Development of red/near-infrared fluorescent molecules and macromolecules for biomedical applications

Yang, Cangjie

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**DEVELOPMENT OF RED/NEAR-INFRARED
FLUORESCENT MOLECULES AND
MACROMOLECULES FOR BIOMEDICAL
APPLICATIONS**

YANG CANGJIE

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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List of Abbreviations

R	red
NIR	near infrared
FR	far red
NP	nanoparticle
ACQ	aggregation caused quenching
AIE	aggregation induced emission
CIE	crystallization induced emission
SPN	semiconducting polymer nanoparticle
FRET	Förster resonance energy transfer
BRET	bioluminescence resonance energy transfer
Qdot	quantum dot
Pdot	polymer dot
OI	optical imaging
FL	fluorescence imaging
UV	ultraviolet
ATRP	atom transfer radical polymerization
RAFT	reversible addition fragment transfer
ROP	ring opening polymerization
DAP	direct arylation polymerization
CP	conjugated polymer
CPE	conjugated polyelectrolyte
PBS	phosphate buffer
FWHM	full width at half maximum
DP	number-average degree
M_n	number-average molecular weight
M_w	Weight-average molecular weight
PDI	polydispersity index
Φ	quantum yield
$\lambda_{abs.max}$	maximum absorption wavelength
$\lambda_{em.max}$	maximum emission wavelength
CMC	critical micelle concentration
ICG	indocyanine green

PPcy	pyrrolopyrrole cyanine
PCL	polycaprolactone
POEGMA	poly[oligo(ethylene glycol) methyl ether methacrylate]
DOX	doxorubicin
CPT	camptothecin
THF	tetrahydrofuran
GSH	glutathione
DTT	dithiothreitol
DAPI	4',6-Diamidino-2-Phenylindole
PFTB	poly(fluorene- <i>alt</i> -(4,7-bis(hexylthien)-2,1,3-benzothiadiazaole))
JAGG	J-aggregated
HAGG	H-aggregated
nanoRED	red-emissive nanoparticle
UM	unimolecular micelle
UMPD	unimolecular micelle based prodrug
NMR	nuclear magnetic resonance
GPC	gel permeation chromatography
DLS	dynamic light scattering
TEM	transmission electron microscopy
SEM	scanning electron microscopy
DFT	density functional theory

Abstract

Red/near infrared (R/NIR) fluorescence imaging based on organic materials has emerged as one of the most effective modalities in clinical practice due to the availability of biocompatible contrast agents, maneuverable instruments, and high spatial/temporal resolution with good sensitivity. To date, many organic fluorophores including organic small molecules and macromolecules have been developed as promising candidates for R/NIR bioimaging. However, the imaging efficacy of this technique has been limited by several challenges such as the lack of highly fluorescent R/NIR contrast agents, aggregation caused quenching (ACQ) effect, poor photostability, non-biodegradability and small Stokes shift. In this thesis, we have developed several strategies to overcome these challenges.

One of the strategies is to precisely control the molecular packing of the structures to improve the optical properties of R/NIR fluorophores. Specifically, **Chapter 2** presents a new crystallization-induced-emission (CIE) fluorophore (denoted as Indigoid-B). A transition from amorphous to crystalline state of Indigoid-B was induced upon ultrasonication, accompanied with an enhancement of red emission at 600 nm. In **Chapter 3**, the aggregated states (H-, J-aggregates) of NIR dye pyrrolopyrrole cyanines (PPcys) in colloidal nanoparticles were investigated by modification of chemical structure, variation of concentration and the treatment of ultrasonication. Nanoparticles containing J-aggregates of PPcy exhibited a narrow emission band at 773 nm, a fluorescent quantum yield comparable to that of indocyanine green.

Another strategy of overcoming ACQ effect is developed in **Chapter 4** and **Chapter 5** by covalently conjugating biodegradable polycaprolactones (PCL) to red-emitting conjugated polymers (denoted as PFTB) and NIR dye PPcy. Two kinds of colloidal nanoparticles composed of PCL-grafted PFTB were prepared in the absence and presence of PCL-tethered PPcy, respectively. The presence of PCL-tethered PPcy enabled colloidal nanoparticles to emit at a longer wavelength through Förster resonance energy transfer (FRET). The introduction of PCL improved the quantum yields of both these two systems as

compared to those without PCL, where FRET colloidal nanoparticles exhibited a NIR emission at 760 nm and a high quantum yield of 46%.

A strong candidate for clinical theranostics also calls for the improvement in optical properties of R/NIR imaging agents. In **Chapter 6**, we developed a kind of theranostic unimolecular micelles by grafting amphiphilic block copolymers from conjugated polymers PFTB. The surrounding amphiphilic scaffold helped to suppress aggregation of the central PFTB fluorophore, resulting in a high quantum yield of 22%. The hydrophobic layer of side chain was used to physically entrap drugs for chemotherapy. To address the potential problem of drug leakage, we utilized amphiphilic random copolymers containing drug-modified units as side chain of conjugated polymers instead in **Chapter 7**. The experiments of *in vivo* bioimaging and therapy demonstrated a good tumor-specific ability and long retention time in tumor.

Overall, this thesis has explored several ways to overcome the currently existing drawbacks of organic R/NIR fluorescent probes, especially aggregation caused quenching, non-degradability, small Stokes shifts, and further integrated the chemotherapy function with the R/NIR emitting unimolecular micelles for promising theranostic applications.

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Chapter 1 Introduction

1.1 Background of biological imaging

Biological imaging, relying on interdisciplinary advances in cellular biology, chemistry, physics, and optical engineering, has emerged as an essential research tool to allow clinicians not only to detect the location and status of diseases, but also to evaluate the activity of the specific molecules (e.g., specific proteins, genes and polysaccharides) and physiological changes regarding biological processes (e.g., metastasis, angiogenesis and apoptosis).¹ Since the first X-ray photograph was obtained by Wilhelm Rontgen, several clinical imaging modalities such as magnetic resonance imaging (MRI), computed tomography (CT), single-photon emission computed tomography (SPECT), positron emission tomography (PET), ultrasound (US) and optical imaging (OI) have been inspired by new discoveries. It is essential to achieve contrast for these imaging techniques, which requires corresponding contrast agents or molecular probes or beacons to monitor physiological changes *in vivo*.

Each aforementioned tomographic imaging modality has its own unique advantages along with intrinsic limitations. CT, MRI, PET, SPECT and US provide functional information about the disease with higher tissue penetrating depth. Among them, CT and MRI offer high spatial resolution of 0.5 mm and 1 mm, respectively, while PET and SPECT have lower resolution but accomplish high sensitivity. However, the adverse effect to hazardous ionizing radiation exists as a potential risk in the application of CT, PET and SPECT. Ultrasound (US) is a low-cost technique, but offers both low resolution and sensitivity for detection. Moreover, SPECT or PET have limitation for long term imaging due to the unfavorable short half-life of the radioactive tracers. There are some other drawbacks for CT, MRI, PET and SPECT such as a time-consuming and complex process, the cost of instruments and the lack of both exogenous and endogenous tracers for molecular imaging.²

In contrast, optical imaging (OI) offers the opportunity to solve these problems. OI, as a non-ionizing radiation technique, can bring the benefits of lower risk to patients, faster analysis process and real-time wide-field image

acquisition.³ Besides, there are the exceptional advantages of OI such as high sensitivity, multi-target monitoring and triggered photophysical and photochemical response to physiological changes. To this end, OI has attracted rapidly increasing attention and efforts to translate into clinical application, even though most of currently available probes possess short photon penetration depth and provide low spatial resolution in most mammalian tissues.

Figure 1.1 Characteristics including advantages and disadvantages of currently available molecular imaging modalities used for biomedical application.⁴

1.2 Fluorescence imaging

In recent decades, OI based on the photophysical process of luminescence and multiphoton absorption at the molecular level has been primarily employed to distinguish tumors or pathological tissue as well as monitor the evolution of disease and outcome of treatment.⁵ To date, fluorescence imaging (FL) serves as a dominant modality of optical imaging due to its abundant tracer library.⁶ It relies on the illumination in the spectral range from ultraviolet (UV) to near

infrared (NIR) of fluorophores in targeted tissues upon the excitation by a light source.

To achieve desired signal output and high signal-to-noise ratio in biological environment, a suitable fluorescent probe is always required for the following features: (i) high molar absorption coefficient and fluorescence quantum yield; (ii) soluble or colloiddally stable in relevant buffer, cell culture medium or body fluids; (iii) large Stoke-shift; (iv) narrow emission band; (v) excellent photostability; (vi) deep penetration of NIR fluorescence into biological tissue; (vii) specific site-targeting ligands; (viii) nontoxicity.⁷

Generally, fluorescent materials including fluorescent proteins,⁸ organic dyes,⁹ quantum dots (QDs),¹⁰ metal complexes,¹¹ have dominated bioimaging application in the past two decades, but there is still a large gap to match the ideal requirements.

1.2.1 Fluorescent proteins

Fluorescent proteins, as natural organic compounds, have become one of the most useful bioimaging tools due to their excellent biodegradability since Shimomura discovered and isolated green fluorescent protein (GFP) from jellyfish *Aequorea victoria* in 1956.⁸ The GFP fluorescent mechanism proposed by Tsien was attributed to the formation of a conjugated fluorophore, p-hydroxybenzylideneimidazolinone, composed of amino acid residues Thr65 or Ser, Gly67 and Tyr66. The emission color of fluorescent protein can be regulated by tailoring the conjugation length of the fluorophore.⁹ Figure 1.2a summarizes the GFP variants that have been developed to emit in the large range from 475 nm to 649 nm.¹² More importantly, Tsien and coworkers have also developed the first NIR fluorescent protein with emission band centered at 708 nm for targeted imaging of liver.¹³ To date, GFP variants have been extensively utilized for specific protein encoding and expressing in living cells and organisms as an intrinsic intracellular indicator. However, there are some drawbacks of fluorescent proteins such as blinking of fluorescence, poor photostability and relatively short emission wavelength.

Figure 1.2 (a) Left: X-ray crystal structure of typical GFP; right: digital photo represents the fluorescence of GFP and its variants upon the excitation at different wavelengths. Copyright © 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.¹² (b) Emission spectra of Alexa Fluor Dyes which are commercial molecular probes in ThermoFisher scientific company. (c) The dependent relationship between emission maxima and sizes of quantum dots with different composition. Inset: Emission spectra for representative materials. Copyright © 2005, American Association for the Advancement of Science¹⁴ (d) Structures of selected emissive metal complexes showing salient chemical or photochemical characteristics. Copyright © 2009, American Chemical Society.¹⁵

1.2.2 Organic chromophores

As compared to GFP and its variants, synthetic organic dyes can be more easily designed by structural engineering to cover the emission range from UV to NIR.^{16,17} The commercial Alexa Fluor dyes as shown in Figure 1.2b with various emission bands have been applied for cell-staining and visualization of morphological details. Since the use of fluorescein as the first label for immunofluorescence in 1953, a myriad of common fluorophores such as rhodamines, xanthene, cyanine etc.¹⁶ with high molar absorption coefficients, comparatively high fluorescence quantum yields, and even narrow emission bands have been designed to be promising candidates for fluorescence imaging. Unfortunately, the π -conjugated planar structures of organic dyes always lead to strong hydrophobicity, which enables them to prefer to aggregate and even precipitate in aqueous environment. To render organic dyes with water solubility or colloidal stability in aqueous medium, the approaches of chemical modification or physical nanofabrication have been developed.

1.2.3 Semiconductor quantum dots (Qdots)

Colloidal semiconductor quantum dots (Qdots) are single crystals with a size of a few nanometers and limited number of electrons in nonaggregated zero-dimensional space.¹⁸ The confinement energy depends on the size of Qdots due to the delocalized nature of the electronic states, resulting in the size-dependent absorption and emission of Qdots. Over the last two decades, many basic properties of Qdots have been comprehensively. The unique advantages of Qdots such as high molar absorption coefficient, narrow emission band and excellent photostability make them promising as candidates for *in vitro* and *in vivo* imaging.^{14,19,20} The emission band of Qdots can be regulated in the spectral range from UV to NIR by varying composition and crystal size from 0 to 10 nm (Figure 1.2c).¹⁴ Among them, the most widely-applied Qdots for biological applications are CdSe and CdTe. However, the challenge of the cytotoxicity from cadmium ions involved motivates chemists to develop III/V group or ternary semiconductors such as InP and InGaP as potential alternatives.

1.2.4 Metal complexes

Due to the outstanding optical properties such as large Stokes shifts and long emission lifetimes ($1\ \mu\text{s} \sim 5\ \text{ms}$),²¹ lanthanide (III) complexes ($\text{Ln} = \text{Sm}, \text{Eu}, \text{Tb}, \text{Dy}, \text{Yb}$) are of potential for bioimaging to achieve high signal-to-background ratio, minimized interference from light scattering and autofluorescence through time-gated acquisition methods.²² Moreover, the excited states of lanthanide complexes are quite stable to molecular oxygen, and the relaxation of energy leads to the unique emission profiles with several extremely narrow bands. To date, most studies on their bioimaging application have focused on Eu(III) and Tb(III) complexes (Fig. 1.2d),^{23,24} as their excited states were less sensitive to vibrational quenching by blocking the pathway to transfer energy to OH, NH, or CH oscillator.

1.3 Fluorescent organic imaging probe

Although metal complexes and Qdots exhibit superior optical properties, their cytotoxicity, especially for Qdots, is a major issue due to the potential leakage of heavy metal. In contrast, the metal-free nature enables organic fluorescent materials as a prior option for bioimaging. Presently, majority of efforts are being devoted to overcoming the disadvantages of organic fluorescence materials such as poor photostability, unsymmetrical emission and even poor biodegradability.

The recent advances of organic fluorescent materials are summarized in the following two sections: (i) “always on” imaging; (ii) activatable imaging. The former refers to those probes that show negligible change of fluorescent signal during the imaging process in living cells and *in vivo*. The review on “always on” imaging is introduced based on the categories of organic fluorescence materials such as small molecular dyes, fluorescent conjugated polymers and biodegradable fluorescent materials. In contrast, smart probes which are able to respond to specific stimuli or organelles or biomolecules in the physiological process with significant change of fluorescent signal, so-called activatable response, are highlighted in the “activatable imaging” section.

1.3.1 “Always on” imaging

1.3.1.1 Small molecular dyes (ACQ\AIE)

The most popular organic emitters are small molecular dyes that are rich in variety. Evidently, most of these dyes are hydrophobic due to the π - π conjugated system.⁷ One strategy to solve this problem is to tune their water solubility via organic substitution (such as sulfonic acid group and quaternary ammonium salt) with minimized influence on the optical properties. However, small molecules can be rapidly excreted from the body and the ionization may lead to unexpected labeling by electrostatic interaction during the blood circulation.

In contrast, fluorescent nanoparticles usually have prolonged intracellular retention due to their larger size, enabling long-term cell tracking. To fabricate organic dye containing nanoarchitectures, supramolecular assemblies driven by self-organization of amphiphilic polymers have been utilized to carry organic dyes. There are two main approaches to prepare polymer encapsulated nanoparticles including: (i) introducing emitters by physical encapsulation based on the supramolecular self-assembly or nanoprecipitation of surfactants; (ii) introducing emitters into amphiphilic polymers via covalent bonding such as grafting-to, end-capping, and backbone-modification.

In the typical process of physical encapsulation, the solution of surfactants and dyes in a good solvent is quickly or slowly added into water, resulting in the formation of nanoparticles with hydrophobic core. As the aggregation caused quenching (ACQ) problem is common for many aromatic fluorophores (Fig. 1.3a), colloidal fluorescent nanoparticles prepared by facile physical encapsulation always show much weaker fluorescence with respect to that in monomeric state. On the other hand, the potential leakage of fluorophores from hydrophobic core remains a serious issue.

To address this problem, researchers synthesized dye-containing water soluble or amphiphilic polymers via strategies of covalent bonding such as polymerization of dye-containing monomers and initiators or post-modification of polymer. (Fig. 1.3b)²⁵ Specifically, the fluorophores can be directly decorated with acrylate, methacrylate, vinyl, norbornene, as well as cyclic carbonate groups, yielding dye containing monomers for advanced

polymerization techniques, i.e. atom transfer radical polymerization (ATRP), reversible addition-fragment transfer (RAFT) polymerization, and living anionic polymerization (LAP), nitroxide mediated radical polymerization (NMP), ring opening metathesis polymerization (ROMP) and ring opening polymerization (ROP).²⁶⁻³⁰

For example, pyrene containing triblock amphiphilic copolymers synthesized by ATRP could self-assemble as micelles with a hydrophobic reservoir to entrap model drug Nile red (Fig. 1.3c).²⁷ Recently, Hedrick and coworkers reported a facile synthetic route of pentafluorophenyl ester functionalized cyclic carbonate monomers,^{31,32} which could be easily substituted with desirable fluorophores or anticancer drugs. It provides a convenient and promising strategy to design diagnostic/therapeutic nanoplatforms based on biodegradable block copolymers. In another example, Shastri and coworkers synthesized biodegradable aliphatic polyesters via ROP of alkyne epoxy ester based monomers which was further post-functionalized with multiple fluorophores by click chemistry.²⁹

On the other hand, fluorophore-cored or fluorophore-end-capped polymers have been also developed by conjugating organic dyes to initiator.³³ Müllen and coworkers utilized perylene-3,4,9,10-tetracarboxydiimide as eight-arm initiator to induce the polymerization of amine-based monomers as shown in Figure 1.3d.^{34,35} The resulting dendritic polymers were soluble in water and formed unimolecular micelles, which prevented perylene-3,4,9,10-tetracarboxydiimide from aggregation. These positively charged fluorescent micelles successfully stained the microstructure of extracellular matrix (ECM) and were able to deliver the double-stranded RNA (dsRNA) towards genetic control of insect pests.

Figure 1.3 (a) Fluorescence photographs of solutions of fluorescein (ACQ dye) in water/acetone mixtures at different fractions of acetone and chemical structure of fluorescein; Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.³⁶ (b) Schematic representation of dye-decorated polymer structures and pathways; (c) Chemical structure of pyrene based triblock copolymers synthesized by ATRP²⁷; (d) Chemical structure of PDI-cored star polymers¹⁷.

The notorious ACQ effect inevitably exists in most fluorescent nanoarchitectures based on self-assembly of linear amphiphilic polymers. This challenge always encourages the researchers to achieve higher quantum yield at the expense of the concentration of dyes, resulting in weakening the brightness of single nanoparticle. As the opposite effect of ACQ, “aggregation-induced emission” (AIE) provides a straightforward solution to ACQ problem. AIE fluorophores refers to a group of special luminogens such as hexaphenylsilole (HPS), tetraphenylethene (TPE), and distyreneanthrathene (DSA) that exhibit no or weak fluorescence in dilute solutions but strong fluorescence in the solid or crystalline state.³⁶⁻³⁸ AIE phenomenon was firstly discovered in the study of HPS by Tang and coworkers in 2001 (Fig. 1.4a). Tang’s group has also proposed the mechanism of restriction of intramolecular motions (RIM) to explain these unique characteristics of AIE fluorophores.

TPE is a typical AIE fluorophore (Fig. 1.4c). In the dilute solution, the phenyl groups in TPE are free to rotate to relax the energy in excited state, which blocks the radiation pathway and results in no fluorescence. When TPE molecules aggregate, the rotation is suppressed, leading to the relaxation in the

way of strong emission with a wavelength of maximum emission (λ_{em}) $\sim$ 480 nm. In the past decade, a myriad of AIE fluorophores have been designed with emission in the spectral region from blue to NIR by fine structure engineering and further employed as promising alternatives of bioprobes.³⁹⁻⁴³ Colloidal nanoparticles composed of AIE fluorophores can be constructed through encapsulation by surfactants or self-assembly of bare AIE electrolyte⁴⁴ (Fig. 1.4b).

The general strategy to design AIE-featured molecules is molecular transformation by conjugating typical AIE fluorophores such as HPS, TPE with available fluorogens regardless of AIE or ACQ. For example, the attachment of two TPE moieties on benzo-2,1,3-thiadiazole (BT) yielded 4,7-bis[4-(1,2,2-triphenylvinyl)phenyl]benzo-2,1,3-thiadiazole (BTPEBT) (Fig. 1.4c), which was also an AIE fluorophore with a red-shifted λ_{em} of 550 nm due to efficient intramolecular charge transfer (ICT). The corresponding AIE nanoparticles showed potential for real-time intravital two-photon imaging of blood vessels. Compared with BTPEBT, the further extension of conjugation has been achieved to give TNB as shown in Figure 1.4c, whose colloidal NPs showed an emission maximum at 616 nm, a high quantum yield of 0.67 and a large Stokes shift of 130 nm.⁴⁵

Figure 1.4 (a) Fluorescence photographs of solutions of hexaphenylsilole (AIE dye) in water/THF mixtures with different fractions of water and chemical structure of hexaphenylsilole. Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.³⁶ (b) Model of fabricating Tat-AIE NPs. Copyright © 2013, American Chemical Society.⁴⁶ (c) Chemical structures of currently reported AIE fluorogens with different emission band.

1.3.1.2 Fluorescent conjugated polymers

As compared to small molecular dyes, fluorescent π -conjugated polymers (CPs) as macromolecular fluorophores generally exhibit higher extinction coefficient and better photostability.⁴⁷⁻⁴⁹ Conventional fluorescent CPs have been developed with emission color covering the range from blue to red as listed in Figure 1.5. To address the similar issue of hydrophobicity with small molecules, conjugated polymers have been also functionalized with ionic side chain to give water-dispersible fluorescent conjugated polymers, so called conjugated polyelectrolytes (CPEs). Since Whitten and coworkers reported that the anionic CPEs were efficiently quenched by cationic quencher dimethyl viologen, CPEs have emerged as promising alternatives of biomarkers.⁵⁰⁻⁵²

Figure 1.5 Chemical structures of conventional highly fluorescence conjugated polymers.

The advances in the development of CPEs based bioimaging probes have been reviewed by Liu and Wang.⁵²⁻⁵⁴ In the last decade, a variety of CPEs with well-defined architectures such as linear, star, brush and hyperbranched have been developed and utilized for bioimaging (Fig. 1.6a-d).⁵⁵⁻⁶¹ To render a specific ability for active targeting, CPEs nanoaggregates can be functionalized with active targeting ligands such as folic acid⁶² etc. by post surface

engineering or pre-modification on ionic side chain. Charged polythiophenes (Fig. 1.6a) were firstly reported by Nilsson and coworkers for both *in vitro* and *in vivo* specific detection of proteins aggregates.⁵⁶

In contrast with linear CPEs, the special structures of star and hyperbranched CPEs allow for the formation of unimolecular micelles in water, whose size can be tuned by controlling segmental flexibility, arm length and generation number.^{60,63-65} For example, fluorene-based hyperbranched CPEs were synthesized with surrounding biocompatible moieties polyethylene glycol, which was grafted to neutralize the surface charges and suppress nonspecific interaction with biological substances. (Fig. 1.6d) Core-shell micellar morphology of fluorene-based hyperbranched CPEs clearly observed in Figure 1.6e were internalized efficiently by cells and tend to accumulate in the cytoplasm.⁶⁰ Impressively, Liu and coworkers fabricated unimolecular micelles composed of POSS-cored star CPEs with size of ~4 nm, which was nucleus permeable with lower cytotoxicity and enabled intracellular fluorescence resonance energy transfer (FRET) imaging of cellular nucleus.⁶⁶⁻⁶⁸

Figure 1.6 Chemical structures of typical linear CPEs (a-c) and hyperbranched CPEs (d). Representative model (e) and TEM images (f) of hyperbranched CPEs in aqueous medium. Copyright © 2009, American Chemical Society.⁶⁰

However, due to the heavy functionalization with ionic side chain, the charged nanoaggregates always cause significant nonspecific labeling by electrostatic interaction. To tackle this problem, McNeill and coworkers demonstrated the formation of hydrophobic conjugated polymers nanoparticles for cellular imaging.⁶⁹⁻⁷⁵ This kind of nanoparticles, so-called semiconducting polymer nanoparticles (SPNs) or polymer dots (Pdots), consist of bare conjugated polymers and can be facilely prepared with controllable sizes and narrow size distribution by nanoprecipitation or miniemulsion techniques.⁴⁸

Figure 1.7 (a) Digital photographs of SPNs composed of commercial conjugated polymers (**PFO**, **PPE**, **PFPV**, **PFBT** and **MEHPPV**); (b) AFM image of small PPE SPNs; (c) Fluorescence images of cells upon incubation with **PPE**, **PFPV**, **PFBT** and **MEHPPV** SPNs, respectively. Scale bar: 10 μm. Copyright © 2008, American Chemical Society.⁷¹ (d) Functionalization and conjugation of semiconducting polymer nanoparticles using click chemistry. Copyright © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.⁷⁶

Commercial conjugated polymers including PFO, PPE, PFPV, MEH-PPV and PFBT etc. have been employed to fabricate SPNs with multicolor fluorescence in the absence of surfactant. (Fig. 1.7a) These densely packed NPs exhibited small particle diameters of nearly 10 nm (Fig. 1.7b), extraordinary fluorescence brightness and low photobleaching.⁷¹ Specifically, as compared to

dyes and quantum dots, SPNs showed much higher emission rates ($\sim 10^8 \text{ s}^{-1}$) and no blinking.

However, it is difficult to prepare these SPNs dispersions quantitatively because a further step of filtration is always needed to remove large precipitates after the nanoprecipitation process. Hence, it is crucial to introduce oligomeric or macromolecular surfactant to enhance the colloidal stability of SPNs. Wu and coworkers chose carboxyl groups functionalized amphiphilic polystyrene as a surfactant to encapsulate PFBT and obtained colloidal-stable SPNs with ultrabright green fluorescence (Fig. 1.7d). Moreover, amphiphilic polymers with various groups such as carboxyl, azide groups also modified the surface of SPNs, providing possibilities for subsequent covalent conjugation to biomolecules such as streptavidin, immunoglobulin (IgG), chlorotoxin (CTX) and glycoprotein via amidation or click reactions. These SPN bioconjugates facilitate the effective and specific cellular imaging.⁷⁴⁻⁷⁷

1.3.1.3 Biodegradable fluorescent materials

The usage of biodegradable materials as biological materials is beneficial to reduce the risk to patient as foreign implants could be degraded, and cleared by the body. Reasonably, biodegradable imaging materials are also highly desirable.

It has never been reported about conventional synthetic fluorescent molecules or macromolecules being intrinsically biodegradable until Yang and coworkers developed a new family of biodegradable photoluminescent polymers (BPLPs) via a facile polycondensation reaction as shown in Figure 1.8a.⁷⁸⁻⁸⁰ Nontoxic monomers such as 1,8-octanediol, citric acid, and α -amino acid were used to react to prepare BPLPs via two-steps of polycondensation. These polymers with robust backbones composed of amino acid units can be completely degraded in PBS buffer within 16 days. Meanwhile, the fluorescence intensity of BPLPs decayed with the increase of incubation time. Representatively, BPLP-Cysteine (BPLP-Cys) and BPLP-serine (BPLP-Ser) exhibited with high quantum yields, controlled degradability and excellent photostability. In detail, BPLP-Cys showed a quantum yield of 62.3%, which

was comparable to the CdTe/ZnS QDs and much higher than GFP. Different from BPLP-Cys, the tunable fluorescent color of BPLP-Ser from blue to near infrared (up to 724 nm) could be achieved by varying the excitation wavelength (Fig. 1.8b). Nanoparticles of BPLP-Ser have been fabricated by nanoprecipitation for *in vivo* imaging. BPLP-Ser nanoparticles were efficiently internalized by cells, resulting in the simultaneous cells labeling with different fluorescence colors (Figure 1.8c-d).

Figure 1.8 (a) Synthesis schematic of biodegradable fluorescent polymer **BPLPs**; (b) Fluorescence spectra of **BPLP-Ser** at different excitation wavelengths; Fluorescence images of **BPLP-Ser** nanoparticle-stained 3T3 fibroblasts cells with FITC filter (c) and with Texas Red filter (d). Copyright © 2009, National Academy of Sciences.⁷⁹

1.3.2 Activatable imaging

In most cases, fluorescent materials used as contrast agents are always fluorescent during the blood circulation and accumulation in tumor or tissues, which may lead to the poor recognition of specific tissues from background. Besides, washing operations are required to remove the remaining materials to improve the quality of molecular imaging. To this end, designing an activatable

probe based on a switching mechanism (variation of fluorescent intensity and/or wavelength upon activation by the specific biological substances) seems a good choice to tackle this issue. Hence, increasing attention has been paid to activatable probes, which can respond to external stimuli such as pH, temperature, polarity, ions, protein, DNA or RNA etc. in biological metastasis process. These probes further provide valuable insights into detection of ions in the body and evaluation of therapeutic efficacy and disease progression.

1.3.2.1 Environment-sensitive

The local microenvironment plays a crucial role in determining the physical or chemical characteristics of fluorescent molecules. The properties of probes at a specific location are significantly influenced by the alteration of several parameters including polarity, pH, temperature, viscosity etc., which are extremely significant factors in regulating diffusion, penetration, accumulation, and intermolecular interactions.⁸¹

Environmental polarity directly reflects the interaction activity of proteins and permeability of membrane compartments at specific site. Donor-acceptor (D-A) based solvatochromic fluorophores, which typically show light absorption and emission properties in response to variation of solvent polarity, have been widely used to estimate the polarity of the environment.⁸² For instance, Schultz and coworkers utilized environment-sensitive proteins encoded with fluorescent amide acids to monitor the unfolding of proteins and study their structures, localizations, and biomolecular interactions.^{83,84} Few of these probes showed strong brightness and emitted in the NIR spectral region. Recently, Klymchenko and coworkers designed a dye based on a squaraine dimer that exhibited no emission in aqueous media due to H-aggregation of squaraine and strong emission at far-red region in organic medium as shown in Figure 1.9a. It was grafted to carbetocin, a ligand of the oxytocin G protein-coupled receptor. A strong receptor-specific signal of this probe was observed upon binding to the specific serum and membrane.⁸⁵

Most polarity-sensitive probes fluoresce in a hydrophobic environment and exhibit a decreased intensity when surrounded by local hydrophilic

biomacromolecules. Surprisingly, Ohwada and coworkers synthesized a new fluorophore 8-methoxy-4-methyl-2H-benzo[g]chromen-2-one with an opposite characteristic. It was almost nonfluorescent in aprotic solvent and strongly fluorescent in protic solvent such as water (Fig. 1.9b).⁸⁶

Figure 1.9 (a) Schematic representation of NIR emissive polarity-sensitive sensor. Copyright © 2015, American Chemical Society.⁸⁵ (b) Chemical structure and fluorescence image in different solvents of 8-methoxy-4-methyl-2H-benzo[g]chromen-2-one. Copyright © 2006, American Chemical Society.⁸⁶ (c) NIR fluorescent pH-sensitive probes with reversible color changes upon responding to pH variation. Copyright © 2011, American Chemical Society.⁸⁷ (d) Structure of two types of pH-sensitive nanoprobe with pH transitions at 6.9 and 6.2 and in vivo imaging with high tumor specificity and efficacy. Copyright © 2013, Nature Publishing Group.⁸⁸

In different cell types, pH and temperature variations are responsible for different cellular functions and reactions at specific locations. Abnormal cells suffered by cancer, viral infection or inflammation can generate excess heat and

lead to more acidic level.⁸⁹ To obtain the information of disease evolution, pH/temperature-sensitive fluorescent materials have been utilized to map intracellular pH/temperature distribution.

Some dyes possess intrinsically pH-sensitive characteristic. Nagano and coworkers presented a series of ratiometric NIR fluorescent pH Probes based on aminocyanine fluorophore (Fig.1.9c). A red shift up to 80 nm of absorption maximum was observed under acidic condition.⁸⁷ In another example, an “off-on-off” type of pH-dependent fluorescent sensor was synthesized by Jiang and coworkers based on BODIPY modified by 8-hydroxyquinoline. The protonated and deprotonated forms of this probe showed the gradual fluorescence quenching in either acidic or basic conditions.⁹⁰

Besides the interruption on the conjugation of fluorophore by pH variation, another strategy to fabricate pH-responsive probe is to choose pH-responsive polymer to carry special fluorophores. Gao and coworker reported a set of ultra-pH-responsive, multicolor nanoprobe composed of self-assembled ionizable diblock copolymers (Fig. 1.9b). The sharp pH response was realized by well tuning the transition from hydrophobicity to hydrophilicity of the ionizable block. The pH change of high fluorescence activation (>50 fold) between off and on states maintained within 2.5 pH units.⁹¹⁻⁹³ Reasonably, the hydrophobicity/hydrophilicity of block copolymer determines the homo-FRET efficiency of fluorophores, resulting in the on/off switchable characteristic. Moreover, they further used one of these NIR probes as a non-toxic reporter for the identification of tumor tissue and detection of acute treatment responses. Specifically, it was silent in the circulation, and then strongly enhanced by >300 folds of fluorescent intensity in response to the low extracellular pH in head, brain, breast, lung tumor (Fig. 1.9b).⁸⁸

There are few fluorophores endowed with intrinsic temperature-sensitive properties. Mostly, thermoresponsive polymers, which always show reversibly switchable phase transition at lower critical solution temperature (LCST), are employed to change the environment of fluorophores by temperature alteration. N-isopropylacrylamide (NIPAm), as monomer of the most studied thermoresponsive polymer (PNIPAm), has been used for copolymerization with

rhodamine modified acrylate monomers. The resulting polymer showed very weak fluorescence under 25 °C and dramatically turned strongly fluorescent at 33 °C. The further increase of temperature to 40 °C induced polymer aggregation and fluorescence quenching in solutions.⁹⁴ Ivai and coworkers designed a type of copolymer composed of polarity-sensitive fluorophore, temperature-sensitive block and pH sensitive units. This special structure rendered it with the dual-responsive characteristic to both temperature and pH input.⁹⁵

1.3.2.2 Ions-sensitive

Metals are essential for sustaining all forms of life, but severe alterations deviated from their cellular homeostasis may lead to human disorders, including cancer, inflammation and neurodegenerative diseases. Smart organic fluorescent materials can report signal change in response to metal ions with high selectivity and sensitivity, providing an opportunity to visualize the metal accumulation and biodistribution in living systems.⁹⁶ Generally, the most critical factor for imaging metal ions is the selectivity of sensor for a specific ion (Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Zn^{2+} , Cu^{2+} , Fe^{3+} , Hg^+ , Pb^{2+} , Cd^{2+}).

Zinc (Zn^{2+}) is the second most abundant heavy metal ion after iron. Nagano and coworkers developed a highly zinc-selective fluorescent sensor molecule ZnAF-1 and ZnAF-2 utilizing N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) as the reporter (Fig. 1.10a).⁹⁷ Upon coordination with Zn^{2+} , the fluorescence was significantly enhanced by 17-fold and 51-fold for ZnAF-1 and ZnAF-2, respectively. Chang and coworkers synthesized a Cu^{2+} -specific fluorescence probe CS by combining a BODIPY reporter and thioether-rich receptor (Fig. 1.10b).^{98,99} The response to the intracellular Cu^{2+} with high selectivity and sensitivity was demonstrated by *in vitro* experiments of CS-labelled human embryonic kidney (HEK) 293T cells.

Heavy-metal poisoning as a widespread issue always threatens human health, which also requires new technique for selective identification of specific heavy-metal in water and in the body.¹⁰⁰ Lippard and coworkers designed an Hg^{2+} sensor (MS1) based on fluorescein scaffold (Figure 1.10c), which exhibited a 5-

fold emission enhancement upon the addition of Hg^{2+} in water and a high selectivity over Cd^{2+} and Pb^{2+} .^{101,102} Peng and coworkers reported a BODIPY-based probe for selective imaging of Cd^{2+} in living cells which could distinguish Cd^{2+} from Zn^{2+} (Fig. 1.10e).¹⁰³

Figure 1.10 Chemical structure of ion-sensor: (a) Zn^{2+} ; (b) Cu^{2+} ; (c) Hg^{2+} ; (d) Pb^{2+} ; (e) Cd^{2+} ; (f) CN^- ; (g) fROS.

Cyanide ion is well-known as a highly toxic species in drinking water.^{104,105} The imaging of cyanide *in vivo* system is increasingly desirable for monitoring the accumulation of cyanide in lungs of CF patients. A NIR fluorescence sensor as shown in Figure 1.10f was developed by Yoon and coworkers and showed a 14-fold increase of intensity upon the addition of CN^- . In addition, it has been used for detection of HCN produced by *Pseudomonas aeruginosa* (PA) 14 in nematodes.^{106,107}

Reactive oxygen species (ROS) are important mediator in many pathological processes. Once suffered from chronic bacterial infections or chronic diseases such as cancer, oschemic stroke, sepsis etc., the mediated system will be out of balance and excessively generate ROS, resulting in strong oxidative stress.^{108,109} Mitochondria has been proved to be the major source of intracellular highly reactive oxygen species (hROS) such as hydroxyl radical (OH), peroxyntirite (ONOO^-) and hypochlorite (OCl^-) etc.¹¹⁰ Nagano and coworkers designed a fluorescent probe, MitoAR, to selectively detect in real-time the hROS generation in mitochondria of living cells. (Fig.1.10g) Recently, Pu and coworkers presented a smart conjugated polymer based nanosensor to

simultaneously and differentially detect *in vivo* the drug-induced ONOO⁻/OCl⁻ and H₂O₂ by integration of fluorescence resonance energy transfer (FRET) and chemiluminescence resonance energy transfer (CRET).¹¹¹

1.3.2.3 Biomolecules/biomacromolecules-sensitive

Selective detection of biomolecules (glutathione (GSH), etc.) and biomacromolecules (protein, polysaccharides, DNA, RNA etc.) is of fundamental importance in basic biological study as well as medical diagnosis. Activatable fluorescent probes, whose emissive features (especially on/off characteristic) only change in response to the intended target biomolecules or biomacromolecules, are particularly useful.

One design principle of biomolecule-sensitive probe relies on the analyte induced interruption of internal charge transfer (ICT) behavior in fluorophores. For example, naphthalimide is well-recognized as a typical fluorescence-switchable biomarker due to its outstanding ICT efficiency and high quantum yield. Considering the cleavage reaction of disulfide by the abundance of cellular free thiols such as GSH¹¹², Kim and coworkers utilized a disulfide bond to link naphthalimide and camptothecin (CPT) (the anti-cancer active agent). Upon the presence of GSH, naphthalimide became active and emitted more brightly at a longer wavelength, accompanied with the release of the free CPT active agent.^{113,114}

The assembly-disassembly transition of supramolecular structure can also be employed to realize the off/on targeting imaging thanks to the aforementioned ACQ and AIE behavior. Hamachi and coworkers presented a protein-specific probe composed of ACQ fluorophores such as BODIPY and tetramethyl rhodamine and specific targeting ligands such as biotin, benzene sulfonamide (SA) etc (Fig. 1.11a). The self-assembled aggregates of these amphiphilic molecules could form in aqueous medium and disassemble driven by recognition of specific proteins (avidin, trypsin). The disassembled process prevented it from fluorescence quenching of fluorophores.¹¹⁵⁻¹¹⁷

Figure 1.11 (a) “off-on” fluorescent probes based on self-assembling for specific protein recognition. Copyright © 2010, American Chemical Society.¹¹⁵ (b) Schematic representation of **Ac-DEV DK-TPE** for caspase activities study to monitor cell apoptosis process. Copyright © 2012, American Chemical Society.¹¹⁸ (c) Illustration of the mechanism of proposed caspase-3/7 and reduction-controlled conversion of **C-SNAF** into **C-SNAF-cycl** through the bioorthogonal intramolecular cyclization reaction, followed by self-assembly into nanoaggregates *in situ*. Copyright © 2014, Nature Publishing Group.¹¹⁹

In the opposite pathway, assembly of AIE fluorophores to activate the emission can be achieved by alternating the hydrophilicity in the process of analyte recognition. Liu and coworkers designed a probe based on AIE to

monitor in real time the cell apoptosis process. This probe so-called DEVDK-TPE was a synthetic oligomer with caspase-identified Asp-Glu-Val-Asp (DEVD) peptide as a hydrophilic chain and a AIE-active unit tetraphenylethene (TPE) as a hydrophobic moiety (Fig. 1.11b). Specifically, it was nonfluorescent in aqueous media but displayed caspase-3/-7 triggered enhancement of emission, where caspase-3/-7 mediated the apoptosis process and was capable of cleaving the DEVD moieties. The cleavage induced the aggregation of TPE, resulting in the fluorescence turn-on behavior.^{118,120,121}

In another example, Ye and coworkers reported a caspase-sensitive nanoaggregation probe (C-SNAF) with Cy5.5 as fluorescent reporter. After the cleavage by caspase *in vitro* and *in vivo*, this probe underwent self-assembly through biorthogonal cyclization chemistry, leading to the formation of nanoaggregates and significant enhancement of NIR fluorescence. This probe is promising to distinguish negative from positive outcomes of tumors upon chemotherapeutic treatment.¹¹⁹

Additionally, selective detection of DNA, RNA by π -conjugated polymer has been successfully accomplished with amplified fluorescence signal.^{47,49} Water-soluble conjugated polymer-polyfluorene containing 2,1,3-benzothiadiazole (BT) units was designed by Pu and coworkers for ssDNA. A change of emission color was observed upon the addition of negatively charged DNA which improved the intramolecular energy transfer efficiency from fluorine units to BT units.⁵⁰ They applied this similar method to detect negatively charged Heparin.¹²² Techniques based on FRET also appeared to make probes smarter. Bazan and coworkers employed cationic poly(fluorine-phenyl)s (PFP) and peptide nucleic acid labeled with fluorescein (PNA-C*) for highly sensitive detection of DNA. FRET effect was activated upon the addition of a target ssDNA that was complementary to the PNA probe.¹²³

1.4 Organic R/NIR fluorescent Probe

Despite the advanced development, *in vivo* and clinical translation of most of these organic probes are still hampered due to their shorter wavelengths (400-600 nm), because the influence of the photons absorption by tissue substances

such as oxy- and deoxyhemoglobin ($\lambda_{max} < 600$ nm), photons scattering by tissue and non-negligible autofluorescence from tissue are significant. As a result, the ideal imaging window referring to red/near infrared (R/NIR) spectral range (600-1700 nm) can afford deeper tissue optical imaging with better signal-to-background (SBR). In this section, the recent advances on R/NIR imaging using organic probes are summarized.

