Ultrafast fluorescence imaging in vivo with conjugated polymer fluorophores in the second near-infrared window

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In vivo fluorescence imaging in the second near-infrared window (1.0–1.7 µm) can afford deep tissue penetration and high spatial resolution, owing to the reduced scattering of long-wavelength photons. Here we synthesize a series of low-bandgap donor/acceptor copolymers with tunable emission wavelengths of 1,050–1,350 nm in this window. Non-covalent functionalization with phospholipid-polyethylene glycol results in water-soluble and biocompatible polymeric nanoparticles, allowing for live cell molecular imaging at >1,000 nm with polymer fluorophores for the first time. Importantly, the high quantum yield of the polymer allows for in vivo, deep-tissue and ultrafast imaging of mouse arterial blood flow with an unprecedented frame rate of >25 frames per second. The high time-resolution results in spatially and time resolved imaging of the blood flow pattern in cardiogram waveform over a single cardiac cycle (~200 ms) of a mouse, which has not been observed with fluorescence imaging in this window before.
In vivo biological imaging in the second near-infrared window (NIR-II, 1.0–1.7 μm) has attracted much interest recently owing to salient advantages over imaging in the visible (400–750 nm) and the conventional near-infrared (NIR, 750–900 nm) regions. Detecting longer-wavelength photons in the NIR-II affords reduced photon scattering in biological tissues accompanied by lower autofluorescence, leading to higher spatial resolution at deeper tissue penetration depths. To date, single-walled carbon nanotubes (SWNTs)\(^1\)–\(^3\),\(^10\)–\(^14\), semi-conducting quantum dots (QDs)\(^5\)–\(^7\),\(^15\)–\(^17\), rare-earth doped nanoparticles\(^9\) and nanoparticles made of small organic molecules\(^18\) are fluorophores used for fluorescence imaging in the NIR-II region. These NIR-II agents can be functionalized with hydrophilic molecules to afford biocompatibility for intravenous administration and can be gradually cleared out from the body without obvious acute or chronic toxicity in animal studies\(^16\),\(^19\),\(^20\). However, shortcomings of existing NIR-II fluorophores include relatively low fluorescence quantum yield\(^1\),\(^2\),\(^21\), unfavourably short emission wavelength\(^18\) and potential toxicity due to heavy metals in QDs\(^6\),\(^7\),\(^15\)–\(^17\). A much wider range of fluorophores could be developed to tackle these problems. An ideal fluorophore developed for NIR-II biological imaging should allow for tunable excitation and emission wavelengths in the >1,000-nm NIR-II region with a high fluorescence quantum yield and high biocompatibility.

Here we report the synthesis of conjugated polymers with intrinsic fluorescence >1,000 nm through donor–acceptor (D–A) alternating copolymerization, an effective way of synthesizing polymers with tunable bandgap energy in the NIR and at even longer-wavelength regions\(^22\)–\(^29\). By copolymerization of an electron-donating monomer benzo[1,2-b:3,4-b']difuran and an electron-withdrawing monomer fluorothieno-[3,4-b]thiophene, we derive a brightly fluorescent copolymer, poly[b(1,2-b: 3,4-b’)-difuran-alt-fluorothieno-[3,4-b]thiophene] (named ‘pDA’) with fluorescence emission at ~1,050 nm with a large Stokes shift of ~400 nm and a high fluorescence quantum yield of ~1.7%, much higher than the quantum yield of typical carbon nanotubes (~0.4%) used previously\(^3\). We non-covalently functionalize the polymer with a PEGylated surfactant to afford water solubility and biocompatibility. We successfully tune the emission wavelength of the conjugated polymer fluorophores from 1,050 to 1,350 nm, and demonstrate the first in vitro and in vivo biological imaging in the >1,000-nm window using conjugated polymer fluorophores.

