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***In vivo* cancer imaging by poly(ethylene glycol)-*b*-poly(ε-caprolactone) micelles containing a near-infrared probe**

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Abstract

Non-invasive near infrared (NIR) fluorescence imaging is a promising technique for the intra-operative assessment of solid tumor removal. We incorporated a lipophilic NIR probe, 1,1'-diiodododecyltetramethyl indotricarbocyanine iodide (DiR), in poly(ethylene glycol)-*b*-poly(ε-caprolactone) (PEG-*b*-PCL) micelles, resulting in DiR solubilization in water, occupying nanoscopic PEG-*b*-PCL micelles. DiR in a self-quenched or non-quenched state showed different kinetics of release from PEG-*b*-PCL micelles *in vitro*; however, both obtained high tumor delineation (tumor-to-muscle ratio of 30–43 from collected organs). These results suggest that PEG-*b*-PCL micelles with DiR are a promising nano-sized imaging agent that will provide a basis for enhanced surgical guidance via NIR visualization of tumors.

Keywords

Near infrared (NIR) fluorescence imaging; Poly(ethylene glycol)-*b*-poly(ε-caprolactone); Polymeric micelles; Self-quenching

Introduction

Fluorescence imaging has been emphasized as a powerful technique for human diagnostic applications, named “tumor painting” for the tracking of solid tumors and metastasis.^{1–3} However, *in vivo* applications of fluorescence optical imaging have limitations such as poor tissue penetration of incident light, high lipophilicity of fluorescent dyes, a lack of stability

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of fluorescent dyes, and low target-to-background fluorescence ratios. Some of the limitations are anticipated to be overcome by using optical probes that absorb and emit light in the NIR range (700–900 nm), allowing deep-tissue penetration.⁴ This spectral region is considered as an “optical transmission window” of biological tissues that allows penetration of light for deep-tissue imaging with relatively high contrast as well as reduced autofluorescence.⁴ Recent interdisciplinary research in optical imaging has focused on “nanocarriers,” e.g. quantum dots, organic dye-doped nanoparticles, and up-conversion nanoparticles for NIR optical imaging.^{5,6} Among these nanocarriers, NIR probe-incorporated polymeric micelles, which have been used widely in drug delivery, may have a number of advantages. First, polymeric micelles have a proven track record of safety in humans.⁷ They provide enhanced aqueous solubility and stability for lipophilic fluorescent dyes. They render multimodal options for imaging by the co-incorporation of agents of different modalities, e.g. PET/SPECT plus fluorescence optical imaging.^{8,9} Upon systemic administration, polymeric micelles preferentially migrate into tumors by the enhanced permeability and retention (EPR) effect, owing to leaky and porous vasculature and poor lymphatic drainage of tumors, and thus polymeric micelles can amplify the signal of probes at tumors.^{10–12}

In a clinical point of view, NIR probe-incorporated polymeric micelles could be used for both diagnosis of tumors and guidance of surgery. Surgical removal of tumor is the best method to treat cancers if the entire tumor can be completely removed. However, complete tumor removal is dependent on how well the tumor is delineated and how impeccably surgery is performed.¹³ Monitoring the presence of tumor tissues remaining in surrounding surgical margins using NIR probe-incorporated polymeric micelles and removal of tumor tissues are prudent courses of action for avoiding recurrence.¹⁴ It is expected that NIR probe-incorporated polymeric micelles can provide decisive guidance for improved surgical removal of tumors. In this study, we studied the optical imaging of cancer by NIR probe-incorporated polymeric micelles in a xenograft model. DiR is a lipophilic carbocyanine dye, and it is a strongly light-absorbing and used as a tool in different research areas, such as lipid membrane-potential sensor, organelle stains for mitochondria and endoplasmic reticulum, and long-term labeling of cells in culture.^{15,16} For optical imaging, DiR absorbs in the NIR region ($\lambda_{\text{max}} = 750 \text{ nm}$), and it has a high quantum yield. For the clinical applications in optical imaging, PEG-*b*-PCL micelles are promising class of nanocarriers for DiR for the following reasons: The preparation of PEG-*b*-PCL micelles that contain DiR is facile. PEG-*b*-PCL micelles increase the water solubility of the highly lipophilic DiR in aqueous solution.^{17–22} PEG-*b*-PCL micelles have prolonged *in vivo* circulation due to the kinetic stability of PEG-*b*-PCL micelles in blood with respect to disassembly and the avoidance of the reticuloendothelial system by PEG-*b*-PCL micelles.²³ As a result, passive tumor targeting of PEG-*b*-PCL micelles has been achieved by the EPR effect^{20,23} in xenograft models. We studied PEG-*b*-PCL micelles with NIR probe, DiR, in a quenched or non-quenched state. While both showed different kinetic profiles for NIR probe release *in vitro* and photophysical properties *in vivo*, both achieved high signals in tumor tissues for optical imaging.