1.4.1 R/NIR probes based on small molecular dyes and conjugated polymers

A number of NIR fluorophores, including borondipyrromethenes (BODIPYs),^{124,125} cyanines,^{126,127} rylenes,¹²⁸ croconiums,¹²⁹ squaraines,¹³⁰ quinoidal¹³¹ have been developed for molecular imaging. Most R/NIR dyes show intrinsically low fluorescence quantum yields, which can be explained by the energy gap law.¹³² In 2008, a new class of NIR fluorophores, pyrrolopyrrole cyanines (PPcy), which contained a pyrrolopyrrole system as the central element of a cyanine-type chromophore, has been reported with emission wavelength in the NIR region up to 1000 nm and higher quantum yield (up to 70%) in good solvent than those of other currently available NIR dyes.¹³³⁻¹³⁵ However, further structural ionization by introducing sulfonic acid group rendered PPcy a sharp reduction of quantum yield to 1% (Fig. 1.12b),¹³⁶ comparable with that of indocyanine green (ICG).

To date, only ICG ($\lambda_{em} \sim 800$ nm) and methylene blue (MB, $\lambda_{em} \sim 700$ nm) have been approved by the US Food and Drug Administration (FDA) for clinical imaging. However, the main drawbacks appear that ICG with negative charge and MB with positive charge can cause the strong non-specific interaction with proteins *in vivo*, resulting in low signal-to-noise ratio.

To minimize the non-specific interaction, two zwitterionic NIR fluorophores (named ZW700 and ZW800) with charge-neutral structure have been synthesized and applied for targeted tumor imaging with improved SBR as compared to conventional NIR fluorophore with net charges (Fig. 1.12a).¹³⁷⁻¹³⁹ There is still a potential problem of the short blood half-life due to its small size in aqueous medium. In contrast, nanoscaled supramolecular structures of

probes facilitate to prolong the circulation time. As mentioned in the previous section, the self-assembled nanoprobe composed of ultra pH-sensitive amphiphilic diblock polymer showed long circulation time and highly effective tumor-specific imaging with $SRB > 300$ owing to the significant NIR signal enhancement in response to the low extracellular pH in the tumor microenvironment.⁸⁸

Figure 1.12 (a) Zwitterionic NIR fluorophores (named ZW800) with charge-neutral structure¹³⁷; (b) Chemical structure of water-soluble PPcy¹³⁶; (c) (d) Chemical structure of

R/NIR AIE fluorogens^{43,140}; (e) Chemical structure of red-emissive CPEs and schematic representation of fabricating corresponding nanoparticles. Copyright © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.⁶² (f) Far-red emitting alternating conjugated polymers (PF-TQ) and Zebrafish microangiography by injection of based SPNs. Copyright © 2015, American Chemical Society.¹⁴¹

The large conjugated system and planar structure of R/NIR dyes always cause more significant ACQ effect. Therefore, R/NIR AIE fluorophores emerge as ideal alternatives to solve the ACQ problem. For example, two TPE pendants have been attached to N,N-dicyclohexyl-1,7-dibromo-3,4,9,10-perylenetetracarboxylic diimide (DDPD) to yield a TPE-DDPD, which transformed ACQ DPPD to AIE TPE-DDPD (Fig. 1.12c).⁴³ The TPE-DPPD containing NPs were R/NIR fluorescent with an emission band centered at around 680 nm and possessed a relatively high quantum yield of 13%. More R/NIR AIE dyes have been synthesized¹⁴² but most of their emission maxima are located below 700 nm.^{36,143,144} Very recently, Liu and coworkers conjugated the typical AIE dye tetraphenylethene (TPE) to strong electron acceptor benzo[1,2-c:4,5-c']bis([1,2,5]thiadiazole) (BBT) to give unique fluorophore with NIR emission maxima of ~815 nm in the aggregated NPs. (Fig. 1.12d)¹⁴⁰ These AIE NPs displayed a relatively high quantum yield of 4.8% and a favorable large Stokes shift of 170 nm. However, NIR AIE probes with high FL quantum yield remain rare.

In contrast with small molecules, rare alternating conjugated polymers emit in the NIR window. In the aggregated state, poly[9,9-bis(6'-hexyl)) fluorenyldivinylene-alt-4,7-(2',1',3',-benzothiadiazole) (PFVBT), poly(fluorene-alt-(4,7-bis(thien)-2,1,3-benzothiadiazole)) (PFTB) derivatives show an emission band near 650 nm with relatively low quantum yield of 5%.^{145,146} PFVBT polymers have been transformed into corresponding PEG-graft CPEs, which formed micelles with a quantum yield of ~ 12% by self-assembly. (Fig. 1.12d)⁶² Recently, a series of quinoxaline based alternating conjugated polymers were reported with emission bands covering the NIR range and a fluorescence quantum yield as high as 47%.¹⁴¹ Especially, PF-TQ based nanoparticles as shown in Figure 1.12e, one of the derivatives, has a $\lambda_{em,max}$ of 710 and a quantum yield of 9%. The *in vivo* macroangiography imaging on

living zebrafish embryos have been performed well using these quinoxaline-based NPs. However, it is still difficult to satisfy both requirements of the preferable higher wavelength and higher quantum yield in the alternating conjugated polymer system.

Figure 1.13 (a) Chemical structure of the NIR-II-emissive conjugated polymer pDA and absorption/emission spectra of pDA based NPs. Copyright © 2014, Rights Managed by Nature Publishing Group.¹⁴⁷ (b) Chemical structure of NIR-II-emitting organic dye, CH1055 and relative absorption/emission spectra in water solution. Copyright © 2015, Nature Publishing Group¹⁴⁸.

Most of aforementioned NIR fluorophores emit photons in the first NIR window (NIR-I, 650-1,000 nm). However, with respect to NIR-I ones, the probes with emission in the second NIR window (NIR-II, 1,000-1,700 nm) have appeared to show more favorable imaging quality with the higher resolution in micro-scale (up to ~3 mm depth) due to the reduced photon scattering tissue and minimum photon absorption by tissue.

So far, few organic NIR-II-emitting fluorophores are available. Dai and coworkers discovered a series of low-bandgap donor/acceptor conjugated polymer (denoted as pDA) with tunable emission wavelength from 1050 to 1350 nm. (Fig. 1.13a)¹⁴⁷ The corresponding phospholipid-polyethylene glycol

functionalized NPs exhibited a high quantum yield of 1.7% and allowed for the first *in vivo* ultrafast biological imaging in the > 1000 nm window. Recently, they also synthesized a water-soluble BBT-based NIR-II probe (CH1055) (Fig. 1.13b) with an emission maximum at 1055 nm for visualizing the mouse lymphatic vasculature at a higher resolution as compared to ICG.¹⁴⁸ Different from the previously reported NIR-II emitting materials such as multiwall carbon tubes and QDs, approximately 90% of injection dose of CH1055 was rapidly excreted through the kidneys within 24 h after intradermal administration. Further conjugation with anti-EGFR Affibody allowed for targeted imaging of tumors *in vivo*.

1.4.2 Inter- and intra-chain energy transfer based NIR probes

Energy transfer (ET) system is a well-established technology that can easily match the requirement on imaging platforms by integrating the advantages of both NIR dyes (longer emission wavelength) and conjugated polymers (high extinction coefficient).¹⁴⁹

Figure 1.14 (a) Schematic illustration of narrow emissive **BODIPY** based conjugated polymer and emission spectra of **BODIPY** based SPNs. Copyright © 2013, American Chemical Society.¹⁵⁰ (b) Preparation of NIR dye-doped SPNs via nanoprecipitation and emission spectra of SPNs with different mass content of NIR dyes. Copyright © 2011, American Chemical Society.¹⁵¹ (c) Preparation of self-luminescing NIR dye-doped BRET-FRET NPs and corresponding emission spectra. Copyright © 2012, Rights Managed by Nature Publishing Group.¹⁵²

One approach based on ET system is to incorporate NIR dye into the conjugated polymer to form part of the backbone as NIR emitting unit, where energy transfer occurs via efficient exciton diffusion along the polymer chains to generate strong NIR emission.^{57,141,153} Boron dipyrromethene (BODIPY), familiar with small full width of half maximum (FWHM) of emission band and high quantum yield, has been incorporated into conjugated backbones by Wu and coworkers via the copolymerization with fluorene monomers. These BODIPY containing SPNs emitted with narrow emission and high brightness (Fig. 1.14a), which were desirable for multiplex detections.^{150,154} They also designed a series of squaraine-based conjugated polymers composed of most fluorene donor units and yielded corresponding SPNs with a narrow emission band near 700 nm, a quantum yield up to 30% and extremely large Stokes shift over 300 nm.¹⁵³

In contrast, the physical doping of NIR dye into visibly emissive matrix seems more convenient to allow for the occurrence of efficient Förster resonance energy transfer (FRET). Recent efforts have been devoted to fabricating FRET system by encapsulating NIR775 dyes into hydrophobic matrix composed of conjugated polymers (poly(9,9-dioctylfluorene-co-benzothiadiazole) (PFBT)¹⁵⁵ (Fig. 1.14b) and poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV))¹⁵⁶ in confined space. These FRET systems exhibited FL quantum yield of approximately 10%. Furthermore, Rao and coworkers integrated both bioluminescence resonance energy transfer (BRET) and FRET.¹⁵² Specifically, they utilized *Renilla luciferase* mutant as BRET donor, SPNs of MEH-PPV as BRET acceptor and FRET donor, and NIR775 as FRET acceptor, respectively (Fig. 1.14c). The visualization of lymphatic networks and vasculature of xenografted tumors

upon activation by intrinsic bioluminescence was realized with high tumor-to-background ratio (>100). These efforts based on FRET NIR systems overcome the challenge of small Stokes shift of NIR dyes and further avoid crosstalk between the excitation and emission signals. However, these probes suffer from the leakage of the dopant molecules from the matrix during storage and over long period of blood circulation, which is still a serious issue.^{157,158}

1.5 R/NIR fluorescence guided cancer therapy

Cancer, arise from the transformation of normal cell into tumor cells, is the second leading cause of mortality today. By 2030, the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer deaths simply due to the growth and aging of the population.¹⁵⁹ However, in the United States, the cancer death rate in 2016 has dropped by 23% since 1991,¹⁶⁰ reflecting the rapid development of technology on early diagnostics and effective therapy of cancer in the past few decades.

In the past decades, different treatment strategies such as surgery, chemotherapy, radiotherapy, phototherapy and immunotherapy have been developed to cure cancer effectively. Among them, chemotherapy emerges as one of the most preferential and effective treatments. However, due to the undesired accumulation of drugs in healthy organs, healthy cells can also be attacked while drugs are slowing down and even stopping the rapid growth of cancer cells. Hence, the suppression of the side effects of toxicity has become an urgent demand in the treatment of chemotherapy. Beyond this, the R/NIR fluorescence modality has been incorporated into drug delivering systems to distinguish tumor lesions from normal tissue and provide detailed pharmacokinetics information of biodistribution of therapeutic agent. A variety of so-called theranostic platforms with both NIR imaging modality and therapeutic agents are reviewed in this section.¹⁶¹⁻¹⁶⁵

Since the commercial introduction of Doxil[®]^{166,167}, supramolecular assemblies driven by self-organization of amphiphilic polymers such as micelles, vesicles, fibers and nanoparticles have been intensively utilized as carriers for delivering chemotherapeutic drugs, triggered release to cells and

reducing the non-specific toxicity of the drug.^{168,169} Due to the hydrophobicity of most R/NIR dyes, it is convenient to carry R/NIR diagnostic agent by these polymeric nanoarchitectures. For instance, ICG has been introduced to the doxorubicin (DOX)-loading liposomes or micelles to impart NIR fluorescence, enabling real-time monitoring of accumulation at the tumor site.^{170,171} Kim and coworkers directly attached drug and folic acid to NIR dye heptamethine cyanines (Cy7s) to yield a small theranostic molecule bearing a disulfide bonds. Upon the stimulation by GSH, the active drug gemcitabine was released, accompanied with the “off/on” switchable NIR fluorescence.¹¹⁴

Figure 1.15 (a) Schematic representation of a pyropheophorbide-lipid porphysome; (b) TEM images of negatively stained porphysomes; (c) Photothermal therapy set-up and

thermal image upon continuous irradiation for 60 s (1.9 W cm^{-2}) on a KB tumor of mice. Copyright © 2011, Rights Managed by Nature Publishing Group.¹⁷²

Porphysomes composed of phospholipid-porphyrin conjugates have been developed as a potential theranostic nanodevices by Zheng and coworkers (Fig. 1.15).¹⁷² The high porphyrin loading capacity rendered porphysomes dual NIR dual fluorescence and photoacoustic properties. However, the self-quenching porphyrin bilayer resulted in the weak fluorescence of porphysomes. Due to the enzymatical biodegradability, minimal toxicity in mice was achieved with an extremely high intravenous dose of 1000 mg/kg. Reasonably, doxorubicin (DOX) can be facily encapsulated in this liposome-like structure, yielding Doxil[®]-like nanomedicine with imaging function. In addition, other theranostic systems based on self-assembly of amphiphilic small molecules or macromolecules were designed to deliver NIR dyes such as squaraine, phthalocyanine, and boron-dipyrromethene (BODIPY) derivatives and therapeutic agents to tumors.¹⁷¹⁻¹⁷⁴

In contrast, it is rarely reported to employ conjugated polymers as diagnostic agent to construct theranostic platform owing to the lack of R/NIR macromolecular fluorophores. Recently, Liu and coworkers synthesized a polyprodrug by conjugating Dox to red-emissive conjugated-polyelectrolyte (PFVBT) though a reactive oxygen species (ROS) cleavable linker.¹⁷⁵ The irradiation with single light source simultaneously induced the on-demand Dox release and the generation of ROS to achieve synergistically chemo- and photodynamic- therapy.

To date, the studies on R/NIR imaging guided therapy remain rare and more developments are further expected to simultaneously minimize the side effect of toxicity and improve imaging quality.

1.6 Objectives and organization of this thesis

Chapter 1 gives an overview on the significant advances of fluorescence probes, which helps us to gain insight into the unique advantages of imaging modality based on organic R/NIR materials. A tremendous number of organic “smart” probes with various optical features and specific responses to various

stimuli have been designed relying on novel chemistry. However, there still exist several challenges as follow:

(i) Aggregation caused quenching (ACQ) effect limits the application of high concentrated R/NIR fluorophores. There are two strategies to solve the ACQ problem. One is to use fluorophores possessing aggregation induced emission (AIE) characteristics. However, currently available AIE fluorophores with R/NIR emission are rare and always require tedious synthesis. Another strategy is to incorporate fluorescence-silent polymers to enlarge the distance among fluorophores and improve their quantum yield. However, it always leads to very dilute solution of fluorophores. Therefore, it is essential to maintain the balance between quantum yield and fluorophore concentration, which hasn't been investigated.

(ii) The most convenient way to fabricate R/NIR probes is to incorporate available R/NIR fluorophores into nanoarchitectures via physical encapsulation or chemical conjugation. At the nanoscopic scale, their optical properties can also be precisely controlled by means of electronic coupling of transition moments, which depend on different molecular packing structures. However, this tool for controlling the stacking states of fluorophores has been rarely explored in aqueous medium.

(iii) The biodegradability of materials such as fluorescent proteins are preferred and can further accelerate the pace of translation into clinical application. However, fluorescent proteins have some limitations such as low brightness and the potential risk of oligomerization and the short emission wavelength (the longest emission wavelength of available fluorescent protein is near 700 nm). Therefore, it is highly desirable to develop new biodegradable fluorescent probes with emission wavelength in the NIR region.

(iv) Theranostic nanoplateforms can be facilely achieved by encapsulating both R/NIR fluorophores and anticancer drugs in supramolecular nanostructures of surfactants. However, a remaining challenge in these delivery systems, normally associated by relatively weak hydrophobic interaction, is the dissociation of supramolecular assemblies during circulation in physiological

environment. It leads to the leakage of fluorophores and drugs, which may further result in a poor imaging contrast and a serious side effect of toxicity. Therefore, the development of new theranostic nanoplateforms with strong R/NIR fluorescence and excellent nanoarchitectural stability is still challenging.

The aim of this thesis is to design and synthesize organic R/NIR fluorophores and develop effective strategies to enhance the brightness of probes, improve the biodegradability and optical stability of probes as well as combine chemotherapeutic functionality with R/NIR fluorescent modality for fabrication of new theranostic platforms.

Chapter 2 describes the discovery of a new crystallization induced red emission fluorophore based on an indigo derivative (Indigoid-B). The spectroscopic and microscopic techniques were employed to characterize the monomeric, aggregated and crystalline states of Indigoid-B. The mechanism of crystallization induced emission behavior of Indigoid-B was elaborately discussed based on the Density Functional Theory calculation and analysis of single crystal structure.

Chapter 3 focused on the NIR-absorbing J-aggregates of pyrrolopyrrole cyanines (PPcy) derivative. The spectral tailoring from J- to H-aggregates was realized by modifying the molecular structure, alternating the concentration and the treatment of ultrasonication. Considering the comparable quantum yield with ICG and narrow emission band, the preliminary experiments of bioimaging were also studied.

In **Chapter 4**, the studies aim at improving the quantum yield of far-red emissive semiconducting polymer nanoparticles (SPN) through covalently conjugated the biodegradable polycaprolactones on sides of conjugated polymers. The balance between quantum yield and concentration of conjugated polymers was investigated by tuning the chain length of PCL. *In vitro* experiment on cellular uptake was also conducted to demonstrate the imaging efficacy.

Chapter 5 extends the study of PCL-incorporated FRET system by importing PCL-tethered PPcy as acceptor into the far red emitting nanoparticles

presented in **Chapter 4**. The effect of chain length of PCL and the donor-acceptor distance on the optical properties of FRET nanoparticles including quantum yield and stability was evaluated. *In vitro* and *in vivo* bioimaging experiments by using optimized FRET probe were studied in detail.

Chapter 6 and **Chapter 7** present two types of theranostic unimolecular micelles with excellent architectural integrity and bright FR/NIR fluorescence. In **Chapter 6**, amphiphilic diblock copolymers was grafted from conjugated polymer backbones via subsequent ROP and ATRP. The hydrophobic layer was utilized to entrap antitumor drug doxorubicin for chemotherapy. In **Chapter 7**, hydrophilic side chains containing camptothecin decorated units was grafted from the same conjugated backbones via RAFT. The structural, morphological and optical properties of both two types of unimolecular micelles were studied. The theranostic efficacy of these unimolecular micelles noncovalently and covalently containing drugs was assessed both *in vitro* and *in vivo*.

Chapter 8 is the summary and outlook of this thesis.

1.7 References

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Scheme 2.1 (a) Molecular structures of Indigoid-B and -A, and the one-step synthesis toward these two compounds from indigo. Reagents and conditions: (i) N-dimethylaminopyrine (DMAP), di-*tert*-butyldicarbonate, dichloromethane, R.T.; (ii) acetyl chloride, pyridine, ethyl acetate, 100 °C.

This chapter presents a novel red-emitting CIE chromophore based on an indigo derivative (denoted as Indigoid-B, Scheme 2.1), which was modified from commercial indigo.¹⁵ Indigo, which can be extracted from the plants *indigofera tinctoria* and *isatis tinctoria*, have been known for centuries as important dyes for industries such as coloring textiles (mostly blue jeans).¹⁶ Recently, indigo has been employed to fabricate biodegradable electronic devices such as field effect transistors with charge carrier mobility up to $0.5 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$.^{15,17,18}

2.2 Results and discussion

2.2.1 CIE-active behaviour of Indigoid-B

Figure 2.1 (a) A digital photograph under the UV light (365 nm) irradiation of Indigoid-B (100 μM) in THF/water mixtures with ultrasonic treatment. (b) FL spectra of Indigoid-B (100 μM) in THF/water mixture. Solid lines: with ultrasonication; dash lines: without ultrasonication. Excitation wavelength: 520 nm. (c) FL relative intensity of Indigoid-B versus the water volume fraction in THF/water mixtures.

Herein, we started from the synthesis of Indigoid-B through a one-step reaction of indigo with di-tert-butylidicarbonate. Indigoid-B shows no photoluminescence in dilute solutions (e.g. in tetrahydrofuran (THF)). Surprisingly, Indigoid-B crystals obtained from recrystallization in ethyl acetate became luminescent under UV light (365 nm) irradiation. This result implies that this indigoid molecule may be AIE and/or CIE active.

To examine further details, different amounts of water (with volume fraction $f_w = 0\sim 95\%$) were added into an aliquot of Indigoid-B with a concentration of 100 μM in THF. Considering the possibility of molecular reorganization induced by ultrasonic treatment,^{8,19} we prepared each of the solutions with various water fractions by the slow addition (22.5 mL/h) of a known volume of water under ultrasonication and the colorimetric and luminescence changes were observed immediately. As shown in Figure 2.1a, when f_w is less than 80%, solutions remain non-luminescent. Interestingly, bright pink luminescence under UV light (365 nm) irradiation was observed when f_w was increased to 80%. The steady-state fluorescence (FL) spectra (Fig. 2.1b) showed that the FL intensity was dramatically enhanced when f_w was increased up to 80%, with a sharp emission peak centered at 596 nm. The dramatic increase of the FL intensity with the increase of f_w from 70% to 80% can be more clearly discerned in Figure 2.1c. In addition, with further increase of f_w from 80% to 95%, there was a blue shift of the emission peak to 589 nm, associated by a slight increase of FL intensity (Fig. 2.1b).

2.2.2 Well-defined microcrystals of Indigoid-B

To investigate the factors that contribute to the AIE and/or CIE effect of Indigoid-B described above, we employed SEM and TEM to study the morphology of the aggregates formed in water/THF mixtures. Figures 2.2a-f show the SEM and TEM images of the aggregates formed by Indigoid-B in water/THF mixtures with ultrasonic treatment. The resulting dispersion at $f_w = 80\%$ was drop-cast on a precleaned silicon substrate and subsequently dried in air at room temperature. We observed well-defined octahedral microcrystals under SEM (Fig. 2.2a). These octahedral microcrystals appeared relatively uniform in size and shape, with an average length of $2.4 \pm 1 \mu\text{m}$. The TEM

image (Fig. 2.2d) of these microcrystals shows morphology consistent with those observed using SEM. Slightly different microcrystals formed in the solution of Indigoid-B with $f_w = 90\%$. A representative SEM image (Fig. 2.2b) shows a majority of elongated octahedral microcrystals with a larger aspect ratio than that of the well-defined octahedral microcrystals formed at $f_w = 80\%$. At $f_w = 95\%$, Indigoid-B formed lamellar microcrystals (Fig. 2.2c) with irregular sizes and shapes.

Figure 2.2 SEM and TEM images of Indigoid-B microcrystals formed in THF/water mixtures with ultrasonic treatment (a, d): $f_w = 80\%$; (b, e): $f_w = 90\%$; (c, f): $f_w = 95\%$. Fluorescence microscopic images of the octahedral microcrystals formed by Indigoid-B in THF/water mixtures. (a): $f_w = 80\%$; (b): $f_w = 90\%$. (Excitation wavelength: 488 nm)

The crystalline structures of the Indigoid-B microcrystals shown in Figure 2.2a-f were further investigated using X-ray diffraction (XRD) spectroscopy. The XRD pattern of the octahedral microcrystals showed intense reflection (Fig. 2.3c), but fewer peaks in comparison to those of simulation and bulk crystals (Fig. 2.3a-b). The XRD profile (Fig. 2.3d) of the elongated octahedral microcrystals formed at $f_w = 90\%$ showed a similar pattern to that at $f_w = 80\%$.

In contrast to the microcrystal samples formed at $f_w = 80\%$ and 90% respectively, the lamellar microcrystals formed at $f_w = 95\%$ show an XRD profile (Fig. 2.3e) more similar to that of the bulk crystals, in which the intensity of peaks at $2\theta = 9.2^\circ$ and 18.5° was enhanced. These results are consistent with the different polymorphs observed using SEM and TEM.

It is worthy to note that the optical properties of Indigoid-B are dependent on the morphology of microcrystals. Firstly, all of the microcrystals formed by Indigoid-B in THF/water mixtures at $f_w \geq 80\%$ showed an obvious red shift of the major absorption band compared to the absorption of Indigoid-B in pure THF (Fig. 2.4). Secondly, with the morphological transformation of Indigoid-B microcrystals from polyhedral to lamellar, blue shifts were observed in both the absorption and emission bands. Such correlation between organic polymorphs and luminescent properties has also been recently reported by Wang et al. in bulk crystals of (4,4'-(thiazolo[5,4-d]-thiazole-2,5-diyl)bis(N,N-diphenylaniline))²⁰ and by Galer et al. in a BF₂ complex with 1-phenyl-3-(3,5-dimethoxyphenyl)-propane-1,3-dione⁷.

Figure 2.3 XRD patterns of Indigoid-B: (a) Simulated pattern of the single crystal; (b) pristine samples from recrystallization in ethyl acetate; (c-e) samples of Indigoid-B microcrystals in THF/water mixture with ultrasonication treatment: (c) $f_w = 80\%$; (d) $f_w = 90\%$; (e) $f_w = 95\%$.

Ultrasonication played a crucial role in the crystallization of Indigoid-B and the resulting AIE and/or CIE phenomenon. Only weak fluorescence (Fig. 2.1b-c) was observed in the samples prepared without ultrasonic treatment while other experimental conditions were maintained the same as described above. Even when f_w was relatively high, e.g. 80~95%, the FL intensity was only slightly enhanced compared to those with f_w below 80%. TEM images of the aggregates formed by Indigoid-B in water/THF mixtures without ultrasonic treatment are shown in Figure 2.5. At an f_w of 90%, well-dispersed relatively uniform tiny dots with an average diameter of 2~3 nm were observed (Fig. 2.5a). When f_w was increased to 95%, irregular amorphous aggregates with larger sizes were observed (Fig. 2.5b).

Figure 2.4 Normalized UV-vis absorption spectra of Indigoid-B (100 μ M) in THF/water mixtures with ultrasonication treatment.

Figure 2.5 TEM images of Indigoid-B aggregates in THF/water mixture without ultrasonication treatment (a) $f_w = 90\%$; (b) $f_w = 95\%$.

2.2.3 Comparison to Indigoid-A

To study the effect of the molecular structure, particularly the *t*-BOC substituting group, on the ultrasound-induced crystallization and the CIE effect described above, we synthesized another indigoid (denoted as Indigoid-A, Scheme 1) functionalized with an acetyl group.²¹ In contrast to Indigoid-B, Indigoid-A showed a bright orange-red emission ($\lambda_{\text{max}} = 601$ nm) in pure THF solutions (Fig. 2.6a-b). The FL intensity dramatically decreased at $f_w = 10\%$, followed by a continuous but more moderate decrease when the water fraction was increased to 95% (v/v) (Fig. 2.6b). When water was added into the Indigoid-A solution (in THF) under ultrasonication, the change of FL intensity at $f_w < 70\%$ was similar with that without ultrasonic treatment. After the water fraction increased to 90% and even higher (e.g. 95%), there was a slight recovery of the FL intensity. In addition, a blue shift of the emission peak from 601 to 595 nm was observed with the increase of f_w from 0 to 90%. The SEM results showed that Indigoid-A formed uniform nanoparticles with an average diameter 42 ± 5 nm with ultrasonication treatment at $f_w = 80\%$ (Fig. 2.7a). When f_w reached 90% and 95%, respectively, lamellar microcrystals (Fig. 2.7b-c) formed in the dispersion in both cases, resulting in the moderate recovery of FL intensity.

Figure 2.6 (a) A digital photograph under the UV light (365 nm) irradiation of Indigoid-A (100 μM) in THF/water mixtures with ultrasonic treatment. (b) FL spectra of Indigoid-A (100 μM) in THF/water mixtures. Excitation wavelength: 540 nm. Solid lines: with

ultrasonication; dash lines: without ultrasonication. (c) FL relative intensity of Indigoid-A versus the water volume fraction in THF/water mixtures.

Figure 2.7 SEM images of Indigoid-A aggregates in THF/water mixture under ultrasonication treatment and with different water fractions: (a) 80%; (b) 90%; (c) 95%.

2.2.4 Mechanism of CIE-active behaviour

The very different CIE effect described above in Indigoid-B and Indigoid-A motivated us to understand the mechanism using DFT calculations. The restricted intramolecular motion (RIM) has been widely reported as a common feature for AIE or CIE chromophores.²²⁻²⁷ To evaluate the effect of torsional motion on the CIE effect observed in the synthesized Indigoids, DFT calculations were performed for single molecules of Indigoid-B and Indigoid-A, respectively. The calculations have been performed using the ab-initio total-energy and molecular-dynamics program VASP (Vienna ab-initio simulation program) developed at the Fakultät für Physik of the Universität Wien.^{28,29} Computational details are provided in the supporting information. As shown in Figure 2.8, the energy difference between the highest energy structure and the ground state for Indigoid-B is 213 kJ/mol. Nevertheless, the calculation shows existence of many torsional configurations in Indigoid-B. Indeed, the biggest energy gap between individual configurations in the energy profile for Indigoid-B is only 40 kJ/mol (from Structure B4 to Structure B5, Fig. 2.8). Such small energy barriers between different torsional configurations may allow torsional motions in dilute solutions (e.g. in THF), and enable nonradiative dissipation pathways of the exciton energy.³⁰ In addition, the twisting of the C=C bonds and the *trans-cis* isomerization may also contribute to the non-radiative relaxation of excited states.^{3,31} As a consequence, Indigoid-B is non-photoluminescent in THF.

In solid crystalline states, the dissipation of exciton energy in Indigoid-B through the configurational relaxation may not be feasible since the motion is restricted, and thus bright fluorescence was observed. More detailed analysis of the crystal packing diagram¹⁵ (Fig. 2.9) shows that Indigoid-B molecules stack in a parallel manner at a π -inter planar distance of 6.77 Å, resulting in no face-to-face π - π interaction (Fig. 2.9a). In addition, there are two intermolecular hydrogen (H)-bonds between adjacent Indigoid-B molecules to form the zig-zag linkage (H-bond distance is 2.45 Å) (Fig. 2.9b), which may increase the overall torsional energy cost and contribute to the CIE behavior.

In contrast to Indigoid-B, the calculated energy difference between the highest and the lowest energy torsional configurations of Indigoid-A is lower (104 kJ/mol), but there are fewer torsional degrees of freedom (Fig. 2.8). Moreover, the torsion of the acetyl group in Indigoid-A is more difficult than the torsion of the *t*-BOC group in Indigoid-B due to the stronger intramolecular H-bonding (H-bond distance is 2.24 Å compared to 2.35 Å in Indigoid-B) (Inset, Fig. 2.8). This type of H-bond may hinder the intramolecular twisting and enhances the radiative decay pathway,³² which may also account for fluorescence of Indigoid-A in THF. For Indigoid-A, the dramatic decrease of fluorescence intensity with the increase of water fraction in mixed THF/water solvent can be attributed to the aggregation caused quenching (ACQ) effect. In the crystalline state (from Cambridge Crystallographic Data Centre), “head-to-tail” planar stacking with a π -inter planar of 3.65 Å is realized for its mutually twisted halves³³ (Fig. 2.9c). The significantly stronger π - π interaction in Indigoid-A than that in Indigoid-B may result in the nonradiative relaxation of excited states and fluorescence quenching in aggregated states. On the other hand, a single intermolecular H-bond between two adjacent Indigoid-A molecules (H-bond distance is 2.52 Å) (Fig. 2.9d), the twisted configuration in crystalline state and the restriction by crystalline lattice may lead to partial reactivation of the radiative pathway in crystalline states, which is proved by the bluer emission of the microcrystals (formed at $f_w = 90$ -95% in THF/water mixture under the ultrasonic treatment) compared to that of the THF solution.

Figure 2.8 Torsional energy profile of Indigoid-B and Indigoid-A. Types of rotation are indicated by arrows. The configurations of A0 and B0 shown in the inset are the most stable structures of single molecule Indigoid-A and Indigoid-B, respectively. Enlarged configurations/structures are shown in the supporting information.

Figure 2.9 Single crystal structure of Indigoid-B: (a) No π - π interaction between continuous molecules of Indigoid-B in the crystalline structure¹. The distance between parallel π -planes is 6.77 Å; (b) The zig-zag intermolecular H-bonding between adjacent molecules of Indigoid-B in the crystalline structure. Each monomer links with two adjacent molecules via 4 H-bonds, with the bond length of 2.45 Å. Single crystal structure of Indigoid-A: (c) The “head-to-tail” π - π interaction between adjacent monomers of Indigoid-

A in the crystalline state (from Cambridge Crystallographic Data Centre). The distance between parallel π -planes is 3.65 Å; (d) The intermolecular H-bonding between adjacent molecules of Indigoid-A in the crystalline structure. Each monomer links with two adjacent molecules via 2 H-bonds, with bond length of 2.52 Å.

2.3 Conclusions

In conclusion, we have presented an indigo-based derivative (Indigoid-B) modified with *t*-BOC groups that shows unique crystallization-induced red emission. Ultrasonication played an important role to induce the crystallization of Indigoid-B in THF/water solvent mixtures, resulting in the formation of well-defined microcrystals with distinct morphologies and crystalline structures at different water contents. Replacement of the *t*-BOC groups with acetyl groups (i.e. Indigoid-A) resulted in dramatic change of the fluorescence properties in THF/water mixtures, i.e. strong emission in the non-aggregated state (e.g. in THF) and a moderate emission in the crystalline state. Our DFT calculation and the analysis of the single crystal structures of these two indigoid derivatives indicate that the CIE effect is related to factors including the torsional relaxation, the intra- and intermolecular hydrogen-bonding, and the packing diagrams in crystalline states. We believe that the facile synthesis and biodegradable nature of indigo derivatives with CIE characteristics will provide new insights for designing AIE-active molecules and will find applications in light-emitting devices, bioimaging, sensing, and so on.

2.4 Experimental Section

Indigo and other reagents were purchased from Aldrich and used without further purification.

Synthesis of Indigoid-B Typically, a mixture of indigo (1.05 g, 4 mmol) and 4-dimethylaminopyridine (DMAP, 0.76g, 6 mmol) were dissolved in 200 mL of CH₂Cl₂. Di-tert-butylidicarbonate ((*t*-Boc)₂O, 3.3g, 15mmol) was added dropwise in the solution with ice bath for cooling, followed by stirring at room temperature for 24 h. The reaction mixture was washed with water to remove excess (*t*-Boc)₂O and BOC-DMAP complexes and further dried over anhydrous MgSO₄. The crude product was obtained after removing solvent by reducing

pressure. It was recrystallized from EtOAc, yielding (80%) of a red crystalline product. ^1H NMR (300 MHz, CDCl_3), δ (ppm): 1.64 (s, 18H), 7.21(m, 2H), 7.61(m, 2H), 7.75(d, 2H), 8.01(d, 2H).

Synthesis of Indigoid-A Based on a modified Blanc's method,²¹ indigo (1.0 g, 4.35 mmol) and pyridine (5 mL) were dispersed in 100 mL ethyl acetate under stirring. Under the protection of nitrogen atmosphere, acetyl chloride (5 mL) was added dropwise into the solution. Then the reaction mixture was heated to 100 °C and reacted for 24 h. The reaction mixture was washed with water for three times and the organic phase was obtained. After concentration, the product was obtained via purification by silica column chromatography using dichloromethane/hexane=9/1 as elution solvent, yielding (18%) red powder. ^1H NMR (300 MHz, CDCl_3), δ (ppm): 2.58 (s, 6H), 7.28(m, 2H), 7.71(m, 2H), 7.80(d, 2H), 8.31(d, 2H).

Preparation of Indigoid aggregates (microcrystals) dispersion in THF/water with different ratios Indigoid-B or Indigoid-A (100 μL of 0.01 M CH_2Cl_2 solution) was dissolved in different amounts of THF (x mL), followed by the slow addition (22.5 mL/h by syringe pump) of a known volume of water ((10-x) mL) with or without treatment in an ultrasonic cleaner (SB-120 DTN, power: 120 W) bath. Through this typical process, we obtain a series of Indigoid-B and Indigoid-A dispersion with different water contents ($f_w = 0\%$, 10%, 20%, 30%, 40%, 50%, 60 %, 70 %, 80%, 90%, 95%).

DFT calculation Total energies of different torsional conformers of Indigoid-A and Indigoid-B were computed using the Perdew-Burke-Ernzerhof functional (DFT-PBE)³, as implemented in the Vienna Ab initio Simulation Package (VASP)^{4, 5}. The calculations were performed using a plane-wave basis with a cut-off kinetic energy of 450 eV. Electron-ion interactions were described by the projector-augmented wave (PAW) method^{6,7}. Indigoid-B and Indigoid-A was modeled in a unit cell of 20 x 20 x 20 Å, and the Brillouin zone was sampled by the Γ point.

2.5 Supporting information

Figure S2.1. ¹H NMR (300 MHz) spectrum of Indigoid-B in CDCl₃.

Figure S2.2. ¹H NMR (300 MHz) spectrum of Indigoid-A in CDCl₃.

Figure S2.3. SEM images of the octahedral microcrystals formed by Indigoid-B in THF/water mixture ($f_w=80\%$) with ultrasonication treatment: (a) high magnification; (b) low magnification.

Figure S2.4. Normalized UV-vis absorption (a) and FL emission (b) spectra of Indigoid-A (100 μM) in THF/water mixture with ultrasonication treatment. (Excitation wavelength: 540 nm).

Figure S2.5 Digital photographs of Indigoid-B and Indigoid-A in THF solution under UV light (365 nm) irradiation at different temperatures: (a) RT; (b) -196°C (liquid nitrogen).

Figure S2.6. Different torsional configurations of Indigoid-B, as shown in Figure 2.9 in Section 2.2. Inserted arrows indicate the types of torsion.

Figure S2.7. Different torsional configurations for Indigoid-A, as shown in Figure 2.9 in Section 2.2. Inserted arrows indicate the types of torsion.