**Results**

**Synthesis of NIR-II fluorescent pDA-PEG nanoparticles.** The pDA polymer was synthesized through a copolymerization reaction of two monomers, 1-(4,6-dibromo-3-fluorothieno-[3,4-b]thiophen-2-yl)monan-1-one and 2, 6-bis(trimethyltin)-4,8-bis (2-ethylhexyloxy)benzo[1,2-b:3,4-b’]-difuran. Fluorine was introduced into the thienothiophene unit to tune the fluorescence to long wavelengths (Fig. 1). The pDA polymer was characterized by \(^1\)H NMR spectroscopy (see Methods and Supplementary Figs 1–4) and gel permeation chromatography. The average molecular weight (\(M_\text{w}\)) of the polymer was ~16 kDa (see Methods and Supplementary Fig. 5). We dissolved the as-made pDA polymer in tetrahydrofuran (THF) and then mixed it with an aqueous solution of a biocompatible surfactant of PEGylated phospholipid (DSPE-mPEG (5 kDa))\(^1\),\(^3\),\(^19\) (see Methods). The mixture was dialysed against water to remove THF, resulting in a stable aqueous solution of supramolecular conjugate comprising the hydrophobic core of pDA and the hydrophilic shell of DSPE-mPEG coating (Fig. 2a; named as pDA-PEG).

Atomic force microscopy (AFM) revealed the size distribution of pDA-PEG nanoparticles (Fig. 2b) with an average size of 2.9 nm when dried on a silicon surface (Supplementary Fig. 6). The UV–vis-NIR absorption spectrum of the as-made pDA-PEG solution in water exhibited an absorption peak at 654 nm, while the fluorescence emission spectrum showed a main emission peak at 1,047 nm, suggesting a large Stokes shift of ~400 nm (Fig. 2c). The pDA copolymer has been reported to have red-shifted absorption and emission compared with the corresponding homopolymers because of the formation of a charge-transfer structure between the electron donor and acceptor units\(^26\),\(^27\),\(^30\). The fluorescence quantum yield of pDA-PEG in a solution (Fig. 2d) under an excitation of 808 nm was ~1.7%, measured against a standard IR-26 dye as a reference\(^31\),\(^32\) (Supplementary Fig. 7a–f, where the excitation of 808 nm was intentionally chosen to balance absorption and scattering to afford maximum penetration depth of excitation light for in vivo imaging). The quantum yield was much higher than that of typical nanotubes (~0.4%; Supplementary Fig. 7g–i). The pDA-PEG exhibited high photostability in phosphate-buffered saline (PBS) and fetal bovine serum with negligible decay under continuous excitation for 1 h (Fig. 2e,f and Supplementary Fig. 8). These results suggested pDA-PEG as an aqueous soluble, photo-stable and high brightness NIR-II fluorescent agent suited for biological imaging.

**Molecular imaging of cells with pDA-PEG in NIR-II.** We investigated pDA-PEG as a fluorescent label capable of targeting specific molecules on cell surfaces for performing molecular imaging of cancer cells through functionalization of pDA with targeting ligands. We made pDA-PEG-NH\(_2\) using an amine-terminated phospholipid surfactant, DSPE-PEG-NH\(_2\) (refs 33,34) and conjugated to pDA-PEG-NH\(_2\) via standard crosslinking reaction between the -NH\(_2\) groups on the polymer and -SH groups on the thiolated antibody (see Methods). Then the pDA-PEG-Erbilix was applied to target the epidermal growth factor receptors (EGFRs) on the cell membranes of EGFR-positive breast tumour MDA-MB-468 cells, while the EGFR-negative brain tumour U87-MG cells were used as a negative control\(^15\). Cell imaging in NIR-II detected >1,000-nm fluorescence of pDA-PEG-Erbilix (Fig. 3) selectively on the EGFR-positive MDA-MB-468 cells and not on the negative U87-MG cells, showing a positive/negative ratio of ~5.8. Thus, the pDA-PEG was made into an NIR-II fluorescent agent capable of recognizing and staining live cells with molecular specificity. This opened up the possibility of molecular imaging with conjugated copolymers in vitro and...
in vivo, which would not be attainable by structural imaging techniques such as ultrasound and optical coherence tomography (OCT)\textsuperscript{35,36}.

**Figure 2 | Characterizations of pDA-PEG nanoparticle.** (a) A schematic of the pDA-PEG nanoparticle showing a hydrophobic polymer core and a hydrophilic PEG shell. (b) A typical AFM image of pDA-PEG nanoparticles deposited on a silicon substrate. Owing to the effect of tip size convolution in AFM\textsuperscript{66}, the height measurement from the AFM micrograph, rather than the lateral size measurement, was used to measure the size of the nanoparticles deposited on the substrate. The lateral scale bar in b indicates 100 nm. (c) Absorption and emission spectra of pDA-PEG, featuring a large Stokes shift of ~400 nm. (d) An NIR-II fluorescence image of an aqueous solution of pDA-PEG taken in the range of 1.0–1.7 μm NIR-II window under an excitation of 808 nm. (e) Fluorescence stability of pDA-PEG in different media including water, PBS and serum. The error bars were obtained by taking the s.d. of all pixel intensities within an region of interest (ROI) covering the solution in the 1-mm cuvette, as shown in Supplementary Fig. 8. (f) Photostability curves of pDA-PEG in water, PBS and serum under continuous 808-nm illumination. pDA-PEG in serum exhibits the lowest degree of photobleaching (<10%) among the three.

