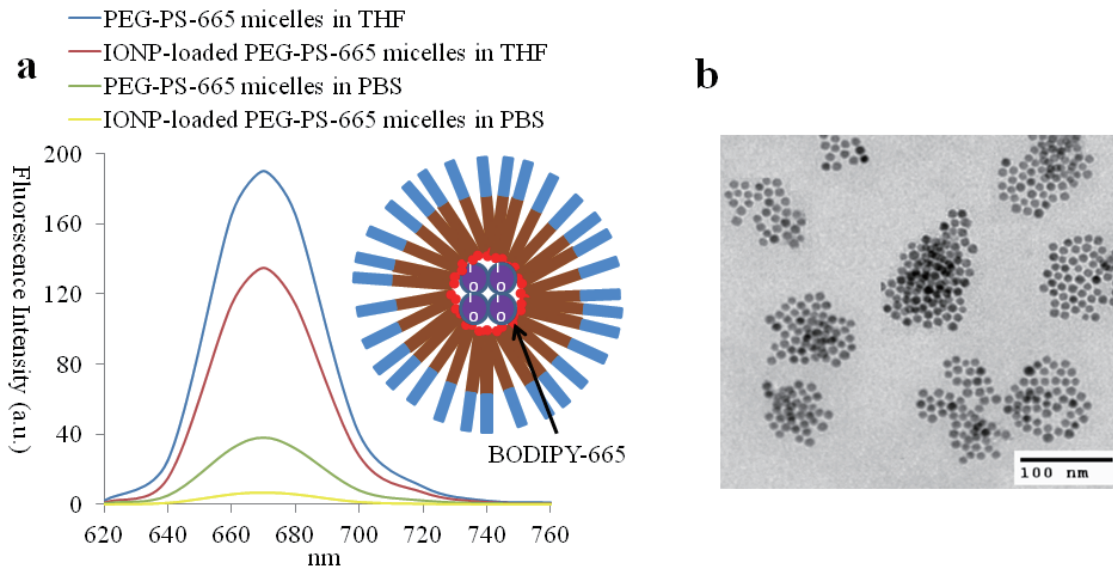
As shown in figure 6a, the formation of core-shell micelles caused quenching of Bodipy-665 fluorescence. The disassembly of PEG-PS-665 micelles ( $5 \mu\text{M}$ ) in THF resulted in a fluorescent intensity (blue line) 5 fold higher than that of intact micelles in PBS (green line). IONP-loaded PEG-PS-665 micelles ( $5 \mu\text{M}$ ) showed a 21.4 fold

higher fluorescence at 670 nm in THF (red line) than in PBS (yellow line). TEM image of IONP-loaded PEG-PS micelles showed clusters of 10 nm IONPs in the cores of PEG-PS-665 micelles (Figure 6b).