2.6 References

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Chapter 3 Robust Colloidal Nanoparticles of Pyrrolopyrrole Cyanines J-Aggregates with Bright Near Infrared Fluorescence in Aqueous Media: From Spectral Tailoring to Bioimaging Application¹

Molecular packing structures play a key role in influencing the optical properties of fluorophores. As presented in **Chapter 2**, Indigoid-B is highly fluorescent in crystalline state but non-fluorescent in amorphous state or dilute solution. However, the bioimaging applications of Indigoid-B microcrystals are limited by the relatively short emission wavelength near 600 nm in crystalline state and crystal size at microscale level ($\sim 10\ \mu\text{m}$). These problems motivated the exploration of the optical properties of fluorophores with longer emission wavelength by controlling their molecular packing structures. Among the available near-infrared (NIR) dyes, pyrrolopyrrole cyanines (PPcy) originally reported by Fischer and his coworkers showed the emission in the NIR region up to 1000 nm and highest quantum yield up to 70% in organic solvents.²⁻⁶ The optical properties of PPcy in colloidal nanoparticles were investigated to determine whether they could be possibly induced by the formation of different aggregated states such as dimeric-, H- and J-aggregates.

3.1 Introduction

Since their first discovery in 1936 by Jelley and Scheibe,⁷⁻¹⁰ J-aggregates (also called Scheibe aggregates), which exhibit strongly red-shifted light absorption and a nearly resonant fluorescence, have attracted considerable attention for various applications.¹¹⁻¹⁴ Such a resonant fluorescence band of J-aggregates with quite narrow bandwidth is highly desirable for simultaneous detection of multiple targets to minimize the spectral overlapping and interference. However, the bioimaging application of existing J-aggregates of dyes is limited by relatively low FL quantum yield, short emission wavelength and poor solubility in aqueous media. For instance, J-aggregates of ICG associated by van der Waals forces in water have been observed.^{13,15} But the colloidal stability of these aggregates without stabilization by surfactants was susceptible to changes of environmental factors such as concentration, ionic

strength, and temperature. Würthner and coworkers¹⁶ reported the J-type aggregation of H-bonding perylene bisimide derivatives with far-red fluorescence and high quantum yield, close to unity. But such self-assembly took place in nonpolar solvents which are not suitable for bioimaging.

Scheme 3.1 Schematic diagram of encapsulation of the PPcy derivatives into H-type and J-type aggregated nanoparticles (HAGG and JAGG) including: (i) molecular structures of **PC**, **PCBF**, **PCBFB** and amphiphilic block copolymers PCL-*b*-POEGMA; (ii) schematic representation of an aggregate and PCL-*b*-POEGMA; (iii) arrangement of PPcy derivatives in H and J aggregates.

In this chapter, we present colloidal NPs of J-aggregates (denoted as **JAGG**) in water with bright NIR fluorescence and narrow bandwidth for bioimaging applications. The key component of the **JAGG** is a brightly fluorescent molecule, **PCBF** (as shown in Scheme 3.1), a PPcy derivative. To render the hydrophobic dye **PCBF** dispersible in water, a synthetic amphiphilic block copolymer PCL-*b*-POEGMA ($M_n = 31,100$; $M_w/M_n = 1.37$, as shown in the supporting information) was employed to encapsulate **PCBF** into the hydrophobic core of NPs via a process of nanoprecipitation as shown in Scheme 3.1.¹⁷ Both H-aggregation and J-aggregation of **PCBF** occur in the corresponding NPs. These NPs exhibited bright NIR fluorescence, which is

mainly contributed by J-aggregating components, and robust photostability that is important for bioimaging application.

3.2 Results and discussion

3.2.1 Synthesis and optical properties of PCBF

The synthetic route to **PCBF** is illustrated in Scheme 3.2, using a modified method reported by Fischer and coworkers.² 3,6-Bis(4-octyldodecyloxyphenyl)-2,5-dihydropyrrolo [3.4-c]pyrrole-1,4-dione (Compound **3**, Scheme 3.2) was prepared by using a condensation of diisopropyl succinate 4-octyldodecyloxybenzonitrile in the solution of potassium tert-butoxide and 2-methyl-2-butanol, where octyldodecyl branch group can provide DPP with a relatively good solubility. Subsequently, condensations of C-H acidic methylene groups from 2-cyanomethylpyridine at the carbonyl group of **3** proceeded in toluene with an excess of phosphoryl chloride to give compound **PC**, respectively. In order to stiffen the chromophores to activate NIR emission, trifluoroborane etherate was added to the dichloromethane solution of **PC** at the presence of Huning base to give **PCBF** with a quantitative yield.

Scheme 3.2 Synthetic route to Compound **PC**, **PCBF** and **PCFBF**.

The UV-Vis-NIR absorption and FL emission spectra of **PCBF** in CHCl_3 (Figure 3.1a) show all the intrinsic features of PPcy. The absorption maximum

of monomeric **PCBF** appears at 700 nm (absorption coefficient: $\epsilon = 1.55 \times 10^5 \text{ cm}^{-1}\text{M}^{-1}$, even higher than that of porphyrin¹⁸) while the FL emission spectrum exhibits a maximum at 715 nm. Thus, **PCBF** in CHCl_3 shows a Stokes shift of 15 nm and a quite high FL quantum yield of 0.69 using ICG in water (QY~0.01) as a standard. The emission band is relatively broader with full-width-at-half-maximum (FWHM) value of 6910 cm^{-1} than that of the absorption band (3849 cm^{-1}) (Fig. 3.1a). The optical properties of **PCBF** observed here are consistent with what was reported by Fischer and coworkers.²⁻⁶

Figure 3.1 (a) UV-Vis-NIR absorption and FL emission spectra of **PCBF** in chloroform. (b) UV-Vis-NIR absorption and FL emission spectra of **PCBF** based **JAGG** NPs with the concentration of 0.4 mM in aqueous media. (c) TEM image of **JAGG** NPs of **PCBF** with the concentration of 0.4 mM in aqueous media; (d) DLS result shows the size distribution of **JAGG** NPs of **PCBF** in aqueous media.

3.2.2 Preparation and optical properties of **JAGG** NPs

To render **PCBF** dispersible in aqueous media, we synthesized an amphiphilic block copolymer with PCL as a biodegradable and hydrophobic block and poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA) as a hydrophilic block. POEGMA as a well-known biocompatible polymer has

been widely used for pharmaceutical and medical applications.¹⁹ As shown in Scheme 3.3, PCL was first prepared by ROP of ϵ -caprolactone using isopropanol as an initiator. The terminal OH groups further reacted with 2-bromoisobutyryl bromide (BIB) to enable atom transfer radical polymerization (ATRP) of OEGMA monomers to proceed. The details about the synthesis and characterization by NMR and GPC are presented in Supporting Information.

In order to prepare colloidal NPs of **PCBF** in water, a mixture of **PCBF** and PCL-*b*-POEGMA codissolved in THF was rapidly injected into large excess water under ultrasonication, followed by evaporation of the organic solvent, resulting in colloidally stable NPs. In this work, a dispersion of NPs based on **PCBF** was obtained at a weight ratio of **PCBF**/PCL-*b*-POEGMA at 3.5 and a concentration of **PCBF** at 0.4 mM. As shown in Figure 3.1c, roseberry-like spherical NPs with an average diameter of 30.0 ± 2.5 nm were observed in the transmission electron microscopy (TEM) image of **JAGG** NPs (Fig. 3.1c). Beneficial from the higher electron density of **PCBF** than the PCL-*b*-POEGMA matrices, dye aggregation inside the NPs gave a clear contrast of **PCBF** aggregates as darker dots in the grey matrices of the polymeric NPs. The hydrodynamic diameter of **JAGG** NPs measured by dynamic light scattering (DLS) is 47.5 nm (Fig. 3.1d), which is larger than that from TEM results due to the hydration of POEGMA corona.

In striking contrast to the optical absorption of monomeric **PCBF** in CHCl_3 , the **JAGG** NPs in water exhibit two new absorption bands as shown in Figure 3.1b. The blue-shifted band with respect to the monomeric band (M-band) appears as a typical signal of the formation of H-type aggregation.²⁰ The maximum of the H-aggregate absorption band (H-band) is centered at 647 nm. As compared to the M-band, another narrow and intense band in the absorption transition shows a 61nm red-shift to 768 nm (Fig. 3.1b), which is a spectral signature of ordered J-aggregates. Furthermore, the FWHM of J-band is narrower (2235 cm^{-1}) than that of **PCBF** in CHCl_3 (3849 cm^{-1}). This spectral-narrowing phenomenon is a known property of coherently coupled J-aggregates. It has been predicted that the absorption spectral width of the aggregate is narrower than that of the monomer by a factor $N^{1/2}$, where N is the

number of monomers in direct communication with each other.²¹ The average J-aggregation number N calculated from the spectral narrowing of **JAGG** NPs is 7.4.

Figure 3.2 (a) UV-Vis-NIR absorption spectra of **PCBF** (0.033 mg/mL) in THF/water mixture without surfactant; (b) DLS data of **PCBF** in THF/water mixture with a water fraction of 50% (by volume).

A control experiment was also conducted to monitor the formation of aggregates of **PCBF** by varying the volume ratio of water/THF mixture in the absence of PCL-*b*-POEGMA. As shown in Figure 3.2a, with the gradual increase water fraction (f_w , by volume) from 0 to 40%, there is no significant change in the absorption spectra. Further increase of f_w to 50% led to the appearance of the J-band, accompanied with a transition from transparent solution to suspension of aggregates which precipitated after standing overnight, implying the poor colloidal stability in the absence of the polymeric surfactant. The DLS result shows an average diameter of 10 μm (Fig. 3.2b). When f_w was increased to 60%, the J-band became stronger and sharper. Obviously, the absence of H-band in the absorption spectra indicates the preferable formation of J-aggregation of **PCBF**. The presence of high fraction of good solvent THF in the mixture enables **PCBF** molecules to slowly self-assemble to reach the more thermodynamically stable state, that is J-aggregation. In contrast, the aforementioned **JAGG** NPs were prepared by a rapid injection of a small amount of **PCBF**/THF solution (1 mL) into water (10 mL), which didn't permit all the **PCBF** molecules to form J-aggregation spontaneously. This leads to the mixture of H-aggregates and J-aggregates in **JAGG** NPs, which were instantly frozen in the nanoprecipitation process.

The FL emission spectrum of **JAGG** NPs dispersion shows a characteristically small Stokes shift of 5 nm, a $\lambda_{\text{max,em}}$ of 773 nm and a smaller FWHM of 2114 cm^{-1} than that of **PCBF** in chloroform (6910 cm^{-1}) as shown in Figure 3.1b, which provides further evidence of J-aggregate formation. Evidently, the emission of monomeric molecules was not observed in Figure 3.1b. It can be explained by the intrinsic Föster resonance energy transfer (FRET) from monomer to J-aggregates in confined space due to the well overlapping between emission band of monomer (Fig. 3.1a) and absorption band of J-aggregates (Fig. 3.1b). The excitation spectrum (Fig. S3.4) of **JAGG** dispersion at the emission wavelength of 773 nm shows emission maxima at the excitation wavelength of 769 nm, with an intensity approximately 4 times higher than that excited at 650 nm. This result suggests that the J-aggregates are the most active component that contributes to the fluorescence. While the calculated FL quantum yield (0.012) of **JAGG** NPs excited at 650 nm in water is much lower than that (0.69) of **PCBF** in CHCl_3 , it is comparable with those of water-soluble PPcy derivatives (0.01)⁴ and ICG (0.01)²² in water. It should be noted that the actual FL quantum yield of **JAGG** is underestimated here, given the fact that only a small fraction of light at 650 nm (i.e. the excitation wavelength) is absorbed by the J-aggregates. Due to the small Stokes shift (5 nm) of the emission band versus the absorption band of the J-aggregates, it is difficult to estimate the FL quantum yield of **JAGG** when the J-absorption band is excited. Nevertheless, the effect of the excitation wavelength on the FL intensity of the J-aggregates is reflected from the FL excitation spectrum shown in Figure S3.4.

The photostability of **JAGG** NPs under continuous irradiation with a 770 nm laser (power density $\sim 0.22 \text{ W/cm}^2$) was investigated using ICG in water as the control, where the absorbance at 770 nm of **JAGG** and ICG is close to 1.4. With the increase of irradiation time, the maximal absorbance and FL emission intensity of ICG aqueous solution decreased quickly, accompanied by the color change of ICG solution from green to colorless (Fig. 3.3a & 3.3b). In contrast, only negligible decrease of absorbance could be observed in **JAGG** solution under the same condition of irradiation (Fig. 3.3c). After 55 min of continuous irradiation, the decrease of the FL intensity at 771 nm of **JAGG** solution was

~12% (Fig. 3.3d), much lower than that (97%) at 805 nm of ICG solution, indicating relatively high photostability of **JAGG** NPs. The excellent photostability could be attributed to the intrinsic structural stability of PPcy² and its isolation from unfavorable influences of the environment.²³⁻²⁵ Hence, these **JAGG** NPs with such a large size are preferable for long-term tracking due to the increase of circulation half-life and excellent photostability compared to water-soluble dyes.^{25,26}

Figure 3.3 UV-Vis-NIR absorption spectra (a) and FL emission spectra (b) of ICG solution in water under continuous irradiation by a 770 nm laser for ~1h at a power density of 0.22 W/cm²; UV-Vis-NIR absorption spectra (c) and FL emission spectra (d) of **PCBF** based **JAGG** dispersion under continuous irradiation by a 770 nm laser for ~1h at a power

density of 0.22 W/cm²; (e) NIR absorbance ratio A/A_0 of ICG solution and **JAGG** dispersion plotted as a function of irradiation time. A_0 is the initial absorption maximum and A is the absorption maximum of the sample at different time points after illumination. (f) NIR fluorescence intensity ratio A/A_0 of ICG solution and **JAGG** dispersion plotted as a function of irradiation time. F_0 is the initial fluorescence maximum and F is the fluorescence maximum of the sample at different time points after illumination.

3.2.3 Concentration effect of JAGG NPs

Figure 3.4 Concentration-dependent absorption spectra (a) and FL emission spectra (b) of **PCBF** based **JAGG** NPs upon the encapsulation of PCL-*b*-POEGMA. (c) Concentration-dependent normalized absorption spectra and zoom-in J-band variation of **PCBF** based **JAGG** NPs. C_{PCBF} corresponds to concentration of **PCBF**.

The effect of the concentration of **PCBF** and the weight ratio of **PCBF**/PCL-*b*-POEGMA on the spectral characteristics were studied. We gradually decreased the feeding amount of **PCBF** when the amount of surfactant was kept constant. The concentration-dependent UV-Vis-NIR spectroscopy revealed that the J-aggregation in **JAGG** NPs was enhanced with the increase of the concentration of **PCBF** from 0.04 to 0.4 mM, accompanied by a gradually sharpening J-band and increasing ratio between J-band and H-band absorbance

(Fig. 3.4a & 3.4c). This result, as expected, indicates that formation of the J-aggregate was favoured by increased concentration of **PCBF**. With the increase of J-aggregate proportion, a slight 4 nm red-shift in the absorption from 764 to 768 nm was observed (Fig. 3.4c). A shoulder peak at longer wavelength can be found in the J-aggregate absorption band of the **JAGG** dispersions at 0.04, 0.08 and 0.16 mM, respectively. Interestingly, the J-band in the absorption spectrum of the NPs at 0.24 mM exhibits two overlapping peaks at 766 and 771 nm, respectively (Fig. 3.4c). This is a clear indication of the two overlapping bands of J-aggregation presumably due to two populations of J-aggregates with different geometrical configurations.^{27,28} Evidently, with the increase of **PCBF** concentrations from 0.04 to 0.4 mM, the intensity of the emission band with $\lambda_{\text{max,em}}$ at 773 nm gradually increased and the bandwidth turned narrower (Fig. 3.4b), which is attributed to the increasing proportion of J-aggregation and configuration corresponding to the longer absorption wavelength.

3.2.4 Comparison to PC and PCBFB

We envisioned that such unusual optical property of the J-aggregate of **PCBF** was encoded in its molecular structure: a planar conjugated core stiffened by difluoroborane group. To confirm this presumption, **PC**, the precursor of **PCBF**, was encapsulated by PCL-*b*-POEGMA to give colloidal dispersion that could be used as a control. In contrast to **PCBF**, the absorption spectra of **PC** dispersion (Fig. 3.5c) with 0.4 mM exhibited two peaks at 648 and 705 nm, which could be typically assigned to the H- and M-band, respectively. The same optical feature was observed at different concentrations of **PC** (Fig. 3.5c), suggesting no formation of J-aggregates. Such dramatically different aggregation behaviours between **PC** and **PCBF** may be rationalized by the more rigid and planar aromatic core and the resonance structure of a merocyanine in the latter, which favors the formation of J-aggregates.

To further understand how the structural details determine the aggregating state, **PCBFB** extended with two benzene rings was synthesized (Scheme 3.2). In contrast to **PCBF**, **PCBFB** showed a higher $\lambda_{\text{max,abs}}$ of 729 nm and $\lambda_{\text{max,em}}$ of 743 nm due to the extension of conjugation (Fig. 3.5b). A high FL quantum yield (0.56) of **PCBFB** in CHCl₃ was also achieved. After encapsulation with

PCL-*b*-POEGMA in water, the absorption spectra of **PCBFB** NPs (0.4 mM) exhibited the typical feature of H-aggregation (Fig. 3.5d). **PCBFB** preferentially formed sandwich-type H-aggregates with significantly quenched fluorescence (quantum yield of 0.0011). The twist between the benzene ring and the PPcy core in **PCBFB** may reduce the planarity of the aromatic component as compared to **PCBF**. These results imply that the planarity and rigidity of the aromatic cores in **PCBF** could possibly facilitate the slipped π - π stacking, and thus the formation of J-aggregates. Nevertheless, a clear picture how **PCBF** molecules stack inside the colloidal nanoparticles remains unclear, and warrants further experimental study, for example, of the crystalline structures of PPcy derivatives and some simulation study in the future.

Figure 3.5 (a) UV-Vis-NIR absorption and FL emission spectra of **PC** in chloroform. (b) UV-Vis-NIR absorption and FL emission spectra of **PCBFB** in chloroform. Concentration-dependent absorption spectra of (c) **PC** and (d) **PCBFB** based **HAGG** NPs upon the encapsulation of PCL-*b*-POEGMA. C_{PC} and C_{PCBFB} correspond to concentration of **PC** and **PCBFB** in stock solutions. (All the samples were diluted by eight folds before test)

Table 3.1 Fluorescence quantum yields of PPcy derivatives solution and NPs dispersions compared to ICG in water.

Sample	PCBF (CF) ^b	PCBFB (CF)	JAGG (0.04 mM)	JAGG (0.4 mM)	HAGG	JAGG (sonication for 200 min)
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QY (%)^a	69.3	56.0	1.07	1.19	0.11	2.50
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Note: ^a Quantum yield of ICG in water has been reported before²² and ICG was used as a reference standard for quantum yield measurement; ^b CF stands for chloroform.

3.2.5 Ultrasonication effect of JAGG NPs

The optical properties of several kinds of J-aggregates based on supramolecular assembly have been reported to be tunable by changing the environmental temperature.^{16,29,30} In contrast, as shown in Figure S3.10, only a slight change around the J-band was observed in the UV-Vis-NIR absorption spectra of **JAGG** dispersion (0.4 mM) with the temperature was gradually increased from 25 to 60 °C.

Figure 3.6 The evolution of UV-Vis-NIR spectra (a) and FL emission spectra (b) of **PCBF**-based **JAGG** NPs (diluted by eight folds from 0.4 mM stock solution with water) after ultrasonication over different periods; arrows indicate the spectroscopic changes with increasing ultrasonication time. (c) UV-Vis-NIR absorption and FL emission spectra of **PCBF** based **JAGG** NPs after sonication for 200 mins. (d) TEM image of **PCBF** based **JAGG** NPs after sonication for 200 mins.

In Chapter 2, we reported that ultrasonication played a role to induce the colloidal crystallization of an indigo derivative.³¹ Here, the colloidal NPs of **PCBF** were also treated with ultrasonication. Upon the ultrasonic treatment for

200 mins, the absorbance of the J-band at 774 nm gradually increased, accompanied with the decrease of the H-band absorbance (Fig. 3.6a). The change can be observed with naked eyes, as the bluish solution of **JAGG** NPs turned green upon ultrasonication (Fig. 3.6a). These results clearly indicate the transition from H-aggregate to J-aggregate.¹⁵ Moreover, the J-band in the absorption spectra exhibits a red-shift of 6 nm (from 768 to 774 nm) upon ultrasonication for 200 mins while the FL emission spectrum shows a $\lambda_{\text{max,em}}$ of 774 nm and negligible Stokes shift (Fig. 3.6b & 3.6c). The FL quantum yield of **JAGG** NPs after ultrasonication for 200 mins was increased to 0.025, consistent with the increase of J-aggregate population. It is noted that there was no obvious change of the morphology and the size of **JAGG** after the ultrasonication (Fig. 3.6d & Fig. S3.11).

3.2.6 Biocompatibility and *In vivo* imaging of **JAGG** NPs

The optical properties of **JAGG** NPs described above, meeting most requirements of a FL imaging probe, encouraged us to examine their potential for *in vivo* imaging. Firstly, the cytotoxicity of **JAGG** NPs against mouse fibroblast cells (L929) was studied by PrestoBlue assays. As summarized in Figure 3.7a, there was no obvious change of the metabolic viability of L929 cells after incubation with **JAGG** for 72 h at a series of concentrations of 0.01, 0.02, 0.05, 0.1, 0.2 mM, respectively, indicating the low cytotoxicity of **JAGG** NPs. In *in vivo* experiment, subcutaneous and intramuscular injections of **JAGG** dispersions (50 μL aliquots, 1 mg/mL) into the left flank (position II in Fig. 3.7b) and left leg (position III in Fig. 3.7b) of a nude mouse, respectively, were administered. The mouse was imaged in a fluorescence mode (excitation filter, 605-640 nm, and emission filter, 740-800 nm). The FL signals collected from both injected regions were clearly observed and the FL intensity emanating from deeper tissue (ROI (III) = 9.856×10^{10} , ROI stands for regions of interest) is comparable with that of the near-skin fluorescence (ROI (I) = 1.029×10^9). As a control, the **HAGG** dispersion of **PCBFB** was subcutaneously and intramuscularly injected to right flank (position IV) and right leg (position V), respectively (Fig. 3.7b). Obviously, the weak fluorescence signals (ROI (IV) = 5.340×10^8 ; ROI (V) = 5.492×10^8) observed here were only slightly

stronger than that of the blank (ROI (I) = 1.571×10^8), due to the quite low FL quantum yield of the **PCBFB** NPs.

3.3 Conclusions

In summary, we have presented a novel type of colloidal NPs based on the J-aggregation of PPcy dyes stabilized with polymeric surfactant in water. The packing configuration of PPcy in NPs is determined by factors including the chemical structure, the concentration and the ultrasonication treatment. The outstanding spectroscopic properties of **JAGG** NPs, such as large light-absorption coefficient, relatively high FL quantum yield, as well as narrow emission band, enable them to be promising candidates for *in vivo* imaging in the near infrared range. These **JAGG** NPs may also be useful for multimodal imaging (fluorescence and photoacoustic)³² and therapeutic (photothermal therapy) applications.^{33,34}

Figure 3.7 (a) Metabolic viability of L929 cancer cells after incubation with **JAGG** NPs at different concentrations. (b) In vivo fluorescence image of **JAGG** and **HAGG** NPs (50 μ L of 1 mg/mL) injected subcutaneously and intramuscularly on each flank of a mouse. Position I: blank control; II: **JAGG** NPs subcutaneous; III: **JAGG** NPs intramuscular; IV: **HAGG** NPs subcutaneous; V: **HAGG** NPs intramuscular.

3.4 Experimental Section

Materials

10×phosphate buffer saline (PBS) buffer with pH = 7.4 (ultrapure grade) is a commercial product of 1st BASE Singapore. MilliQ water (18.2 MQ) was used to prepare the buffer solution from the 10×PBS stock buffer. 1×PBS consists of NaCl (137 mM), KCl (2.7 mM), Na₂HPO₄ (10 mM) and KH₂PO₄ (1.8 mM). Chloroform-D (99%) was purchased from Cambridge Isotope Laboratories, Inc. All other chemicals and reagents were purchased from Aldrich or Merck and used as received unless specified. Copper(I) bromide was purified by washing with acetic acid and methanol three times, respectively, then stored in a glove box. Olig(ethylene glycol) methyl ether methacrylate (OEGMA, M_n = 500 g/mol) was passed through a column of activated basic alumina to remove inhibitors before use.

Characterization

The samples were dissolved with chloroform-d for ¹H NMR and ¹³C NMR measurements on a Bruker AV300 MHz NMR spectrometer. Emission spectra of solutions were measured by fluorescence spectrophotometry on a Horiba Fluolog 3 spectrofluorometer at 25 °C. The fluorescence quantum yield of these materials were measured using indocyanine green (ICG) solution in water as the standard ($\Phi_F = 0.01$). UV-Vis-NIR spectra of the samples were measured on a SHIMADZU UV-2450 spectrophotometer. The photostability of ICG solution and **JAGG** dispersion subjected to the continuous irradiation by a 770 nm laser (power density ~ 0.22 W/cm²) over different periods was monitored by the UV-Vis spectrometer and fluorometer. TEM measurements were performed with a TEM Carl Zeiss Libra 120 Plus at an acceleration voltage of 120 kV. A 5 μ L droplet of diluted samples was directly dropped onto a copper grid (300 mesh) coated with a carbon film, followed by drying at room temperature. The size distribution of resulting nanoparticles was determined by dynamic light scattering (DLS) using a BI-200SM (Brookhaven, USA) with angle detection at 90°. Ultrasonic treatment of **JAGG** dispersion was performed in an ultrasonic cleaner (SB-120 DTN, power: 120 W) bath. Temperature-dependent variation on absorption spectra of **JAGG** dispersions was monitored by the UV-Vis spectrometer from 25 to 60 °C. Each temperature point was kept for 1 h to treat the sample.

Synthesis of Compound 1

Triphenylphosphine (26 g, 0.1 mol) was dissolved in dichloromethane (100 mL). Then, bromine (16 g, 0.1 mol) was added dropwise at 0 °C. After completion of bromine addition, the reaction mixture was stirred half an hour at room temperature. 2-Octyl-1-dodecanol (30 g, 0.1 mol) was added slowly. The reaction mixture was stirred at room temperature overnight. Then the reaction mixture was poured into water (200 mL) and extracted with dichloromethane (3 × 50 mL). The extracts were combined and washed with water and saturated sodium bicarbonate solution then dried over anhydrous magnesium sulfate. After filtration, the filtrate was concentrated to 100 mL under reduced pressure. Petroleum ether (200 mL) was added to the mixture and stirred. The resulting triphenylphosphine oxide precipitate was filtered off and the filtrate was concentrated to 100 mL. Added another 100-mL petroleum ether and filtered. This operation was repeated till no precipitate was observed, and the filtrate was evaporated to dryness. The product 2-octyl-1-dodecyl bromide was obtained in a yield of 92% and used in subsequent reactions without further purification.

Synthesis of Compound 2

4-Cyanophenol (1.19 g, 10 mmol) and potassium carbonate (5.52 g, 40 mmol) were dissolved in 60 mL acetone. After being purged for 15 min, the mixture was heated to 100 °C for half an hour. Then, 2-octyl-1-dodecyl bromide (4 g, 11 mmol) was added to the mixture by syringe. The reaction mixture was stirred at 100 °C for 30 h. After cooled to room temperature, the reaction mixture was added to 100 mL of water and extracted with ethyl acetate. The extracts were combined and washed with water before being dried over anhydrous magnesium sulfate. The crude product was purified by flash chromatography (petroleum ether/dichloromethane from 10: 0 to 10:1) to yield target product as a light yellow viscous liquid in a yield of 90%. ¹H NMR (300 MHz, CDCl₃): 7.58 (2H, d), 6.97 (2H, d), 3.88 (2H, d), 1.81 (1H, m), 1.25-1.50 (32H, broad), 0.90 (6H, t).

Synthesis of Compound 3

Potassium tert-butoxide (1.68 g, 15 mmol) was added to 2-methyl-2-butanol (15 mL), and the mixture was heated to reflux. When the alkali was dissolved, Compound **2** (4 g, 10 mmol) was added in one portion. Then diisopropyl succinate (1.01 g, 5 mmol) was added over three hours with a dropping funnel. After heating for another three hours at 110 °C, the mixture was cooled down and slowly added to a mixture of 100 mL ethanol (containing 2 mL concentrated hydrochloric acid). The red precipitate is filtered and washed with ethanol. The solid was digested in boiling ethanol, filtered and washed with ethanol. This procedure was repeated until the filtrate was clear. Drying in vacuum yielded 2.1 g (24 %) orange solid. ¹H NMR (300 MHz, CDCl₃): 8.42 (2H, s), 8.26 (4H, d), 7.08 (4H, d), 3.96 (4H, d), 1.84 (2H, m), 1.25-1.50 (64H, broad), 0.90 (12H, m).

Synthesis of Compound **4**

Compound **3** (0.88 g, 1 mmol) and 2-cyanomethylpyridine or 2-cyanomethylpyridine (0.295 g, 2.5 mmol) were heated to reflux in absolute toluene (20 mL) under nitrogen. Phosphoryl chloride (0.75 mL, 8 mmol) was then added. The reaction was monitored by thin-layer chromatography. As soon as Compound **3** was used up, the reaction mixture was cooled down, quenched by water and washed with a saturated sodium bicarbonate solution. Water was separated and extracted with chloroform. The combined organic layer was dried over anhydrous sodium sulfate. After filtration, the volatiles were removed under reduced pressure. The crude product was purified by column chromatography on silica, eluting with dichloromethane/petroleum ether (from 1:5 to 1:1) to give Compound **4** as a bluish green solid (173 mg, 16%). ¹H NMR (300 MHz, CDCl₃): 13.6 (2H, s), 8.45 (2H, d), 7.65 (8H, m), 7.12 (4H, d), 7.01 (2H, t), 3.97 (4H, d), 1.84 (2H, m), 1.25-1.50 (64H, broad), 0.89 (12H, m).

Synthesis of Compound **5**

Compound **4** (25 mg, 0.02 mmol) and N,N-diisopropylethylamine (0.2 mL, 1.2 mmol) were dissolved in dichloromethane (2 mL). Trifluoroborane etherate (0.2 mL, 1.6 mmol) was added and the mixture was stirred at room temperature for 2 hours. The reaction mixture was washed with water and dried over anhydrous

sodium sulfate. After removing the solvent, the crude product was purified by column chromatography, eluting with dichloromethane to give Compound 5 as a green solid (26 mg, quantitative yield). ¹H NMR (300 MHz, CDCl₃): 8.36 (2H, d), 7.88 (2H, t), 7.65 (6H, m), 7.22 (2H, t), 7.08 (4H, d), 3.95 (4H, d), 1.83 (2H, m), 1.25-1.50 (64H, broad), 0.91 (12H, m).

Synthesis of Compound 7

5-Bromo-2-cyanomethylpyridine was used to replace 2-cyanomethylpyridine as the raw material. The procedure to synthesize Compound 8 was similar to that of Compound 2 and the similar yield was achieved. ¹H NMR (300 MHz, CDCl₃): 8.43 (2H, d), 7.91 (2H, d), 7.64 (4H, d), 7.55 (2H, d), 7.07 (4H, d), 3.95 (4H, d), 1.83 (2H, m), 1.25-1.50 (64H, broad), 0.91 (12H, m).

Synthesis of Compound 8

Compound 8 (133.5 mg, 0.1 mmol), phenylboronic acid (36.6 mg, 0.3 mmol) and K₂CO₃ (1.38g) was dissolved in degassed mixture of toluene (10 mL), water (2 mL) and ethanol (2 mL) under nitrogen atmosphere. It was followed by the addition of a catalytic amount (1% m/m) of Pd(PPh₃)₄. The resulting mixture was heated at 100 °C for 6 h, then cooled down to room temperature. The organic layer was washed by water for three times and concentrated under reduced pressure. The product was purified by column chromatography on silica using dichloromethane/hexane (1/1) as the solvent and dried under vacuum at 80 °C overnight. Finally, we obtained 121.4 mg dark green powder (yield 91.3%). ¹H NMR (300 MHz, CDCl₃): 8.61 (2H, s), 8.09 (2H, d), 7.70 (6H, m), 7.49 (10H, m), 7.10 (4H, d), 3.96 (4H, d), 1.84 (2H, m), 1.25-1.50 (64H, broad), 0.90, (12H, m).

Synthesis of PCL₃₇-*b*-POEGMA₆₀

Scheme 3.3 Synthetic route to PCL₃₇-*b*-POEGMA₆₀ block copolymer.

Synthesis of Polymer 9 PCL was synthesized via ring opening polymerization of ϵ -caprolactone. A mixture of isopropyl alcohol (3 mmol, 0.292 g), ϵ -caprolactone (67.5 mmol, 8.456 g), SnOct₂ (3 mg) was placed in a Schlenk tube and stirred for 24 h at 110 °C under N₂ atmosphere. For purification of PCL, bulk PCL was first dissolved in a small amount of CH₂Cl₂ and then precipitated in cold diethyl ether. The precipitated PCL was then dried in vacuum at 50 °C.

Synthesis of Polymer 10 PCL (5 g, $M_{n,NMR}=3,500$ g/mol), TEA (0.244 mL, 1.75 mmol) and DMAP (0.964 g, 2.63 mmol) were added in a three-neck round bottom flask. THF (50 mL) was added to the flask via a syringe after purging with N₂ gas, followed by cooling to 0 °C. BIB (0.542 mL, 4.39 mmol) was then added via a micro syringe and the temperature of the flask was slowly raised to room temperature. The reaction was continued for 24 h under stirring. The solution was filtered and precipitated in cold diethyl ether. PCL macroinitiator was obtained after drying in vacuum at 50 °C.

Synthesis of Polymer 11 Macroinitiator PCL-Br (0.25 g), oligo(ethylene glycol) methyl ether methacrylate (OEGMA) (1.25 g, 2.5 mmol), CuBr (7 mg, 0.05 mmol), PMDETA (9 mg, 0.05 mmol) and 2 mL anisole were added to a Schlenk tube in the glove box under N₂ atmosphere. After three freeze-and-thaw cycles for removal of oxygen, the tube was then placed in an oil bath thermostated at 65 °C for 12 h to allow atom transfer radical polymerization (ATRP). A solution of the crude product was passed through an alumina column to remove the catalyst and precipitated in diethyl ether. The final polymer was then dried in vacuum at 50 °C and obtained as a waxy solid (yield: 0.92 g, 61.3%).

Preparation of nanoparticles in the presence of surfactant as stabilizer

Taking **JAGG** NPs with 0.4 mM as representative example, **PCBF** (200 $\mu\text{L} \times 0.01 \text{ M}$ THF solution) and amphiphilic copolymer PCL-*b*-POEGMA (100 $\mu\text{L} \times 6.67 \text{ g/L}$ THF solution) were mixed in 200 μL of THF. This mixture was rapidly injected into 5 mL of deionized water under ultrasonication. THF was evaporated by exposure in open air overnight. Similarly, **JAGG** dispersions with concentration of 0.24, 0.16, 0.08 and 0.04 mM were prepared by using 120, 80, 40 and 20 μL of 0.01M THF solution of **PCBF**, respectively, while the amount of PCL-*b*-POEGMA was kept unchanged. **HAGG** dispersions with various concentrations were obtained by the similar procedure.

Monitoring the aggregation of PCBF in mixture of THF/water in the absence of surfactant as stabilizer

PCBF (100 μL of 1 mg/mL THF solution) was mixed with different amounts of THF ($x \text{ mL}$), followed by the direct addition of a known volume of water ($(3-x) \text{ mL}$) with any treatment. Through this typical process, we obtain a series of **PCBF** dispersion with different water fractions ($f_w = 0\%, 10\%, 20\%, 30\%, 40\%, 50\%, 60\%$).

Cell culture and cytotoxicity study

L929 mouse fibroblast cells were cultured in folate-free Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified environment containing 5% CO_2 . Before experiments, the cells were precultured until confluence was reached. The cytotoxicity of **JAGG** NPs against L929 cancer cells was evaluated by PrestoBlue (PB) assay. Briefly, L929 cells were seeded in 96-well plates (Costar, IL, USA) at an intensity of $5 \times 10^4 \text{ cells mL}^{-1}$. After 12 h incubation, the cells were exposed to a series of doses of **JAGG** NPs at 37 °C. After the designated time intervals, the wells were washed twice with $1 \times \text{PBS}$ buffer and 100 μL of freshly prepared PB solution in culture medium was added into each well. The PB medium solution was carefully removed after 1 h incubation in the incubator. The absorbance of PB at 570 nm and 600 nm was monitored by the microplate reader (Genios Tecan). Cell viability was

expressed as the ratio of the percent PB reduction of the cells incubated with **JAGG** NPs suspension to that of the cells incubated with culture medium only.

***In vivo* fluorescence imaging**

The care and use of laboratory animals were performed according to the approved protocols of the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore. 50 μ L of **JAGG** solution of **PCBF** and **HAGG** solution of **PCBFB** (1 mg/ml) were subcutaneously injected to the left or right side of flanks of 8-week-old NCr nude mice (Invivos Pte Ltd), respectively. Next, 50 μ L of the same **JAGG** dispersion of **PCBF** and **HAGG** dispersion of **PCBFB** were intramuscularly injected to left or right side of rear legs, respectively. The efficacy of dye as imaging agents was then examined using an IVIS SpectrumCT *in vivo* imaging system (PerkinElmer). The wavelengths of excitation and emission filters used were 605-640 nm, and 740-800 nm, respectively. The auto-fluorescence of the skin was removed using a spectral unmixing software.

3.5 Supporting information

Figure S3.1 ^1H NMR (300 MHz, CDCl_3) spectra of iPA-PCL₃₇, PCL₃₇-Br and PCL₃₇-b-POEGMA₆₀.

Figure S3.2 GPC curves of PCL₃₇-Br and PCL₃₇-*b*-POEGMA₆₀ polymers.

Figure S3.3 TEM image of PCBF based JAGG NPs under different magnifications.

Figure S3.4 Fluorescence excitation spectra of **PCBF** based **JAGG** dispersion (emission wavelength: 800 nm).

Figure S3.5 UV-Vis-NIR absorption and FL emission spectra and **PCBF** based **JAGG** NPs diluted from the 0.04 mM solution.

Figure S3.6 Quantum Yield Measurements. (a) UV-Vis-NIR absorption spectra and (b) NIR emission spectra of a series of the ICG reference solutions in water with different concentrations; (c) Integrated NIR fluorescence intensity plotted as a function of absorbance at 650 nm for ICG reference solutions based on the measurements in (a) and

(b); (d) UV-Vis-NIR absorption spectra and (e) NIR emission spectra of a series of **PCBF** in chloroform with different concentrations. (f) Integrated NIR fluorescence intensity plotted as a function of absorbance at 650 nm for **PCBF** in chloroform based on the measurements in (d) and (e); (g) UV-Vis-NIR absorption and (h) NIR emission spectra of a series of **JAGG** dispersion with different concentrations by dilution from 0.04 mM **JAGG** sample. (i) Integrated NIR fluorescence intensity plotted as a function of absorbance at 650 nm for **JAGG** dispersion based on the measurements in (g) and (h).

Figure S3.7 Quantum Yield Measurements. (a) UV-Vis-NIR absorption and (b) NIR emission spectra of a series of **JAGG** reference dispersion with different concentrations by dilution from 0.04 mM **JAGG** sample. (c) Integrated NIR fluorescence intensity plotted as a function of absorbance at 650 nm for **JAGG** dispersion based on the measurements in (a) and (b). (d) UV-Vis-NIR absorption and (e) NIR emission spectra of a series of **JAGG** dispersion with different concentrations by dilution from 0.4 mM **JAGG** sample. (f) Integrated NIR fluorescence intensity plotted as a function of absorbance at 650 nm for **JAGG** dispersion based on the measurements in (d) and (e). (g) UV-Vis-NIR absorption spectra and (h) NIR emission spectra of a series of **JAGG** dispersion with increasing concentrations by dilution from 0.4 mM **JAGG** solution after 200 min sonication. (i) Integrated NIR fluorescence intensity plotted as a function of absorbance at 650 nm for **JAGG** dispersion based on the measurements in (g) and (h).