**Figure 3 | Molecular cell imaging with pDA-PEG-Erbitux.** (a) A schematic showing the structure of pDA-PEG-Erbitux bioconjugate, where the anti-EGFR antibody (Erbitux) selectively targets EGFR on the cell membrane of an MDA-MB-468 cell. (b,c) White-light (b) and NIR-II (c) fluorescence images of EGFR-positive MDA-MB-468 cells incubated with the pDA-PEG-Erbitux bioconjugate, showing positive staining of cells. (d,e) White-light (d) and NIR-II (e) fluorescence images of EGFR-negative U87-MG cells incubated with the pDA-PEG-Erbitux bioconjugate, without obvious staining of the cells. The scale bar in e indicates 40 μm, which applies to all images shown in b–e. (f) Average NIR-II fluorescence of EGFR-positive MDA-MB-468 cells and negative U87-MG cells, showing a positive/negative ratio of ~5.8. The error bars in f were obtained by taking the s.d. of average fluorescence intensity from 20 cells in each NIR-II fluorescence image.

**Ultrafast blood flow tracking with pDA-PEG in NIR-II.** Next, we performed in vivo mouse blood vessel imaging by detecting the >1,000-nm fluorescence of intravenously injected pDA-PEG
solutions. Owing to the bright fluorescence of pDA-PEG with a fourfold higher quantum yield (1.7%) than nanotube fluorophores (quantum yield ~0.4%), we were able to dynamically image and track real-time arterial blood flow in the mouse hindlimb with a much shorter exposure time (~20 ms) than previously possible (~100 ms) in the NIR-II window. Immediately following a tail vein injection of 200 µl solution of pDA-PEG, we performed video-rate imaging of the left hindlimb of a mouse in the supine position with an ultrafast frame rate of 25.6 frames per second (fps) compared with the previously achievable 5.3 fps (ref. 3). Such a high imaging speed enabled clear observation of the fast-moving flow front in the femoral artery as the NIR-II emitting pDA-PEG entered the hindlimb (Supplementary Movie 1 and Fig. 4a). The average femoral blood flow velocity was quantified by plotting the distance travelled by the flow front as a function of time, showing an overall linear increase with an average blood velocity of 4.36 cm s⁻¹ (Fig. 4b), consistent with measurement using ultrasound (4.36 cm s⁻¹) (ref. 3). This represented the first in vivo blood flow velocity measurement by direct tracking through real-space imaging of fast-moving flow front on the millisecond scale using NIR-II fluorescence.

Interestingly, high time-resolution analysis of blood flow front position versus time revealed periodic variations in instantaneous velocity over time with a period of 150–200 ms, showing oscillations between the highest instantaneous blood velocity of ~8 cm s⁻¹ and the lowest blood velocity of ~2 cm s⁻¹, corresponding to the ventricular ejection (systolic) and ventricular relaxation (diastolic) phases, respectively, of a single cardiac cycle (Fig. 4c). The observation of such a rapid periodic oscillation in the femoral blood velocity was entirely owed to the high time resolution of 20 ms, much shorter than the cardiac cycle of ~200 ms. The fast arterial blood flow (average speed ~4.36 cm s⁻¹) passed through the entire femoral artery (length ~2 cm) within two cardiac cycles (~400 ms). At longer times post injection, the same hindlimb imaged at 39 s post injection showed full perfusion of the injected NIR-II pDA-PEG fluorescent polymer into the femoral artery (Fig. 4d).