Methods

Preparation and characterization of PEG-*b*-PCL micelles with DiR

DiR loaded PEG-*b*-PCL micelles were prepared by dissolving 4.0–200 mg of PEG-*b*-PCL (5k:10k, Mw/Mn: 1.3), (Polymer Source, Dorval, Quebec) and 0.10–0.50 mg of DiR (Invitrogen, Carlsbad, CA) in acetone (2.0 mL) followed by the drop-wise addition of 0.9% sodium chloride (2.0 mL) by a syringe at a flow rate of 1.0 mL sec^{-1} with vigorous mixing. Acetone was evaporated from the aqueous solution by stirring at room temperature for 1 hr. The PEG-*b*-PCL solution was centrifuged for 5 min at 10,000 g to remove unincorporated

DiR that was insoluble. The aqueous micelle solution was filtered through 0.45 μm nylon syringe filter (National Scientific, Rockwood, TN). The aqueous solubility of DiR was determined by UV-visible measurements on a Cary 100 Bio UV-visible spectrophotometer (Varian, Palo Alto, CA). Particle size distributions of PEG-*b*-PCL micelles with DiR were determined by dynamic light scattering measurement, using a Zetasizer Nano-ZS (Malvern Instruments, UK) at a detection angle of 173° and a He–Ne ion laser ($\lambda = 633 \text{ nm}$) for the incident beam. Fluorescence measurements on DiR were conducted on AMINCO-Bowman Series 2 spectrofluorometer (Thermo Scientific, Rockford, IL) with excitation and emission wavelengths at 745 and 800 nm, respectively. For fluorescence measurements on DiR, absorbances were kept below 0.3 to preclude inner filter effects. The fluorescence self-quenching of DiR as a function of its loading in PEG-*b*-PCL micelles was evaluated by monitoring its fluorescence emission relative to its fluorescence emission upon 100% DiR release from PEG-*b*-PCL micelles; this was realized by freeze-drying of an aliquot of PEG-*b*-PCL micelles, followed by dissolution of the solid sample in acetone and measurement of DiR fluorescence.

***In vitro* DiR release profile for PEG-*b*-PCL micelles**

Aqueous micelle solutions (2.5 mL) were loaded into 20,000 MWCO Slide-A-Lyser® dialysis cassettes (Thermo Scientific, Rockford, IL) and cassettes ($n = 4$) were then placed in 0.9% sodium chloride solution (2.0 L) at 37 °C with stirring. The sodium chloride solution was refreshed every 3 hrs to ensure sink conditions. Samples of 100 μL were withdrawn from cassettes, and cassettes were replenished with 100 μL fresh sodium chloride solution. Sampling intervals were 1, 2, 3, 6, 12, 24, 48, 72, and 96 hrs. Withdrawn samples at each time point were analyzed for the amount of DiR release on a Cary 100 Bio UV-visible spectrophotometer (Varian, Palo Alto, CA).

Xenograft model

LS180 human colon carcinoma cells (kindly provided by Dr. Jamey Weichert, University of Wisconsin-Madison) were cultivated in minimum essential medium including Earle's salts (Cellgro, Manassas, VA), supplemented with 2% sodium bicarbonate, 1% non essential amino acids, 1% sodium pyruvate, 10% fetal bovine serum, 1% L-glutamine, and 100 U mL^{-1} penicillin-streptomycin solution. Cultivated cells were kept at 37 °C under an atmosphere of 5% CO_2 in a humidified incubator. About one week prior to imaging, 1×10^6 LS180 cells were inoculated subcutaneously into the right flanks of anesthetized female 6-8 week-old athymic nude mice (Laboratory Animal Resources, Madison, WI). General anesthesia was induced with 1.5% isoflurane/oxygen, and anesthesia was maintained with 1% isoflurane/oxygen. All animal experiments were performed with human care of animals and approved by UW-Madison's institutional animal care and use committee guidelines, and procedures were in accordance with the institutional guidance.