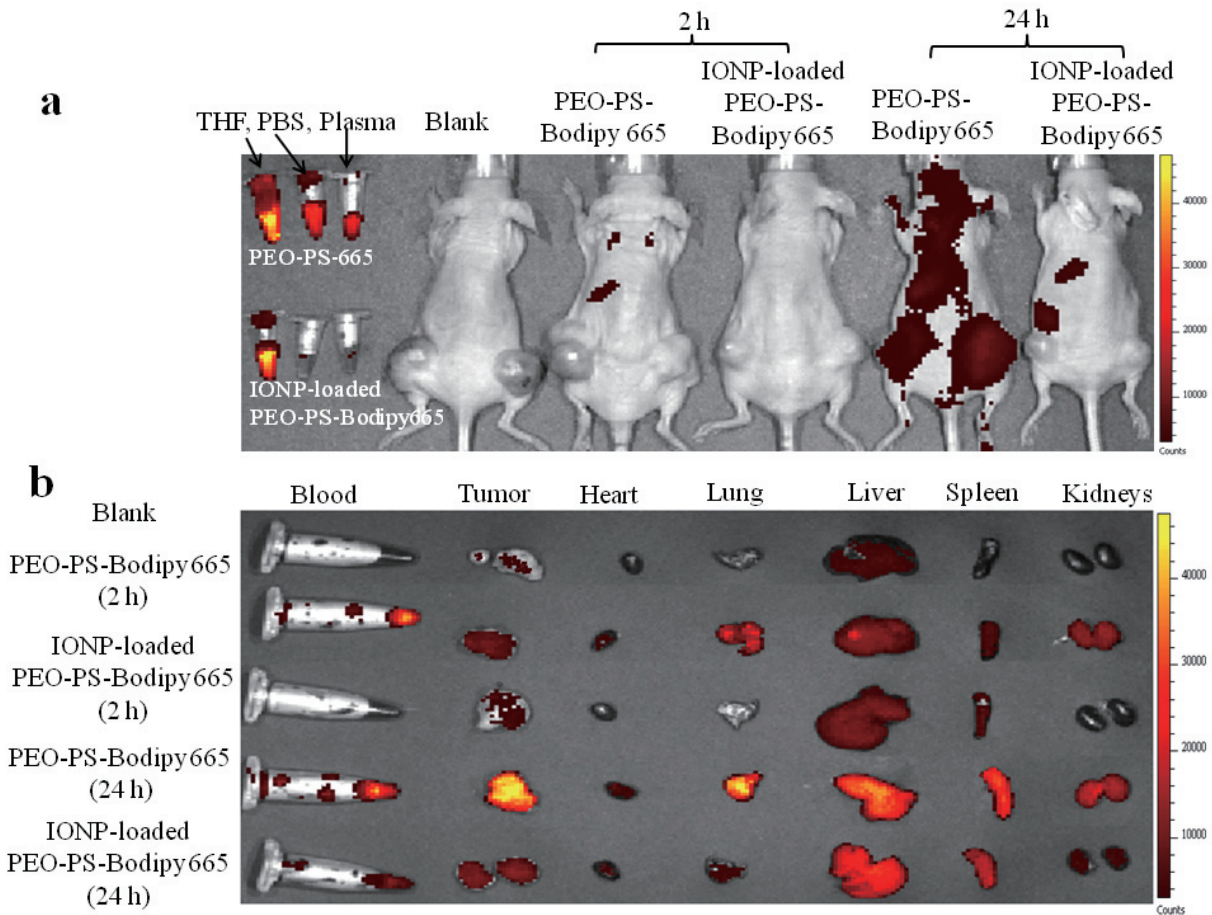
#### *In vivo* micelle disassembly in xenograft mice

Formation of compact PEG-PS-665 micelles and incorporation of IONPs into PEG-PS-665 micelles dramatically quenched fluorescence of Bodipy-665. Hence, the recovery of Bodipy-665 fluorescence was used to detect *in vivo* disassembly of micelles. As shown in figure 7a, PEG-PS-665 micelles and IONP-loaded PEG-PS-665 micelles in PBS and mouse plasma exhibited much lower fluorescence than in THF, suggesting that both PEG-PS-665 micelles and IONP-loaded PEG-PS-665 micelles kept their integrity in PBS and mouse plasma.

PEG-PS-665 micelles and IONP-loaded PEG-PS-665 micelles were *i.v.* injected to nude mice with xenograft PC-3 tumors. At 2 h following the injection of PEG-PS-665 micelles, due to very limited disassembly of micelles, only very low fluorescence was detectable (Figure 7a). At 2 h, fluorescence was not detectable from the mouse treated with IONP-loaded PEG-PS-665 micelles. However, at 24 h, both the mouse treated with PEG-PS-665 micelles and the mouse treated with IONP-loaded PEG-PS-665 micelles showed dramatically increased fluorescence. The large particle sizes of IONP-loaded PEG-



**Figure 6** Characterization of PEG-PS-665 micelles and IONP-loaded PEG-PS-665 micelles. (a) The formation of micelles in PBS caused fluorescence quenching and the disassembly of micelles in THF caused recovery of fluorescence; (b) TEM of IONP-loaded PEG-PS-665 micelles.