To further glean the oscillating blood flow front with high time resolution, we selected a region of interest of the femoral artery

![Figure 4](https://example.com/figure4.png)
and analysed the NIR-II fluorescence intensity as a function of time (Fig. 5a). We observed evenly spaced intensity humps over a linearly increasing baseline (Fig. 5b), with the humps corresponding to the systolic phases of cardiac cycles of the mouse and the increasing baseline due to increased overall perfusion of pDA-PEG fluorophore into the artery vessel. By subtracting the linear rise from the intensity versus time curve, we observed clear consecutive intensity spikes in the time course due to periodic ventricular ejections (Fig. 5c). Further, the linear-baseline-subtracted fluorescence intensity versus time was made into a movie that clearly showed the blood flow variations over the two phases of each heartbeat (Fig. 5d–j and Supplementary Movie S2). The blood flow versus time showed a similar cardiogram waveform (Fig. 5c) as previous Doppler ultrasound measurement, demonstrating that fluorescence imaging could help visualize rapid blood flow changes in real space and real time within a single cardiac cycle of the mouse.

It is also interesting to note that the dots observed along the femoral artery in the baseline-subtracted images (Fig. 5f–h) were at fixed positions over time and corresponded to local higher NIR-II intensity regions along the femoral artery. These high-intensity local regions were found to coincide with locations along the vessel with slightly larger vessel diameters (by ~20%) of the lumen measured with the vessel image. That is, more NIR-II pDA-PEG fluorophores in the blood were filled into a larger lumen and thus exhibited higher NIR-II intensity (Supplementary Fig. 9). From the NIR-II fluorescence oscillations with an average period of 206.7 ms per cardiac cycle (Fig. 5k), we measured a

Figure 5 | Resolving waveform blood flow pattern with pDA. (a,b) An NIR-II fluorescence image (a) of the mouse femoral artery, where the fluorescence intensity inside the ROI red box is integrated and plotted as a function of time in b, showing an increasing profile with humps corresponding to ventricular ejections of cardiac cycles. The scale bar in a indicates 5 mm. (c) NIR-II fluorescence intensity plotted as a function of time, after a linear increasing baseline is subtracted from the plot shown in b, featuring five cardiac cycles in the plot. (d–j) Time course NIR-II fluorescence images of the red box area shown in a, after subtraction of a time-dependent linearly increasing background given by the baseline in b. Note that these seven images correspond to a complete cardiac cycle from 312 to 546 ms. See Supplementary Movie 2 for a video showing the real-time evolution of the linear-background-subtracted fluorescence intensity. The scale bar in d indicates 1 mm, which applies to all images of d–j. (k) Time point of NIR-II fluorescence spikes corresponding to ventricular ejections shown in c, plotted over several heart pulses (black squares). The data are fitted to a linear function with its slope of 206.7 ms per pulse corresponding to the period of each cardiac cycle.
heart rate of 290 beats per minute for the mouse, which agreed with previous results via cardiac gating. Thus, the high temporal and high spatial resolution NIR-II imaging afforded by the high brightness of the conjugated polymer led to remarkably rich details of blood flows and cardiac cycles in vivo.

Discussion

In vivo fluorescence imaging of live animals in the NIR-II region benefits from deeper penetration of up to a few millimetres inside the body and reduced scattering of photons that scales inversely proportional with wavelength as $\sim \lambda^{-w}$ ($w=0.22-1.68$) in biological tissues. Conjugated polymers have been widely used in organic solar cells, light-emitting diodes, and organic electronics. Fluorescent imaging with conjugated polymers has been limited to emission wavelength <$900\text{ nm}$ due to packing constraints. Our current work developed the NIR-II agent with $>1,000\text{ nm}$ fluorescence based on conjugated polymers for biological imaging both in vitro and in vivo. The facile synthetic route of donor–acceptor copolymers could allow further tuning of the optical properties of the polymers through modifying the donor–acceptor structures (Supplementary Fig. 10) and the length of the copolymer. A more electron-donating donor and a more electron-withdrawing acceptor typically result in smaller band gap of the copolymer, and a longer copolymer exhibits reduced band gap due to a greater delocalization of $\pi$-electrons. This could lead to a library of polymers with tunable excitation and emission wavelengths for NIR-II imaging (see Supplementary Fig. 10 for several polymers synthesized thus far). With such development, we envisage fluorophores with different emission wavelengths in the 1.0- to 1.7-µm NIR-II window, allowing for multicolour molecular imaging of different biomarkers. In addition, further improved penetration depth could be achieved in vivo by tuning the emission wavelength.