Animal surgery and tissue excision

Animals were treated in a sterile environment. Ninety-six hrs after injection of PEG-*b*-PCL micelles with DiR, mice were sacrificed by CO_2 asphyxia. A midline incision was done with microsurgical instruments under white light illumination, and organs such as liver, lung, heart, spleen, kidneys, and muscle tissue were removed. The skin of mice was also removed over the tumor, and tumor excision was performed.

NIR fluorescence imaging of DiR

For imaging, DiR-loaded PEG-*b*-PCL micelles, representing 10 μg of DiR, were injected in the tail vein of anesthetized animals when sizes of tumors reached to 80–100 mm^3 . Whole-body fluorescence images were recorded 10 minutes, 1, 4, 9, 12, 24, 48, 72, and 96 hrs after

intravenous administration using Xenogen IVIS® 200 Series (Caliper Life Sciences, Hopkinton, MA) equipped with 150 W Quartz halogen lamp and 1 mW power scanning laser. Mice ($n = 3$ for each micelle formulation) were placed in the dorsal and lateral positions to obtain whole-body optical images. Tumors, organs, and tissues removed at 96 hrs post injection were scanned before dehydration began and their *ex vivo* images were obtained. All images were acquired by back-thinned, back-illuminated grade 1 CCD camera with the following parameters: exposure time = 1 sec; binning = medium; f/stop 2. Filter sets were fixed with the following parameters for DiR: excitation at 745 nm and emission at 800 nm. Acquired images were measured and analyzed with Living Imaging® software. The distribution of DiR in the whole body was quantified by average radiant efficiency, total photons per second per square centimeter per steradian in the irradiance range (microwatts per square centimeter): $[\text{p s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}]/[\mu\text{W cm}^{-2}]$. The tumor-specific accumulation of DiR was evaluated by signal-to-noise ratio: average radiant efficiency at the region of interest (ROI) was drawn around the tumor to average radiant efficiency at the opposite region indicating background tissue (left flank). Horizontal radiant efficiency maps were drawn by Living Imaging® software according to the fluorescence intensity of DiR at the horizontal line passing through the center of a tumor. DiR uptake values in organs and tumors were calculated by its fluorescence relative to its fluorescence in muscles after tissue excision. *In vitro* fluorescence measurements of DiR-loaded PEG-*b*-PCL micelles seeded in 96 well black fluorescence microplates were also performed using Xenogen IVIS® 200 Series.

Results

Characterization of DiR-loaded PEG-*b*-PCL micelles

Table 1 shows the results on the solubilization of DiR by PEG-*b*-PCL micelles at varied ratios of DiR to PEG-*b*-PCL. DiR is practically insoluble in water ($< 1.0 \mu\text{g/mL}$). PEG-*b*-PCL micelles readily increased the water solubility of DiR from 44 to 230 $\mu\text{g/mL}$ with an increase in the quantity of PEG-*b*-PCL. The loading efficiency ($\text{wgt DiR}_{\text{PEG-}b\text{-PCL}}/\text{wgt DiR}_{\text{initial}}$) was ca. 90%. At a low DiR to PEG-*b*-PCL ratio (1:400), PEG-*b*-PCL micelles had an average hydrodynamic diameter of 32 nm with a low polydispersity index (PDI). At a higher ratio of DiR to PEG-*b*-PCL ratio (1:40), PEG-*b*-PCL micelles had a higher average hydrodynamic diameter of 47 nm and higher polydispersity index (PDI), possibly due to a higher average number of DiR per micelle. Assuming that the association number of PEG-*b*-PCL micelles is 200,¹⁹ there were about 7 DiR per PEG-*b*-PCL micelle at the DiR to PEG-*b*-PCL ratio of 1:400, whereas there were about 74 DiR per PEG-*b*-PCL micelle at the DiR to PEG-*b*-PCL ratio of 1:40.