Figure S3.8 Quantum Yield Measurements. (a) UV-Vis-NIR absorption spectra and (b) NIR emission spectra of a series of the **PCBF** reference solutions in chloroform with increasing concentrations; (c) Integrated NIR fluorescence intensity plotted as a function of absorbance at 650 nm for the **PCBF** reference solutions in chloroform based on the measurements in (a) and (b); (d) UV-Vis-NIR absorption spectra and (e) NIR emission spectra of a series of **PCBFb** in chloroform with increasing concentrations. (f) Integrated NIR fluorescence intensity plotted as a function of absorbance at 650 nm for **PCBFb** in chloroform based on the measurements in (d) and (e); (g) UV-Vis-NIR absorption spectra and (h) NIR emission spectra of a series of **PCBFb** based **HAGG** dispersion in water with increasing concentrations. (i) Integrated NIR fluorescence intensity plotted as a function of absorbance at 650 nm for **PCBFb** based **HAGG** dispersion based on the measurements in (g) and (h).

Figure S3.9 UV-Vis absorption and FL emission spectra of **PCBF** based **HAGG** NPs in aqueous medium.

Figure S3.10 Temperature-dependent UV-Vis spectra of **PCBF** based **JAGG** NPs in water (0.4 mM diluted by 8 folds).

Figure S3.11 TEM image of **PCBF** based **JAGG** NPs after sonication for 200 mins. Inset: DLS result of **PCBF** based **JAGG** NPs in water after sonication for 200 mins.

Figure S3.12 ¹H NMR (CDCl₃, 300 MHz) spectra of Compound 2.

Figure S3.13 ¹³C NMR (CDCl₃, 300 MHz) spectra of Compound 2.

Figure S3.14 ^1H NMR (CDCl_3 , 300 MHz) spectra of Compound 3.

Figure S3.15 ^1H NMR (CDCl_3 , 300 MHz) spectra of Compound 4.

Figure S3.16 ^{13}C NMR (CDCl_3 , 300 MHz) spectra of Compound 4.

Figure S3.17 ^1H NMR (CDCl_3 , 300 MHz) spectra of Compound 5.

Figure S3.18 ^{13}C NMR (CDCl_3 , 300 MHz) spectra of Compound 5.

Figure S3.19 ^1H NMR (CDCl_3 , 300 MHz) spectra of Compound 7.

Figure S3.20 ^{13}C NMR (CDCl_3 , 300 MHz) spectra of Compound 7.

Figure S3.21 ^1H NMR (CDCl_3 , 300 MHz) spectra of Compound 8.

Figure S3.22 ^{13}C NMR (CDCl_3 , 300 MHz) spectra of Compound 8.

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Chapter 4 Hydrophobic-Sheath Segregated Macromolecular Fluorophores: Colloidal Nanoparticles of Polycaprolactone-Grafted Conjugated Polymers with Bright Far-Red/Near-Infrared Emission for Biological Imaging¹

As presented in **Chapter 3**, colloidal nanoparticles containing J-aggregates of PPcy showed unique optical properties such as extremely narrow emission band and comparable quantum yield with indocyanine green. However, they suffer from the limitation of small Stokes shift (< 5 nm) which can cause crosstalk between the excitation and emission signals. One strategy of overcoming this limitation is by Förster resonance energy transfer (FRET).² Generally, the high energy transfer of a FRET pair requires a donor with high quantum yield and a good overlap between emission spectra of donor and absorption spectra of acceptor. Therefore, due to the high absorption extinction coefficient and broad red emission, we chose conjugated polymer poly(fluorene-*alt*-(4,7-bis(hexylthien)-2,1,3-benzothiadiazole)) (PFTB) as a donor to transfer energy to acceptor PPcy. However, the aggregation of PFTB and PPcy also leads to the fluorescence quenching. To address this issue, we utilized biodegradable polymers polycaprolactone (PCL) to modify PFTB and PPcy and obtain PCL-grafted PFTB (PFTB-g-PCL) and PCL-tethered PPcy (PCL-PPcy-PCL). This strategy facilitates the enlargement of the fluorophore-fluorophore distance and weakens the π - π interactions in conjugated polymer nanoparticles. In this chapter, the optical properties of colloidal nanoparticles composed of donor PFTB-g-PCL were first investigated.

4.1 Introduction

Colloidal semiconducting polymer nanoparticles in aqueous solutions can be directly obtained via miniemulsion or nanoprecipitation techniques using small molecular or polymeric surfactants as stabilizers.³⁻¹⁰ These nanoparticles, also called polymer dots (Pdots), primarily consisted of densely packed hydrophobic

conjugated polymers in the core, and exhibited tunable particle sizes, relatively high fluorescence quantum yield and excellent photostability.¹¹⁻¹⁵ However, dramatic FL quenching in aqueous media was still observed due to the inter-chain aggregation within the hydrophobic core that resulted in non-radiative pathways.

To minimize the aggregation caused quenching (ACQ) effect, the concentration of fluorescent conjugated polymers relative to the matrix materials were kept as low as possible, for instance, at about 1% in poly(D,L-lactide-*co*-glycolide acid)¹⁶ and 8.5% in phospholipids.¹⁷ Despite the good photostability of these colloidal nanoparticles (NPs), the per-particle FL brightness was limited. Therefore, a more effective strategy for suppressing the ACQ behavior is highly desirable.

Scheme 4.1 (a) Schematic presentation of conjugated polyelectrolyte (CPE), conjugated polymer (CP) with short alkyl side chains and conjugated polymer brush (CPB). (b) Chemical structure of PFTB-*g*-PCL with different hexyl side positioning on thiophene rings. (c) Schematic illustration of **nanoREDs** formed by coprecipitation of PFTB-*g*-PCL and PCL-*b*-POEGMA as the stabilizer.

In this chapter, we report a series of red-emitting polycaprolactone-*grafted* poly(fluorene-*alt*-(4,7-bis(hexylthien)-2,1,3-benzothiadiazole)) (denoted as PFTB-*g*-PCL) with better-defined alternating conjugated backbones than the aforementioned statistical copolymers. These PFTB-*g*-PCL polymers as fluorescence imaging agents show significantly enhanced FL quantum yield in aggregating states such as colloidal NPs. While PFTB-based alternating conjugated polymers were originally developed as red-emitting materials for applications in organic light-emitting devices,^{18,19} they have been recently processed into NPs for fluorescent biological labeling and sensing, but still suffer from the ACQ effect.^{20,21} To address this FL quenching issue, an effective strategy is to encapsulate hydrophobic conjugated polymers by amphiphilic molecules and separate chromophores apart to minimize the aggregation-caused FL quenching. To that end, we have grafted a well-known biodegradable polymer, polycaprolactone (PCL), from the backbone of PFTB via ring-opening polymerization (ROP).

Two kinds of conjugated polymer brushes (denoted as PFTB^{out}-*g*-PCL and PFTBⁱⁿ-*g*-PCL) (Scheme 4.1b), with the same backbone but distinct location of hexyl groups on the thiophene, were synthesized and characterized. To render these hydrophobic polymers dispersible in water, we also employed the synthetic amphiphilic block copolymer PCL-*b*-POEGMA as a surfactant to encapsulate the PFTB-*g*-PCL polymers into the hydrophobic core of NPs via a process of coprecipitation (also called nanoprecipitation).^{14,22} (Scheme 4.1c) The resulting NPs, denoted as **nanoREDs**, consist of PFTB-*g*-PCL as the hydrophobic core and POEGMA as the hydrophilic shell. The covalently bond PCL brushes with proper chain lengths enable perfect isolation of PFTB backbones from each other, and thus significantly higher FL quantum yield in aqueous media compared to the NPs of conjugated polymers bearing short alkyl chains. Moreover, the semicrystalline

feature of PCL from the co-collapsed hydrophobic core of **nanoREDS** is expected to enhance the colloidal stability of these NPs against potential leaching, compared to previously reported Pdots stabilized by small molecular surfactants.^{23,24} We further demonstrate that these **nanoREDS** with strong FR/NIR fluorescence, tunable sizes, good colloidal stability and biocompatibility can be internalized by cells for bioimaging applications.

4.2 Results and discussion

4.2.1 Synthesis of PFTB-g-PCL

The synthetic route to the target conjugated polymer bottle brush, PFTB-g-PCL, is shown in Scheme 4.2. The conjugated backbone was synthesized via a previously reported protocol²⁵ called C-H direct arylation polymerization (DAP) that enables the synthesis of the target polymer with fewer synthetic steps and without highly flammable and/or toxic organometallic reagents.²⁵⁻²⁹

Scheme 4.2 Synthetic route to PFTB^{out} conjugated polymer and PFTB^{out}-g-PCL conjugated polymer brushes. The same synthetic route was used to synthesize PFTBⁱⁿ-g-PCL.

Our initial trial of DAP between Compound **1** and dibromofluorene derivative without protecting the -OH group in the side chains resulted in polymers with relatively low molecular weight ($M_n = 6,500$, $M_w/M_n = 2.56$). As a consequence, a typical OH-protective group 3,4-dihydro-2H-pyran (DHP) was end-capped to the side chains of the dibromo-fluorene monomer (Compound **3**). Under our previous optimized reaction condition of DAP,²⁵ conjugated polymers (denoted as PFTB^{out}-DHP and PFTBⁱⁿ-DHP) were obtained with yield as high as 90%. We also synthesized two conjugated polymers (denoted as PFTB^{out} and PFTBⁱⁿ) with non-functionalized side chains (i.e. octyl) on fluorene units as control (Scheme 4.2).

Table 4.1 Molecular weights of PFTB, PFTB-DHP, PFTB-g-PCL and PCL-*b*-POEGMA

<i>Polymers^b</i>	<i>M_n, NMR^c</i> (Da)	<i>M_n, GPC^d</i> (Da)	<i>M_w /M_n^e</i>
PFTB ^{out} ₂₀	—	17,600	1.9
PFTB ⁱⁿ ₉	—	7,700	2.54
PFTB ^{out} ₁₀ -DHP	—	11,100	2.58
PFTB ⁱⁿ ₁₀ -DHP	—	12,400	2.69
(PFTB ^{out} -g-(PCL ₁₃) ₂) ₁₀	40,740	40,900	1.89
(PFTB ^{out} -g-(PCL ₅₉) ₂) ₁₀	145,837	115,500	1.61
(PFTB ^{out} -g-(PCL ₈₈) ₂) ₁₀	232,009	177,200	1.68
(PFTB ⁱⁿ -g-(PCL ₂₄) ₂) ₁₀	67,120	51,100	2.23
(PFTB ⁱⁿ -g-(PCL ₉₁) ₂) ₁₀	219,880	165,600	1.85
PCL ₃₇ - <i>b</i> -POEGMA ₆₀	24,382	31,100	1.37

Note: ^a DP_n of conjugated alternating units (FTB) was calculated by GPC results; DP_n of CL units was calculated by ¹H NMR results based on DP_n of FTB; ^b $M_{n,NMR}$ was calculated by ¹H NMR results; ^{c,d} $M_{n,GPC}$ was calculated by GPC results.

The details about the structural characterization of the synthesized polymers are presented in Table 4.1. The results of gel permeation chromatography (GPC) using linear polystyrene as standards gave number average molecular weight $M_n = 11,100$ g/mol, $M_w/M_n = 2.58$ for PFTB^{out}-DHP and $M_n = 12,400$ g/mol, $M_w/M_n = 2.69$ for PFTBⁱⁿ-DHP. Further hydrolysis of PFTB^{out}-DHP and PFTBⁱⁿ-DHP in a mixture of hydrochloric acid, THF and methanol resulted in complete removal of DHP protecting groups and exposure of -OH groups. The hydrolyzed products were utilized as macroinitiators to induce the ring opening polymerization (ROP) of ϵ -caprolactone using SnOct₂ as the catalyst to obtain targeting PCL-grafted conjugated polymers.

The chemical structures of the obtained PCL-grafted conjugated polymers were characterized by ¹H NMR spectroscopy. Taking synthesis of (PFTB^{out}-g-(PCL₁₃)₂)₁₀ as an example, the ¹H NMR spectra of all target polymer products corresponding to each synthetic step are shown in Figure 4.1. In detail, in the ¹H NMR spectrum of the precursor PFTB^{out}₁₀-DHP (Fig. 4.1a), signals at 7.5~8.5 ppm could be assigned to the protons of aromatic rings from the backbone and signal at 4.59 ppm was attributed to protons on chiral carbons (labeled by Δ , Fig. 4.1a). Meanwhile, multiple peaks at 3.2-4.0 ppm correspond to the methylene groups next to the chiral carbon. The disappearance of the peak at 4.59 ppm and the appearance of triplet resonance at $\delta = 3.6$ ppm associated with the protons of -OH group in the ¹H NMR spectrum (Fig. 4.1b) of hydrolyzed products indicate the complete removal of the THP groups.

After growth of PCL from the exposed hydroxyl groups from the pendant chains of PFTB derivatives, two sharp peaks at $\delta = 2.32$ and 4.08 ppm appeared in the ¹H NMR spectrum of (PFTB^{out}-g-(PCL₁₃)₂)₁₀. These two peaks correspond to the protons from the two methylene groups next to the ester moiety of PCL. Meanwhile the peaks at $\delta = 7.5\sim 8.5$ ppm corresponding to aromatic protons can be observed. Similarly, the ¹H NMR spectra (Fig. S4.5) confirm the successful synthesis of (PFTBⁱⁿ-g-(PCL₂₄)₂)₁₀ copolymers. The absolute number-average

degree of polymerization (DP_n) of PCL can be determined from the integration ratio of the peak at 2.32 ppm over those from 7.5 to 8.5 ppm.

Figure 4.1 ^1H NMR (CDCl_3 , 300 MHz) spectra of (a) PFTB^{out}₁₀-THP, (b) PFTB^{out}₁₀-OH and (c) (PFTB^{out}-g-(PCL₁₃)₂)₁₀.

The controllable growth of PCL from the PFTB backbone was monitored by GPC analysis. The results shown in Figures 4.2a, 4.2b and S4.6 clearly indicate the well-defined structures of the PFTB-g-PCL copolymers, where chromatograms from both RI and UV detectors shifted to lower elution volume with the increasing DP_n of the grafted PCL.

Figure 4.2 GPC traces of a series of PFTB^{out}-g-PCL polymers and their precursor, PFTB^{out}₁₀-DHP, using (a) RI and (b) UV-vis ($\lambda_{\text{abs}} = 550$ nm) detectors.

4.2.2 Preparation and characterization of nanoREDs

The synthetic block copolymer, denoted as PCL₃₇-*b*-POEGMA₆₀, is expected to serve as a surfactant and facilitate the encapsulation of PCL-grafted conjugated polymers through a previously established nanoprecipitation process (**Scheme 4.1c**),^{24,30,31} in which a mixture of PFTB-g-PCL and PCL₃₇-*b*-POEGMA₆₀ codissolved in THF was rapidly injected into a large excess water, followed by evaporation of the organic solvent. More details of the sample preparation are described in the Experimental section. The resulting well-dispersed and colloidally stable NPs (denoted as **nanoREDs**) have both PFTB-g-PCL and the PCL block of PCL₃₇-*b*-POEGMA₆₀ in the hydrophobic core, and POEGMA in the hydrophilic shell. In contrast, PFTB-g-PCL immediately formed a precipitate after the rapid injection of (PFTB^{out}-g-(PCL₁₃)₂)₁₀ THF solution into deionized water in the absence of amphiphilic block copolymer such as PCL₃₇-*b*-POEGMA₆₀ as the stabilizer. The experimental details and results are listed in Table 4.2.

Table 4.2 Characterization of **nanoREDs** vs. **Pdots**

<i>Entry^a</i>	<i>Polymers</i>	$\lambda_{\text{max,abs}}$ (nm)	$\lambda_{\text{max,em}}$ (nm)	<i>Stokes shift</i> (nm)	Φ^b (%)	D_h^c (nm)	w_{CPs}^d (%)
Pdot^{out}	PFTB ^{out} ₂₀	521	640	119	4.68	28.0	100
nanoRED1^{out}	(PFTB ^{out} -g-(PCL ₁₃) ₂) ₁₀	518	632	114	18.1	52.9	20.9
nanoRED2^{out}	(PFTB ^{out} -g-(PCL ₅₉) ₂) ₁₀	520	635	115	24.8	45.4	7.4
nanoRED3^{out}	(PFTB ^{out} -g-(PCL ₈₈) ₂) ₁₀	526	633	107	25.6	43.9	4.8
Pdotⁱⁿ	PFTB ⁱⁿ ₉	476	628	152	8.44	19.8	100
nanoRED1ⁱⁿ	(PFTB ⁱⁿ -g-(PCL ₂₄) ₂) ₁₀	470	622	152	20.7	50.4	18.7
nanoRED2ⁱⁿ	(PFTB ⁱⁿ -g-(PCL ₉₁) ₂) ₁₀	467	620	153	19.9	46.1	5.8

Note: ^a **nanoREDs** were prepared upon the weight ratio (*r*) of PFTB-g-PCL / PCL₃₇-*b*-POEGMA₆₀ = 10/3 and **Pdots** were prepared without amphiphilic block copolymers; ^b the

Quantum yields of solutions were measured by the standard method using Rhodamine 6G in ethanol ($\Phi_F = 0.95$); ^c hydrophilic diameter of nanoparticles was measured by DLS; ^d w_{CPs} stands for the weight percentage of conjugated backbone in **nanoREDS**.

The size of **nanoREDS** can be tuned by varying the weight ratio (r) of PFTB-*g*-PCL/PCL₃₇-*b*-POEGMA₆₀. Taking (PFTB^{out}-*g*-(PCL₁₃)₂)₁₀ as an example, when a mixture of these two polymers with $r = 10:3$ and $m((\text{PFTB}^{\text{out}}\text{-g-(PCL}_{13}\text{)}_2)_{10}) = 0.67$ mg in THF (1 mL) was quickly added into 5 mL of water under vigorous sonication, particles with an average diameter of 53 nm (measured by dynamic light scattering (DLS)) were obtained (Fig. 4.3b). As shown in Figure 4.4a, adding more PCL₃₇-*b*-POEGMA₆₀ to reach r of 10:4 resulted in smaller NPs with an average diameter of 45 nm. In addition, decreasing the r to 10:8 and 10:15 produced slightly larger NPs (i.e. 64 and 83 nm, respectively). This could be attributed to that more amphiphilic macromolecules participate in the formation of each particle. Due to the stretched state of POEGMA chains in water, more POEGMA chains surrounding the particle cannot influence the thickness of corona. In contrast, more PCL segments involve the inter-chain aggregation and intra-chain collapse in the hydrophobic core, resulting in the gradual increase of the size of core as well as that of particles. The particle size further increased to 228.7 nm when r is increased to 10:2 while other conditions were maintained the same as above (Fig. 4.4b). Moreover, NPs with an average diameter of 437 nm were obtained at $r = 10:1$.

The different sizes of the NPs prepared at different ratios of PFTB-*g*-PCL/PCL₃₇-*b*-POEGMA₆₀ can be rationalized that less PCL₃₇-*b*-POEGMA₆₀ cannot effectively reduce the interfacial energy between the PCL-core and water, resulting in the formation of larger particles with lower interfacial area. It should be noted that no precipitates were observed in the preparation process of **nanoREDS** described, even for particles with diameter > 400 nm. In contrast, some precipitates were observed in the nanoprecipitation of Pdots composed of PFTB^{out}₂₀ and PFTBⁱⁿ₉ at the same concentration without amphiphilic block copolymers. After

filtration through a 0.2 μm cellulose membrane filter, **Pdot^{out}** and **Pdotⁱⁿ** exhibit average diameter of 28 and 20 nm, respectively, smaller than those of **nanoREDs**.

The morphologies of the produced NPs with sizes ranging from 50 to 450 nm are shown in Figure 4.3a and Figure S4.7. The NPs exhibited low polydispersity with PDI of less than 0.2 from the DLS results (Table S4.1). They are all spherical independently of the different sizes, as observed by transmission electron microscope (TEM). The sizes of **nanoREDs** from TEM are consistent with those from DLS analysis (Fig. 4.4a & S4.7). Those **nanoREDs** with sizes from 40 to 200 nm showed good colloidal stability in PBS 7.4 as shown in Figure 4.4b. However, the sample with an r of 10:1 and the size of 436.0 nm tended to form larger aggregates upon further aging under ambient conditions (Fig. 4.4b).

Figure 4.3 (a) Representative TEM image and (b) DLS histogram of **nanoRED1^{out}** at a weight ratio (r) of PFTB-*g*-PCL / PCL₃₇-*b*-POEGMA₆₀ = 10:3 (diameter = 50 nm). The inset in figure (b) shows the evolution of the size of **nanoRED1^{out}** incubated in PBS 7.4.

Figure 4.4 (a) Size distribution of **nanoREDs** prepared with different weight ratios between (PFTB^{out}-g-(PCL₁₃)₂)₁₀ and PCL₃₇-b-POEGMA₆₀ measured by DLS with a concentration of (PFTB^{out}-g-(PCL₁₃)₂)₁₀ at 0.33 mg/mL. (b) Size evolution of different **nanoREDs** incubated in PBS 7.4 over different aging periods.

It is known that the size of NPs affects their accumulation, penetrations, internalization into cells.³² Particularly, sub-100-nm micelles or NPs exhibited enhanced tissue penetration and tumor inhibition *in vivo*.³³ Hence, we focused on **nanoREDs** with an *r* of 10:3 due to their suitable size (*d* = 53 nm) and relatively higher concentration of the conjugated backbones per particle. Based on this consideration, **nanoRED2^{out}**, **nanoRED3^{out}**, **nanoRED1ⁱⁿ** and **nanoRED2ⁱⁿ** corresponding to (PFTB^{out}-g-(PCL₅₉)₂)₁₀, (PFTB^{out}-g-(PCL₈₈)₂)₁₀, (PFTBⁱⁿ-g-(PCL₂₄)₂)₁₀ and (PFTBⁱⁿ-g-(PCL₉₁)₂)₁₀, respectively, were also prepared at *r* = 10:3 and all of them showed similar diameters of ~50 nm as shown in Table 2.

4.2.3 Photophysical properties of NanoREDs

PFTB^{out}₂₀ in toluene shows a maximum absorption wavelength ($\lambda_{\text{max,abs}}$) at 516 nm, a maximum emission wavelength ($\lambda_{\text{max,em}}$) at 616 nm (Figure 4.5a and 4.5b) and a FL quantum yield (Φ) of 0.30 (measured using Rhodamine 6G in ethanol as the standard). For polymer dots **Pdot^{out}** consisting of PFTB^{out}₂₀ in aqueous medium, both the absorption and the emission peaks red shifted to 520 and 640 nm, respectively. Moreover, the Φ of PFTB^{out}₂₀ in the form of colloidal NPs showed a dramatic decrease to 0.05. Such changes of optical properties can be attributed to the intermolecular aggregation of conjugated polymer chains, which results in the

formation of less-emissive species (e.g. excimers and exciplexes) and significant luminescence quenching of the polymers.³⁴

The ACQ effect described above in PFTB^{out}₂₀ with relatively short alkyl side chains can be effectively suppressed when PCL is grafted from the conjugated backbone. Similar to what we previously observed in PCL-DPP-PCL polymers,³¹ the chain length of PCL plays a critical role to determine the fluorescence properties of PFTB in aggregated states. As shown in Figure S4.8, it is clear to see that polymers (PFTB^{out}-g-(PCL₁₃)₂)₁₀ and (PFTB^{out}-g-(PCL₅₉)₂)₁₀ and (PFTB^{out}-g-(PCL₈₈)₂)₁₀ all exhibit bright red fluorescence in bulk solid states, in contrast to the dim red fluorescence of PFTB^{out}₂₀ bearing short alkyl (i.e. hexyl) chains. These results encouraged us to fabricate colloidal NPs with bright FR/NIR emission using these conjugated polymer brushes, as discussed below.

As shown in Table 4.2, when the number average degree of polymerization (DP_n) of grafted PCL is 13, the Φ of **nanoRED1^{out}** (corresponding to (PFTB^{out}-g-(PCL₁₃)₂)₁₀) is 0.18, approximately 4 times higher than that of **Pdot^{out}** (Φ = 0.05), indicating that the grafted PCL side chains effectively segregate the PFTB backbones and enhance the FL intensity of the colloidal NPs. More direct evidence is shown in Figure S4.8, in which the dispersion of **nanoRED1^{out}** showed strong red fluorescence under irradiation of UV light, while the dispersion of **Pdots** appeared weakly fluorescent. At the same time, **nanoRED1^{out}** in water exhibits a maximum emission at 632 nm, which is 8 nm blue-shifted as compared to that for **Pdot^{out}**. Such blue shift of the maximum absorption of **nanoRED1^{out}** vs. **Pdot^{out}** further reflects the reduced intermolecular aggregation of PFTB backbones in the former. In addition, the emission tail of **nanoRED1^{out}** is extended to 850 nm (Figure 4.5b). While the FL emission of **nanoREDs^{out}** above 750 nm appears relatively weak, the significantly improved FL quantum yield of PFTB^{out}-g-PCL in the far-red (FR) region (ca. 650-700 nm) assures FL imaging of cells as shown in the following section and also for future *in vivo* imaging in near-infrared (NIR) region (> 700 nm).

To understand the effect of PCL chain length on the optical properties of PFTB^{out}-g-PCL, we used two more polymer brushes, (PFTB^{out}-g-(PCL₅₉)₂)₁₀ and (PFTB^{out}-g-(PCL₈₈)₂)₁₀, to fabricate **nanoRED2^{out}** and **nanoRED3^{out}**, respectively, following the same procedure of preparing **nanoRED1^{out}**. The weight ratio of PFTB^{out}-g-PCL/PCL₃₇-*b*-POEGMA₆₀ was kept at 10:3 for both **nanoRED2^{out}** and **nanoRED3^{out}**. While the length of PCL brushes does not have an obvious effect on the shape of the absorption and the emission spectra (Fig. 4.5b), the Φ of **nanoREDs** was notably enhanced from 0.18 (**nanoRED1^{out}**, (PFTB^{out}-g-(PCL₁₃)₂)₁₀) to 0.25 (**nanoRED2^{out}**, (PFTB^{out}-g-(PCL₅₉)₂)₁₀) with the increase of the PCL chain length in PFTB-g-PCL. When the chain length of grafted PCL was further increased (e.g. (PFTB^{out}-g-(PCL₈₈)₂)₁₀), there was no significant improvement of the FL quantum yield compared to that of (PFTB^{out}-g-(PCL₅₉)₂)₁₀. Figure 4.5e summarizes the variation of FL quantum yield of **nanoREDs^{out}** with different chain lengths of grafted PCL on PFTB backbones. We can conclude that the length of PCL block in (PFTB^{out}-g-(PCL₅₉)₂)₁₀ is long enough to effectively separate neighboring conjugated backbones at the molecular level and minimize the FL quenching caused by inter/intra-chain transfer and/or ACQ effect. Additionally, the Stokes shift of **nanoRED3^{out}** slightly decreased to 107 nm compared with those of **nanoRED1^{out}** (114 nm) and **nanoRED2^{out}** (115 nm), which could arise from a greater rigidity of the PFTB backbone in the former in which grafted PCL chains are longer.³⁵

Figure 4.5 UV-Vis absorption (a, c) and FL emission (b, d, excited at the maximum absorption wavelength) spectra of PFTB-based **Pdots** and **nanoREDS**. (e-f) Dependence of FL quantum yield of **nanoREDS** on chain length of PCL grafted from PFTB backbones. Unit numbers in (e) (f) represent n in the formula $(\text{PFTB-g-(PCL}_n)_2)_{10}$ of conjugated polymer brush.

In the case of PFTBⁱⁿ, the Φ of **nanoRED1ⁱⁿ** (corresponding to $(\text{PFTB}^{\text{in}}\text{-g-(PCL}_{24})_2)_{10}$) is 0.207, which is 2.5 times higher than that (~ 0.08) of **Pdotⁱⁿ** consisting of bare PFTB₆ⁱⁿ but still lower than that in toluene (~ 0.45). **NanoRED2ⁱⁿ** consisting of $(\text{PFTB}^{\text{in}}\text{-g-(PCL}_{91})_2)_{10}$ showed a Φ (0.199) comparable to that of **nanoRED1ⁱⁿ**, indicating that the further increase of the chain length of PCL does not result in improvement of the FL quantum yield. Compared with **NanoRED^{out}**, **NanoREDⁱⁿ** showed a higher FL quantum yield, larger Stokes shift of 152 nm and

a lower maximum emission wavelength, which was also observed by Chan and coworkers³⁶ in PFBT-DBSIC₆X statistical copolymers. Such difference could be attributed to the fact that PFTBⁱⁿ with two alkyl side chains inwards is likely to introduce more steric hindrance and further prevent the close interchain stacking of the conjugated backbones.³⁷ Interestingly, in the presence of grafted PCL brush, **nanoRED1ⁱⁿ** exhibits a Φ comparable to that of **nanoRED1^{out}** (~0.18), but a bluer emission band centered at 622 nm (Fig. 4.5d). It suggests that PFTB^{out} is more vulnerable to ACQ and self-quenching probably due to its planar structure and smaller Stokes shift. As a consequence, the grafting of a PCL sheath of PFTB^{out} results in a more significant recovery of FL as compared to PFTBⁱⁿ.

To better understand the enhanced FL quantum yield of **nanoREDS** as compared to **Pdots**, we also measured the FL lifetimes (τ) for these NPs. The FL radiative decay rate constant (k_r) and nonradiative rate constant (k_{nr}) can be estimated according to the following equations³⁸:

$$\Phi = k_r / (k_r + k_{nr})$$

$$\tau = (k_r + k_{nr})^{-1}$$

Figure 4.6 shows the FL decay curves of **Pdot^{out}**, **Pdotⁱⁿ**, **nanoRED1^{out}** and **nanoRED1ⁱⁿ** in water. From the fitted lifetime decay results (Fig. S4.9), the FL of **Pdot^{out}** and **Pdotⁱⁿ** shows lifetimes of 0.72 and 1.68 ns, respectively. Compared to **Pdotⁱⁿ**, the closer interchain stacking of the PFTB^{out} conjugated backbones in **Pdot^{out}** may lead to the stronger nonradiative recombination and shorter FL lifetime. In contrast, the incorporation of PCL brush endows **nanoRED1^{out}** and **nanoRED1ⁱⁿ** with longer lifetimes of 3.86 and 3.39 ns, respectively. These results clearly demonstrate the significant nonradiative recombination of the excited states of **Pdots** due to the self-quenching behavior in aggregated states. For **nanoRED1^{out}** and **nanoRED1ⁱⁿ**, the longer lifetimes should result from the blocking of nonradiative decay pathways of PFTB backbones which are segregated from each other by the grafted PCL. As a consequence, these four kinds of NPs show similar FL radiative rates of $5 \times 10^7 \sim 6 \times 10^7 \text{ s}^{-1}$. In contrast to the relatively low

nonradiative rates of $2.12 \times 10^8 \text{ s}^{-1}$ for **nanoRED1^{out}** and $2.37 \times 10^8 \text{ s}^{-1}$ for **nanoRED1ⁱⁿ**, the FL of **Pdot^{out}** and **Pdotⁱⁿ** show nonradiative rates as high as 1.32×10^9 and $5.45 \times 10^8 \text{ s}^{-1}$, respectively. It indicates that relatively weak nonradiative recombination of **nanoREDS** leads to the remarkable decrease of k_{nr} and the enhanced FL quantum yields. Meanwhile, the lower nonradiative rate of **Pdotⁱⁿ** compared to that of **Pdot^{out}** provides a supporting evidence to explain why **Pdotⁱⁿ** exhibits a higher FL quantum yield.

Figure 4.6 Fluorescence lifetime measurements of PFTB-based **Pdots** and **nanoREDS** in aqueous media. The black dot line represents the internal reference (IRF). The lifetime of **Pdot^{out}**, **Pdotⁱⁿ**, **nanoRED1^{out}**, **nanoRED1ⁱⁿ** solutions are 0.72, 1.68, 3.86 and 3.39 ns, respectively.

4.2.4 Cellular imaging and cytotoxicity

To demonstrate the application of the highly fluorescent **nanoREDS** described above for bioimaging, **nanoRED1^{out}** and **nanoRED1ⁱⁿ** were chosen as examples in the following cellular imaging and cytotoxicity experiments due to the high concentration of fluorescent backbones and high quantum yield. Fluorescence imaging of live fibroblast cell from mouse (L929) and cervical cancer cell (HeLa) was investigated using **nanoREDS** as contrast agents under confocal laser scanning microscopy (CLSM). After incubating the cells with the **nanoRED1^{out}** dispersion

(25 µg/mL) for 12 h, the cells were fixed and their nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). For fluorescence imaging of cells labeled with **nanoRED1^{out}**, the excitation wavelength was fixed at 488 nm, and the FL emission signals in the range 530 to 700 nm were collected. Figure 4.7a shows the CSLM images of L929 cells stained by **nanoRED1^{out}**. In order to determine whether **nanoRED1^{out}** are internalized into cells, 35 optical sections were measured in incremental steps of 1000 nm from regions around the upper surface of the cells to the lower surface. As shown in the ortho-view images of z-stack (Fig. S4.10a), a uniform distribution of **nanoRED1^{out}** with strong fluorescence throughout the cellular cytoplasm was observed for L929 cells, suggesting the efficient accumulation of **nanoRED1^{out}** in the cytoplasm of these cells.

In addition, HeLa cells were chosen as representative cancer cells to uptake **nanoREDS**. The CLSM images of HeLa cells stained by **nanoRED1^{out}** are presented in Figure 4.7b. The preferable distribution of **nanoRED1^{out}** in the cytoplasm of HeLa cells indicated efficient intracellular uptake of formulated NPs. The FL imaging results of L929 cells and HeLa cells treated with **nanoRED1ⁱⁿ** are shown in Figure S4.11. Similar to **nanoRED1^{out}**, bright dots of **nanoRED1ⁱⁿ** were observed to mainly cluster in cytoplasm zone of L929 cells and HeLa cancer cells. These confocal FL images provide clear evidence for **nanoREDS** as promising fluorescence probes in cellular imaging.

As low toxicity is crucial for biological applications, the cytotoxicity of **nanoREDS** against mouse fibroblast cells (L929) was studied by Presto blue assays. Figure 4.7c summarizes the *in vitro* L929 cell viability after incubation with **nanoRED1^{out}** at a series of concentrations of 5, 10, 25, 50, 75 µg/mL for 24 and 48 h, respectively. It is noteworthy that the concentrations of these **nanoREDS** are much higher than that used for practical cell imaging (0.1 µg/mL). There was no obvious change of the metabolic viability of L929 cells after incubation with **nanoRED1^{out}** even at a concentration as high as 75 µg/mL, indication of the low cytotoxicity of **nanoRED1^{out}**. Similarly, the metabolic viability of L929 cells after incubation with **nanoRED1ⁱⁿ** for 24 and 48 h was not affected under the same

experimental conditions (Fig. 4.7d). These results demonstrate the good biocompatibility of these **nanoREDs** for FR/NIR intracellular imaging.

Figure 4.7 Confocal fluorescence microscope images of (a) L929 cells and (b) HeLa cells stained with **nanoRED1^{out}**. The concentration of **nanoREDs** is 25 $\mu\text{g/mL}$. The fluorescence of DAPI and NPs is pseudo-labeled with blue and red, respectively. The scale bar represents 100 μm . Metabolic viability of L929 cancer cells after incubation with (c) **nanoRED1^{out}** and (d) **nanoRED1ⁱⁿ** at different concentrations for 24 and 48 h, respectively.

4.3 Conclusions

In conclusion, we have presented an effective strategy of hydrophobic-sheath segregated macromolecular fluorophores to fabricate colloidal nanoparticles with strong FR/NIR emission for bioimaging applications. Specifically, we synthesized and characterized a series of π -conjugated polymeric bottle brushes (PFTB-*g*-PCL) with PFTB as the backbone from which biodegradable polymers such as PCL with tunable chain lengths are grafted. These hydrophobic PFTB-*g*-PCL polymers were further encapsulated with amphiphilic block copolymers PCL-*b*-POEGMA via nanoprecipitation to form colloidally stable nanoparticles (so called **nanoREDS**) in aqueous media. The chain length of the PCL side chains in PFTB-*g*-PCL played a critical role to determine the fluorescence properties in both bulk solid states and the colloidal nanoparticles. The **nanoREDS** composed of PFTB-*g*-PCL polymers with long enough PCL side chains ($DP_{n,PCL} > 13$ for PFTB^{out}-*g*-PCL, and $DP_{n,PCL} > 24$ for PFTBⁱⁿ-*g*-PCL) showed remarkably improved FL quantum yield above 18% in aqueous media, in contrast to the dramatic FL quenching in the control of **Pdots** composed of PFTB with short alkyl chains. Moreover, these fluorescent **nanoREDS** with an average diameter of 50 nm could be efficiently uptaken by cells such as HeLa and L929 cells with good biocompatibility for FL imaging applications. Confocal fluorescence microscope images showed that these **nanoREDS** preferred to localize in the cytoplasm of L929 cells and HeLa cancer cells. We anticipate that this strategy of hydrophobic-polymer sheathed fluorophores can be applied to a broad range of π -conjugated polymers for bioimaging applications.

4.4 Experimental section

Materials 10 $\times$ phosphate buffer saline (PBS) buffer with pH = 7.4 (ultrapure grade) is a commercial product of 1st BASE Singapore. MilliQ water (18.2 M Ω) was used to prepare the buffer solution from the 10 $\times$ PBS stock buffer. 1 $\times$ PBS consists of NaCl (137 mM), KCl (2.7 mM), Na₂HPO₄ (10 mM) and KH₂PO₄ (1.8 mM). Chloroform-D (99%) was purchased from Cambridge Isotope Laboratories, Inc. All other chemicals and reagents were purchased from Aldrich or Merck and used as received unless specified. 4,7-bis(3-hexylthien)-2,1,3-benzo-thiadiazole

(Compound 1) and 4,7-bis(4-hexylthien)-2,1,3 -benzo-thiadiazole monomers was prepared according to the reference.^{39,40}

Characterization The samples were dissolved in chloroform-d for ¹H NMR measurements on a Bruker AV300 MHz NMR spectrometer. Molecular weights of polymers were measured using gel permeating chromatography (GPC) (Agilent 1260, USA). The eluent was THF at a flow rate of 1.0 mL/min. A series of low polydispersity polystyrene standards were employed for the GPC calibration. Emission spectra of solutions were measured by fluorescence spectrophotometry on a PerkinElmer LS-55 at 25 °C. UV/Vis spectra of the samples were measured on a SHIMADZU UV-2450 spectrophotometer. Quantum yields of solutions were measured by the standard method using Rhodamine 6G in ethanol (Φ_F = 0.95). In order to minimize re-absorption effects, absorbances in the 10-mm fluorescence cuvette didn't exceed 0.1 at the excitation wavelength of 488 nm. Fluorescence lifetime measurements were performed on a Horiba Fluolog 3 spectrofluorometer. PL decays were fitted to sums of three exponential terms. The fitting results are shown in the Supporting Information. TEM measurements were performed with a TEM Carl Zeiss Libra 120 Plus at an acceleration voltage of 120 kV. A 5 μ L droplet of diluted samples was directly dropped onto a copper grid (300 mesh) coated with a carbon film, followed by drying at room temperature. The size distribution of resulting nanoparticles was determined by dynamic light scattering (DLS) using a BI-200SM (Brookhaven, USA) with detection angle at 90°.