A salient advantage of the pDA-PEG polymer as an NIR-II fluorophore for in vivo live animal imaging is its much higher quantum yield ($\sim 1.7\%$) than SWNTs ($\sim 0.4\%$) previously used for hindlimb blood flow tracking. This allows for much faster (20-ms exposure time versus previous 100 ms) video-rate imaging of dynamic changes in the blood flow labelled by NIR-II fluorophore, pushing the limit of temporal resolution to a previously unattainable level of $>25\text{ f.p.s.}$ (the instrument frame rate limit is 50 f.p.s. due to an overhead time of 19 ms). Although the pDA-PEG polymer has similar quantum efficiency ($\sim 1.7\%$) to nanoparticles of small organic molecules ($\sim 1.8\%$), since the major peak of the small organic molecule nanoparticles is located at $\sim 920\text{ nm}$ while the major peak of pDA-PEG polymer is at $\sim 1,050\text{ nm}$, the pDA-PEG polymer has a greater portion of emitted photons in the $>1,000\text{- nm}$ NIR-II region ($\sim 70\%$) than the small molecule nanoparticles ($\sim 40\%$). Taken together, the high fluorescence quantum yield, red-shifted fluorescence emission in the NIR-II region and low density of the pDA polymer require $10 \times 100 \times$ lower mass dose of injection than previous NIR-II fluorophores (such as SWNTs$^3$–$3,9$–$12$, QDs$^6,7,15$–$17$, rare-earth doped nanoparticles$^8$ and nanoparticles incorporating organic molecules$^{18}$) to reach the same in vivo imaging quality, and allow for dynamic fluorescence imaging with much shorter exposure time and higher temporal resolution. This high temporal resolution well exceeds the heart rate of mice ($\sim 5$ beats per second or $\sim 200\text{ ms per cycle}$) by five times, eliminating the need for cardiac gating typically required for low-frame-rate cardiovascular imaging techniques.$^{38,49}$

Previous fluorescence imaging and tracking of blood flow usually require the removal of scattering tissue over the vessels of interest to afford higher spatial resolution and gating devices to eliminate image blurring due to cardiac and respiratory motions on a faster timescale than the temporal resolution of image acquisitions ($\sim 10 \text{ f.p.s.}$). Ultrasonic imaging and OCT with ultrafast image acquisition rate ($\text{kHz-\text{MHz}}$) have shown the advantages of measuring fast blood flow and cardiac cycles; however, the spatial resolution (in the $10\text{ µm} \sim 1\text{ mm}$ range) and signal-to-noise ratio are suboptimal due to long wavelengths and speckles. Here by utilizing NIR-II fluorescence with reduced tissue scattering and an ultrafast frame rate of $>25\text{ f.p.s.}$, we have enabled simultaneous blood velocity and cardiac cycle measurements as well as high-resolution imaging of blood vessels $1-2\text{ mm}$ deep under otherwise highly scattering skin tissues. We have also proved the concept that the polymer-based NIR-II fluorophores can be used to track blood flow in capillaries with sub-$10\text{ µm}$ diameters (Supplementary Fig. 11a), which are well below the spatial resolutions of traditional ultrasound and OCT. On the basis of the capillary blood flow-tracking images, we measured the capillary blood velocity to be $\sim 5.2\text{ mm s}^{-1}$ (Supplementary Fig. 11b), in good agreement with previous studies.$^{58,59}$ The dynamic range of blood velocity measurement in our NIR-II imaging system using the pDA fluorophores is derived as $0-640\text{ nm s}^{-1}$ based on the brightness of the fluorophore and the detector sensitivity (Supplementary Note 1). In addition, we have shown that the pDA-PEG fluorophores can be used to track regional blood flow and redistribution as a result of increased metabolic demand after heat-induced inflammation of the tissue (Supplementary Fig. 12), providing a direct diagnostic tool for visualizing the metabolic difference of the tissue.

The heavy-metal-free nature of NIR-II fluorescent copolymers bodes well for potentially low-toxicity agents for in vivo applications. In a cellular toxicity study, we found that the toxicity of pDA-PEG nanoparticles depended on the surfactant coating, and a surfactant with branched PEG exhibited alleviated toxicity (see Methods). The mice injected with pDA-PEG were monitored over a period of up to 2 months without showing obvious toxic effects or health problems. We also attempted tuning the size of the pDA-PEG nanoparticles and obtained the smallest pDA-PEG with an average hydrodynamic size of $<6\text{ nm}$ (Supplementary Fig. 13, and also see Methods for more information). Systematic investigation is required to study the long-term fate and toxicology of pDA-PEG nanoparticles, which is beyond the scope of the current work.