Self-quenching of DiR in PEG-*b*-PCL micelles

Figure 1A shows the relative fluorescence intensity of DiR at varied ratios of DiR to PEG-*b*-PCL. At a ratio of 1:400, the fluorescence of DiR incorporated in PEG-*b*-PCL micelles was close to the fluorescence of DiR after 100% release from PEG-*b*-PCL micelles in acetone, suggesting that DiR in the core region exists in a non-self quenched state (monomers). As the content of DiR increased in PEG-*b*-PCL micelles, there was a loss in quantum yield, presumably due to DiR-self quenching at higher levels of multiply-occupied PEG-*b*-PCL micelles. At a ratio of 1:40 the fluorescence of DiR incorporated in PEG-*b*-PCL micelles was less than 10% relative to the acetone control. At about 74 DiR per PEG-*b*-PCL micelle, DiR self-quenching is evident, consistent with multiply-occupied PEG-*b*-PCL micelles that have cores with diameters less than 10 nm and presence of ground state species of DiR. The absorption spectra of DiR at varied ratios of DiR to PEG-*b*-PCL are consistent with DiR self-quenching in PEG-*b*-PCL micelles at high loading (Figure 1B). As the ratio increased, absorption bands at lower wavelengths relative to the major absorption band of DiR at 745

nm increased. The absorption bands at 650 and 692 nm signify the self-association of DiR in PEG-*b*-PCL micelles, resulting in the self-quenching of fluorescence. The *in vitro* fluorescence intensity of DiR detected by IVIS® 200 Series in a 96 well plate is shown in Figure 1C. At a ratio of 1:400, the fluorescence of DiR loaded in PEG-*b*-PCL micelles showed the highest intensity, whereas at a ratio of 1:40, the fluorescence of DiR loaded in PEG-*b*-PCL micelles had a lower intensity than DiR solubilized in acetone at the same level. The fluorescence intensities of DiR in PEG-*b*-PCL micelles and acetone were concentration-dependent. In summary, PEG-*b*-PCL micelles could effectively raise the water solubility of DiR, and DiR existed in either a quenched or non-quenched state in the cores of PEG-*b*-PCL micelles, depending on the extent of DiR incorporation.

***In vitro* DiR release kinetics for PEG-*b*-PCL micelles**

The release kinetics of DiR for PEG-*b*-PCL micelles at 1:40 (self-quenched) and 1:400 (non-quenched) ratios were investigated (Figure 1D). At a 1:40 ratio, DiR was gradually released from PEG-*b*-PCL micelles with the half-life for DiR release at ca. 20 hrs. On the other hand, the release of DiR from PEG-*b*-PCL micelles at a ratio of 1:400 was biphasic, and most of the DiR was released from PEG-*b*-PCL micelles over 6 hrs, followed by a more gradual release profile over 24 hrs. The half-life for non-quenched DiR release was ca. 1 hr. After 48 hrs, ca. 70% of DiR had been released from PEG-*b*-PCL micelles irrespective of the quenched state of DiR. It is assumed that self-quenched DiR molecules in PEG-*b*-PCL micelles at a 1:40 ratio are aggregated and slowly transform into monomers as function of time. This release profile for DiR from PEG-*b*-PCL micelles was further verified with *in vivo* optical imaging experiments.

NIR fluorescence imaging of DiR-loaded PEG-*b*-PCL micelles in tumor-bearing mice

Tumor-specific accumulation of PEG-*b*-PCL micelles that contain DiR was determined using non-invasive optical imaging based on fluorescence intensity of DiR. The time-dependant whole-body biodistribution of PEG-*b*-PCL micelles that have quenched or non-quenched DiR was monitored by radiant efficiency (Figures 2A and 2B). In a quenched state (1:40), the fluorescence of DiR gradually emerged from the dorsal and lateral portions of the mice over several hrs (Figure 2E). On the other hand, DiR in a non-quenched state (1:400) showed a rapid onset of fluorescence intensity that was much stronger than that of DiR in a quenched state. While the fluorescence signal of DiR slowly decreased after ca. 10 min, the fluorescence signal of quenched DiR increased over time, reaching a similar value at dorsal and lateral sites after ca. 24-48 hrs. Figures 2C and 2D demonstrate the fluorescence intensity of DiR following a horizontal line passing through tumors. Radiant efficiency maps of DiR taken at various time points after injection of PEG-*b*-PCL micelles that contain quenched DiR showed gradual tumor-specific accumulation of DiR as function of time; the strongest signal from the tumor region was observed after 12 hrs, and the lowest signal from DiR was detected at 10 min post injection. On the other hand, maps for PEG-*b*-PCL micelles that contain non-quenched DiR showed an opposite tendency; the strongest fluorescence signal of DiR was observed within 1 hr after injection, whereas the lowest signal of DiR was obtained after 72 hrs. Tumor to muscle ratios for DiR indicate that the tumor specific accumulation efficiency were not significantly different between quenched and non-quenched groups. The ratios were calculated to be approximately 2; however, the ratios calculated from 1:40 group were slightly higher than the ratios for the 1:400 group (Figure 2F). DiR solubilized in 20% DMSO solution was also injected into xenograft mice through the tail vein, but no tumor specific accumulation could be observed. Moreover, the whole-body signal was so weak that the biodistribution of DiR could not be visualized with the same fluorescence intensity scale that was used for PEG-*b*-PCL micelles (supplementary material). These results suggest that DiR-incorporated PEG-*b*-PCL micelles are superior to