Synthesis of 4,7-bis(4-hexylthien)-2,1,3-benzo-thiadiazole³⁹

Scheme 4.3 Synthetic route to 4,7-bis(4-hexylthien)-2,1,3-benzo-thiadiazole

3-Hexyl-thiophene (1.68 g, 10 mmol) was dissolved in 100 mL anhydrous THF in a round flask under nitrogen atmosphere. n-Butyllithium (2.4 M in hexanes, 5 mL, 12 mmol) was added dropwise at -78 °C. After stirring for 1 h, trimethyltinchloride

(1 M in THF, 12 mL, 12 mmol) was added dropwise, and the mixture was allowed to warm to room temperature. After stirring for 6 h, 4,7-dibromo-2,1,3-benzothiadiazole (1.18 g, 4 mmol) and $\text{PdCl}_2(\text{PPh}_3)_2$ (0.14 g, 0.2 mmol) were added to this mixture. After flushing with nitrogen for 10 min, the reactants were heated to reflux for 24 h. After cooled to room temperature, the reaction mixture was poured into water. The product was extracted with ethyl acetate (3×50 mL). The extracts were washed with water and brine then dried with anhydrous magnesium sulfate. After filtration, the solvent was removed under reduced pressure. The residue was purified via column chromatography (silica gel) using hexane as eluent, to give compound 1 as orange powder (1.5 g, 80%). ^1H NMR (300 MHz, CDCl_3 , ppm): δ = 8.0 (d, 2H), 7.85 (s, 2H), 7.06 (s, 2H), 2.72 (t, 4H), 1.6-1.8 (m, 4H), 1.2-1.5 (m, 12H), 0.92 (t, 6H).

Synthesis of 4,7-bis(3-hexylthien)-2,1,3-benzo-thiadiazole⁴⁰

Scheme 4.4 Synthetic route to 4,7-bis(3-hexylthien)-2,1,3-benzo-thiadiazole.

After 3-hexylthiophene (3 g, 1.78 mmol) was dissolved in 45 mL of glacial acetic acid, N-bromosuccinimide (3.17 g, 1.78 mmol) was added and the reaction was left for 3 h. After that, 50 mL of water was added and the mixture was extracted with 50 mL of diethyl ether. The organic phase was collected and washed several times with NaOH (2 M) and brine. After drying over MgSO_4 , ether was evaporated under reduced pressure. Finally, the crude product as a clear oil was purified through column chromatography (silica gel) using hexane as eluent. (3.74 g, 85%). ^1H NMR (300 MHz, CDCl_3 , ppm): δ = 7.20 (d, 1H), 6.80 (d, 1H), 2.58 (t, 2H), 1.59 (m, 2H), 1.2-1.5 (m, 6H), 0.92 (t, 3H).

2-Bromo-3-hexylthiophene (3.58 g, 14.5 mmol) was dissolved in 100 mL anhydrous THF in a round flask under nitrogen atmosphere. n-Butyllithium (2.4

M in hexanes, 6.6 mL, 16 mmol) was added dropwise at -78 °C. After stirring for 1 h, trimethyltinchloride (1 M in THF, 14.5 mL, 14.5 mmol) was added dropwise, and the mixture was allowed to warm to room temperature. After stirring for 6 h, 4,7-dibromo-2,1,3-benzothiadiazole (1.70 g, 5.8 mmol) and PdCl₂(PPh₃)₂ (0.14 g, 0.2 mmol) were added to this mixture. After flushing with nitrogen for 10 min, the reactant was heated to reflux for 24 h. After being cooled to room temperature, the reaction mixture was poured into water. The product was extracted with ethyl acetate (3 × 50 mL). The extracts were washed with water and brine, then dried with anhydrous magnesium sulfate. After filtration, the solvent was removed under reduced pressure. The residue was purified via column chromatography (silica gel) using hexane as eluent, to obtain a light orange liquid (1.9 g, 71%). ¹H NMR (300 MHz, CDCl₃, ppm): δ = 7.66 (d, 2H), 7.45 (d, 2H), 7.12 (d, 2H), 2.68 (t, 4H), 1.5-1.75 (m, 4H), 1.1-1.4 (m, 12H), 0.83 (t, 6H).

Synthesis of Compound 2 11-Bromo-1-undecanol (41.4 mmol, 10 g) and 3, 4-dihydro-2H-pyran (62.1 mmol, 5.4 mL) were stirred in 0 mL THF at room temperature, followed by the slow addition of 10 mL of *p*-toluenesulfonic acid monohydrate (0.25 mmol, 50 mg) in THF solution. After having reacted for 4 h, the mixture was concentrated via rotary evaporation and purified via column chromatography (silica gel) using hexane/EA (20/1) as eluent. The compound DHP-C₁₁-Br was obtained as a colorless oil (13.35 g, quantitative yield). ¹H NMR (300 MHz, CDCl₃, ppm): δ = 4.59 (t, 1H), 3.6-4.0 (m, 2H), 3.25-3.55 (m, 4H), 1.25-2.0 (m, 24H). ¹³C NMR (300 MHz, CDCl₃, ppm): δ = 98.84, 67.67, 62.34, 57.06, 34.03, 32.83, 30.79, 29.75, 29.53, 28.76, 28.17, 26.23, 25.51, 19.71. MS (ESI): calcd. for C₁₆H₃₁BrO₂ [M+H]⁺ m/z 335.15, found 335.01.

Synthesis of Compound 3 A mixture of 2,7-dibromofluorene (3.09 mmol, 1 g), KOH (30.9 mmol, 1.73 g) and KI (0.309 mmol, 51 mg) was stirred overnight in 10 mL of DMSO at 60 °C under N₂ atmosphere. Compound 2 (6.8 mmol, 2.27 g) in 5 mL of DMSO was added slowly by syringe. After cooling, the solution was poured into excess ethyl acetate. The organic phase was concentrated under reduced pressure and purified via column chromatography (silica gel) using hexane/toluene

(20/1) as eluent. The colorless oil (5.0216 g) was obtained with a yield of 97.36%. ¹H NMR (300 MHz, CDCl₃, ppm): δ = 7.55 (d, 2H), 7.45 (m, 4H), 4.59 (t, 2H), 3.6-4.0 (m, 4H), 3.25-3.55 (m, 4H), 0.59 (br, 4H), 1.0-2.0 (m, 48 H). ¹³C NMR (300 MHz, CDCl₃, ppm): δ = 152.54, 139.07, 130.15, 126.16, 121.46, 121.14, 98.83, 67.70, 62.37, 55.69, 40.15, 30.79, 29.86, 29.74, 29.51, 29.19, 26.22, 25.52, 23.63, 19.70. MS (ESI): calcd. for C₄₅H₆₈Br₂O₄ [M+H]⁺ m/z 833.35, found 833.97.

Representative synthesis of Polymer 4 Under a nitrogen atmosphere, fluorene monomer (Compound 3) (1 eq, 0.1 mmol), Compound 1 (1 eq, 0.1 mmol), Pd₂(dba)₃ (0.05 eq, 0.005 mmol, 4.59 mg), (*o*-MeOPh)₃P (0.1 eq, 0.001 mmol, 3.5 mg), K₂CO₃ (3 eq, 0.3 mmol, 41.4 mg), PivOH (0.5 eq, 0.05 mmol, 5.1 mg) and 0.5 mL of xylene was added in a reaction vial with a magnetic stirring bar. The vial was sealed with a rubber cap and heated in a 100 °C oil bath for 24 h. After being cooled to the room temperature, the reaction mixture was diluted with chloroform and then precipitated in cold methanol three times. The precipitates were collected by filtration and dried under vacuum at 60 °C overnight to yield the target polymers (yield: 101 mg, 88%).

Synthesis of Polymer 5 PFTB-DHP (100 mg) was dissolved in the mixture of THF (90 mL) and methanol (10 mL). The solution was heated to 60 °C and then 0.5 mL of HCl (12 M) aqueous solution was added. After reaction for 6 h, the solvent was evaporated under reduced pressure. Solids obtained were redissolved in minimal amount of THF and then precipitated in cold methanol. The solid of PFTB-OH was obtained as red solid (yield: 80 mg, 94%).

Synthesis of Polymer 6 A mixture of PFTB-OH (25 mg), ϵ -caprolactone (0.3 g, 1 g, 2 g), SnOct₂ (3 mg) was placed in Schlenk tube and stirred for 24 h in 3 mL of anhydrous toluene at 120 °C under N₂ atmosphere. After cooling to room temperature, the reaction mixture was poured into cold diethyl ether. The product was obtained after further purification by precipitation in diethyl ether three times (yield: 81 mg, 162 mg and 183 mg, respectively).

Preparation of Pdots and nanoREDs For **Pdots**, a THF solution of PFTB with a concentration of 2000 ppm was prepared first. An aliquot (1 mL) of this solution was injected into 10 mL of water rapidly under ultrasonication. After evaporation of THF, a dispersion of PFTB NPs was obtained after filtration through a 0.2 μm cellulose membrane filter to remove trace amount of precipitates. For preparation of **nanoREDs**, PFTB-g-PCL ($100\ \mu\text{L} \times 6.67\ \text{g/L}$) and amphiphilic copolymer PCL-*b*-POEGMA ($x\ \mu\text{L} \times 6.67\ \text{g/L}$, $x = 10, 20, 30, 40, 80, 150$) were mixed in $(900-x)\ \mu\text{L}$ THF. This mixture was rapidly injected into 5 mL of deionized water under ultrasonication. THF was evaporated by exposure in open air overnight.

Cell culture L929 mouse fibroblast cells and HeLa immortal cancer cells were cultured in folate-free Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified environment containing 5% CO₂. Before experiments, the cells were precultured until confluence was reached.

***In vitro* cellular uptake** L929 and HeLa cells were seeded in 12-well plates at 37 °C (Costar, IL, USA). After 80% confluence, the medium was removed and the adherent cells were washed twice with $1 \times \text{PBS}$ buffer. The **nanoREDs** in DMEM medium at 25 $\mu\text{g/mL}$ were then added in different wells, respectively. After incubation for 12 h, the cells were washed five times with $1 \times \text{PBS}$ buffer and then fixed with 75% ethanol for 10 min. After washing three times, nucleus was stained by DAPI for 10 min at room temperature. Then samples were washed three times, added in fresh $1 \times \text{PBS}$ and imaged by confocal laser scanning microscopy (CLSM), sequentially. Lasers of 405 and 517 nm were used to excite DAPI and **nanoREDs**, respectively. The corresponding fluorescence emissions were recorded through a confocal microscope (LSM 780, Carl Zeiss, Germany) using a band-pass filter combination including 421-481 nm, 530-700 nm for imaging in two individual channels (Objective: LD Plan-Neofluar 20x/0.4 Korr M27).^{41,42}

Cytotoxicity study The cytotoxicity of **nanoREDs** against L929 cancer cells was evaluated by Presto blue (PB) assay. Briefly, L929 cells were seeded in 96-well

plates (Costar, IL, USA) at an intensity of 1×10^4 cells mL^{-1} . After 24 h incubation, the cells were exposed to a series of doses of **nanoREDs** at 37 °C. After the designated time intervals, the wells were washed twice with $1 \times$ PBS buffer and 100 μL of freshly prepared PB solution in culture medium was added into each well. The PB medium solution was carefully removed after 1 h incubation in the incubator. The absorbance of PB at 570 nm and 600 nm was monitored by the microplate reader (Genios Tecan). Cell viability was expressed as the ratio of the percentage of PB reduction of the cells incubated with **nanoREDs** suspension to that of the cells incubated with culture medium only.

4.5 Supporting information

Figure S4.1 ^1H NMR (300 MHz, CDCl_3) spectrum of DHTBT^{out}.

Figure S4.2 ^1H NMR (300 MHz, CDCl_3) spectrum of DHTBTⁱⁿ.

Figure S4.3 ^1H NMR (300 MHz, CDCl_3) spectrum of 11-bromo-1-undecanol-DHP.

Figure S4.4 ^1H NMR (300 MHz, CDCl_3) spectrum of F-DHP.

Figure S4.5 ^1H NMR (300 MHz, CDCl_3) spectra of $\text{PFTB}_{10}^{\text{in}}$ -DHP, $\text{PFTB}_{10}^{\text{in}}$ -OH and $(\text{PFTB}_{10}^{\text{in}}\text{-g-(PCL}_{24})_2)_{10}$.

Figure S4.6 GPC traces of a series of PFTBⁱⁿ-g-PCL polymers with (a) RI and (b) UV-vis (550 nm) detectors.

Table S4.1 Size distribution data of nanoRED^{out} with different sizes from DLS measurement.

Mean size (nm)	Width (nm)	PDI
80.2	26.6	0.110
64.3	18.1	0.079
44.7	15.9	0.127
52.9	16.9	0.102
228.7	7	0.001
436.0	7.3	0.0003

Figure S4.7 Representative TEM images of nanoRED^{out}s with an average diameter of (a) 80 nm, and (b) 450 nm which were prepared with different weight ratios of (PFTB^{out}-g-(PCL₁₃)₂)₁₀/PCL₃₇-*b*-POEGMA₆₀ at 10/15 and 10/1, respectively.

Figure S4.8 Photographs of conjugated polymer brushes (A) (a) PFTB^{out}₂₀, (B) (b) (PFTB^{out}-g-(PCL₁₃)₂)₁₀, (C) (c) (PFTB^{out}-g-(PCL₅₉)₂)₁₀ and (D) (d) (PFTB^{out}-g-(PCL₈₈)₂)₁₀ in bulk solid state and NPs dispersion under ambient light (top) and UV light (bottom, excited at 365 nm).

Figure S4.9 Fluorescence decay profiles for PFTB-based Pdots and nanoREDs in aqueous media. (a) nanoRED1^{out}; (b) nanoRED1ⁱⁿ, (c) Pdot^{out} and (d) Pdotⁱⁿ solutions.

Figure S4.10 Ortho-view fluorescence (a, c) and white-contrast (b, d) images of z-stack for **nanoRED1^{out}**-stained L929 (a, b) and HeLa (c, d) cells, showing **nanoREDs** inside the cells. The scale bar represents 50 μm .

Figure S4.11 Confocal fluorescence microscope images of (a) L929 cells and (b) HeLa cells labeled with **nanoRED1ⁱⁿ**. The concentrations of **nanoREDs** are 25 $\mu\text{g/mL}$. The fluorescence of DAPI and NPs is pseudo-labeled with blue and red, respectively. The scale bar represents 50 μm .

Figure S4.12 Ortho-view fluorescence images of z-stack for **nanoRED1^{out}**-stained L929 (a) and HeLa (b) cells, showing **nanoREDs** inside the cells. The scale bar represents 50 μm .

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Chapter 5 Tunable Förster Resonance Energy Transfer in Colloidal nanoparticles of Polycaprolactone-Tethered Donors and Acceptors: Enhanced Near-Infrared Emission for Biological Imaging

Chapter 4 has reported that the introduction of polycaprolactone (PCL) effectively improved the quantum yield of donor PFTB in colloidal nanoparticles. This chapter extends the study on FRET system by importing PCL-PPcy-PCL into PFTB-g-PCL matrix and explores the influence of incorporated PCL on their optical properties.

5.1 Introduction

FRET has been reported in the physical blends of NIR dyes as acceptors mixed with an emissive matrix of small molecular¹ or polymeric^{2,3} fluorophores as donors. For instance, an NIR fluorescent dye, silicon 2,3-naphthalocyanine bis(trihexyldilyloxi) (denoted as NIR775), as an acceptor was formulated together with a visibly fluorescent π -conjugated polymer as donor into colloidal nanoparticles in aqueous media. However, the efficiency of the energy transfer in these nano-scale physically blended FRET systems is limited by the small overlap between the donor emission and the acceptor absorption, the aggregation-induced fluorescence quenching of both the donor and the acceptor, as well as the leaching of small-molecular NIR dye due to the unfavorable compatibility of the donor and the acceptor.^{4,5}

Scheme 5.1 (a) Chemical structure of PPcy, PCL-PPcy-PCL and PFTB-g-PCL; (b) Schematic illustration showing NIR fluorescent NPs. Facilely encapsulating PCL-PPcy-PCL with PCL-*b*-POEGMA by co-precipitation affords NPs with J-aggregation enhanced NIR emission (middle). Using same method, PFTB-g-PCL conjugated polymer brushes are incorporated for co-precipitation to afford FRET NPs (right) with unusual strong NIR emission and large Stokes shift.

In this chapter, we present a strategy of polymer-tethered donor/acceptor to overcome the aforementioned limitations in FRET, particularly in the NIR region that is ideal for noninvasive *in vivo* bioimaging. Specifically, we employed red-emissive conjugated polymer brushes polycaprolactone-*grafted* poly(fluorene-*alt*-(4,7-bis(hexylthien)-2,1,3-benzothiadiazole)) (PFTB-*g*-PCL) as the donor and PCL-tethered pyrrolopyrrole cyanines (PCL-PPcy-PCL) as the acceptor. These hydrophobic PCL-decorated donor and acceptor were encapsulated into the hydrophobic compartment of colloidal nanoparticles (NPs) formed by a synthetic amphiphilic block copolymer polycaprolactone-*b*-poly[oligo(ethylene glycol) methyl ether methacrylate] (PCL-*b*-POEGMA) in aqueous media (Scheme 5.1). PCL plays a crucial role in not only minimizing the aggregation-induced fluorescence quenching of the donor and the acceptor, but also enhancing the interfacial compatibility, physical stability, and biocompatibility. The obtained FRET NPs exhibit excellent optical properties including large Stokes shift of ~233 nm, high FL quantum yield of ~45 %, maximum emission wavelength of 760 nm, an emission full width at half maximum (FWHM) of ~53 nm. Especially, its FL quantum yield doubles that of the FRET platform without incorporation of PCL. Furthermore, an excellent colloidal stability was observed in PCL-decorated FRET system, in contrast to the significant leakage of the acceptor in the absence of PCL. We further demonstrated the application of these FRET-based NPs for NIR *in vitro* cellular imaging as well as animal imaging at both *in vivo* and *ex vivo* level.

5.2 Results and discussion

Our criteria of selecting the donor and the acceptor to achieve a FRET probe with bright and stable NIR emission was based on the equation below on the rate of energy transfer ($k_T(r)$)⁶:

$$k_T(r) = \frac{1}{\tau_D} \left(\frac{R_0}{r} \right)^6 = \frac{Q_D \kappa^2}{\tau_D r^6} \left(\frac{9000 (\ln 10)}{128 \pi^5 N n^4} \right) J(\lambda) \quad (1)$$

Where τ_D and Q_D are the natural lifetime and fluorescence quantum yield, respectively R_0 is the Förster distance; r is the donor-to-acceptor distance, κ^2 is the

orientation factor, N is Avogadro's number, n is the refractive index, $J(\lambda)$, the overlap integral expresses the degree of the spectral overlap between the donor emission and the acceptor absorption.

Firstly, we chose PFTB-*g*-PCL as the donor for the FRET based on our previous discovery in which the colloidal NPs (called “**nanoREDs**”) composed of PFTB-*g*-PCL showed a FL quantum yield four times higher than that of bare PFTB as well as significantly improved photostability.^{7,8} Therefore, we expected that PFTB-*g*-PCL with the preserved FL intensity and enhanced photostability would serve as an ideal donor for efficient FRET. Secondly, we chose a family of PPcy fluorophores as the acceptor for FRET owing to their tunable emission wavelengths in the NIR region (up to 1000 nm) and relatively high fluorescence quantum yield (up to 70%).⁹⁻¹¹ Thirdly, the relatively high extent of spectral overlap between the emission of PFTB as the donor and the absorption of PPcy as the acceptor would facilitate highly efficient FRET. We further synthesized PCL-PPcy-PCL in order to minimize the aggregation-induced fluorescence quenching of PPcy and enhance its interfacial compatibility with the donor, PFTB-*g*-PCL.^{12,13} Finally, the bioresorbable nature of PCL¹⁴ in the present donor/acceptor system is expected to improve the biocompatibility and biodegradability of the imaging probes for future clinical applications.

5.2.1 Structural characterization of PFTB-*g*-PCL and PCL-PPcy-PCL

This section describes the synthesis and characterization of the acceptor PCL-PPcy-PCL and the macromolecular surfactant PCL-*b*-POEGMA. Particularly, the synthetic route to conjugated polymer bottlebrush PFTB-*g*-PCL and PCL-*b*-POEGMA has been described in Chapter 4.⁷ In this chapter, we chose two polymer brushes (PFTB-*g*-(PCL₁₃)₂)₁₀ and (PFTB-*g*-(PCL₅₉)₂)₁₀ (denoted as **B1** and **B2**, respectively, Scheme 5.1) with different lengths of grafted PCL chains as the donor matrix. The results of gel permeation chromatography (GPC) using linear polystyrenes as standards gave a number average molecular weight $M_n = 40,900$

g/mol, $M_w/M_n = 1.89$ for **B1**, $M_n = 115,500$ g/mol, $M_w/M_n = 1.61$ for **B2**, and $M_n = 31,100$ g/mol, $M_w/M_n = 1.37$ for PCL-*b*-POEGMA (Table 5.1).

Scheme 5.2 Synthetic route to PCL-PPcy-PCL polymers.

Table 5.1 Molecular weights and polydispersity indices (PDI) of a series of polymers including PCL-PPcy-PCL and PFTB-*g*-PCL

Entry	Sample ^a	$M_{n,\text{NMR}}^b$	$M_{n,\text{GPC}}^c$	PDI ^d
P0	PPcy	--	--	--
P1	PCL ₂₅ -PPcy-PCL ₂₅	6,861	6,700	1.30
B0	PFTB ₁₆		17,600	1.90
B1	(PFTB- <i>g</i> -(PCL ₁₃) ₂) ₁₀	40,700	40,900	1.89
B2	(PFTB- <i>g</i> -(PCL ₅₉) ₂) ₁₀	145,800	115,500	1.61

Note: ^a DP_n of conjugated alternating units (FTB) was calculated by GPC results, DP_n of CL units was calculated by ¹H NMR results based on PPcy or DP_n of FTB; ^b $M_{n,\text{NMR}}$ was calculated by ¹H NMR results; ^{c,d} $M_{n,\text{GPC}}$ was calculated by GPC results

The general synthetic routes to the modified acceptor PCL-PPcy-PCL are illustrated in Scheme 5.2. We have reported the synthesis of bromo-functionalized

PPcy (Compound **1** in Scheme 5.2) in Chapter 3,¹¹ which was utilized to react with 4-benzyl alcohol boronic acid by Suzuki cross-coupling to afford OH-functionalized PPcy (Compound **2**). The hydroxyl groups on PPcy served as initiating sites to induce the ring opening polymerization of ϵ -caprolactone in the presence of stannous octoate ($\text{Sn}(\text{Oct})_2$) as the catalyst, resulting in the target polymer PCL-PPcy-PCL.

The chemical structures of Compound **1**, **2** and PCL-PPcy-PCL were characterized using ^1H NMR spectroscopy (Fig. S5.1). After introducing the initiator of ROP, the signals at $\delta \sim 4.78$ ppm appears in the spectrum of Compound **2**, which could be assigned to the ethylene protons of the benzyl alcohol group. The grafting of PCL brought two strong peaks at $\delta = 2.32$ and 4.08 ppm, while the signals at $\delta = 6.5 \sim 9$ ppm corresponding to aromatic protons could be clearly observed in the spectrum of PCL-PPcy-PCL. The appearance of these two peaks was typically assigned to the protons of the two methylene groups near the ester moiety. According to the integration ratio of the signal at 2.32 ppm over that at 8.51 ppm, the absolute number-average degree of polymerization (DP_n) of PCL was determined as 25. The GPC analysis of $\text{PCL}_{25}\text{-PPcy-PCL}_{25}$ (denoted as **P1**) gave $M_n = 6,700$, and $\text{PDI} = 1.30$ as shown in Table 5.1 and Figure S5.2.

5.2.2 FRET in Colloidal NPs of **B1P1**

In dilute chloroform solutions, **P1** exhibits the typical spectroscopic feature of pyrrolopyrrole cyanine chromophores (Figure 5.2a). Specifically, an absorption maximum ($\lambda_{\text{abs,max}}$) of **P1** was observed at 732 nm corresponding to the $\text{S}_0\text{-S}_1$ transition, while its emission band centered at 745 nm ($\lambda_{\text{em,max}}$) is a mirror image of the $\text{S}_0\text{-S}_1$ absorption band. Both the absorption and the emission bands are quite narrow with full-width-at-half-maximum (FWHM) values of 33 nm and 46 nm, respectively. Interestingly, the FL quantum yield of **P1** in chloroform was determined as 0.78, which is obviously higher than that of PPcy in the absence of PCL (0.56)¹¹. This behavior may result from the restriction of intramolecular motion of PPcy backbone by the bulky PCL chains, leading to the blockage of the nonradiative pathways and the opening of the radiative channel accordingly.¹⁵ It

indicates that **P1** is a promising candidate of guest to relax the resonance energy from the host mainly in the form of radiation.

The **B1** and **B2** based colloidal NPs reported in our previous work can be excited in the green wavelength region (e.g., 520 nm) and emit fluorescence at 635 nm with a high quantum yield of 18.1% and 24.8% for **B1** and **B2**, respectively, where the longer PCL side chains resulted in a higher FL quantum yield.⁷ Hence, conjugated polymer brushes **B1** and **B2** offer a better choice to serve as host matrix than bare PFTB (FL quantum yield (Φ) of PFTB NPs: $\sim 4.68\%$).⁷ Besides, the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor also determines the efficiency of FRET. As shown in Figure 5.2a, the intersection of the shadowed area suggests that the emission spectrum of the **B1** host overlaps well with the absorption spectrum of **P1**.

Figure 5.2 (a) Normalized emission and absorption spectra of **B1** based NPs (nanoREDs) and **P1** in chloroform. The intersection of shadow part in (a) represents the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor; (b)

Optical absorption spectra, (c) FL emission spectra of **B1P1** FRET NPs with increased mass ratios between **B1** and **P1** polymers. The shadow patterned spectra for comparison in (b) corresponds to the absorption spectra of **P1** in chloroform; (d) the dependence of the fluorescence intensity on mass ratios between **B1** and **P1**. The dotted box in (d) represents NPs with a mass B1/P1 mass ratio of 100:12.5 as called B1P1^{1/2} whose FL intensity (I_{760}) at 760 nm nearly doubles that (I_{630}) at 630 nm.

The aforementioned characteristics of the donor **B1** and the acceptor **P1** implies an appropriate host-guest pair to enhance the NIR fluorescence through FRET. To examine this hypothesis, we prepared a series of colloidal NPs composed of different ratios of **B1/P1** in aqueous media which are suitable for bioimaging applications. Similar to the method developed to obtain **nanoREDs** particles in our previous work,⁷ a mixture of **B1**, **P1**, PCL-*b*-POEGMA co-dissolved in 1 mL of THF was rapidly injected into 10 mL of water, followed by exposure in air at room temperature overnight to remove THF. The mass ratio of **P1** to **B1** was varied from 0:100 to 15:100 gradually while the amounts of **B1** and PCL-*b*-POEGMA were fixed.

The optical properties of the as-prepared **B1/P1** NPs were characterized using both absorption and fluorescence spectroscopies. As shown in Figure 5.2b, with increasing amounts of **P1**, the absorbance at 738 nm increased. A red shift (6 nm) of $\lambda_{abs,max}$ related to **P1** was observed with respect to that of **P1** in chloroform, which could be interpreted by the less excitonic structure distortions of the PPcy backbones (Fig. 5.2b).¹⁶ The emission intensity of PFTB and PPcy was also modulated as the mass concentration of **P1** was varied (Fig. 5.2c). With increasing mass ratio of **P1/B1** from 0/100 to 4/100, the PFTB emission centered at 630 nm is gradually quenched, while the PPcy emission band at 756 nm is gradually enhanced. However, further increase in the mass ratio of **P1/B1** in FRET NPs from 4/100 to 15/100 lead to reduced NIR emission intensity while the emission from PFTB kept declining. The weakening NIR emission might be attributed to the occurrence of homo resonance energy transfer (HRET). HRET is a common phenomenon for fluorophores which display small stokes shift.^{6,17,18} With the increased number of **P1** in the interior of NPs, HRET is gradually predominant to

quench the NIR emission due to the smaller distance separating the PPcy molecules. The variation tendency was more clearly demonstrated in Figure 5.2d, which showed the dependence of emission intensity at 635 and 750 nm on the mass concentration of **P1**.

5.2.3 **B1P1^{1/2}** NPs with the optimal ratio for FRET

The results shown in Figures 5.2(c-d) suggest that an optimal ratio of **P1/B1** by mass to achieve the acceptor-dominating fluorescence is 12.5/100, at which the FL intensity (I_{760}) at 760 nm with a FWHM of 53 nm nearly doubles that (I_{630}) at 630 nm (Fig. 5.3a). Such a narrow emission band with a balanced maximal intensity at 760 nm and minimal interference from the donor emission will be ideal for bioimaging presented later, particularly for multiplexed fluorescence imaging. For simplification, the sample of the colloidal NPs with this ratio is denoted as **B1P1^{1/2}**.

As a consequence, we carried out a detailed characterization as following for the **B1P1^{1/2}** NPs before we explored their potential for bioimaging applications. The excitation spectrum of **B1P1^{1/2}** NPs as shown in Figure 5.3a depicts that the FL intensity at 760 nm from PPcy was enhanced by 7 folds via energy transfer from PFTB as compared to that upon direct excitation at 730 nm, indicating the high efficacy to transfer energy to enhance the NIR emission.

Lifetime measurements were also conducted for **B1** based NPs in the absence and the presence of **P1** (Fig. 5.3e and 5.3f). Before doping **P1**, the maximum emission of **B1** based NPs at 630 nm exhibits an initial fluorescence lifetime of 3.86 ns. For **B1P1^{1/2}** FRET NPs, the lifetime of PFTB decreased to 1.28 ns. Dealing here with a solution of donor-acceptor pairs which are not free to move randomly in confined space and thus are separated by a fixed distance,^{6,19} the energy transfer efficiency (η) calculated according to Equation (3) below is 69%:

$$\eta = 1 - \tau_{DA}/\tau_D \quad (2)$$

where τ_{DA} denotes the average lifetime of the donor in the presence of acceptor. Besides, the Forster radius (R_0) of this FRET pair is calculated to be 5.4 nm according to Equation (3):

$$R_0^6 = \frac{9000(\ln 10)\kappa^2 Q_D}{128\pi^5 N n^4} J(\lambda) = 8.79 \times 10^{-5} (\kappa^2 n^{-4} Q_D J(\lambda)) \quad (3)$$

where Q_D of the donor **B1** NPs is 18.1%, n is taken to be 1.33 for water, $J(\lambda)$ is estimated to be $7.33 \times 10^{15} \text{ M}^{-1}\text{cm}^{-1}\text{nm}^4$ according to Equation (4):

$$J(\lambda) = \int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda = \frac{\int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda} \quad (4)$$

where $F_D(\lambda)$ is the normalized fluorescence spectrum of donor **B1** based NPs and $\varepsilon_A(\lambda)$ is the extinction coefficient of the conjugated core of **P1**, i.e. PPcy-B¹¹ with its chemical structure shown in Figure S5.

Moreover, further increase of **P1** fraction to a mass ratio of **P1/B1** ~15/100 led to a shorter lifetime of 0.54 ns, where a high energy transfer efficiency of 86% was achieved. We have observed a simultaneous decrease of the lifetime of PPcy emission at 760 nm from 3.10 to 2.06 ns upon the increase of **P1/B1** ratio from 12.5/100 to 15/100. This result is convincing evidence for the significant HRET phenomenon which results in the quenching of NIR emission at 760 nm with the increase of **P1** concentration in the matrix.²⁰

The FL quantum yield (Φ) of **B1P1**^{1/2} FRET NPs was estimated based on the integration of the emission intensity in the range of 700 to 850 nm, which is mainly contributed by the NIR emission of PPcy. **P0** (chemical structure is shown in Scheme 5.1) in chloroform with Φ of 0.69 as reported in our previous work was chosen as the standard. The Φ of **B1P1**^{1/2} FRET NPs in aqueous solutions was determined to be 0.46, which is the highest FL quantum yield in currently reported NIR probes with emission near 800 nm to the best of our knowledge. Importantly, **B1P1**^{1/2} FRET NPs shows a much larger Stokes shift of 233 nm ($\lambda_{ex,max} = 517$ nm and $\lambda_{em,max} = 750$ nm), thus reducing possibility of the crosstalk between the excitation source and the NIR fluorescence emission.

To assess the stability of the **B1P1**^{1/2} FRET NPs, the evolution of the FL intensity was monitored during incubation with phosphate buffer solution (PBS) at 37 °C over different periods. As shown in Figure 5.3b, there was no obvious change in the FL intensity of **B1P1**^{1/2} FRET NPs and the $I_{760\text{nm}}/I_{630\text{nm}}$ ratio was remarkably stable after 7-day incubation with 1×PBS at 37 °C, revealing that the optical properties of **B1P1**^{1/2} FRET NPs were not affected by increasing the aging time. As the transfer efficiency and FL intensity is quite sensitive to the proportion of acceptors, the extremely low leakage of the acceptor in this FRET system could be directly reflected by little change of the spectral features. Such excellent stability can be ascribed to reliable entanglement and poor chain mobility of donor and acceptor endowed by the semicrystalline PCL chains,^{21,22} resulting in strongly “locking” the guest in the interior. Additionally, TEM image (Fig. 5.3c) showed that **B1P1**^{1/2} FRET NPs existed as spherical NPs in aqueous medium with an average diameter of 45.1 ± 10.9 nm, which is lower than that (60 nm) from DLS result (Fig. 5.3d). The size of these colloidal NPs can be further mediated simply by varying the feed ratio of PCL-*b*-POEGMA over **B1P1** in the nanoprecipitation process.⁷

Figure 5.3 (a) FL emission and excitation spectra of **B1P1**^{1/2} FRET NPs with I_{620}/I_{760} close to 0.5; (b) Evolution of the FL intensity of **B1P1**^{1/2} FRET NPs in PBS buffer at 37 °C over 7 days; (c-d) TEM images (c) and DLS histogram (d) of **B1P1**^{1/2} FRET NPs dispersed in water. (e-f) Fluorescence decays at (e) 620 nm and (f) 760 nm in **B1P1** FRET NPs with different **B1/P1** mass ratios of 100/0, 100/12.5 and 100/15, respectively. Excitation wavelength: 530 nm.

To investigate the effect of PCL length on the FL quantum yield of FRET NPs, we also prepared **B2P1** FRET NPs by replacing **B1** with **B2**, where **B2** possess longer PCL as side chains (Tab. 5.1). Figure S5.5 shows the variation of emission

spectrum of **B2P1** FRET NPs by changing the P1 concentration. When the mass ratio of **P1/B2** reached 5/100, the value of I_{630}/I_{758} of 1/2 was achieved (Fig. S5.5a). In the excitation spectra of **B2P1**^{1/2} FRET NPs, the FL intensity at 760 nm upon the excitation at 530 nm is only 2.8 times higher than that excited at 730nm (Fig. S5.5b). It may be attributed to the bulkier PCL moiety in **B2**, results in enlarging the inter-distance between PPcy cores, suppressing the self-quenching and further enhancing the emission of **P1** in **B2P1** as compared to **B1P1**. As shown in Table 5.2, the FL quantum yields of **B2P1**^{1/2} FRET NPs were determined to be 0.57, which is slightly higher than that of **B1P1**^{1/2}. This behavior is likely attributed to the higher quantum yield of **B2** host matrix.⁷ However, since the PCL chains on **B2** is approximately five times longer than that on **B1**, the per-particle brightness of **B2P1**^{1/2} NPs would be much lower than that of **B1P1**^{1/2} NPs.

5.2.4 FRET in B0P0 colloidal NPs

To examine the role of the conjugation of PCL to donor and acceptor, bare PFTB (**B0**) and PPcy (**P0**) was employed as a colloidal dispersion for a control. First, we used the same calculation procedure as discussed above and estimated the Förster radius (R_0) of this pair to be 4.5 nm, mainly due to the lower quantum yield of **B0** based NPs. The emission spectra of conjugated polymer NPs of **B0** and the absorption spectra of **P0** in chloroform also showed a good overlap with a comparable overlap integral $J(\lambda)$ of $8.95 \times 10^{15} \text{ M}^{-1}\text{cm}^{-1}\text{nm}^4$ as shown in Figure S5.6.

We firstly prepared NPs consisting of the mixture of **B0** and **P0** in the absence of macromolecular surfactant via nanoprecipitation^{23,24} to give a series of dispersions with different **P0** fractions. Unfortunately, during the preparation process, some precipitates were found in the dispersion, indicating a poor colloidal stability in the absence of surfactant. It was observed that a high **P0** content at the **P0/B0** ratio of 30/100 was required to quench the emission of **B0** to reach the I_{630}/I_{738} ratio of 1/2 (Fig. 5.4b & 5.4c). Interestingly, a small shoulder peak at a longer wavelength of 772 nm was observed in the emission spectra of **B0P0** dispersion at **P0/B0**=30/100, indicating the coexistence of J-aggregates of **P0** with

monomeric **P0** in the interior.¹¹ With the further increase of **P0** mass fraction, the emission from monomeric **P0** continued to attenuate while the peak related to J-aggregates became stronger. It could be confirmed by the typical signature of J-band centered at 771 nm in the absorption spectra (Fig. 5.4a), accompanied by the appearance of the H-band of **P0** at 653 nm, which is in accordance with the spectral feature of **P0** based NPs (called **JAGG** NPs, as shown in Fig. S5.7a) reported in our previous work¹¹ (**JAGG** NPs was facilely prepared by encapsulating **P0** with the same macromolecular surfactant). It indicates the excitonic energy from individual **PFTB** monomers also can be transferred to H-aggregates and even J-aggregates, since the absorption spectra of **JAGG** NPs, especially H-band and M-band, overlaps well with the emission spectra of the **B0** matrix (Fig. S5.7a).

We previously found that the NIR emission of **JAGG** NPs is mainly contributed by J-aggregates of **P0** owing to the extremely weak fluorescence of H-aggregates and the FRET from monomer to J-aggregates.¹¹ In contrast, for the **B0P0** system at **P0/B0**=30/100 (w/w), the FL intensity of J-aggregates is lower than that of monomeric **P0** (Fig. 5.4c). It is likely due to the extremely small fraction of J-aggregates in the **B0** matrix, limited resonance with **B0** and large distance from monomeric **P0** as explained in Figure S5.7c. However, upon excitation at 635 nm, the dispersion showed a sharp resonance emission of J-aggregates at 773 nm (Fig. 5.4c). These results suggest the poor interfacial compatibility between **B0** and **P0**. Owing to the mixed fluorescence from monomeric and J-aggregated **P0**, **B0P0** dispersion at **P0/B0**=30/100 exhibits a large FWHM of 70 nm.

Figure 5.4 (a) Absorption and (b) emission spectra spectra of **B0P0** FRET NPs in the absence of surfactant with increased mass ratios between **B0** and **P0**; *H*, *M* and *J* in (a) correspond to H band, M band and J band assigned to H-aggregated, monomeric and J-aggregated state of **P0**, respectively. (c) emission ($\lambda_{\text{ex}} = 520$ nm and 635 nm) and excitation ($\lambda_{\text{em}} = 735$ nm) spectra of

B0P0^{1/2} FRET NPs in the absence of surfactant whose $F_{630\text{nm}}/F_{735\text{nm}}$ is close to 0.5 upon the mass ratio **P0/B0** of 30/100; (d) Fluorescence intensity evolution of **B0P0**^{1/2} FRET NPs in the absence of surfactant was tested in PBS buffer at 37 °C for 7 days. (e) Absorption and (f) Emission spectra spectra of **B0P0** FRET NPs in the presence of surfactant with increased mass ratios between **B0** and **P0**. (g) emission ($\lambda_{\text{ex}} = 520 \text{ nm}$ and 635 nm) and excitation ($\lambda_{\text{em}} = 735 \text{ nm}$) spectra of **B0P0**^{1/2} FRET NPs in the presence of surfactant whose $F_{630\text{nm}}/F_{735\text{nm}}$ is close to 0.5 upon the mass ratio **P0/B0** of 2/100; (h) Fluorescence intensity evolution of **B0P0**^{1/2} FRET NPs in the presence of surfactant was tested in PBS buffer at 37 °C for 4 days. The insets in (a) and (e) represent the architecture model of **B0P0** in the absence and presence of macromolecular surfactant, respectively.