Real-time haemodynamic imaging could be of central importance to better understand various cardiovascular diseases and to design treatment strategies. An ideal NIR-II fluorescent agent capable of achieving haemodynamic imaging with high spatial and temporal resolutions should have tunable emission wavelength in the $>1,000\text{- nm}$ region to minimize tissue scattering, high fluorescence quantum efficiency ($>5\%$) for ultrashort imaging exposure time. The current work opens up future research to achieve these goals and could eventually lead to NIR-II fluorescence agents suitable for clinic use.

Methods

Synthesis of pDA copolymer. The synthesis of monomers M1 and M2 can be found in previous publication.$^{62,64}$ For the synthesis of the pDA copolymer, 0.091 g of monomer M1 (0.2 mmol), 0.148 g of the monomer M2 (0.2 mmol) and 15 ml of anhydrous toluene were mixed in a two-neck flask. The solution was flushed with $N_2$ for 10 min, and 15 mg of Pd(PPh3)$_4$ was added into the flask. Then the solution was flushed with $N_2$ for another 25 min. The two-neck flask was placed in an oil bath and heated up carefully to $110^\circ$C. The mixture was stirred for 24 h at $110^\circ$C under $N_2$ atmosphere. After the reaction was complete, the mixture was allowed to cool down to room temperature, and the polymer was precipitated by adding 100 ml of methanol and filtered through a Soxhlet thimble. Soxhlet extraction was performed with methanol, hexanes and chloroform. Eighty milligrams of the polymer were obtained as a green solid from the chloroform fraction by rotary evaporation and dried under vacuum overnight with a moderate yield of 56%.

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Characterizations of monomers and pDA copolymer. All compounds, including the monomers M1 and M2, and the pDA copolymer, were characterized using nuclear magnetic resonance (NMR) spectroscopy. Typical (1H NMR) 1H NMR 1H-NMR 1H-NMR 1H-NMR 1H-NMR spectra were measured on a Bruker AV-400 spectrometer at room temperature. Chemical shifts were described as δ values (p.p.m.), where tetramethylsilane was used as an internal reference. Splitting patterns were labelled as singlet (s), doublet (d), triplet (t), multiplet (m) and broad (broad). As such, the NMR spectral assignments for the two monomers M1 and M2, as well as the product polymer, are given as follows:

Monomer M1: 1H NMR (400 MHz, CDCl3): δ 2.95 (t, 2 H), 1.76 (m, 2 H), 1.39–1.30 (m, 10 H), 0.91 (t, 3 H). 13C NMR (100 MHz, CDCl3): δ 42.0, 23.1, 14.0 ppm. 

Monomer M2: 1H NMR (400 MHz, CDCl3): δ 7.06 (s, 2 H), 4.35 (d, 4 H), 1.78 (m, 2 H), 1.37–1.70 (m, 16 H), 0.96–1.03 (m, 12 H), 0.14 (s, 3 H). 

Polymer pDA: 1H NMR (400 MHz, CDCl3): δ 6.80 (br, 2 H), 4.31 (br, 4 H), 3.06 (br, 2 H), 2.01–2.11 (br, 30 H), 0.81–1.21 (br, 4 H). 

Elemental analysis of the pDA copolymer was performed on a Flash EA 1112 elemental analyser. Elemental composition of pDA copolymer was calculated based on the molecular formula C13H31N6O3S1 as C: 69.26%; H: 7.80%; O: 11.25%, and compared with measured elemental composition by the elemental analyser: C: 70.08%; H: 7.73%; O: 11.09%. 

Dynamic light scattering (DLS) analysis of pDA-PEG. The pDA-PEG polymers and SWNTs in aqueous solutions too. Then all dynamic light scattering measurements were carried out for pDA-PEG polymers and SWNTs in aqueous solutions too. The dynamic light scattering data were collected on a BI-9000AT Light Scattering Spectrometer (Brookhaven Instruments, Inc.) 