free DiR for visualizing tumor tissues in living mice. No notable toxicity of DiR-loaded PEG-*b*-PCL micelles during the animal experiments was found.

Ex vivo NIR fluorescence imaging of DiR-loaded PEG-*b*-PCL micelles in excised tissues

Color-coded NIR fluorescence images of excised tissues (lung, heart, spleen, tumor, kidneys, liver, and muscle) (Figures 3A and 3B) were obtained after 96 hrs. Fluorescence images showed that the strongest signal of DiR was observed in tumor tissues, followed by the liver and spleen, for both quenched and non-quenched groups of DiR-loaded PEG-*b*-PCL micelles. The optical signal of DiR from the lungs was also relatively strong for PEG-*b*-PCL micelles with non-quenched DiR (Figure 3B). The fluorescence intensity ratio of tumor to muscle was ca. 30 for PEG-*b*-PCL micelles with quenched DiR, whereas the fluorescence intensity ratio of tumor to muscle was ca. 43 for PEG-*b*-PCL micelles with non-quenched DiR (Figure 3C).

Residual tumor detection after surgical removal

Xenograft animals were sacrificed 96 hrs post injection and their dorsum was opened guided by strong fluorescence signal of DiR. Tumor tissue was selectively visualized in the incised animal body due to NIR fluorescence emitted by DiR-loaded PEG-*b*-PCL micelles. (Figure 4A)

The completeness of tumor removal depends on how fine the tumor is delineated before and during surgery. The LS180 human colon carcinoma solid tumor was removed as completely as possible from the xenograft animal under white light, and residual tumor was detected by NIR fluorescence imaging of DiR (Figure 4B). A weak fluorescence signal from DiR was detectable at the site of tumor resection, pointing to remaining tumor margins that were not clearly observable by the naked eye during the surgical removal of the primary tumor (Figure 4C).

Discussion

In this study, DiR was successfully incorporated into PEG-*b*-PCL micelle by a simple nanoprecipitation method, and the average diameter of DiR-loaded PEG-*b*-PCL micelles was about 30–50 nm. PEG-*b*-PCL micelles substantially increased the water solubility of DiR, enabling optical imaging experiments in mice. As shown in Table 1, the content of DiR in PEG-*b*-PCL micelles could be controlled, resulting in PEG-*b*-PCL micelles that are multiply-occupied by DiR. At low occupancy (1:400), photophysical experiments on DiR suggest that the dye exists in a non-quenched state in cores of PEG-*b*-PCL micelles. At high occupancy (1:40), DiR exists in a quenched state in the cores of PEG-*b*-PCL micelles, probably due to the existence of ground state species of DiR. Carbocyanine dyes tend to self-associate and exist as H-aggregates. H-aggregates are one-dimensional array of dyes in which the transition moments of individual monomers are aligned parallel to each other, but perpendicular to the line joining their centers (face-to-face arrangement).^{24–28} H-aggregates of DiR do not emit typical fluorescence, and their excited states are rapidly deactivated by internal conversion and intersystem crossing. H-aggregates show lower fluorescence intensity than monomers and display blue-shifted absorption spectrum (Figures 1 A and 1B)^{29,30}. At a ratio of 1:40, DiR molecules are positioned closely and exist predominately as H-aggregates in cores of PEG-*b*-PCL micelles, whereas at a ratio of 1:400, DiR molecules are widely separated and exist predominately as monomers in the cores of PEG-*b*-PCL micelles, resulting in a strong fluorescence signal compared to DiR in acetone (Figure 1C).