In order to improve the colloidal stability of the **B0P0** NPs described above, we further prepared another batch of colloidal NPs with the same polymers in the presence of a macromolecular surfactant PCL-*b*-POEGMA, and studied their optical properties in comparison with the bare NPs aforementioned without extra stabilizer. A series of transparent dispersions were obtained without precipitates. The variation of fluorescence spectra of **B0P0** FRET dispersions dependent on different fractions of **P0** was shown in Figure 5.4f. Different from that in the absence of surfactant, the emission from **B0** host was dramatically quenched by a small mass fraction of **P0** thanks to the stabilization of surfactant, where **B0P0**^{1/2} FRET NPs with $I_{630}/I_{738} = 1/2$ was achieved at **P0/B0** = 2/100. When **B0P0**^{1/2} FRET NPs were excited at 635 nm, the emission spectrum was almost identical with the monomeric one in chloroform (Fig. 5.4g), indicating that the uniformly distribution of **P0** in form of monomers in the host matrix. The absorption spectra of **P0** component always show the typical monomeric feature even at the high mass ratio **P0/B0** of 5/100 as shown in Figure 5.4e. In the excitation spectrum of **B0P0**^{1/2} FRET dispersion, the FL intensity at 735 nm excited at 530 nm is up to 35 times higher than that excited at 720 nm (Fig. 5.4g). Evidently, the energy transfer in the **B0P0** system is more efficient than that in the **B1P1** system. This is due to the direct D/A contact in the absence of PCL, resulting in the short distance for excitons on **PFTB** to encounter the quencher molecules.

However, the **B0P0**^{1/2} FRET NPs in the presence and absence of surfactant possess a much lower quantum yield (0.26 and 0.22, respectively) than that of **B1P1**^{1/2} FRET NPs owing to the low quantum yield of both donor and acceptor in the aggregated states in the former. Besides, we applied the same method to incubate **B0P0**^{1/2} FRET NPs in PBS solutions at 37 °C over different periods and observed the serious leaching of entrapped **P0**. As shown in Figure 5.4d & 5.4h, with increasing of incubation time, the fluorescence of the donor PFTB gradually recovered while the NIR emission band with maximum at 735 nm decreased. Especially for **B0P0** with surfactant, the NIR emission completely vanished after only 4 days of incubation (Fig. 5.4h). Simultaneously, upon the excitation at 635 nm, the emission at 735 nm also sharply decreased after 24 h of incubation at 37 °C, accompanying the disappearance of the absorption band of PPcy in the corresponding absorption spectra (Fig. S5.9a). These results imply that PPcy probably escaped from the NPs. The IVIS (in vivo image system) fluorescence images as shown in Figure 5.5a further illustrated that the fluorescence signal of the **B0P0**^{1/2} dispersion was much weaker than that of the **B1P1**^{1/2} and **B2P1**^{1/2} dispersions at the same mass concentration of 1 mg/mL due to the low quantum yield and leakage of donor. Therefore, we can conclude that the absence of PCL chain grafted to PFTB and PPcy led to the distortion of the emission spectra, the reduction of quantum yield and poor stability with respect to **B1P1** system.

Table 5.3 Stokes shift and fluorescence quantum yields of FRET NPs series.

Sample	P0 (CHCl ₃)	P1 (CHCl ₃)	B1P1 ^{1/2} (water)	B2P1 ^{1/2} (water)	B0P0 ^{1/2} (water)
<i>Stokes shift/nm</i>	15	13	233	228	204
Φ / % ^a	69.0	77.7	45.5	56.7	26.1 (w ^b) 22.0 (wl ^c)

Note: ^a Quantum yields of solutions were measured by the standard method using PPcy in chloroform (Φ_F = 0.69, which is determined by using ICG as reference reported in our previous work). Here, we used integrated fluorescence intensity from 700 nm to 850 nm instead of full range for calculation of quantum yield because

this range mainly is contributed by acceptors. ^{b,c} *w* and *w*/*l* represent the B0P0 system with and without surfactant, respectively.

5.2.5 *In vitro* and *in vivo* imaging with B1P1^{1/2} NPs

Figure 5.5 (a) A digital photograph of **B0P0**^{1/2}, **B1P1**^{1/2} and **B2P1**^{1/2} FRET NPs at 1 mg/mL in 1× PBS at pH=7.4 over 1 day post preparation under ambient light (top) and the IVIS imaging system (bottom, $\lambda_{ex} = 535 \text{ nm}$, $\lambda_{em} = 760 \text{ nm}$). (b) Viability values (%) of L929 cells estimated

by PrestoBlue assay versus concentrations of **B1P1**^{1/2} FRET NPs after incubation for 24 and 48 h, respectively. Data represent mean $\pm$ standard error (n=5). (c) CLSM photos of L929 cells incubated with **B1P1**^{1/2} FRET NPs. The scale bar represents 50 μ m. (d) Whole-body NIR imaging with **B0P0**^{1/2} and **B1P1**^{1/2} FRET NPs at time points from 0.5 h to 72 h post injection. (e) *Ex vivo* NIR imaging with **B1P1**^{1/2} FRET of major organs and tumor tissues at 72 h post intravenous injection (λ_{ex} = 535 nm, λ_{em} = 760 nm). (f) The distribution profiles of **B0P0**^{1/2} and **B1P1**^{1/2} FRET NPs in excised organs based on the mean fluorescence intensity at 72 h post injection. Data are given as mean $\pm$ standard error (n=2).

Considering the advantages of high stability, high quantum yield, long emission maximum and Stokes shift, we chose **B1P1**^{1/2} FRET NPs for *in vitro* and *in vivo* imaging application. Firstly, the cytotoxicity of **B1P1**^{1/2} FRET NPs against mouse fibroblast cells (L929) was studied by PrestoBlue assays. Figure 5.5b summarizes the *in vitro* L929 cell viability after incubation with **B1P1**^{1/2} FRET NPs at the concentration of 10, 20, 40, 60, 80 μ g/mL for 24 and 48 h, respectively. No obvious change of the metabolic viability of L929 cells was observed after incubation with **B1P1**^{1/2} FRET NPs even at a high concentration of 80 μ g/mL, indicating the low cytotoxicity of **B1P1**^{1/2} FRET NPs.

Fluorescence imaging based on **B1P1**^{1/2} FRET NPs was investigated with confocal laser scanning microscopy (CLSM). After incubating the L929 cells with the **B1P1**^{1/2} FRET NPs solution (50 μ g/mL) for 12 h, the cells were fixed and their nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Figure 5.5c shows the images of L929 cells stained by **B1P1**^{1/2} NPs from CLSM, where **B1P1**^{1/2} NPs were well internalized by L929 cells. Especially in the ortho-view images of z-stack (Fig. S5.10), a uniform distribution of **B1P1**^{1/2} NPs with strong red fluorescence throughout the cellular cytoplasm was observed for L929 cells, implicating that **B1P1**^{1/2} NPs are efficiently accumulated in the cytoplasm of L929 cells.

To demonstrate the feasibility of using **B1P1**^{1/2} FRET NPs for tumor imaging in living mice as well as the advantages compared with **B0P0**^{1/2}, a $\sim$ 100 μ L intradermal injection of **B1P1**^{1/2} and **B0P0**^{1/2} (1 mg/mL) at 24 h post preparation was performed on the tail of immunodeficient nude mice (Fig. 5.5d) with shoulder

xenograft HepG2 tumours (n=2). Immediately following the injections of NPs, fluorescence images of the whole body via IVIS were acquired continuously. At 30 min post-injection, as the NPs were introduced rapidly throughout the bloodstream, the mice were brightly lightened up by **B1P1**^{1/2} NPs and the fluorescence intensity in the tumor area was stronger than that in neighboring area, implying that **B1P1**^{1/2} NPs with a relatively small size of 60 nm prefer to penetrate into tumor via enhanced permeability and retention (EPR) effect.²⁵

To monitor the biodistribution of **B1P1**^{1/2} NPs in the mice after the tail-injection, the whole-body bright-field and fluorescence images were collected at multiple time points ranging from 30 min to 72 h post injection (Fig. 5.5d). After 9 h of post injection, strong fluorescence signals emanating from tumor and bladder were observed. At 24 h post injection, the decay of fluorescent signal was observed, indicating the clearance of the NPs prevailed over their accumulation.²⁶ However, the retention of NPs in tumor in the period from 24 to 72 h post-injection still allowed us to distinguish tumor from normal tissues based on the stronger fluorescence in tumor, providing a possibility for long-term tumor-specific imaging. As a control, the fluorescence image of mice administered with **B0P0**^{1/2} dispersion in PBS showed a large contrast with that of **B1P1**^{1/2}, where the fluorescence signal for **B0P0**^{1/2} was much weaker, although the accumulation of **B0P0**^{1/2} NPs could be also recognized.

At 72 h post injection, the mice were sacrificed and the *ex vivo* biodistribution was quantified based on the fluorescence images of the harvested organs. As expected, the liver and the tumor were the main source of fluorescence signals over the 72 h treatment period (Fig. 5.5e). Figure 5f showed that the mean fluorescence intensity in tumor is comparable with that in liver, and 3-5 times higher than those in other organs. Thus, these results clearly declare the potential of these PCL-tethered FRET imaging probes for *in vivo* tumor imaging.

5.3 Conclusions

In summary, we have presented a type of highly fluorescent colloidal nanoparticles with a maximum emission at 760 nm and a remarkably enhanced fluorescence quantum yield of 46% and good structural integrity. The colloidal nanoparticles are composed of polycaprolactone-tethered donor (PFTB-*g*-PCL) and acceptor (PCL-PPcy-PCL) as a FRET pair which co-collapse with a macromolecular surfactant PCL-*b*-POEGMA to form the hydrophobic core, stabilized with POEGMA as the corona that interfaces with the aqueous media. The hydrophobic sheath of PCL chains grafted from both the donor and the acceptor not only minimizes the aggregation-caused fluorescence quenching of both fluorophores, but also improves the interfacial compatibility and structural integrity of the FRET pair to enable both high efficiency of energy transfer and a high fluorescence quantum yield in the near infrared spectral window. The FRET efficiency in this system was further adjusted by tuning the D/A distance, resulting in **B1P1**^{1/2} FRET NPs with a fluorescence quantum yield as high as 46% and a small FWHM of 35 nm. Moreover, the semicrystalline nature of PCL chains grafted from both the donor and the acceptor suppressed the leakage of the NIR fluorophore from NPs, leading to significantly improved structural and optical stability. The good biocompatibility and imaging efficacy of **B1P1**^{1/2} NPs were demonstrated in both *in vitro* and *in vivo* bioimaging experiments. The FRET-enhanced NIR fluorescence, the remarkable tumor-specific ability and the long retention in tumor render **B1P1**^{1/2} NPs as a promising imaging probe for long-term tumor targeting and monitoring in a noninvasive way.

5.4 Experimental Section

Materials: 10×Phosphate buffer saline (PBS) buffer with pH = 7.4 (ultrapure grade) is a commercial product of 1st BASE Singapore. MilliQ water (18.2 MQ) was used to prepare the buffer solution from the 10×PBS stock buffer. 1×PBS consists of NaCl (137 mM), KCl (2.7 mM), Na₂HPO₄ (10 mM) and KH₂PO₄ (1.8 mM). Chloroform-D (99%) was purchased from Cambridge Isotope Laboratories, Inc. All other chemicals and reagents were purchased from Aldrich or Merck and used as received unless other specified. Block copolymers PCL-*b*-POEGMA,

conjugated polymer brush PFTB-g-PCL and Compound 1 were obtained according to our previous work.^{7,11}

Characterization: The samples were dissolved with chloroform-d for ¹H NMR measurements on a Bruker AV300 MHz NMR spectrometer. Molecular weight determinations for polymers were made using gel permeating chromatography (GPC) analyses (Agilent 1260, USA). The eluent was THF at a flow rate of 1.0 mL/min. A series of low polydispersity polystyrene standards were employed for the GPC calibration. Emission spectra of solutions were measured by fluorescence spectrophotometry on a PerkinElmer LS-55 at 25 °C. UV/Vis spectra of the samples were measured on a SHIMADZU UV-2450 spectrophotometer. Fluorescence lifetime measurements were performed on a Horiba Fluorolog 3 spectrofluorometer. Quantum yields of solutions were measured by the standard method using A₀ in chloroform ($\Phi_F = 0.69$). In order to minimize re-absorption effects, absorbances in the 10-mm fluorescence cuvette didn't exceed 0.1 at the excitation wavelengths of 517 nm for FRET NPs and 650 nm for A₀. The actual quantum yields might be underestimated or overestimated due to the difference Stokes shift and different excitation wavelengths. TEM measurements were performed with a TEM Carl Zeiss Libra 120 Plus at an acceleration voltage of 120 kV. A 5 μ L droplet of diluted samples was directly dropped onto a copper grid (300 mesh) coated with a carbon film, followed by drying at room temperature. The size distribution of resulting nanoparticles was determined by dynamic light scattering (DLS) using a BI-200SM (Brookhaven, USA) with angle detection at 90°. Fluorescence images of FRET NPs dispersions were acquired with an IVIS® Spectrum CT imaging system.

Synthesis of Compound 2 Compound 1 (267 mg, 0.2 mmol), 4-benzyl alcohol boronic acid (91.2 mg, 0.6 mmol) and K₂CO₃ (2.76 g) was dissolved in degassed mixture of toluene (20 mL), water (4 mL) and ethanol (4 mL) under nitrogen atmosphere. It is followed by addition of a catalytic amount (1% m/m) of Pd(PPh₃)₄. The resulting mixture was heated at 100 °C overnight, then cooled down to room temperature, and solvents were removed under reduced pressure at

80 °C. The products were purified by column chromatography on silica using dichloromethane as the solvent and dried under vacuum at 80 °C overnight. Finally, we obtained 243.7 mg of a red powder (yield 85.1%).

Synthesis of PCL-PPcy-PCL A mixture of compound 9 (0.02 mmol, 27.9 mg), ϵ -caprolactone (1 g), SnOct₂ (3 mg) was placed in a Shlenk tube and stirred for 24 h in 3 mL anhydrous toluene at 120 °C under N₂ atmosphere. After cooling, the solution was poured into cold diethyl ether. The product was obtained after further purification by precipitation in diethyl ether for three times and dried under 50 °C vacuum.

Preparation of nanoparticles The preparation of the **B1P1** FRET NPs is presented as a representative case. Polymer **B1** (100 μ L $\times$ 6.67 g/L), polymer **P1** (**w** μ L $\times$ 6.67 g/L, **w**=1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12.5, 15) and amphiphilic copolymer PCL-*b*-POEGMA (100 μ L $\times$ 6.67 g/L) were dispersed in (1000-200-**w**) μ L THF. These solutions were rapidly injected into 9 mL rapidly under ultrasonication. After evaporation of excess THF, solutions were adjusted by 1 mL 10 $\times$ PBS buffer. Other FRET series were prepared by using the same procedure.

Cell culture L929 mouse fibroblast cells were cultured in folate-free Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified environment containing 5% CO₂. Before experiments, the cells were precultured until confluence was reached.

In vitro cellular uptake L929 cells were seeded in 12-wells plates at 37 °C (Costar, IL, USA). After 80% confluence, the medium was removed and the adherent cells were washed twice with 1 $\times$ PBS buffer. The **B1P1**^{1/2} FRET NPs in MEM medium at 25 μ g/mL were then added to different chambers, respectively. After incubation for 12 h, the cells were washed five times with 1 $\times$ PBS buffer and then fixed with 75% ethanol for 10 min. After washing three times, the nucleus was stained by DAPI for 10 min at room temperature. Then the samples were washed three times, added to fresh 1 $\times$ PBS and imaged by confocal laser scanning microscopy (CLSM), sequentially. Lasers of 405 and 517 nm were used to excite

DAPI and FRET NPs, respectively. The corresponding fluorescence emissions were recorded through a confocal microscope (LSM 780, Carl Zeiss, Germany) using a band-pass filter combination including 421-481 nm, 650-750 nm for imaging in two individual channels (Objective: LD Plan-Neofluar 20x/0.4 Korr M27).

Cytotoxicity study The cytotoxicity of FRET NPs against L929 cells was evaluated by Presto blue (PB) assay. Briefly, L929 cells were seeded in 96-well plates (Costar, IL, USA) at an intensity of 1×10^4 cells mL^{-1} . After 24 h incubation, the cells were exposed to a series of doses of FRET NPs at 37 °C. After the designated time intervals, the wells were washed twice with $1 \times$ PBS buffer and 100 μL of freshly prepared PB solution in culture medium was added into each well. The PB medium solution was carefully removed after 1 h incubation in the incubator. The absorbance of PB at 570 nm and 600 nm was monitored by the microplate reader (Genios Tecan). Cell viability was expressed as the ratio of the percent PB reduction of the cells incubated with FRET NPs suspension to that of the cells incubated with culture medium only.

Tumor mouse model The care and use of laboratory animals were performed according to the approved protocols of the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore. All animal experiments were performed in compliance with the Guidelines established by the Institutional Animal Care and Use Committee (IACUC), SingHealth. To establish tumor models in 4-week-old female nude mice, 1×10^6 HepG2 cancer cells in 100 μL of supplemented MEM (10% fetal bovine serum, 1% pen/strep (100 U/ml penicillin and 100 mg/mL streptomycin) were injected subcutaneously in the right flank region of the mouse. Tumors were allowed to grow to $\sim 150 \text{ mm}^3$ before being used for *in vivo* imaging experiments

***In vivo* and *ex vivo* optical imaging** After the nude mice were anesthetized using 2% isoflurane in oxygen, **B1P1**^{1/2} (100 μL , 1 mg/mL) (n=2) or **B0P0**^{1/2} (100 μL , 1 mg/mL) (n = 2) was systematically injected through the tail vein using a

microsyringe. Fluorescence whole animal imaging was performed using a IVIS® Spectrum CT whole animal imaging system. The fluorescence distribution was monitored at 0, 0.5, 6, 24, 48, 72 h after NPs administration using the in vivo imaging system with appropriate wavelength ($\lambda_{\text{ex}} = 535 \text{ nm}$, $\lambda_{\text{em}} = 760 \text{ nm}$). Mice were sacrificed by cervical dislocation under deep isoflurane anesthesia at 72 h post injection. The liver, tumor, stomach, spleen, kidney, heart and intestine were harvested for *ex vivo* fluorescence imaging to estimate the tissue distribution of the NPs.

5.5 Supporting information

Figure S5.1 ^1H NMR spectra of compound 1, 2 (as shown in supporting information) and P1 in CDCl_3 (300 MHz, 25 °C).

Figure S5.2 GPC traces of **P1** with RI and UV-vis ($\lambda_{\text{abs}}=600$ nm) detectors (elution solvent: THF; 25 °C).

Figure S5.3 (a) Excitation spectra of **B1P1** FRET NPs with increased mass ratios between **B1** and **P1** polymers. (b) The ratio of the fluorescence intensity of the **B1** based nanoREDs without **P1** doping (F_0) and with **P1** doping.

Figure S5.4 (a) Chemical structure of PPcy-B, which possesses a similar conjugated core with **P1**. (b) absorption spectra of PPcy-B in chloroform (concentration: 7.52×10^{-6} M). As it is difficult to determine the extinction coefficient of **P1**, the extinction coefficient of PPcy-B was chosen for the calculation of the overlap integral ($J(\lambda)$) instead of **P1**.¹¹

Figure S5.5 Emission spectra of (a) **B2P1** with increased mass ratios between **B2** and **P1** polymers. Emission and excitation spectra of (b) **B2P1**^{1/2} FRET NPs at the mass ratio **P1/B2** of 5/100 whose intensity ratio of 620 nm and 760 nm ($F_{620\text{nm}}/F_{760\text{nm}}$) is close to 0.5.

Figure S5.6 (a) Normalized emission and absorption spectra of PFTB NPs and monomeric **P0** in chloroform. Overlapping shadow part in (a) represents the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor.

Figure S5.7 (a) Normalized emission and absorption spectra of PFTB NPs and **JAGG** NPs in aqueous medium.^{7,11} Overlapping shadow part in (a) represents the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor. (b) representative model corresponding to monomeric, H-aggregated and J-aggregated state of **P0**. (c) schematic diagram for explaining the optical features of **B0P0** FRET NPs in the absence of surfactant.

Figure S5.8 The ratio of the fluorescence intensity of the **B0** based conjugated polymer NPs without **P0** doping (F_0) and with **P0** doping.

Figure S5.9 (a) Absorption and FL emission spectra of **B0P0**^{1/2} FRET NPs upon the excitation at 635 nm before and after 24 h of incubation at 37 °C.

Figure S5.10 Ortho-view images of z-stack for **B1P1**^{1/2} FRET NPs stained L929, showing **B1P1**^{1/2} FRET NPs inside the cells. The scale bar represents 50 μm .

Figure S5.11 The distribution profiles of **B0P0^{1/2}** and **B1P1^{1/2}** FRET NPs in excised organs based on the total fluorescence intensity at 72 h post injection. Data are given as mean $\pm$ standard error (n=2).

5.6 References

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Chapter 6 Theranostic Unimolecular Micelles of Highly Fluorescent Conjugated Polymer Bottlebrushes for Far Red/Near Infrared Bioimaging and Efficient Anticancer Drug Delivery¹

In **Chapter 2-5**, a series of R/NIR colloidal nanoparticles with improved optical properties have been developed. However, these colloidal nanoparticles lack the ability to carry anticancer drugs due to the densely-packed structure of their hydrophobic core. Therefore, it is still challenging to develop a new drug delivery system with the following features: (i) the aggregation caused quenching effect of R/NIR agents is effectively suppressed, leading to the strong R/NIR emission; (ii) the high drug loading content is achieved, leading to high therapeutic efficacy.

6.1 Introduction

Nanoscale organic drug delivery systems (ODDSs) such as liposomes, micelles, dendrimers, with at least one dimension in the range of 1 to 100 nm, have drawn much attention from both basic research and the biomedical/pharmaceutical industries.^{2,3} Particularly, a variety of ODDSs integrated with both imaging modalities and anti-cancer drugs, also called “theranostics”, have been recently developed for imaging-guided therapy for cancer treatment.⁴⁻⁸ Among a variety of imaging contrast agents, organic and polymeric fluorophores have been widely used in both *in vitro* and *in vivo* bioimaging studies owing to their high sensitivity and resolution, tunable optical properties for multiplexed imaging, capability for long-term tracking^{9,10} and relatively low toxicity compared to inorganic materials.¹¹⁻¹³ For instance, indocyanine green (ICG) has been introduced to the doxorubicin (DOX)-loading liposomes or micelles to impart NIR fluorescence, enabling real-time monitoring of accumulation at the tumor site.^{14,15} In addition, other theranostic systems based on self-assembly of amphiphilic small molecules or macromolecules were designed to deliver NIR dyes such as cyanine, squaraine, phthalocyanine, porphyrin and boron-dipyrromethene (BODIPY) derivatives and

therapeutic agents to tumours.¹⁵⁻¹⁹ Particularly, far red/near infrared (FR/NIR) fluorescent conjugated polymers with premium optical properties such as their high absorption efficiency, bright fluorescence and robust photostability have attracted considerable attention for bioimaging applications.²⁰⁻²⁵ Nevertheless, the integration of conjugated polymers for FR/NIR fluorescence imaging into a theranostic platform remains rarely reported.²⁶

Despite the aforementioned advances, the self-assembled nanoscale carriers such as micelles formed by linear block copolymers are still limited by their poor stability. It is well known that critical micelle concentration (CMC) is an important parameter determining the thermodynamic stability of micellar aggregates.^{27,28} Although polymeric micelles often exhibit better stability with CMC values in the μM range compared to the micelles formed by small molecular surfactants,²⁹ after intravenous administration, the external factors such as temperature, pH, ionic strength, large shear forces during circulation in physiological systems will affect the structural stability of micelles, resulting in the dissociation of micellar aggregates.³⁰ This might further lead to undesired drug leaching, reduced therapeutic efficacy, and toxic side effects on the healthy tissues.

One strategy to enhance the stability of the aforementioned micellar drug carriers is to employ unimolecular micelles (UMs) formed by individual amphiphilic macromolecules such as dendrimers, hyperbranched polymers, bottle-brush-like grafted copolymers, and multiarm star-like polymers.³¹⁻³⁷ We previously reported a strategy of unimolecular micelles with well-defined chemical structure composed of amphiphilic cyclodextrin-core star-like poly(lactic acid)-*b*-poly(ethylene glycol) (PLA-*b*-PEG) block copolymers.³⁶ These unimolecular micelles showed high drug loading content and sustainable drug-release behavior. Nevertheless, most of the previous unimolecular micelles as drug carriers lack intrinsic fluorescence in the far-red (FR) and near infrared (NIR) region that is ideal for deep tissue bioimaging and noninvasive diagnosis of diseases such as cancers.^{35,38}

In this chapter, we report a new type of unimolecular micelles formed by amphiphilic bottle-brush-like grafted copolymers integrated with dual

functionalities of fluorescence-based imaging and anticancer drug delivery. Each of these polymers (denoted as PFTB-*g*-(PCL-*b*-POEGMA), Scheme 6.1a) consists of a π -conjugated backbone with intrinsic strong FR/NIR fluorescence, and poly(caprolactone)-*b*-poly[(oligo ethylene glycol) methyl ether methacrylate] (PCL-*b*-POEGMA) as the side chains that are able to encapsulate hydrophobic drugs such as DOX in the middle block of PCL, while the POEGMA in the corona of the unimolecular micelles provide sufficient water-solubility and colloidal stability (Scheme 6.1b).

Scheme 6.1 (a) Synthetic route to PFTB based polymer bottlebrushes PFTB-*g*-POEGMA and PFTB-*g*-(PCL-*b*-POEGMA). (b) Schematic illustration of UMs utilized as anticancer drug carrier and (c) their endocytosis by cancer cells.

The present strategy of unimolecular micelles based on amphiphilic diblock copolymer bottlebrushes shows the following advantages over the previously reported **nanoREDS**³⁹ which were fabricated from PFTB-*g*-PCL via a nanoprecipitation process: (i) As PFTB-*g*-(PCL-*b*-POEGMA) is amphiphilic, no extra surfactants are needed to render them soluble or dispersible into water; (ii)

The micellar size, concentration and colloidal stability are more controllable in PFTB-*g*-(PCL-*b*-POEGMA) micelles compared to **nanoREDs** prepared by nanoprecipitation. A series of PFTB-*g*-(PCL-*b*-POEGMA) polymers were synthesized by grafting hydrophilic blocks of POEGMA from presynthesized PFTB-*g*-PCL as macroinitiators via atom transfer radical polymerization (ATRP). The resulting amphiphilic diblock copolymer bottlebrushes showed well-defined molecular structures and tunable solubility and optical properties that could be finely controlled by changing the chain lengths of PCL and POEGMA side chains. The PCL blocks in PFTB-*g*-(PCL-*b*-POEGMA) not only segregate the PFTB backbones from each other, but also serve as the reservoir for encapsulation of hydrophobic anticancer drugs such as DOX. As a consequence, we expected that each macromolecule of PFTB-*g*-(PCL-*b*-POEGMA) bottle brush would form a unimolecular micelle with DOX loaded in the hydrophobic PCL domain, and demonstrate robust integrity and dual functionality of theranostics for cancer imaging and therapy (Scheme 6.1(b-c)).

6.2 Results and discussion

The following sections start with the synthesis and characterization of a series of amphiphilic PFTB-*g*-(PCL-*b*-POEGMA) polymers with tunable chain lengths of PCL and POEGMA. In aqueous solution, well-defined polymer bottlebrushes were expected to form structurally stable UMs consisting of a FR/NIR emissive PFTB backbone in the core that is surrounded by a hydrophobic PCL inner layer and a hydrophilic POEGMA outer corona. The effect of the PCL chain length on the morphologies and optical properties of UMs was investigated. DOX was chosen as a model drug to be encapsulated by UMs to afford theranostic DOX@UMs. The controlled-release properties and cytotoxicity of DOX@UMs were investigated. Finally, in vitro fluorescence imaging experiments were also carried out to evaluate the internalization and distribution of these UMs by different types of cells.

6.2.1 Synthesis of PFTB-*g*-(PCL-*b*-POEGMA)

Table 6.1 Molecular weights of macroinitiator PFTB-OH, PFTB-g-PCL-Br, hydrophilic PFTB-g-POEGMA, amphiphilic polymer bottlebrushes PFTB-g-(PCL-*b*-POEGMA) and characterization of corresponding UMs.

Entry	Polymer ^a	$M_{n,NMR}^b$ ($\times 10^3$)	$M_{n,GPC}^c$ ($\times 10^3$)	PDI ^d	$\lambda_{max,abs}$ (nm)	$\lambda_{max,em}$ (nm)	D_{UM}^e (nm)	Φ_{UM}^f (%)
I0	PFTB ₁₀ -OH	--	9.1	2.54	516 ^g	616 ^g	--	30.0 ^g
I1	(PFTB-g-(PCL ₈ -Br) ₂) ₁₀	28.9	35.8	1.91	--	--	--	--
I2	(PFTB-g-(PCL ₃₅ -Br) ₂) ₁₀	90.5	70.7	1.61	--	--	--	--
I3	(PFTB-g-(PCL ₅₉ -Br) ₂) ₁₀	145.2	111.9	1.56	--	--	--	--
P0	(PFTB-g-(POEGMA ₂₇) ₂) ₁₀	280.7	64.6	1.56	528	639	20.2	16.7
P1	(PFTB-g-(PCL ₈ - <i>b</i> -POEGMA ₁₆) ₂) ₁₀	188.9	115.1	1.35	518	635	26.8	22.0
P2	(PFTB-g-(PCL ₃₅ - <i>b</i> -POEGMA ₂₉) ₂) ₁₀	380.5	143.7	1.60	518	629	30.6	22.7
P3	(PFTB-g-(PCL ₃₅ - <i>b</i> -POEGMA ₁₁₈) ₂) ₁₀	1270.5	234.6	2.22	518	639	54.2	19.8
P4	(PFTB-g-(PCL ₅₉ - <i>b</i> -POEGMA ₃₆) ₂) ₁₀	505.2	193.2	1.41	518	630	32.3	24.8

Note: ^a DP_n of conjugated alternating units (FTB) and OEGMA units in PFTB-g-POEGMA was calculated by GPC results; DP_n of CL units and OEGMA units in PFTB-g-PCL-Br and PFTB-g-(PCL-*b*-POEGMA) was calculated by ¹H NMR results based on DP_n of FTB; ^b $M_{n,NMR}$ was calculated by ¹H NMR results; ^{c,d} $M_{n,GPC}$ was calculated by GPC results; ^e hydrodynamic diameter of nanoparticles was measured by DLS; ^f The FL Quantum yields of UM in aqueous medium were measured using rhodamine 6G in ethanol as the standard ($\Phi_F=0.95$); ^g Measured in toluene.

Scheme 6.1a presents the synthetic route to the well-defined amphiphilic multiarm block copolymers, PFTB-g-(PCL-*b*-POEGMA). PFTB functionalized with OH groups (PFTB-OH) was synthesized according to a similar method reported previously.³⁹ It was used to induce ring opening polymerization (ROP) of

ϵ -caprolactone in the presence of $\text{Sn}(\text{Oct})_2$ as the catalyst at 100 °C. The following esterification reaction with 2-bromoisobutylryl bromide (BIBB) and the subsequent atom transfer radical polymerization (ATRP) of oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA) from macroinitiator (PFTB-*g*-PCL-Br) led to a series of PFTB-*g*-(PCL-*b*-POEGMA) block copolymers with tunable chain lengths of PCL and POEGMA. In addition, we synthesized macroinitiator PFTB-Br via the esterification reaction of pendant hydroxyl groups of PFTB-OH and BIBB. The hydrophilic polymer bottlebrushes, PFTB-*g*-POEGMA, as control were synthesized through an ATRP procedure similar to the synthesis of PFTB-*g*-(PCL-*b*-POEGMA). The details about the average molecular weights and molecular weight distribution of all the synthetic polymers are listed in Table 6.1.

Figure 6.1 ^1H NMR (CDCl_3 , 300 MHz) spectra of (a) (PFTB-*g*-(PCL₃₅)₂)₁₀, (b) (PFTB-*g*-(PCL₃₅-Br)₂)₁₀ and (c) (PFTB-*g*-(PCL₃₅-*b*-POEGMA₂₉)₂)₁₀. After reaction with BIBB, the signal at 3.65 ppm corresponding to the methylene protons adjacent to the hydroxyl group of the (PFTB-*g*-(PCL₃₅)₂)₁₀ shifted to the lower field of 4.19 ppm (peaks are labeled by Δ in (a) and (b)).

Figure 6.1 depicts the representative ^1H NMR spectra of polymer (PFTB-*g*-(PCL₃₅)₂)₁₀, (PFTB-*g*-(PCL₃₅-Br)₂)₁₀ and (PFTB-*g*-(PCL₃₅-*b*-POEGMA₂₉)₂)₁₀. The signal at 3.65 ppm corresponding to the methylene protons adjacent to the hydroxyl

group in the ^1H NMR spectrum of the $(\text{PFTB-g-(PCL}_{35})_2)_{10}$ completely shifted to 4.19 ppm (labeled by Δ in Fig. 6.1a&b) while a new methyl signal (peak f) at 1.9 ppm appeared in the ^1H NMR spectra of the $(\text{PFTB-g-(PCL}_{35}\text{-Br})_2)_{10}$. Moreover, in Figure 6.1b, the integral ratio of peak c (aromatic protons on benzene ring of fluorine units) to peak h (methyl protons adjacent to bromide) was calculated to be 1/6. All these results indicate the complete conversion of PFTB-g-PCL to PFTB-g-PCL-Br as the ATRP macroinitiator. The number-average degree of polymerization (DP_{PCL}) of PCL side chains could be determined from the integral ratio of peak f to peak c in Fig. 6.1b. As shown in the ^1H NMR spectrum of $(\text{PFTB-g-(PCL}_{35}\text{-b-POEGMA}_{29})_2)_{10}$ (Fig. 6.1c), the signals at 3.40 and 3.66 ppm could be assigned to the methylene protons adjacent to oxygen and the protons of the methoxy end groups on the OEGMA units, respectively, while the signals at 2.32 and 4.07 ppm from the PCL are still present. Similarly, the DP of the POEGMA block ($\text{DP}_{\text{POEGMA}}$) can be determined by the integral ratio of peak a to peak f in Figure 6.1c. In addition, the ^1H NMR spectra of PFTB-Br and PFTB-g-POEGMA shown in Figure S6.1 confirm the complete conversion from PFTB-OH to PFTB-Br and successful synthesis of hydrophilic polymer bottlebrushes PFTB-g-POEGMA.

The gel permeation chromatography (GPC) traces of all diblock polymer bottlebrushes in Figure 6.2 depicted monomodal molar mass distribution with moderate polydispersity indices ($\text{PDI}=1.35\sim2.20$). The number-average molecular weight and PDI of the obtained polymers measured by GPC using linear polystyrenes as standards are shown in Table 6.1. Chromatogram peaks of PFTB-g-POEGMA (**P₀**) and PFTB-g-(PCL-b-POEGMA) (**P₁-P₄**) from the refractive index (RI) detector and UV-vis detector all shifted to lower elution volume due to the chain extension (Fig. 6.2), compared with the elution peaks of their respective macroinitiators (**I₀-I₃**).

Figure 6.2 GPC traces of a series of PFTB-g-(PCL-*b*-POEGMA) polymer bottlebrushes and their precursor (macroinitiator) using RI detector and UV-vis detector.

6.2.2 Size and Morphological Characterization of Unimolecular Micelles

The solubility of the PFTB-g-(PCL-*b*-POEGMA) polymers (i.e. **P₁-P₄**) is determined by the relative chain lengths of PCL and POEGMA. First of all, **P₀** with the long hydrophilic POEGMA brushes and the absence of hydrophobic PCL is well soluble in aqueous media. Such good solubility of **P₀** in water enables

convenient preparation of bioimaging probes without use of any organic solvent. Among **P1-P4**, only **P1** with the shortest PCL side chains could be directly dissolved in water under stirring or shaking. For polymers **P2-P4**, the longer PCL chains limited their solubility in water. As a consequence, in order to obtain unimolecular micelles (UMs) of **P2-P4**, it was necessary to dissolve them first in a common solvent such as THF before addition of water as the selective solvent for POEGMA.⁴⁰ THF was removed by evaporation in air, resulting in apparently well dispersed UMs with a co-collapsed core of PFTB and hydrophobic PCL, and a corona of the hydrated POEGMA.

The particle sizes of **P0-P4** in water and in THF were measured with dynamic light scattering (DLS). While **P0** in water and THF both exhibit the similar micellar size of 20 nm as shown in Figure 6.3b and Figure S6.2a, the particle size of **P1** in THF (27.7 nm) is slightly larger than that (26.8 nm) in water (Fig. S2b). The decrease of size could be attributed to the collapse of the inner PCL layer in water. DLS results (Fig. 6.3) of the amphiphilic polymer bottlebrushes **P2-P4** show that these polymers in aqueous solutions form monodisperse micelles with an average hydrodynamic diameter of 30.6, 54.2 and 32.3 nm, respectively. The increase of the micellar size from **P2** to **P3** is consistent with the increase of the chain length of the POEGMA corona, while the chain length of PCL is maintained the same. The number-average hydrodynamic diameter for **P2**, **P3** and **P4** increased from 30.6, 54.2 and 32.3 nm in water to 34.1, 56.1 and 42.9 nm in THF, respectively (Fig. S6.2a-e and Tab. S6.2), which should also be attributed to the swelling of the inner PCL cores in THF. Based on the results above, we conclude that **P1-4** all existed as unimolecular micelles (denoted as UM-P1, UM-P2, UM-P3 and UM-P4, respectively) in aqueous media.^{41,42} The sizes of these UMs in water can be facilely tuned by varying the chain lengths of PCL and POEGMA blocks, especially the latter. Besides, these UMs exhibit good stability, evidenced by their consistent DLS results after being stored at 5 °C for over three months as shown in Figure S6.2f-j.

The morphologies of UM-(P0-P4) in the dry state were characterized by transmission electron microscopy (TEM). The sizes of all these UMs in dry states

were smaller than those measured by DLS in aqueous media in which the POEGMA corona was hydrated. In detail, spherical micelles were observed in TEM images of UM-P0 and UM-P1 (Fig. 6.3a and Fig. 6.3c). The average diameter of both UM-P0 and UM-P1 from TEM images was determined to be 10.8 ± 2.5 and 18.9 ± 4.2 nm, respectively (Fig. 6.3b and Fig. 6.3d). Evidently, in the dry state, the shrinkage of POEGMA corona with high density enables UM-P0 and UM-P1 to form solid spheres due to the absence of PCL core and short PCL length, respectively, as shown in Fig. S6.3. With the increase of the $DP_{n,PCL}$ from 8 to 35 and $DP_{n,POEGMA}$ from 16 to 29, UM-P2 exhibits narrow size distribution but flower-like morphologies (Fig. 6.3e). Such morphological transition from UM-P1 (Fig. 6.3c) to UM-P2 (Fig. 6.3e) could be attributed to the larger PCL core, leading to the decreased PCL/POEGMA interfacial curvature and lower density of POEGMA corona.⁴³⁻⁴⁶ The collapse of POEGMA chains in the dry state under TEM might result in the micellar morphology like a flower with short petals, i.e. with a loose corona instead of solid one. Further increasing the $DP_{n,POEGMA}$ to 118 (UM-P3) resulted in formation of flower-like micelles with longer and clearer petals (Fig. 6.3g, h). Due to the relatively broad molar-mass distribution of the PFTB backbones, those amphiphilic bottlebrushes with longer PFTB backbones formed larger micelles with more petals as shown in the inset of Fig. 6.3g. The strong steric effect of ultra long POEGMA chains in **P3** may force the stretching and branching of both PCL and POEGMA arms, resulting in the flower-like morphology of UM-P3 with longer petals.²⁹ The average width (3.6 ± 0.8 nm) of each brush is much larger than the cross section of single POEGMA chain, suggesting some fusion of adjacent POEGMA chains in the drying process (Fig. 6.3h). **P4** containing longer PCL blocks ($DP_n = 59$) and relatively short POEGMA ($DP_n = 36$) formed irregular butterfly-like micelles in water, as shown in Figure 6.3i. Reasonably, the further loosened POEGMA corona caused by the larger dense PCL core provides the possibility to observe the core-shell structure of the unimolecular micelles (Fig. S6.3).

The DLS and TEM results described above prove the formation of unimolecular micelles for **P0-P4** in water. The chain lengths of both PCL and POEGMA arms

played a role in determining the sizes and the morphologies of the micelles. When the PFTB backbone was maintained the same, the longer side chains of PCL and POEGMA led to stronger steric effect that forces the stretching of the side chains, resulting in formation of flower-like unimolecular micelles.