**Figure 7** *In vivo* and *ex vivo* fluorescent images of xenograft mice administered with PEG-PS-665 micelles and IONP-loaded PEG-PS-665 micelles. PEG-PS-665 micelles or IONP-loaded PEG-PS-665 micelles in an eppendorf tube ( $10 \mu\text{M}$  in THF, PBS or mouse plasma) were imaged with mice. Excitation 640 nm, emission 680 nm.

PS-665 micelles ( $167 \pm 43$  nm) caused rapid elimination from the mouse body, which might explain the lower fluorescence than that of the mouse treated with PEG-PS-665 micelles. The data showed that micelle disassembly within 2 h was very limited and most micelles gradually disassembled in mice.

The mice were sacrificed at 2 h and 24 h following the injection

of micelles to collect the blood and tissues. Figure 7b showed the image of blood and tissue samples collected from blank control mouse, the mice treated with PEG-PS-665 micelles, and the mice treated with IONP-loaded PEG-PS-665 micelles. At 2 h after the injection of IONP-loaded micelles, compared with blank control, slight fluorescence increase was observed only in spleen and liver,

which suggested that micelles kept integrity in blood circulation, tumor, heart, lung and kidneys. The disassembly in liver and spleen might be due to rapid uptake and digestion by macrophages. At 24 h post the injection of IONP-loaded micelles, increased fluorescence was detected in blood and tissues, indicating gradual disassembly of micelles in mouse over 24 h. Consistently, blood and tissues collected from the mice treated with PEG-PS-665 micelles showed dramatically increased fluorescence from 2 h to 24 h, suggesting that a majority of PEG-PS micelles disassembled between 2-24 h and there was no burst disassembly of micelles.

## DISCUSSION

To achieve targeted drug delivery and sustained drug release, it is essential to understand the mechanisms of burst release and reduce the burst release. In this study, rapid Dil release from 5-FAM labeled PEG-PS micelles to cancer cells was observed prior to the cellular internalization of micelles (Figure 1 and figure 2). Similar rapid lipophilic cargo release from PEG-PCL micelles and PEG-PDLLA micelles was previously reported<sup>[6,27]</sup>. *In vivo* burst release of lipophilic dyes from PEG-PDLLA micelles was observed in mice<sup>[5]</sup>. A majority of dyes was released at 15 min post i.v. injection. Our recent study showed that the *in vivo* release half-life of lipophilic dyes from PEG-PS micelles was only 9.2 min (unpublished results). The rapid release is unlikely caused by disassembly of micelles since polymer concentrations in incubation and blood circulation were much higher than their CMC. Furthermore, another study revealed that a majority (80%) of PEG-PCL micelles kept their integrity even 20 h after their internalization into bladder cancer cells<sup>[8]</sup>, indicating disassembly is not the major cause of rapid lipophilic cargo release.

One of the reasons for limited understanding of *in vivo* integrity of micelles is that currently there are no practical experimental methods available to detect the disassembly of micelles without micelle modification<sup>[3]</sup>. Various fluorescent dyes such as tetramethylrhodamine (TAMRA)<sup>[17,19]</sup> and sulforhodamine 101<sup>[18]</sup> have been used to label the hydrophobic end of block copolymers. Although the formation of micelles was observed, the cationic dyes could not form a compact core and efficient fluorescence quenching was not achieved. In our experiment, PEG-PS was labeled with 5-FAM or TAMRA, IONPs entrapped in the core of micelles were not able to quench 5-FAM or TAMRA (data not shown). In contrast, neutral and lipophilic Bodipy dye-labeled PEG-PS formed micelles with a compact core, which allowed FRET effect between Bodipy-FL and Bodipy-TMR. The encapsulation of IONPs could dramatically quench the fluorescence of Bodipy-665.