Most researchers have endeavored to avoid dye aggregation and loss of fluorescence quantum yield for optical imaging.⁹ However, we noticed differences in the *in vitro* kinetics

of DiR release for PEG-*b*-PCL micelles, depending on the quantity of incorporated DiR (1:40 versus 1:400) and speculated that *in vivo* optical imaging of DiR in a xenograft model would depend on the release kinetics of DiR from PEG-*b*-PCL micelles. Presumably, H-aggregates of DiR in cores of PEG-*b*-PCL micelles at 1:40 ratio are responsible for the slow release pattern of DiR obtained *in vitro* (Figure 1D), whereas monomers of DiR in cores of PEG-*b*-PCL micelles at 1:400 ratio are quickly released over the first few hrs. Thus, it was expected that monomeric DiR in PEG-*b*-PCL micelles would be highly fluorescent after injection and during release from polymeric micelles *in vivo*. In contrast, it was expected that aggregated DiR in PEG-*b*-PCL micelles would have a low fluorescence signal after injection and that the optical signal would increase over time due to the release of DiR, which results in the loss of H-aggregates in PEG-*b*-PCL micelles and released monomeric DiR that is highly fluorescent.

Real time *in vivo* fluorescence images of DiR injected in either an aggregated state (1:40) or monomeric state (1:400) support these two mechanisms of NIR fluorescence imaging (Figures 2A and 2B). At a 1:40 ratio, the whole body fluorescence signal from DiR was low and gradually emerged, especially near vascular tissue near the heart region and noticeably at the LS180 human colon carcinoma solid tumor after 4 hr. At a 1:400 ratio, the whole body fluorescence signal from DiR was high even after 10 min and gradually lost intensity. However, the tumor in the flank region could be readily discerned after 4 hrs and quite apparent after 9-24 hrs after an increase in tumor signal and loss in background signal. Horizontal radiant efficiency maps going from the left flank to the tumor also showed that the NIR signal from DiR in an aggregated state was low in the left flank and the tumor and over time the fluorescence signal built up at the tumor. At a 1:400 ratio, the fluorescence signal from DiR was strongest in the tumor and left flank after 10 min and selectivity for the tumor is gained over time, especially after 9 hrs. While the kinetics of *in vivo* fluorescence imaging of DiR depended on its aggregation state in PEG-*b*-PCL micelles, the whole body optical imaging of tumors was quite similar in terms of selectivity as shown in Figures 2E and 2F. The tumor to muscle ratio was about 2 after 9 hrs at a 1:40 ratio and about 1.7 at a ratio of 1:400 at several time points. The tumor to muscle ratio indicates a measure of selectivity of an optical imaging agent comparing tumor accumulation to non-target tissue (muscle). A tumor to muscle ratio greater than 1 suggests that agent has distributed selectively to tumor³¹. The Prosense 680 (Visen Medical, Woburn, MA), a commercially available NIR probe in preclinical development, has shown a tumor to muscle ratio of about 2.3 *in vivo*³². Therefore, DiR-incorporated PEG-*b*-PCL micelles gave equal or better tumor delineation. The tumor selectivity of DiR evidenced by optical imaging probably reflects the EPR effect, enabled by PEG-*b*-PCL micelles. However, pharmacokinetic experiments on DiR and PEG-*b*-PCL micelles are required to fully prove this hypothesis.

Surgical resection of various tissues at 96 hrs after the injection of DiR-loaded PEG-*b*-PCL micelles, followed by optical imaging of DiR confirmed the selectivity of the dye for tumor tissue regardless of the aggregation state of DiR in loaded PEG-*b*-PCL micelles (Figures 3A and 3B). It is noted that the average radiant efficiency of DiR is very high at the solid tumors, ca. $6.0 \times 10^{10} \text{ p s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1} / [\mu\text{W cm}^{-2}]$, reflecting the selective tumor accumulation of DiR-loaded PEG-*b*-PCL micelles, high quantum yield, and dye stability even after 96 hrs *in vivo*. In the excised organs, DiR had a remarkably high tumor to muscle ratio of 30-43³¹. In this experiment, optical imaging is done without the interference of overlying tissue, e.g. skin, blood vessels, and it more closely resembles intra-operative procedures in surgical oncology, as opposed to whole body optical imaging that might have greater diagnostic value.