Figure 6.3 TEM images of UMs formed in aqueous media: (a) UM-P0, (c) UM-P1, (e) UM-P2, (g) UM-P3 and (i) UM-P4. (b), (d), (f), (h) and (j) give the corresponding DLS histograms of UM-P0, UM-P1, UM-P2, UM-P3 and UM-P4 UMs, respectively. Insets in (b), (d), (f), (h)

and (j) represent unimolecular model of UM-P0, UM-P1, UM-P2, UM-P3 and UM-P4, respectively, in which the light-blue part corresponds to POEGMA domain, the orange part corresponds to PCL domain, and the red rod in the center corresponds to single PFTB backbone.

6.2.3 Optical Properties of Unimolecular Micelles

It is well-known that the optical properties including absorption/emission bands and FL quantum yields of red-emitting conjugated polymers can be significantly affected by the status of inter-chain aggregation. Specifically, the inter-chain aggregation of conjugated polymers normally results in the formation of less-emissive species (e.g. excimers and exciplexes) and significant luminescence quenching.⁴⁷⁻⁵⁰ We expected that the polymers (**P0-P4**) in the present work, each of which contains a conjugated PFTB backbone surrounded by hydrophilic POEGMA or amphiphilic PCL-*b*-POEGMA side chains, would exist in separated state with minimized aggregation-caused FL quenching.

To examine this hypothesis, the optical properties of the series of unimolecular micelles described in the former section were characterized. The UV-vis absorption and FL emission spectra of UM-(P0-P4) are presented in Figure 6.4. UM-P0 shows an absorption maxima at 528 nm and an emission maxima at 639 nm, both of which are higher than those of UM-P0 in dilute toluene ($\lambda_{\text{max,abs}} = 512$ nm, $\lambda_{\text{max,em}} = 616$ nm) (Fig. 6.4a & 6.4b). Meanwhile, a decrease of FL quantum yield (Φ) was observed from 0.30 of PFTB in toluene to 0.17 of UM-P0 in water (Table 6.1). Such FL quenching and bathochromic shift may be attributed to the donor-acceptor (D-A) configuration of PFTB and the enhanced polarity-driven charge separation that lead to a decrease of energy difference to ground states and a corresponding increase of the nonradiative pathways in relaxation of excited states.⁵¹⁻⁵³ Unimolecular micelles formed by **P1** with the shortest PCL block ($\text{DP}_n = 8$) showed a moderately increased FL quantum yield to 0.22, with the absorption and emission bands centered at 518 and 635 nm, respectively (Fig. 6.4c & 6.4d). These results suggest that the presence of PCL block serves as a hydrophobic sheath to minimize the negative influence caused by aqueous environment on quantum yield.^{39,51}

Figure 6.4 (a) UV-Vis absorption spectra of **P0** with the same optical absorbance (~ 0.67) in toluene and in water at 512 and 528 nm, respectively. (b) The FL emission spectra of **P0** in toluene and in water excited at 512 and 528 nm, respectively. Insets in (a) and (b) are digital photographs of **P0** in toluene and in water under ambient light and UV light (365 nm), respectively. (c-d) UV-Vis absorption (c) and FL emission (d) (excited at 488 nm) spectra of PFTB in toluene and UMs in aqueous media. The chemical structure of PFTB is shown in Figure S6.5 in Supporting Information.

When the DP_n of PCL block was increased to 35, UM-P2 showed a FL quantum yield of 0.23, comparable to that of UM-P1. However, while the longer PCL brushes did not have an obvious effect on the shape of the absorption and the emission spectra (Fig. 6.4c & 6.4d), UM-P4 showed a slightly higher FL quantum yield of 0.25, which is close to that (0.30) of PFTB in toluene. In contrast, UM-P3 with hydrophilic POEGMA block 3-5 times longer than those of amphiphilic polymers mentioned above exhibits a lower FL quantum yield of 0.20 and a red-shifted emission band centered at 639 nm. Such phenomenon could be attributed to the over-stretching of PCL blocks by the ultra-long hydrophilic POEGMA₁₁₈

blocks, which weakens the sheath-like protective role of PCL hydrophobic blocks as also suggested by the TEM images (Fig. 6.3g).

Figure 6.5 (a-b) FL emission spectra of UM-P2 (a) and UM-P0 (b) in water after being irradiated with white LED light over different periods. The insets of (a) and (b) are the corresponding digital photographs of the samples under ambient light and UV light (365 nm) before and after the white LED light irradiation. (c) Photostability of UM-P2 vs. UM-P0 upon

continuous irradiation by white LED light for 105 min. F_0 is the initial fluorescence intensity and F is the fluorescence intensity of the sample at different time points after irradiation.

The photostability of UMs under continuous irradiation with strong white LED light (power: 19 W) was also investigated using UM-P0 and UM-P2 as examples. After 105 min of continuous irradiation, the FL intensity of UM-P2 solution decreased by ~40% (Fig. 6.5), which was obviously lower than that (by ~76%) of UM-P0 solution. We also observed the blue shift of emission band of UM-P0 from 636 to 610 nm. Simultaneously, as shown in absorption spectra (Fig. S6.4), the absorbance at 528 nm of UM-P0 sharply decreased by 70% upon the 105 min irradiation while the absorbance at 518 nm of UM-P₂ only dropped by 35%. Both of their spectra showed blue shift of absorption band after irradiation, indicating that the conjugation of backbones may be partially disrupted. These results suggest that the presence of inner PCL layer can improve the photostability of UMs to some extent.¹⁰ Overall, these UMs show broad emission band covering the far-red (FR) region (ca. 650-700 nm) and near-infrared (NIR) region (> 700 nm) with relatively high FL quantum yields in the range of 0.17~0.25.

6.2.4 Biocompatibility of Unimolecular Micelles in Water

Due to the bioresorbable nature of PCL, the biocompatibility of POEGMA, and the low weight-fraction of PFTB backbones, we predicted these UMs would show low cytotoxicity for biological applications. UM-P1 was chosen as an example to evaluate the cytotoxicity against normal cells, i.e. mouse fibroblast cells (L929), using PrestoBlue assays. Figure 6.6 summarizes the *in vitro* L929 cell viability after incubation with UM-P1 at a series of concentrations of 10, 50, 100, 200, 400 $\mu\text{g/mL}$ for 24, 48 and 72 h, respectively. There was no obvious change of the metabolic viability of L929 cells after being cultured with UM-P1 within 48 h as shown in Figure 6.6. More specifically, a high cell viability of ~88% was observed after incubation with UM-P1 at a concentration up to 400 $\mu\text{g/mL}$ for 72 h, indicating the negligible cytotoxicity of these polymers.

Figure 6.6 Metabolic viability of L929 cancer cells after incubation with UM-P1 at different concentrations for 24, 48 and 72 h, respectively.

6.2.5 Drug Loading, Release and in vitro Cytotoxicity

The inner hydrophobic core of PFTB-*g*-(PCL-*b*-POEGMA) UMs provides a reservoir for loading hydrophobic anticancer drugs such as DOX. UM-P1, UM-P2 and UM-P4 were selected to encapsulate DOX to give three theranostic unimolecular micelles DOX@UM-P1, DOX@UM-P2 and DOX@UM-P4, respectively. UM-P3 was not tested here for further drug loading due to its ultralong and bulky POEGMA blocks which result in over-stretching of PCL blocks as shown in Figures 6.3(g-k) and expected lower drug-loading content compared to the other three polymers (i.e. **P1**, **P2** and **P4**). The drug loading content and encapsulation efficiency of unimolecular micelles were listed in Table S6.3. UM-P2 showed the highest DOX loading content of 9.9% as compared to those of UM-P1 (7.3%) and UM-P4 (9.5%). It appeared that the longer PCL blocks in UM-P2 provided a larger space for loading more drugs than UM-P1. Further increasing the length of PCL chains in UM-P4 resulted in a drug loading content comparable to that of UM-P2, which implies that the drug-loading capacity reaches a limit once the PCL block is long enough. It should be noted that the semicrystalline feature of the PCL blocks in the present PFTB-*g*-(PCL-*b*-POEGMA) polymers may also limit their drug-loading capacities. The replacement of the semicrystalline PCL with other amorphous polyesters such as poly(δ -decalactone) may lead to a further increase of the drug-loading capacity.⁵⁴ After drug encapsulation, the hydrodynamic diameter of UM-P1 slightly increased from

26.8 to 29.4 nm (Fig. S6.6). A similar size variation of UM-P2 and UM-P4 was observed upon loading DOX.

Figure 6.7 (a) DOX release profiles recorded for DOX@UM-P1, DOX@UM-P2 and DOX@UM-P4 solution in PBS buffer with pH=7.0 and 5.5 at 37 °C. The concentration of each DOX@UM sample was 1 mg/mL. (b) Schematic cross-section structures of DOX@UM-P1 vs. DOX@UM-P2, where the longer POEGMA chains of DOX@UM-P2 lead to a relatively loose hydrophobic core of the unimolecular micelle and easier release of drugs from micelles at both pH = 5.0 and 7.4. (c-e) Viability of HeLa cells incubated for 24 h (c), 48 h (d) and 72 h (e) with carrier-free DOX, DOX@UM-P1 and DOX@UM-P2, respectively, in aqueous solution at varying DOX concentrations.

In vitro drug release profiles of DOX@UMs were characterized under simulated physiological condition (PBS, pH 7.4 and 5.0, 37 °C). As shown in Figure 6.7a, the release of DOX in DOX@UMs system in a mildly acidic environment was

significantly faster than that in a neutral environment, which was also observed in other DOX-loaded carriers.^{55,56} Upon 48 h incubation of DOX@UM-P1 at pH 5.0, approximately ~60% cumulative release of DOX in a controlled manner was achieved. Furthermore, more than 77% DOX was released when the incubation time was extended to 120 h. In contrast, less than 22% cumulative release of DOX was observed in a neutral environment after 120 h incubation, implying low drug leakage during blood circulation. In case of DOX@UM-P2, up to 93% cumulative DOX was released after 120 h incubation at pH 5.0. However, its 46% cumulative release of DOX at pH 7.4 may pose potential toxicity to normal tissues and cells. The higher cumulative drug release observed in DOX@UM-P2 may be attributed to the longer hydrophilic POEGMA blocks that forced the stretching of hydrophobic cores and enhanced diffusion of DOX into the water media (Fig. 6.7b). Compared to DOX@UM-P1 and DOX@UM-P2, DOX@UM-P4 displayed a lower cumulative release of 60% upon 120 h incubation at pH 5.0, which was expected and could be due to the diffusion barrier caused by the densely packed core of relatively long PCL segments.

In vitro cytotoxicity of DOX@UM-P1 and DOX@UM-P2 was then evaluated against HeLa cells using PrestoBlue assay, using carrier-free DOX as a control. At 24 h of incubation, both DOX@UM-P1 and DOX@UM-P2 showed little toxicity to the HeLa cells, as shown in Figure 6.7c. And the cytotoxicity was independent on the concentration of DOX (from 5 to 25 $\mu\text{g/mL}$) encapsulated in the micelles, which suggested little release of DOX from the micelles into the cells during this incubation period. Only ~15% of cells in average were killed by either DOX@UM-P1 or DOX@UM-P2, in contrast to ~40% of cells that were killed by carrier-free DOX over the same incubation time (Fig. 6.7c). When the incubation time was extended to 48 h, some obvious cytotoxicity of DOX@UM-P1 and DOX@UM-P2 was observed at the highest DOX concentration of 25 $\mu\text{g/mL}$ (Fig. 6.7d), but not at a lower concentration of DOX (e.g. in the range of 5 to 15 $\mu\text{g/mL}$). In addition, the cytotoxicities of DOX@UM-P1 and DOX@UM-P2 are comparable under all the tested concentrations of DOX, but still lower than the cytotoxicity of carrier-free DOX with the same effective concentration. Dramatic cytotoxicity of DOX@UM-

P1 and DOX@UM-P2 was observed when the incubation time was extended to 72 h (Fig. 6.7e) under all the tested concentrations of DOX. In particular, the unimolecular micelles containing 25 $\mu\text{g/mL}$ of DOX showed significant cytotoxicity, inducing over 90% death of HeLa cells. This cytotoxicity was comparable to that of carrier-free DOX with the same concentration, which is consistent with the drug release profiles shown in Figure 6.7a.

To examine the cytotoxicity of DOX@UMs to normal cells, L929 cell line was chosen as an example for incubation with DOX@UM-P1 and DOX@UM-P2, respectively. As shown in Figure S6.7, free DOX showed a much higher cytotoxicity than that of DOX@UM-P1. Even after 72 h incubation with DOX@UM-P1 and DOX@UM-P2 containing 25 $\mu\text{g/mL}$ of DOX, the relatively low cytotoxicity was reflected from a relatively high cell viability of $\sim 55\%$ and 35% , which are 2–3 times higher than that of free DOX. Compared to DOX@UM-P1, DOX@UM-P2 showed higher toxicity to L929 cells after 72 h incubation, which can be attributed to its higher cumulative release of DOX. We note that selective toxicity of both DOX@UM-P1 and DOX@UM-P2 to cancer cells as described above, which has been rarely reported in previous passive-targeting anticancer drug delivery systems,⁵⁷ is encouraging for future animal studies and clinical trials.

The results above demonstrate that the chain lengths both PCL and POEGMA blocks in PFTB-*g*-(PCL-*b*-POEGMA) polymers affects the drug-loading capacity and cumulative release of drugs. The drug-loading capacity of the present polymers reached the limit of 9–10% by weight when the DP_n of PCL is up to 35. The increase of POEGMA chain length (DP_n from 16 to 29) led to stretching of PCL blocks (as shown in Figure 6.3(c,g,j,k)) and a relatively loose hydrophobic core of the unimolecular micelles (e.g. UM-P2, Fig. 6.7b) that released the drugs faster. A further increase of the POEGMA chain length from $\text{DP}_n = 29$ to 36 retarded the release of drugs, presumably due to the bulkiness of the hydrophilic corona that induced a diffusion barrier for the drugs. Overall, the chain lengths of both PCL ($\text{DP}_n = 8$) and POEGMA ($\text{DP}_n = 16$) blocks in UM-P1 appears optimal regarding

its single-micelle FL brightness and drug release kinetics, despite its moderate drug-loading capacity (ca. 7 wt%). The fundamental study based on DOX here could also provide guidance for the theranostic system with other hydrophobic drugs such as paclitaxel and camptothecin.

6.2.6 Cellular Imaging Using Fluorescent Unimolecular Micelles

Considering the FL brightness, the drug-loading capacity, and the drug-release kinetics of the single unimolecular micelle described above, UM-P1 and UM-P2 versus UM-P0 as the control were chosen as examples to examine their performances in cellular imaging. Live fibroblast cell from mice (L929) and cervical cancer cell (HeLa) were selected as representative normal and cancer cells, respectively. The cellular internalization of three kinds of UMs by L929 and HeLa cells was then examined under confocal laser scanning microscopy (CLSM). After incubating the cells with UMs dispersion (50 $\mu\text{g/mL}$) for 12 h, the cells were fixed and their nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). For fluorescence imaging of cells labeled with UMs, the excitation wavelength was fixed at 514 nm, and the FL emission signals in the range of 530 to 735 nm were collected.

Figure 6.8 and S6.8 show the CSLM images of HeLa cells and L929 cells, both stained by UM-P0, UM-P1 and UM-P2, respectively. Apparently uniform distribution of UM-P0, UM-P1 and UM-P2 with strong fluorescence throughout the cellular cytoplasm and the perinuclear region was observed for both L929 and HeLa cells, suggesting the efficient accumulation of UMs in the cytoplasm of these cells. Especially in HeLa cells, no cluster of UMs could be observed in the cytoplasm, which implies that UMs with the sub-30 nm size were well dispersed in the cytosol after endocytosis. These fluorescence images demonstrate the potential of these UMs as fluorescent probes for cell imaging. It is well-known that the ultimate goal of cancer diagnosis is to differentiate tumours from normal tissues in the early stages of disease.⁵⁸ Considering the advantages of UMs such as sub-30 nm size, free charges and excellent micellar stability during blood circulation, these

UMs are expected to achieve efficient passive tissue targeting and deep penetration through enhanced permeability and retention (EPR) effect.⁵⁹

Figure 6.8 Confocal fluorescence microscope images of HeLa cells stained with (a) UM-P0, (b) UM-P1, (c) UM-P2. The concentration of UMs is 50 $\mu\text{g/mL}$. The fluorescence of DAPI and UMs is pseudo-labeled with blue and red, respectively. The scale bar represents 100 μm .

6.3 Conclusions

In conclusion, we have presented a series of amphiphilic copolymer bottlebrushes, PFTB-*g*-(PCL-*b*-POEGMA) with well-defined molecular structures and theranostic functionalities of both strong FR/NIR fluorescence for bioimaging and controllable drug loading and release for effective treatment of cancer cells. In

aqueous media, PFTB-*g*-(PCL-*b*-POEGMA) polymer bottlebrushes exist as stable unimolecular micelles (UMs). The sizes and the morphologies of these UMs could be facilely tuned by varying the side-chain lengths of PCL and POEGMA. The increase of chain lengths of PCL and POEGMA arms led to enhanced steric effect that forces the stretching of the side chains, resulting in the formation of butterfly- or flower-like unimolecular micelles. These PFTB-*g*-(PCL-*b*-POEGMA) micelles showed improved fluorescence quantum yield (Φ) above 22% as well as enhanced photostability in aqueous media, compared to the unimolecular micelles formed by PFTB-*g*-POEGMA ($\Phi = 16.7\%$) in absence of the hydrophobic PCL core. On the one hand, these fluorescent UMs could be efficiently internalized by cells such as HeLa and L929 cells with good biocompatibility for fluorescence imaging. On the other hand, the PCL blocks in these polymers not only play a critical role to preserve the fluorescence properties but also serve as a reservoir for loading hydrophobic drugs such as DOX. High loading content and encapsulation efficiency of these UMs were achieved. These DOX@UMs show characteristics of pH-responsive sustainable drug release, resulting in significant cytotoxicity of DOX@UMs against HeLa cancer cells. Importantly, these DOX@UMs exhibited a relatively low cytotoxicity to normal cells such as L929 cells. We expect that these multifunctional unimolecular micelles formed by the amphiphilic conjugated polymer bottlebrushes will provide new avenues for design of theranostic nanocarriers with strong FR/NIR fluorescence for imaging-guided cancer therapy.

6.4 Experimental section

Materials

10×phosphate buffer saline (PBS) buffer with pH = 7.4 (ultrapure grade) is a commercial product of 1st BASE Singapore. MilliQ water (18.2 MQ) was used to prepare the buffer solution from the 10×PBS stock buffer. 1×PBS consists of NaCl (137 mM), KCl (2.7 mM), Na₂HPO₄ (10 mM) and KH₂PO₄ (1.8 mM). Chloroform-D (99%) was purchased from Cambridge Isotope Laboratories, Inc. Oligo-(ethylene glycol methyl ether methacrylate) (OEGMA, $M_n = 500$ g/mol) were

purchased from Aldrich and used after passing through a column of activated basic alumina to remove inhibitors. Copper(I) bromide was purified by washing with acetic acid and methanol three times, respectively, then stored in glove box. All other chemicals and reagents were purchased from Aldrich or Merck and used as received unless specified. Bare conjugated polymer PFTB and hydroxyl group modified conjugated polymer PFTB-OH were synthesized according to our previous report.^{39,60}

Characterization

The samples were dissolved with chloroform-d for ¹H NMR measurements on a Bruker AV300 MHz NMR spectrometer. Molecular weight determinations for polymers were made using gel permeating chromatography (GPC) analyses (Agilent 1260, USA). The eluent was THF at a flow rate of 1.0 mL/min. A series of low-polydispersity polystyrene standards were employed for the GPC calibration. UV/Vis spectra of the samples were measured on a SHIMADZU UV-2450 spectrophotometer. Emission spectra of solutions were measured by fluorescence spectrophotometry on a Horiba Fluolog 3 spectrofluorometer at 25 °C. Fluorescence quantum yield of the UMs in water was measured by using the rhodamine 6G in ethanol ($\Phi_F = 0.95$) as the reference. For reference calibration, a stock solution of 1 mg/mL rhodamine 6G dissolved in ethanol was diluted to five ethanol solutions with different absorbance values below ~0.10 at 488 nm. The emission was collected in the fluorescence spectrophotometry with an excitation light of 488 nm. The same absorption and emission measurements were also carried out for UMs in aqueous solutions. The integrated fluorescence intensity was plotted against absorbance at the excitation wavelength of 488 nm. Two slopes, one obtained from the reference of rhodamine 6G in ethanol and the other from the UMs, were employed in the calculation of the quantum yield of the sample, based on equation (1):

$$QY_{UM} = QY_{ref} \times \frac{slope_{UM}}{slope_{ref}} \times \left(\frac{n_{UM}}{n_{ref}} \right)^2 \quad (1)$$

where n_{UM} and n_{ref} are the refractive indices of water and ethanol, respectively.

TEM measurements were performed with a TEM Carl Zeiss Libra 120 Plus at an acceleration voltage of 120 kV. A 5 μL droplet of diluted samples was directly dropped onto a copper grid (300 mesh) coated with a carbon film, followed by drying at room temperature. The size distribution of resulting nanoparticles was determined by dynamic light scattering (DLS) using a BI-200SM (Brookhaven, USA) with angle detection at 90°. The photostability of UMs subjected to the continuous radiation of white LED light (SMD5050, 19 W) over different periods was monitored by the UV-vis spectrometer and fluorometer.

Synthesis of ATRP macroinitiator PFTB-Br

PFTB-OH (0.158 g), triethylamine (TEA, 0.053 mg, 0.523 mmol) and DMAP (0.080 g, 0.655 mmol) were mixed in a three-neck round bottom flask. THF (10 mL) was added to the flask via a syringe after purging with N_2 gas, followed by cooling to 0 °C. α -Bromoisobutyryl bromide (BIBB) (0.474 mL, 2.6 mmol) was then added via a micro syringe and the temperature of the flask was slowly raised to room temperature. After stirring for 24 h, the solution was filtered and precipitated in cold methanol to purify ATRP macroinitiator. Macroinitiator was then dried in vacuum at 50 °C and obtained as a dark red powder with a quantitative yield.

Synthesis of polymer bottlebrushes PFTB-g-POEGMA

In a N_2 protected glove box, macroinitiator PFTB-Br (0.025 g), OEGMA (1 g, 2 mmol), copper bromide (6.5 mg, 0.046 mmol), $\text{N,N,N',N'',N'''}\text{-pentamethyldiethylenetriamine}$ (PMDETA, 8 mg, 0.046 mmol) and 7 mL of toluene were added into a Schlenk tube. The mixture was immediately frozen in liquid nitrogen after being taken away from the glove box. After three freeze-thaw cycles for removal of oxygen, the tube was then placed in an oil bath thermostated at 60 °C for 3 h to allow ATRP. A solution of the crude product was passed through an alumina column to remove the catalyst and precipitated in cold diethyl ether.

The final polymer bottlebrush was then dried in vacuum at 50 °C and obtained as a red sticky fluid (311.9 mg, 30.4% yields).

Synthesis of polymer bottlebrushes PFTB-*g*-PCL

A mixture of PFTB-OH (50 mg), ϵ -caprolactone (0.5 g, 1.125 g, 2 g), stannous octoate ($\text{Sn}(\text{Oct})_2$) (3 mg) was placed in Schlenk tube and stirred for 24 h in 3 mL of anhydrous toluene at 120 °C under N_2 atmosphere. After cooling, the product was obtained after further purification by precipitation in diethyl ether for three times (yield: 121 mg, 215 mg and 505 mg, respectively).

Synthesis of ATRP macroinitiator PFTB-*g*-PCL-Br

Similar with the synthesis route of macroinitiator PFTB-Br, PFTB-*g*-PCL (1 eq. of hydroxyl group), TEA (2 eq.) and DMAP (2.5 eq.) were put in a three-neck round bottom flask. THF (10 mL) was added to the flask via a syringe after purging with N_2 gas, followed by cooling to 0 °C. BIBB (10 eq.) was then added via a micro syringe and the temperature of the flask was slowly raised to room temperature. After stirring for 24 h, the solution was filtered and precipitated in cold methanol to purify the PCL macroinitiator. The PCL macroinitiator was then dried in vacuum at 50 °C and obtained in near-quantitative yield.

Synthesis of polymer bottlebrushes PFTB-*g*-(PCL-*b*-POEGMA)

In a glove box under N_2 , macroinitiator PFTB-*g*-PCL-Br, oligo(ethylene glycol) methyl ether methacrylate (OEGMA), CuBr, PMEDTA and a fixed volume of toluene were added into a Schlenk tube. In all cases, ATRP proceeded by choosing the proper monomer/initiator ratios, a low monomer concentration, a moderate temperature (60 °C) and low catalyst content (initiator: CuBr: PMEDTA = 1:1:1, by moles). The detailed amount of monomer, initiator and catalyst were shown in Table S1. The mixture was immediately frozen in liquid nitrogen after being taken away from glove box. After three freeze-thaw cycles for removal of oxygen, the tube was then placed in an oil bath thermostated at 60 °C for 2~3 h to allow ATRP. A solution of the crude product was passed through an alumina column to remove

the copper catalyst and precipitated in cold diethyl ether. The final polymer bottlebrush was then dried in vacuum at 50 °C.

Preparation of UMs in aqueous medium

Polymer bottlebrushes **P0** and **P1** (5 mg) can be directly dissolved in 5 mL of water or PBS buffer (pH=7.4). For other polymer bottlebrushes, 1 mL of THF was used to dissolve the polymers (5 mg). After that, 5 mL of water or PBS buffer was added to dilute the solutions and exposed in open air for the evaporation of THF overnight to give 1 mg/mL UMs in water.

Preparation of DOX@UMs

Typical procedures employed for the encapsulation of therapeutic drugs into UMs are as follows. DOX·HCl (4.0 mg), 100 µL of triethylamine and PFTB-*g*-(PCL-*b*-POEGMA) polymer bottlebrushes (20 mg) were dissolved in 5.0 mL of DMF, and the mixture was stirred at room temperature for 2 h. The organic solvent and free DOX was removed by dialysis against deionized water for 72 h at room temperature (MWCO 3500) against DI water (1L×5). The concentration of resulting drug loading UMs was adjusted to 1 mg/mL for further experiments. The loading content (LC %) and entrapment efficiency (EE %) of DOX was calculated by the following equations:

$$LC (\%) = \frac{\text{weight of drug in UMs}}{\text{weight of UMs}} \times 100\%$$

$$EE (\%) = \frac{\text{weight of drug in UMs}}{\text{weight of drug in feed}} \times 100\%$$

***In vitro* drug release measurement**

Typically, 2 mL of DOX@UM solution (1 mg/mL) in PBS buffer solution (1×PBS, pH 7.4) was placed in a dialysis bag (MWCO 3500) and immersed into 40 mL of fresh PBS medium (pH=7.4 or 5.0) under gentle shaking at 37 ± 1 °C. At pre-determined time intervals, 1 mL of the external buffer was withdrawn and it was replaced with 1 mL of fresh PBS 7.4 or 5.0. Then release concentration of free

DOX was calculated based on an emission at 586 nm against a standard calibration curve. Above release experiments were tested in triplicate.

Cell culture

L929 mouse fibroblast cells and HeLa immortal cancer cells were cultured in folate-free Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified environment containing 5% CO₂. Before experiments, the cells were pre-cultured until confluence was reached.

***In vitro* cellular uptake**

L929 and HeLa cells were seeded in 12-wells plates at 37 °C (Costar, IL, USA). After 80% confluence, the medium was removed and the adherent cells were washed twice with 1×PBS buffer. The UMs in DMEM medium at 50 µg/mL were then added to different chambers, respectively. After incubation for 12 h, the cells were washed five times with 1×PBS buffer and then fixed with 75% ethanol for 10 min. After washing three times, the nucleus was stained by DAPI for 10 min at room temperature. Then samples were washed three times, added in fresh 1×PBS and imaged by confocal laser scanning microscopy (CLSM), sequentially. Lasers of 405 and 517 nm were used to excite DAPI and UMs, respectively. The corresponding fluorescence emissions were recorded through a confocal microscope (LSM 780, Carl Zeiss, Germany) using a band-pass filter combination including 421-481 nm, 530-735 nm for imaging in two individual channels (Objective: LD Plan-Neofluar 20x/0.4 Korr M27).

Cytotoxicity study

The cytotoxicity of DOX@UM against HeLa cancer cells was evaluated by PrestoBlue (PB) assay. Briefly, HeLa cells were seeded in 96-well plates (Costar, IL, USA) at an intensity of 5×10^4 cells mL⁻¹. After 12 h for attachment, the cells were exposed to free DOX and DOX@UM with different concentration at 37 °C. After the designated time intervals, the wells were washed twice with 1×PBS

buffer and 100 μL of freshly prepared PB solution in culture medium was added into each well. The absorbance of PB at 570 nm (reference wavelength is 600 nm) was monitored by the microplate reader (Genios Tecan). Cell viability was expressed as the ratio of the percent PB reduction of the cells incubated with free DOX or DOX@UM to that of the cells incubated with culture medium only. Data was analyzed according the protocol. Meanwhile, the cytotoxicity of UM-P0 and DOX@UMs was evaluated as a control against L929 cells through the similar method and protocol. All samples were tested in five replicates.

6.5 Supporting information

Table S6.1 Experimental details for synthesis of polymer brushes PFTB-*b*-POEGMA and PFTB-*g*-(PCL-*b*-POEGMA) via ATRP

Entry	Macroinitiator	[monomer]/ [initiator]	[monomer] [M]	Toluene /mL	[initiator]/[CuBr]/[PMDETA]
P0	I0	100:1	0.5	4	1:1:1
P1	I1	100:1	0.5	4	1:1:1
P2	I2	100:1	0.4	4.5	1:1:1
P3	I2	200:1	0.4	3	1:1:1
P4	I3	150:1	0.15	20	1:1:1

Figure S6.1 ^1H NMR (CDCl_3 , 300 MHz) spectra of PFTB₁₀-Br (a), (PFTB-*g*-(POEGMA₂₇)₂)₁₀ (b).

Figure S6.2 DLS histograms of UMs formed in THF: (a) UM-P0, (b) UM-P1, (c) UM-P2, (d) UM-P3, (e) UM-P4; and UMs in aqueous medium after being stored at 5 °C for over three months: (f) UM-P0, (g) UM-P1, (h) UM-P2, (i) UM-P3, (j) UM-P4.

Table S6.2 Comparison of hydrodynamic diameters between unimolecular micelles in water and THF from DLS results

D_h (nm)	P0	P1	P2	P3	P4
Water	20.2	26.8	30.6	54.2	32.3
THF	19.9	27.7	34.1	56.1	42.9

Figure S6.3 Schematic presentation of the morphology transition of UMs from aqueous solution to solid states.

Figure S6.4 (a) Absorption spectra of UM-P2 for different irradiation time using LED light; (b) absorption spectra of UM-P0 for different irradiation times using white LED light.

Figure S6.5 Chemical structure of PFTB reported in our previous work.⁶⁰

Table S6.3 Loading content (LC) and encapsulation efficiency (EE) of UM-P1, UM-P2 and UM-P4

Sample	UM-P1	UM-P2	UM-P4
LC (%)	7.3	9.9	9.5
EE (%)	44.6	55.3	59.7

Figure S6.6 DLS results of DOX@UMs in aqueous media: (a) DOX@UM-P1, (b) DOX@UM-P2, (c) DOX@UM-P4.

Figure S6.7 Viability of L929 cells incubated for 24 h (a), 48 h (b) and 72 h (c) with free DOX, DOX@UM-P1 and DOX@UM-P2 in aqueous solution at various DOX concentrations.

Figure S6.8 Confocal fluorescence microscope images of L929 cells stained with (a) UM-P0, (b) UM-P1, (c) UM-P2. The concentration of UMs is 50 $\mu\text{g/mL}$. The fluorescence of DAPI and UMs is pseudo-labeled with blue and red, respectively. The scale bar represents 100 μm .

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Chapter 7 Amphiphilic Red Fluorescent Conjugated Polymers Grafted with Disulfide-Linked Camptothecin: Ultrasmall Theranostic Unimolecular Micelles for Sensitive Reduction-Triggered Drug release

In **Chapter 6**, we reported a series of intrinsically red-emitting unimolecular micelles (UMs) composed of conjugated polymer brushes.¹ The UMs with an anticancer drug, doxorubicin (DOX), loaded in the hydrophobic PCL layer demonstrate robust structural integrity. However, concerns with the drug leakage under physiological conditions remain due to the relatively weak non-covalent association between the encapsulated drugs and UM carriers.²⁻⁴ On the other hand, most of current drug delivery platforms are excreted slowly and are largely retained within the reticuloendothelial system. Both these two limitations can cause severe side effect of toxicity to healthy tissues, hindering the clinical translation of theranostic agents. To overcome them, we further modified the design of theranostic UMs by covalent conjugation of drugs and reducing the micellar size in this chapter.

7.1 Introduction

In this chapter, we report a type of unimolecular micelle based polyprodrugs (denoted as **UMPD**) with intrinsic far red/near infrared (FR/NIR) fluorescence from PFTB backbone, from which covalently-bound camptothecin (CPT) prodrugs and hydrophilic POEGMA are grafted. The resulting polymerbrushes form bifunctional unimolecular micelles with significantly improved structural integrity of both the drugs and the carriers as well as stimuli-responsive release of drugs under reducing physiological conditions that are important for tumour targeting while reducing the toxic side effects against healthy tissues and cells.^{5,6}

Scheme 7.1 (a) Synthetic route to unimolecular micelle based polyprodrugs (**UMPD**). (b) Schematic illustration of **UMPD** utilized as anticancer drug carrier and their endocytosis mechanism by cancer cells.

7.2 Results and discussion

Scheme 7.1a depicts this drug delivery system based on the conjugated polymer bottlebrushes, which combine the intrinsic fluorescence of conjugated polymers PFTB with the redox-triggered release behavior of CPT prodrug. PFTB as backbones of conjugated polymer brushes in this work shows FR/NIR fluorescence with a quantum yield as high as ~ 0.2 .^{1,7} In order to graft OEGMA and CPT prodrug monomers from the PFTB backbone in a controllable manner via

reversible addition-fragmentation chain-transfer (RAFT) polymerization, chain-transfer agent (CTA), 4-cyano-4-(thiobenzoylthio)pentanoic acid, was conjugated to PFTB through the esterification between hydroxyl groups on PFTB and the acid in CTA. We hypothesized that, in physiologically reductive environment, the disulfide bonds that link the CPT prodrug units to the polymers could be cleaved upon exposure to reductive agents such as L-glutathione (GSH) (**Scheme 1b**), further resulting in release of CPT drugs.⁸ Finally, the oligo(ethylene glycol) (OEG) units are employed to provide biocompatibility and water-solubility and facilitate the formation of UMs.

The sections below first present the synthesis and characterization of the target polymers PFTB-*g*-(PCPTA_x-*co*-POEGMA_y) and their self-assemblies in aqueous media to form UMs. Then the optical response of the **UMPDs** upon exposure to dithiothreitol (DTT) as a reducing agent was characterized and the reduction-induced release of CPT drugs was studied. Finally, the performance of these theranostic **UMPDs** in fluorescence-based imaging and therapeutic treatment of cancers was examined in both cellular and animal studies.

7.2.1 Synthesis of unimolecular micelles based prodrugs (UMPDs)

The synthetic route to **UMPDs** is illustrated in Scheme 7.1. As reported in our previous work, conjugated polymer PFTB functionalized with hydroxyl group ($M_n = 9.1$ kDa, PDI = 2.54, using linear polystyrene as standards) was synthesized via sequentially direct arylation polymerization and hydrolysis reaction. Chain transfer agent (CTA), 4-cyano-4-(thiobenzoylthio)pentanoic acid, was conjugated to PFTB through the esterification between hydroxyl groups on PFTB and the acid in CTA molecule. The CTA modified polymer can be utilized as macroinitiator to induce reversible addition fragmentation chain transfer (RAFT) copolymerization. Nuclear magnetic resonance (NMR) and gel permeation chromatography (GPC) were conducted to analyze the chemical structures of the polymers. Compared with the precursor PFTB-OH, a new signal at 4.06 ppm appears in the ¹H NMR spectrum of PFTB-CTA as shown in Figure 7.1a, which could be assigned to the protons on the ethylene group neighboring to the ester group. Simultaneously, other signals at

7.39, 2.64, 2.42 and 1.93 ppm corresponding to specific protons on the initiator were observed. The conversion from hydroxyl group to CTA reaches a yield of 60%, which is determined by the integral of the peaks at 3.60 ppm and 4.06 ppm in Figure 7.1b. The GPC traces of PFTB-CTA gave M_n and PDI values of 9.8 kDa and 2.65, respectively, comparable to those of the precursor.

Figure 7.1 (a) ^1H NMR (CDCl_3 , 300 MHz) spectra of (a) PFTB₉-OH, (b) (PFTB-g-CTA_{1.2})₉ and (c) UMPD1. GPC traces of UMPD1 and its precursor (macroinitiator) using (d) RI detector and (e) UV-vis detector (wavelength: 500 nm).

Finally, PFTB-CTA was utilized as macroinitiator to induce the RAFT copolymerization of acrylate-decorated CPT (CPTA) and OEGMA in the presence of 2, 2-azobisisobutyronitrile (AIBN). The experimental details and data of RAFT

polymerization are listed in Table 7.1. A series of conjugated polymer bottlebrushes **UMPD1-4** and **UMWD** (stands for unimolecular micelles without drug) were synthesized by varying the feed ratio of CPTA and OEGMA monomers. Taking the ^1H NMR spectrum of **UMPD1** as an example (Fig. 7.1c), no signal corresponding to vinyl protons was observed, indicating the complete removal of residual monomers. The typical signals from POEGMA appeared at 3.39 and 3.65 ppm, while the signals from CPT were found in the range from 5.5 to 8.5 ppm. Based on the NMR results, the drug loading contents of **UMPD1**, **UMPD2** and **UMPD3** were quantified as high as 15.0%, 11.9% and 10.7%, respectively. The GPC chromatograms of **UMPDs** from both RI and UV detectors shifted to lower elution volume as compared to those of the macroinitiator (Fig. 7.1d & 7.1e). It also showed that M_n of **UMPD1-4** are 48.7, 57.1, 44.4 and 69.4 kDa with moderate PDI of 2.00, 1.67, 2.10 and 1.20, respectively (Fig. S7.2). These results confirm the successful synthesis of the target polymers.

Table 7.1 Characterization of conjugated polymer bottlebrushes PFTB-g-P(CPTA-co-OEGMA) via RAFT polymerization

Entry	Polymer	[OEGMA]:[CPTA]: [CTA]:[AIBN]	$M_{n,NMR}^e$ ($\times 10^3$)	$M_{n,GPC}^f$ ($\times 10^3$)	PDI ^g	DL (%) ^h	d (nm) ⁱ
	PFTB ₉ -OH ^a	---	---	9.1	2.54		
	(PFTB-g-CTA _{1.2}) ₉ ^b	---	---	9.8	2.65		
UMWD	(PFTB-g-(POEGMA _{14.5}) _{1.2}) ₉ ^c	15:0:1:0.33	88.1	44.4	1.30	0	
UMPD1	(PFTB-g-(PCPTA _{2.1} -co-POEGMA _{10.5}) _{1.2}) ₉ ^c	15:3:1:0.33	79.7	48.7	2.00	15.0	8.6
UMPD2	(PFTB-g-(PCPTA _{2.1} -co-POEGMA ₁₆) _{1.2}) ₉ ^c	22.5:3:1:0.33	109.4	57.1	1.67	11.9	9.6
UMPD3	(PFTB-g-(PCPTA _{1.2} -co-POEGMA ₁₂) _{1.2}) ₉ ^c	15:1.5:1:0.33	82.1	44.4	2.10	10.7	7.7
UMPD4	(PFTB-g-(PCPTA-co-POEGMA) _{1.2}) ₉ ^d	45:3:1:0.33	---	69.4	1.20	---	25.5

Note: ^a DP_n of conjugated alternating units (FTB) was calculated by GPC results; ^b DP_n of CPTA units and OEGMA units in PFTB-g-P(CPTA-co-OEGMA) were calculated by ^1H NMR results based on DP_n of FTB; ^d DP_n of UMPD cannot be quantified due to the extremely weak NMR signals on the PFTB backbones; ^e $M_{n,NMR}$

was calculated by ^1H NMR results; ^{f,g} $M_{n,\text{GPC}}$ and PDI was calculated by GPC results. ^h Drug loading of CPT, defined as the weight ratio of drug to UMs. ⁱ average diameter characterized by DLS.