The incubation of FL/TMR FRET micelles with PBS showed that micelles kept integrity in PBS within 24 h, which was consistent with previous report<sup>[8]</sup>. In RPMI 1640 media containing 10% of FBS and 100% of FBS, FRET ratios of micelles only slightly decreased, suggesting that most micelles kept integrity and the decrease of FRET ratio was likely due to particle size changes in different media. In a previous report<sup>[8]</sup>, incubation with RPMI 1640 media and 100% of FBS for 24 h caused 36% and 74% integrity loss of poly(caprolactone)-b-poly(ethylene oxide) (PEG-PCL) micelles. However, the micelles disassembly was probably overestimated since this method was based on the activation of a dye F-5-CADA (to cleave an ester of the fluorescent dye to generate fluorescence). Considering the dynamic equilibrium between PEG-PCL unimers and micelles in the incubations, endogenous esterases in FBS could rapidly activate F-5-CADA even without the disassembly of micelles. There exists some evidence that enzymes possibly penetrate the hydrophilic shell of the micelles. For example, it was observed

that a micelle consisting of PEG-PCL was slowly degraded in the presence of lipase K<sup>[28]</sup>. Additionally, compared with PEG-PCL, PEG-PS polymer has a much lower CMC and higher glass transition temperature of polystyrene (107°C)<sup>[29]</sup>, which might explain the less disassembly of PEG-PS micelles in FBS.

FRET has been utilized to investigate lipid exchange between micelles and between liposomes<sup>[30,31]</sup>. In current study, the FRET between Bodipy-FL and Bodipy-TMR was used to monitor micelles disassembly. The FRET confocal imaging showed that PEG-PS micelles kept integrity during cellular internalization and the micelles gradually lost integrity in endosomes/lysosomes of cancer cells. This was consistent with a previous study, in which 20% of internalized PEG-PCL micelles were disrupted after incubation with bladder cancer cells for 20 h<sup>[8]</sup>. Ideally, FRET effect between Bodipy dyes can be used to visualize the *in vivo* disassembly of micelles. Unfortunately, a pair of near-infrared Bodipy dyes (emission > 700 nm) is still not commercially available. The available Bodipy dye with the longest emission is Bodipy-665. Although IONP-loaded PEG-PS-665 micelles are able to detect micelle disassembly in dissected mouse tissues, poor tissue penetration limits its application to noninvasive imaging of deep tissues. Furthermore, the autofluorescence from tumors and liver detected at emission 680 nm (Figure 7b, blank control) affects accurate detection of micelle disassembly in these two tissues.

The exclusion of micelle disassembly as a major cause of burst release from PEG-PS polymeric micelles has its implications for reducing burst release. Various strategies have been employed to reduce burst release by preventing micelle disintegration, such as varying the lengths of hydrophilic and hydrophobic blocks of the block copolymers, crosslinking of the core, and/or crosslinking the shell. These approaches decrease CMC of micelles and prevent micelle disintegration in the bloodstream but might not necessarily eliminate burst release. On the other hand, a drug release kinetics study implied that the release of lipophilic drugs from micelles could be triggered by ubiquitous nanoscale acceptors in the body such as cell membranes and other lipids<sup>[32]</sup>. Hence, increasing lipophilicity of both the core of micelles and encapsulated drugs might be a more efficient approach to reduce burst release. For example, burst release of paclitaxel in mice was successfully reduced using lipophilicity-enhanced PEG-PS micelles<sup>[33]</sup>. Specifically, lipophilicity of paclitaxel was increased by conjugating lipid anchors to the drug. The lipid-paclitaxel prodrugs and phospholipids were coencapsulated into PEG-PS nanoparticles. The reduced burst release of paclitaxel by incorporating lipids into micelles suggests that lipophilicity of micelle core plays a more important role in burst release of lipophilic drugs than micelle disassembly.

Our *in vitro* FRET imaging and *in vivo* fluorescence quenching study showed that no burst disassembly of PEG-PS micelles was observed and internalized micelles gradually disassembled in cells. Because micelle integrity depends on many factors such as lipophilicity of core-forming block, the ratio of hydrophilic and lipophilic blocks, and the degradation rate of polymer, extrapolations of the herein observed integrity of PEG-PS micelles cannot be generalized. Rather, the integrities of block copolymer micelles made of different polymers need to be investigated separately.

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