Surgical incision of mice followed by optical imaging of DiR revealed the clear delineation of tumor tissues (Figures 4A). After the removal of skin overlying the tumor region and

tumor resection, it was apparent under white light that tumor resection was complete (Figure 4B). However, NIR optical imaging by DiR clearly provided evidence of remaining tumor margins (Figure 4C). These *in vivo* results suggest that DiR-loaded PEG-*b*-PCL micelles can provide intra-operative guidance for surgical removal of solid tumors. One limitation of this work is the use of a subcutaneous xenograft model rather than an orthotopic xenograft model that undergoes metastases. Future work will focus on the evaluation of DiR-loaded PEG-*b*-PCL micelles in an orthotopic xenograft model that will allow tests on the optical imaging of metastases, feasibility of their surgical removal, survival, and a better delineation of the importance of DiR aggregation state on optical imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Fluorescence intensity of DiR-loaded PEG-*b*-PCL micelles relative to free DiR, and (B) Normalized absorbance spectra of DiR-loaded PEG-*b*-PCL micelles at varied ratios of DiR to PEG-*b*-PCL. (C) Fluorescence images of DiR (0.6, 0.3 and 0.2 μ M) in a multi-well format obtained by Xenogen IVIS® 200 Series; DiR-loaded PEG-*b*-PCL micelles (1:40 and 1:400), DiR in acetone, and water (autofluorescence). (D) *In vitro* release profiles of DiR from PEG-*b*-PCL micelles (1:40 and 1:400).

Figure 2.

Real time *in vivo* fluorescence whole-body images of mice with LS180 human colon carcinoma xenografts. Time-dependent images of (A) DiR-loaded PEG-*b*-PCL micelles (1:40) (B) DiR-loaded PEG-*b*-PCL micelles (1:400) in dorsal (upper) and lateral (below) views. Time-dependent radiant efficiency maps according to horizontal lines passing through the center of tumors for (C) DiR-loaded PEG-*b*-PCL micelles (1:40) and (D) DiR-loaded PEG-*b*-PCL micelles (1:400). (E) Calculated average radiant efficiency of DiR-loaded PEG-*b*-PCL micelles (1:40 and 1:400) in dorsal portions and lateral views as a function of the time based upon Figures 2A and B (Expanded figure from 0 to 4 hr time

point was inserted). (F) Relative radiant efficiency of DiR distributed in tumors in comparison to muscles over time in living mice.

Figure 3.
Ex vivo optical images of tissues for (A) DiR-loaded PEG-*b*-PCL micelles (1:40) and (B) DiR-loaded PEG-*b*-PCL micelles (1:400). (C) Relative radiant efficiency of DiR distributed in excised organs in comparison to muscle after 96 hrs.

Figure 4. Detection of tumor and margins before and after surgery by DiR-loaded PEG-*b*-PCL micelles (1:400). (A) Before surgery, (B) surgical tumor removal, and (C) after surgery.

Table 1

Z-average diameters of DiR-loaded PEG-*b*-PCL micelles and aqueous solubility of DiR. Data are expressed as mean $\pm$ SD ($n = 3$).

Micelles (DiR:PEG- <i>b</i> -PCL) (weight:weight)	Z-average diameter (nm)	PDI	Aqueous solubility of DiR in micelle formulations ($\mu\text{g/mL}$)
1:40 (Self-quenching)	47.06 $\pm$ 1.80	0.154 $\pm$ 0.017	44.52 $\pm$ 4.05
1:25	41.98 $\pm$ 0.44	0.123 $\pm$ 0.014	56.37 $\pm$ 8.01
1:33	41.76 $\pm$ 3.39	0.088 $\pm$ 0.065	137.9 $\pm$ 5.49
1:50	37.80 $\pm$ 0.88	0.044 $\pm$ 0.035	150.5 $\pm$ 6.33
1:80	36.61 $\pm$ 0.75	0.062 $\pm$ 0.029	175.7 $\pm$ 2.98
1:100	32.29 $\pm$ 3.46	0.055 $\pm$ 0.024	176.9 $\pm$ 4.15
1:400 (Non-quenching)	31.90 $\pm$ 1.07	0.044 $\pm$ 0.054	229.9 $\pm$ 3.07

* PDI (polydispersity index)