AFM imaging of pDA-PEG. AFM image of pDA-PEG was acquired with a Nanoscope IIIa multimode AFM in the tapping mode. The sample for imaging was prepared by drop-drying a highly diluted aqueous solution of pDA-PEG polymer at a mass concentration of 750 ng ml\(^{-1}\) on the SiO\(_2\)/silicon substrate without any post-processing steps. To plot the distribution of the pDA-PEG polymer, 100 nanoparticles were measured from the AFM images and their heights measured in terms of the size distribution. The measured size distribution convoluted in AFM was plotted against absorbance at the excitation wavelength.
1,000-nm long-pass filter (Thorlabs) so that the intensity of each pixel in the camera represented fluorescence in the 1.9- to 1.7-μm NIRR-II region. The exposure time was 3 s for all fluorescence images.

**Mouse handling.** All vertebrate animal experiments were performed under the approval of the Stanford University’s Administrative Panel on Laboratory Animal Care. Eight-week old female BALB/c mice were obtained from Charles River and housed at the Research Animal Facility of Stanford under our approved animal protocols. Before hindlimb vessel imaging, all mice were anesthetized in a rodent anesthetic machine with isoflurane mixed with 3% Isoflurane. Then the hair over the hindlimb skin was carefully removed using Nair to avoid causing wounds to the skin. Tail vein injection of the pDA-PEG contrast agent was carried out in dark and synchronized with the camera that started continuous image acquisition simultaneously. The injected dose was a 200 μl bolus of the pDA-PEG in a 1 × PBS solution at a mass concentration of 0.25 mg ml⁻¹. During the time course of imaging the mouse was kept anesthetized by a nose cone delivering 1.51 mm⁻³ O₂ gas mixed with 3% Isoflurane.

**Ultrafast video-rate NIR-II vessel imaging.** Video-rate NIRR-II vessel imaging was carried out in a similar manner as our previous publication. In brief, imaging was carried out on a home-built imaging set-up consisting of a 2D InGaAs camera (Princeton Instruments, 2D OMA-V). The excitation was provided by an optical fibre-coupled 808-nm diode laser (RMPC Lasers), intentionally chosen to balance absorption and scattering to afford maximum penetration depth of excitation light for in vivo imaging. The high fluorescence quantum yield of the pDA-PEG also ensured sufficient emission for ultrafast in vivo imaging despite the non-resonant excitation at 808 nm. The light was collimated by a collimator with a focal length of 4.5 mm (ThorLabs) and filtered by an 850- and a 1,000-nm longpass filter (ThorLabs). The power density of the excitation laser at the imaging plane was 140 mW cm⁻², significantly lower than the reported safe exposure limit of 329 mW cm⁻² at 808 nm. The emitted fluorescence from the mouse was allowed to pass through a 900- and a 1,000-nm long-pass filter (ThorLabs) and to be focused on the InGaAs detector by a lens pair consisting of two NIR achromats (200 and 75 mm; ThorLabs). The distance between the two NIR achromats was adjusted to have only one hindlimb of the mouse included in the field of view. The camera was set to expose continuously using the LabVIEW software with an exposure time of 20 ms. Each in vivo imaging was acquired with a frame rate of 25.6 fps. Due to a 19 ms overhead time during readout.

**In vitro toxicity study of pDA-PEG.** We determined the in vitro toxicity of pDA-PEG by MTS assays using a CellTiter96 kit (Promega) on human breast cancer MDA-MB-468 cells. To evaluate how the surfactant coating of the pDA polymer affects toxicity of branched PEG and changing the initial pDA concentration and PEG molecular weights (2 and 5 kDa) and changing the initial pDA concentration in the THF solution (0.025–0.075 mg ml⁻¹) see the **preparation of pDA-PEG and pDA-PEG-NH₂ section in Methods** before mixing with 1.1 mg ml⁻¹ DSPE-mPEG in water. After the pDA-PEG nanoparticles were made in each synthesis condition, cell toxicity analysis was performed to evaluate each sample’s hydrodynamic diameter as a function of initial pDA concentration in the THF solution and the molecular weight of PEG in the surfactant (please see the **dynamic light scattering (DLS) analysis of pDA-PEG section in Methods**). The dependence of pDA-PEG hydrodynamic size on initial pDA concentration and PEG molecular weight is plotted in Supplementary Figure S13, showing that both smaller PEG and lower initial concentration of pDA favour the formation of smaller pDA-PEG nanoparticles in aqueous solution. However, it is noteworthy that DLS was not able to discriminate the ‘empty’ nanoparticles without any pDA molecules loaded inside, and therefore the size distribution of the measured hydrodynamic diameters below ~4 nm could be due to the micelles formed by the DSPE-mPEG surfactants only.

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