7.2.2 Preparation and characterization of unimolecular-micelle based prodrugs (UMPDs)

A co-solvent evaporation method was employed to prepare unimolecular micelles. **UMPDs** (10 mg) were dissolved in 2 mL of tetrahydrofuran (THF), followed by addition of 10 mL of distilled water to the **UMPDs**/THF solutions. After the evaporation of THF, the obtained water solutions of **UMPDs** (1 mg/mL) were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). The DLS result revealed the formation of **UMPD1** (polydispersity index, PDI $\sim$ 0.04) with average size of roughly 8.6 nm (Fig. 7.2b). As shown in Figure 7.2a, spherical micelles of **UMPD1** in the dry state were observed by TEM and the average diameter was determined to be 7.3 ± 1.5 nm. In contrast, **UMPD2** exhibited a slightly higher average hydrodynamic diameter of 9.6 nm while **UMPD3** showed a slightly lower one of 7.7 nm (Fig. S7.3). Reasonably, the average hydrodynamic diameter of **UMPD4** from DLS data was approximately 3-fold higher than that of **UMPD1**, which agreed well with the variation of chain length of brushes. The DLS result further reveals that the hydrodynamic diameters of **UMPD** in THF are comparable with those in aqueous media (Fig. S7.3), indicating the formation of covalently interconnected unimolecular micelles and highly stretched copolymerized brushes in aqueous medium.

Figure 7.2 (a) TEM image of air-dried **UMPD1**; (b) DLS result of **UMPD1** in water (concentration of **UMPD1** = 0.01 mg/mL); (c) UV-vis absorption and FL emission spectra (λ_{ex} = 365 nm) of **UMPD1** in water.

Next, we investigated the optical properties of **UMPDs** by UV-vis absorption and fluorescence spectroscopies. As shown in Figure 7.2c, **UMPD1** shows two absorption maxima at 360 and 520 nm, respectively. The former refers to the overlapping absorption of CPT and fluorene units in PFTB while the latter refers to 4,7-bis(hexylthien)-2,1,3-benzothiadiazole (HTBT) units in PFTB. Similarly, two emission bands are observed with maxima at 420 and 630 nm, corresponding to the emission of CPT and PFTB backbones, respectively. Considering the high extent of spectral overlap of the emission spectrum of CPT and absorption spectrum of PFTB backbones (Fig. 7.3b), Förster energy resonance transfer (FRET) from the CPT donor to the PFTB acceptor in single UM was predicted to occur. Compared to **UMPD1**, **UMPD2** possesses the same ratio between CPT and PFTB but more OEGMA units on each side chain. The emission spectrum of **UMPD2** shows a higher I_{420}/I_{630} than **UMPD1** (Fig. S7.7a). This difference is likely due to the lower energy transfer efficiency from CPT to PFTB caused by the enlarged average host/guest distance in **UMPD2**.

To demonstrate the reductive milieu-triggered cargo release, the CPT release kinetics from **UMPD1s** was investigated in the PBS solutions in the presence of dithiothreitol (DTT) at a series of concentrations (C_{DTT}): 0 mM, 2 μM , 0.1 mM, 1 mM and 5 mM. At $C_{\text{DTT}} \leq 2 \mu\text{M}$, about 10% of CPT was released within 73 h, suggesting that the **UMPD1s** are largely stable in extracellular environment; in contrast, upon the treatment of 0.1 mM, 1 mM and 5 mM of DTT, nearly 100% of the loaded CPT was released during the initial 2 h from the **UMPDs**, due to the reductive activated cleavage of disulfide bond (Fig. 7.3a & S7.5). Obviously, the present **UMPDs** showed a faster rate of CPT release than those of self-assemblies based on CPT-containing amphiphilic polyprodrugs or silica nanoparticle prodrugs and behaved like water-soluble CPT-S-S- based small molecules as previously reported.⁹⁻¹¹ This pronouncing behavior of **UMPDs** could be largely attributed to the flexible and highly permeable nature of brush-like polymers as well as the substantial OEGMA copolymerized units, which contributes to the easy access of DTT and reduction-induced the cleavage of disulfide bond.

Figure 7.3 (a) *In vitro* CPT release profiles of UMPD1 in PBS buffer upon treating with DTT of varying concentrations: (■) 5 mM DTT; (●) 2 μ M DTT; (▲) 0 mM DTT. (b) Normalized emission and absorption spectra of CPTA and UMWD in PBS buffer. The inset of (b) represents the digital graphs of CPTA and UMWD in PBS buffer under the irradiation of UV (354nm). The intersection of shadow part in (b) represents the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor. FL emission spectra (c) and excitation spectra (d) of UMPD1 recorded in the presence and absence of DTT (10 mM). The inset of (c) represents the digital graphs of UMPD1 with/without DTT under the irradiation of UV (354nm). (e) representative model of UMPD, where FRET occurs without the treatment of cytosol and disappears with the treatment of cytosol.

Figure 7.4 FL emission spectra (a) and excitation spectra (b) of **UMPD4** recorded in the presence and absence of DTT (10 mM). The inset of (a) represents the digital graphs of **UMPD4** with/without DTT under the irradiation of UV (354nm). (c) representative model: different Donor/Acceptor distances in **UMPD1** and **UMPD4** lead to FRET manners with different transfer efficiencies.

To investigate whether the release of CPT from **UMPDs** would influence the energy transfer behavior and further cause significant changes of their optical properties, two aqueous solutions of **UMPD1** (1 mL, 1 mg/mL) was treated with 9 mL of PBS and DTT/PBS solution (10 mM) for 1 h, respectively. Upon the addition of DTT, the emission intensity at 420 nm increased while the intensity at 630 nm almost drops to half. The excitation spectra depict that the intensity at 366 nm significantly decreased in the presence of DTT while the intensity at 520 nm remained almost unchanged. As shown in the inset of Figure 7.3c, upon the irradiation of UV light (354 nm), the **UMPD1** solutions in the absence of DTT exhibits a bright purple fluorescence and turns blue fluorescence after treated by DTT. These results are consistent with our FRET hypothesis described previously, which was the FRET-to-nonFRET transition caused by the addition of DTT as illustrated in Figure 7.3e. The FRET phenomenon disappears with the redox-triggered release of CPT. In addition, the red-shift of CPT emission band from 420

to 434 nm (Fig. 7.3c) after the addition of DTT may be attributed to the aggregation of released CPT due to its poor water solubility. Also, the red shift of excitation band from 366 to 377 nm (Fig. 7.3d) suggests fluorene units in PFTB contribute to the whole emission of PFTB when CPT drugs escape from **UMPDs**.

Moreover, DLS measurements on **UMPD1** in the presence of DTT showed a slightly larger hydrodynamic diameter of 9.6 nm than that in the absence of DTT (Fig. S7.6). This is likely due to the removal of CPT from UMs caused by the amphiphilic-to-full hydrophilic transformation, leading to the further stretching of the polymer chains. Similar to **UMPD1**, we monitored the emission variation of **UMPD2** and **UMPD3** in the presence of 10 mM DTT. For **UMPD2**, the increment of fluorescence intensity at 420 nm after the treatment of DTT was found to be ~29% (Fig. S7.7a), lower than that of **UMPD1** (~53%). These results indicate again that the energy transfer efficiency of **UMPD2** is indeed weakened owing to the dilution of CPT units in brushes by increasing the fraction of POEGMA moiety. As expected, due to the longest length of brushes, the intensity difference of CPT fluorescence from **UMPD4** in the presence and absence of 10 mM DTT was found to be negligible. As shown in the inset of Figure 7.4a, both of **UMPD4** with and without DTT exhibit blue fluorescence by naked eye, where, evidently, the large donor-to-acceptor distance lead to the extremely weak FRET. These results provide a powerful evidence for that CPT effectively escape from the UM host through cleavage of disulfide bond in the presence of DTT.

7.2.3 *In vitro* imaging and cytotoxicity

On the basis of the successful controlled drug release in solution, experiments of FR/NIR imaging and chemotherapy were further carried out against HepG2 cancer cells. Considering the highest drug loading content of **UMPD1**, we firstly characterized the intracellular localization of **UMPD1** internalized in HepG2 cells for 2 h as compared to free CPT using confocal laser scanning microscope (CLSM). The images of HepG2 cells stained by free CPT and **UMPD1** (Fig. 7.5) were acquired upon excitation of 405 nm, 514 nm and 633 nm to collect the fluorescence of CPT, PFTB and Alexa Fluor® 633 phalloidin, respectively. The

corresponding fluorescence emission was recorded using a band-pass filter combination including 410-546 nm, 546-735 nm and 638-735 nm for imaging. As shown in Figure 7.5a, the free drug CPT mainly accumulated in the cellular cytoplasm and perinuclear region while some CPT penetrated the nuclear envelope to enter the nucleus. This phenomenon may be attributed to the formation of dispersible aggregates of CPT due to its nature of strong hydrophobicity especially in acidic environment such as tumor microenvironment.

The well-separated FL from CPT and PFTB enabled the simultaneous tracking of penetration pathway of the prodrug and the polymeric carrier. Figure 7.5b shows the cellular uptake and localization of **UMPD** and released CPT. After 2 h incubation of **UMPD1** with HepG2 cells, the strong fluorescence of PFTB backbones throughout the cellular cytoplasm was observed. In contrast, CPT dots also mainly localized in the cytoplasm but some of them traverse the nuclear pore complex into the nucleus by passive diffusion, which could be easily observed in the merged image (Fig. 7.5b). These results indicate the fast triggered-release of CPT after the **UMPDs** were internalized by HepG2 cells. After further incubation for 4 and 9 h, respectively, a more uniform distribution of fluorescence signals in the cytoplasm (Fig. 7.5c & 7.5d) was observed, where the FL intensity of both CPT and PFTB is much higher as compared to that in Figure 4b, suggesting the higher internalization of **UMPDs** by HepG2.

Figure 7.5 CLSM images of HepG2 cells after coincubating with aqueous dispersion of free CPT at a CPT equivalent dosage of 10 $\mu\text{g/mL}$ for (a) 2 h and aqueous dispersion of **UMPD1** for (b) 2 h, (c) 6 h, and (d) 11 h.

HeLa and MCF-7 cancer cells were also treated by **UMPD1**. As shown in Figure S7.9 & S7.10, the clear visualization of cellular uptake can also be observed for HeLa and MCF-7 cells, which further reveal the efficient internalization of **UMPDs** by cancer cells.

Figure 7.6 Cell viability assay performed on HepGs cells in the presence of **UMWD** (a), **UMPD1** (b) and free CPT (b). (c) Cell viability assay performed on HeLa cells in the presence of **UMPD1** and free CPT. Cell viability was measured by PrestoBlue assays after 24 h, 48 h and 72 h incubation at 37 °C. Data show the normalized mean and standard error of five independent experiments.

The cytotoxic effect of **UMPD1** was examined against HepG2 cells. Cell viability was evaluated by PrestoBlue assay after being incubated with **UMPD1** over 24 h, 48 h and 72 h, respectively (Fig. 7.6), employing CPT-free carrier

(**UMWD**) and carrier-free CPT as controls. Figure 7.6a summarizes the HepG2 viability after incubation with **UMWD** at different concentrations of 10, 50, 100, 200, 400 $\mu\text{g/mL}$ for 24, 48 and 72 h, respectively. It displayed a quite high cell viability close to 100% upon incubation with **UMWD** within 72h, indicating the low cytotoxicity of CPT-free carrier. In contrast, upon 24 h incubation with **UMPD1**, the HepG2 cell viability even at 10 $\mu\text{g/mL}$ of CPT was determined to be $\sim 76\%$ (Fig. 7.6b). Increasing cytotoxicity was observed for the **UMPD1** with the increasing incubation time, where it exhibited a dramatic reduction of HepG2 cell viability from 76% to 41% upon 72 h incubation with the CPT concentration of 10 $\mu\text{g/mL}$ (Fig. 7.6b). Reasonably, the increase of CPT concentration leads to enhanced cytotoxicity. More importantly, **UMPD1** shows a comparable cytotoxicity with free CPT, providing a powerful evidence for that CPT drugs were released quite fast in the reductive-milieu environment. The similar results were observed in the experiment of cytotoxicity against HeLa cells (Fig. 7.6c).

These preliminary studies in living cells have proved that **UMPD1** can be considered as a promising theranostic nanoplatform to provide the feasibility to simultaneously achieve FR/NIR imaging and cancer therapy, which inspires us to translate it to *in vivo* application.

7.2.4 *In vivo* and *ex vivo* tumor targeting and long-term monitoring

Due to the absence of surface engineering with targeting ligands on **UMPDs**, passive tumor accumulation through the enhanced permeability and retention effect (EPR) would dominate the specific targeting for cancer treatment. To investigate tumor specificity, human xenograft colon carcinomas (liver cancer cells HepG2) have been successfully inoculated near the right foreleg of mice. Subsequently, the **UMPD1** solution with a polymer dose of 25 $\mu\text{g/g}$ was intravenously injected to mice via tail vein and the real-time imaging of **UMPDs** in the tumor-bearing mice was monitored over the course of 168 h by collecting the FR/NIR fluorescence from PFTB backbones. During the whole-body imaging, mice were placed under anesthesia. Immediately following the bolus injection of **UMPD**, FR/NIR fluorescence was observed from the whole body of mice (Fig. 7.7a). After 30 mins

of administration, the tumor region showed slightly stronger fluorescence than peripheral normal tissues, indicating the gradual accumulation in the tumor. The signals at the tumor site became stronger after 24 h and were the most intense after 48 h, which facilitated to identification of the tumor from the normal tissues. The demand of long-term tracking encourages us to collect the fluorescence images at extended time points of 72, 96, 120, 144 and 168 h postinjection. It was observed that the fluorescence intensities of tumor remained relatively stable even at the time point of 168 h. Additionally, the area of the intensive emissive region gradually expanded, which is associated with the proliferation of cancer cells.

However, from the whole-body fluorescence images, it is difficult to identify the organs and further understand the time-dependent biodistribution of **UMPDs** due to the limited tissue penetration depth of FR/NIR fluorescence. Hence, *ex vivo* biodistribution of **UMPDs** were investigated at different time intervals. Specifically, the mice were sacrificed after administration of **UMPD1s** for 24, 72, and 168 h (n = 2 per group). The fluorescence images of the harvested tumor and organs (liver, spleen, stomach, intestine, kidneys and heart etc) showed that tumor emanated the most intensive fluorescence at all time points (Fig.7.7b). After 24 h postinjection of **UMPD1**, the mean fluorescence intensities of liver and spleen were quantified and found to be comparable even lower than those of stomach, intestine and kidney. More importantly, tumor exhibited the brightest fluorescence, whose mean intensity was at least ~ 9 folds higher than those of all organs (Fig.7.7b), indicating the excellent tumor-specific ability of **UMPD1**. Even at the time point of 168 h postinjection, the fluorescence emitted from tumor remained 7 folds and 37 folds stronger than those in liver and spleen (Fig.7.7b), respectively. This manner makes **UMPD1** a promising candidate for long-term tumor-specific monitoring.

These results were quite different with the typical biodistribution of most of micelles or nanoparticles with relatively large sizes (hydrodynamic diameters larger than 8 nm) which preferred to substantially accumulate in reticuloendothelial systems (RES) including liver and spleen.^{12,13} For example, the accumulations of

free ICG and ICG-carried nanoparticles with size of 20-40 nm were mainly in liver and nanoparticles were slowly excreted through hepatic clearance.¹⁴

Figure 7.7 (a) Long term FR/NIR fluorescence imaging: whole-body images at time points from 0.5 h to 168 h postinjection of **UMPD1**. (b) Ex vivo FR/NIR imaging of tumor and major organs at 1d, 3d and 7d postinjection of **UMPD1** ($\lambda_{ex} = 535$ nm, $\lambda_{em} = 660$ nm). (c) The biodistribution profiles of **UMPD1** in tumor and harvested organs based on the mean fluorescence intensity at different time point after postinjection. Data are represented as average $\pm$ standard error (n = 2).

Figure 7.8 (a) Long term FR/NIR fluorescence imaging: whole-body images at time points from 1 h to 168 h postinjection of **UMPD4**. (b) Ex vivo FR/NIR imaging of tumor and major organs at 1d and 7d postinjection of **UMPD4** ($\lambda_{ex} = 535$ nm, $\lambda_{em} = 660$ nm). (c) The biodistribution profiles of **UMPD4** in tumor and harvested organs based on the mean fluorescence intensity at different time point after postinjection. Data are represented as average $\pm$ standard error (n = 2).

In general, the substantial accumulation and long retention of biomaterials in RES hinder the clinical translation. Previously, a very high tumor-to-organs signal ratio was also found in the study of CH1055-PEG by Dai and coworkers, where CH1055-PEG was a PEGylated NIR-II fluorophore.¹⁵ Such coincidence of biodistribution may be due to the similar structures of CH1055-PEG and **UMPD1** with small micellar size and flexible PEG chains grafting from the π -conjugated backbone. The time-dependent biodistribution profiles of **UMPD1** showed that the fluorescence signals significantly decayed in the tumor and organs with the increasing period of treatment, indicating that **UMPDs** could be gradually excreted from the body probably through renal and fecal pathway.¹⁵

We speculated that this phenomenon of remarkably specific tumor-targeting was likely due to the ultra-small size of **UMPD1** and soft “stealth” of POEGMA. The main composition of **UMPD1** is oligo(ethylene glycol) units, which has been reported as a robust stealth of nanoparticles to show reduced nanospecific interactions with proteins through its hydrophilicity and steric repulsion effects, reduced opsonization.¹⁶ To investigate the size effect of **UMPD** on the micellar accumulation and biodistribution, **UMPD4** with a size of 25.5 nm in water was chosen as a control and intravenously injected at a same polymer dose of 27 $\mu\text{g/g}$. Not surprisingly, during the process of long-term monitoring for 168 h, the mouse showed weak NIR fluorescence (Fig. 7.8a) owing to the low concentration of fluorophores in **UMPD4**. It is difficult to clearly distinguish tumor from normal tissues due to the slight difference of fluorescence intensity between them. The *ex vivo* imaging results of dissected tumor and organs showed that tumor, stomach and intestine exhibited stronger fluorescence than other organs. At 24 h postinjection, the mean fluorescence intensity in tumor is comparable with that of the intestine and 30% lower than that of the stomach (Fig. 7.8b). Intriguingly, after the distribution process for 168 h, the fluorescence from the tumor significantly decayed while stomach and intestine remained intensively fluorescent (Fig. 7.8b). These data clearly demonstrate that **UMPD4** possessed a poor tumor-specificity ability and a long retention in the stomach and intestine.

The comparison between **UMPD1** and **UMPD4** reveals that **UMPD1** with a smaller size is more suitable for fluorescence-guided chemotherapy due to the higher drug/fluorophore content and the more excellent ability of escape from hepatic filtration, tissue diffusion and extravasation.

7.2.5 Real-time fluorescence-guided chemotherapy

It was given that the intracellular concentration of redox potential (glutathione) in the tumor mass, associated with cellular proliferation and metastatic activity, is 100- to 1,000-fold higher than that in the blood and the extracellular level in normal tissues. Thereby we expected enhanced cleavage of redox-sensitive disulfide-bond locally inside the tumors and the *in-situ* release of CPT for cancer therapy. Xenograft HepG2 tumors were inoculated to mice and allowed to grow to $\sim 0.150\text{ cm}^3$. Subsequently, tumor bearing mice were intravenously injected with **UMPD** (CPT dose of $4\text{ }\mu\text{g/g}$), **UMWD** (polymer dose of $27\text{ }\mu\text{g/g}$) and phosphate buffer solution ($1 \times \text{PBS}$ dose of $200\text{ }\mu\text{L}$) as control via tail vein every 3 days for 18 days ($n = 3$ per group). During this period of treatment, tumor volume and body weight of tumor-bearing mice were monitored every 3 days to evaluate the therapeutic efficacy, accompanied with simultaneous real-time fluorescence imaging to assess the ability of guidance (Fig. 7.9). At the end of the experiments, the tumor volumes in mice treated with **UMPD1** were much smaller than those in mice treated with PBS and **UMWD**. The tumor growth profile for **UMWD** almost showed a same fast-growing tendency of tumor volume with that for PBS, demonstrating no significantly therapeutic efficacy and the good compatibility of **UMWD** (Fig. 7.9c). In contrast, the tumor volume after 18 days' treatment of **UMPD** is 47.06% of that for PBS and 45.89% for that for **UMWD**, indicating the predominant tumor growth inhibitory efficacy of **UMPD**. These observations are in accordance with the results of *in vitro* evaluations.

Figure 7.9 (a) Photograph under ambient light (up) and IVIS fluorescence images (bottom, $\lambda_{ex} = 535$ nm, $\lambda_{em} = 660$ nm) of HepG2 tumor bearing mice treated with **UMPD1** at different time points. (b) Photograph under ambient light of HepG2 tumor bearing mice treated with PBS (left) and **UMWD** (right) at different time points. variation of tumor volume (c) and body weight (d) after intravenous injection of PBS, **UMWD**, **UMPD1** in HepG2 tumor bearing mice. (n = 3 per group).

In addition, negligible changes were observed in the time dependence of body weight of mice. After 18 days-treatment, mice were sacrificed and tumors were dissected and photographed. The tumor inhibitory rate (TIR) was calculated from tumor weight. Compared with that for PBS, the TIR for **UMPD** is 50.35% which is significantly higher than that of 4.59% for **UMWD** (Fig. 7.10c). Moreover, the results of fluorescence monitoring showed that the fluorescence emanating from tumor remained more intense than that of normal tissues and kept turning brighter due to the continuous intravenous injection of **UMPD1** (Fig. 7.9a). Evidently, this imaging function is beneficial to visualize the improvement of drug targeting, the growth of tumor and response to therapeutic treatment. These results demonstrate the importance of intrinsically fluorescent prodrug to achieve effective, site-specific and personalized tumor chemotherapy.

Figure 7.10 (a) Photograph of representative tumors separated from mice after 18 days' treatment with PBS, **UMWD** and **UMPD1**. (b) Mean weight of tumors separated from mice and (c) tumor inhibitory rate (TIR) after 18 days' treatment with PBS, **UMWD** and **UMPD1**, where $TIR = 100\% \times (\text{mean tumor weight of control group} - \text{mean tumor weight of$

experimental group)/mean tumor weight of control group. Data are represented as average $\pm$ standard error (n = 3).

7.3 Conclusions

In conclusion, we have developed a new type of theranostic polyprodrugs based on conjugated polymer bottlebrushes with intrinsically FR/NIR fluorescence from the conjugated backbones. Specifically, we have shown that: (i) the well-defined bottlebrush structure with sufficient hydrophilic moiety of POEGMA enables it to form unimolecular micelles with excellent structural integrity; (ii) the controllable triggered CPT release in the active form from bottlebrushes was achieved in the presence of reductive milieu, especially tumor intracellular cytosol reductive milieu; (iii) the random copolymerization of adjustable content of CPT within hydrophilic coronas facilitates the attack by reductive agents, resulting in improved release rate of CPT; (iv) the inherent backbone PFTB with FR/NIR fluorescence endows these small unimolecular micelles the capability to monitor the tumor performance; (v) the *in vitro* cytotoxicity of **UMPD1** is comparable with the carrier-free CPT; (vi) the *in vivo* and *ex vivo* experiments demonstrated that **UMPD1** presented a remarkable tumor-specific imaging ability, long retention in tumor and potential rapid clearance from body; (vii) the real-time fluorescence trafficking and effective antitumor treatment make UMPD a promising candidate of theranostic drug-delivery system. The design strategy outlined here could have broad applications in the area with combination of therapeutics and diagnostics by way of nanotheranostics. In this regard, our unimolecular micellar polyprodrugs (UMPDs) would have the potential to localize into the tumor cells and to deliver CPT, which could be fully monitored by *in vitro* and *in vivo* real-time fluorescence imaging.

7.4 Experimental section

Materials 10 $\times$ phosphate buffer saline (PBS) buffer with pH = 7.4 (ultrapure grade) is a commercial product of 1st BASE Singapore. MilliQ water (18.2 MQ) was used to prepare the buffer solution from the 10 $\times$ PBS stock buffer. 1 $\times$ PBS

consists of NaCl (137 mM), KCl (2.7 mM), Na₂HPO₄ (10 mM) and KH₂PO₄ (1.8 mM). Chloroform-D (99%) was purchased from Cambridge Isotope Laboratories, Inc. All other chemicals and reagents were purchased from Aldrich or Merck and used as received unless otherwise specified. Conjugated polymer **PFTB-OH** and **BHD-CPT** were synthesized according to our previous report.^{7,10}

Characterization The samples were dissolved with chloroform-d for ¹H NMR measurements on a Bruker AV300 MHz NMR spectrometer. Molecular weight determinations for polymers were made using gel permeating chromatography (GPC) analyses (Agilent 1260, USA). The eluent was THF at a flow rate of 1.0 mL/min. A series of low polydispersity polystyrene standards were employed for the GPC calibration. Emission and excitation spectra of solutions were measured by a Horiba Fluolog 3 spectrofluorometer. UV/Vis spectra of the samples were measured on a SHIMADZU UV-2450 spectrophotometer. TEM measurements were performed with a TEM Carl Zeiss Libra 120 Plus at an acceleration voltage of 120 kV. A 5 μ L droplet of diluted samples was directly dropped onto a copper grid (300 mesh) coated with a carbon film, followed by drying at room temperature. The size distribution of resulting nanoparticles was determined by dynamic light scattering (DLS) using a BI-200SM (Brookhaven, USA) with angle detection at 90°.

Scheme 7.2 Synthesis route of monomer **CPTA**

Synthesis of Monomer CPTA **BHD-CPT** (1.89 mmol, 1 g) and TEA (2.83 mmol, 0.39 mL) were mixed in a round bottom flask. THF (50 mL) was added to the flask via a syringe after purging with N₂ gas, followed by cooling to 0 °C. Acrylyl chloride (2.27 mmol, 0.242 g) was then added via a micro syringe drop wise and

the temperature of the flask was slowly raised to room temperature. After reaction for 24 h, the solution was filtered and the solvent was evaporated. The solid was dissolved in 50 mL of DCM and washed by water (50 mL×3). The organic layer was dried by anhydrous MgSO₄. After filtration, the mixture was concentrated via rotary evaporation and purified via column chromatography (silica gel) using hexane/EA (1/1) as eluent. The compound A-CPT was obtained as white powder (0.35 g, 31.8 % yield)

Scheme 7.3 Synthesis route of conjugated polymer bottlebrushes based polyprodrug.

Synthesis of RAFT macroinitiator PFTB-CTA PFTB-OH (120 mg), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (137.45 mg, 0.492 mmol) and 4-(dimethylamino)pyridine (DMAP) (3 mg, 0.0246 mmol) were put in a two-neck round bottom flask. CH₂Cl₂ (30 mL) was added to the flask via a syringe under N₂ atmosphere, followed by cooling to 0 °C. Dicyclohexylcarbodiimide (DCC) (0.344 mL, 1.5 mmol) was then dissolved in 5 mL of CH₂Cl₂ and added via a micro syringe. The temperature of the flask was slowly raised to room temperature. The reaction was continued for 24 h under stirring. The solution was filtered and

precipitated in cold methanol to purify the product. The RAFT macroinitiator was then dried in vacuum at 50 °C to afford 123 mg of dark red powder.

Synthesis of Polymer Bottlebrushes PFTB-g-POEGMA UMPD1 was taken as representative example. The macroinitiator PFTB-CTA (20 mg, molar of CTA = 0.031 mmol, 1 eq.), olig(ethylene glycol) methyl ether methacrylate (OEGMA) (232.5 mg, 0.465 mmol, 15 eq.) and A-CPT monomer (54.2 mg, 0.093 mmol, 3 eq.) were dissolved in 1 mL of THF in a Schlenk tube. AIBN in THF solution (1 mL $\times$ 0.01 mol/L, 0.33 eq.) was added to the mixture. After three freeze -thaw cycles for removal of oxygen, the tube was then placed in an oil bath at 70 °C for 48 h to allow RAFT. A solution of the crude product was precipitated in cold diethyl ether for three times. The final polymer brush **UMPD1** was then dried in vacuum at 35 °C and obtained as a sticky fluid (272 mg). **UMPD2** and **UMPD3** were synthesized by the similar protocol.

Preparation of Unimolecular Micelles in Aqueous Medium 1 mL of THF was used to dissolve the UMPDs (5 mg). After that, 5 mL of water or PBS buffer was directly added to dilute the solutions. After the exposure in open air for the evaporation of THF overnight, solutions with a concentration of 1 mg/mL were obtained and diluted to the desired concentration for further experiment.

***In Vitro* Drug Release of UMPDs** As described above, the aqueous dispersions of UMPDs were obtained. The dispersion (1 mg/mL) was placed in 10 mM PBS buffer at pH 7.4 (0 mM DTT, 2 μ M DTT, 2 mM DTT or 5 mM DTT) and transferred to a dialysis tube (molecular weight cutoff: 3,500 Da) immersed in the same buffered media at 37 °C. After varying time intervals, aliquots of the external medium were withdrawn and replaced with the same volume of fresh buffer solution. The fluorescence spectroscopy was employed to assay the CPT content to determine the CPT release rate based on a calibration curve with an excitation wavelength at 365 nm. The loading content (LC %) of CPT was calculated by the following equations:

$$LC (\%) = \frac{\text{Molecular weight of CPT in UMPDs}}{\text{Molecular weight of UMPDs}} \times 100\%$$

Cell Culture HepG2 human liver cancer cell, HeLa immortal cancer cells and MCF-7 breast cancer cells were cultured in folate-free Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified environment containing 5% CO₂. Before experiments, the cells were pre-cultured until confluence was reached.

In Vitro Cellular Uptake HepG2, HeLa and MCF-7 cells were seeded in 12-wells plates at 37 °C (Costar, IL, USA). After 80% confluence, the medium was removed and the adherent cells were washed twice with 1×PBS buffer. The free CPT (10 µg/mL) and UMPDs (concentration of CPT ~ 10 µg/mL) in DMEM medium were then added to different chambers, respectively. After incubation for 2h, 6h and even 11 h, the cells were washed five times with 1×PBS buffer and then fixed with 75% ethanol for 10 min. After washing them by 1×PBS buffer for three times, UMPD stained cells were directly recorded by a confocal laser scanning microscopy (LSM 710, Carl Zeiss, Germany). The lasers of 405, 514 nm were used to excite CPT and PFTB, respectively. The emission signals were collected by using a band pass filter combination 410-546, 546-735 nm for imaging in two individual channels. For free CPT stained cells, they were further incubated for 30 min in 1×PBS containing 1% (wt/vol) BSA. Then Alexa Fluor® 633 phalloidin diluted 20 times according to the protocol in 1×PBS was added to stain filamentous actin (F-actin) cytoskeleton for 1 h at room temperature. The cells were further washed by 1×PBS buffer and ready for characterization by CLSM (Objective: LD Plan-Neofluar 20x/0.4 Korr M27). Another laser of 633 nm was used to excite Alexa Fluor® 633 phalloidin.

Cytotoxicity study The cytotoxicity of UMPDs against HepG2 and HeLa cancer cells was evaluated by Presto blue (PB) assay. Briefly, HepG2 and HeLa cells were seeded in 96-well plates (Costar, IL, USA) at an intensity of 1×10⁴ cells mL⁻¹. After 24 h incubation, the cells were exposed to a series of doses of UMPDs at 37 °C. After the designated time intervals, the wells were washed twice with 1×PBS

buffer and 100 μ L of freshly prepared PB solution in culture medium was added into each well. The PB medium solution was carefully removed after 1 h incubation in the incubator. The absorbance of PB at 570 nm and 600 nm was monitored by the microplate reader (Genios Tecan). Cell viability was expressed as the ratio of the percent PB reduction of the cells incubated with UMPDs suspension to that of the cells incubated with culture medium only.

7.5 Supporting information

Figure S7.1 ^1H NMR (CDCl_3 , 300 MHz) spectrum of monomer A-CPT.

Figure S7.2 GPC traces of conjugated polymer bottlebrushes PFTB-*g*-P(ACPT-*co*-OEGMA) (a) UMPD2 and (b) UMPD3 using RI and UV-vis detectors.

Figure S7.3 DLS results of (a) **UMWD**, (b) **UMPD2**, (c) **UMPD3**, (d) **UMPD4** in aqueous media and (e) **UMPD1**, (f) **UMPD2**, (g) **UMPD3** in THF.

Figure S7.4 TEM image of air-dried **UMPD2** (a) and **UMPD3** (b).

Figure S7.5 (a) *in vitro* CPT release profiles of **UMPD1** in PBS buffer upon treating with DTT of varying concentrations: (■) 0.1 mM DTT; (●) 1 mM DTT.

Figure S7.6 DLS result of **UMPD1** in water recorded in the presence of DTT (10 mM).

Figure S7.7 UV-vis absorption and FL emission spectra of (a) **UMPD2** and (b) **UMPD3** in water. (excitation wavelength of 365 nm in FL emission spectra). FL emission spectra and excitation spectra of (c) (e) **UMPD2** and (d) (f) **UMPD3** recorded in the presence and absence of DTT (10 mM).

Figure S7.8 CLSM images of HepG2 cells at lower magnification after incubation with aqueous dispersion of free CPT at a CPT equivalent dosage of 10 μg/mL for 2 h and aqueous dispersion of **UMPD1** for 2 h, 6 h, and 11 h.

Figure S7.9 CLSM images of HeLa cells after incubation with aqueous dispersion of free CPT at a CPT equivalent dosage of 10 μg/mL for 2 h and aqueous dispersion of **UMPD1** for 2 h, 6 h.

Figure S7.10 CLSM images of MCF-7 cells after incubation with aqueous dispersion of free CPT at a CPT equivalent dosage of 10 μg/mL for 2 h and aqueous dispersion of **UMPD1** for 2 h, 6 h.

Figure S7.11. The distribution profiles of **UMPD1** (a) and **UMPD4** (b) in excised organs based on the total fluorescence intensity at different time point postinjection. Data are given as mean $\pm$ standard error (n=2).

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Chapter 8 Summary and outlook

Noninvasive molecular imaging based on organic R/NIR fluorescent materials is emerging as a versatile tool for diagnosis and imaging guided therapy. This thesis gives the introduction of background and literature overview on the recent advances of organic R/NIR fluorescence materials in **Chapter 1** to gain insights into their diversity and superiority. In the end of **Chapter 1**, we describe the existing challenges in this field which are also the objectives of this thesis. To overcome these challenges, several strategies have been developed here through controlling the molecular packing structures, evaluating the structure-property relationship and introducing foreign materials to influence the interaction among fluorophores.

As the aggregation behavior is strongly related to the optical properties of fluorophores, it is crucial to control the aggregated state of the molecules. **Chapter 2** describes the facile synthesis and characterization of two indigo derivatives bearing tert-butyloxycarbonyl groups and acetyl groups at imide site of indigo, so called Indigoid-B and Indigoid-A, respectively. Indigoid-B was non-fluorescent in tetrahydrofuran (THF) while Indigoid-A was highly red fluorescent in THF. With the increase of water fraction (f_w) in THF solution upon ultrasonication, Indigoid-B showed strong emission near 600 nm at $f_w > 80\%$. This behavior was attributed to the formation of well-defined microcrystals of Indigoid-B, which was confirmed by SEM, TEM and XRD analysis. In contrast, the emission of Indigoid-A almost disappeared at $f_w \sim 70\%$ and a moderate recovery of emission was observed at $f_w > 90\%$. Interestingly, in the absence of ultrasonication, both Indigoid-B and Indigoid-A dispersions at $f_w > 80\%$ were non-fluorescent due to the formation of amorphous aggregates. Evidently, the ultrasonication plays a key role in inducing the amorphous-to-crystalline transition of Indigoid-B and Indigoid-A. The discovery of Indigoid-B, serving as a fresh member with unprecedented aromatic system in AIE family, provides new insights for the design R/NIR-emissive AIE fluorophores in a facile and cheap way.

In **Chapter 3**, we extended the investigation of molecular packing structures of NIR-absorbing pyrrolopyrrole cyanines (PPcy) derivatives in aqueous medium. Specifically, hydrophobic PPcy derivative PCBF was encapsulated with the assistance of synthetic amphiphilic block copolymers to give colloidal nanoparticles (denoted as **JAGG** NPs). The absorption spectrum of JAGG dispersion showed H-, M- and J- bands centered at 647, 708 and 768 nm, respectively. The control experiment of monitoring the spectral evolution of PCBF at different water/THF ratios confirmed that the J-aggregates are thermodynamically more stable than H-aggregates. These results also helped to explain that the treatment of ultrasonication of **JAGG** dispersion facilitated the transition from H-aggregates to J-aggregates. The **JAGG** dispersion exhibited an emission band at 771 nm, a full width at half maximum (FWHM) of 19 nm and a comparable quantum yield with ICG. Moreover, the biocompatibility and *in vivo* imaging using **JAGG** NPs were preliminarily assessed.

The strategy to tune optical properties by controlling molecular packing structures is disabled for many fluorophores, especially macromolecular fluorophores. Hence, the incorporation of fluorescence-silent materials to suppress the aggregation caused quenching effect of R/NIR macromolecular fluorophores emerges as an effective solution. **Chapter 4** describes a series of hydrophobic sheath segregated macromolecular fluorophores PFTB-g-PCL, where biodegradable polycaprolactone (PCL) with tunable chain length are grafted from the conjugated backbones. After encapsulation by surfactant PCL-*b*-POEGMA, the obtained nanoparticles, so-called **nanoREDs**, showed remarkably improved quantum yield above 18% with respect to that in the absence of grafted PCL. With the increasing $DP_{n,PCL}$ of grafted PCL from 13 to 88, a slight enhancement of the relative quantum yield of **nanoREDs** from 18% to 24%. The *in vitro* experiments demonstrated their good biocompatibility and efficient cellular uptake as evidenced by cytotoxicity and cellular imaging results.

In **Chapter 5**, we further imported PCL-tethered PPcy (denoted as PCL-PPcy-PCL) as acceptor into **nanoREDs** to enable the occurrence of efficient Föster

resonance energy transfer (FRET). The emission spectra of **nanoREDs** and absorption spectrum of PCL-PPcy-PCL in diluted solution showed a good overlap. Similarly, the hydrophobic sheath-like PCL moiety assures the homogeneous nature of hydrophobic core, leading to a high quantum yield as a NIR fluorescent probe and a good optical stability due to the effective suppression of leakage of fluorophores by entanglement of PCL. The optimized NPs, so-called **B1P1^{1/2}** FRET NPs, with a high quantum yield of 46% and a small FWHM of 35 nm, were prepared by adjusting the D-A distances. The excellent fluorescence and photostability of **B1P1^{1/2}** FRET NPs make it possible to implement in vivo tracking of the tumor growth.

To integrate the cancer chemotherapeutic function for fabricating theranostic formula, the intrinsically fluorescent unimolecular micelles are considered as a good nanocarrier for antitumor drugs due to the stable micellar structure and tunable micellar size. In **Chapter 6**, the chain extension on the precursor PFTB-g-PCL proceeded via atom transfer radical polymerization of OEGMA. The introduction of the hydrophilic block POEGMA enabled the formation of unimolecular micelles (UMs) in aqueous medium. The controllable sizes of these UMs were achieved by adjusting the chain length of PCL and POEGMA while distinct morphologies such as butterfly- or flower-like were observed. Due to the absence of self-quenching phenomenon of the PFTB backbone, all these UMs showed high quantum yield of approximately 22%. Moreover, the hydrophobic PCL layer was utilized to entrap doxorubicin (DOX). The obtained theranostic UMs showed comparable cytotoxicity against HeLa cancer cells with free DOX and relatively low cytotoxicity against L929 normal cells.

To overcome the potential leakage of drugs from the UM host, in **Chapter 7**, unimolecular micelles based polyprodrugs (**UMPD**) were prepared by grafting from PFTB backbones via reversible addition fragment transfer (RAFT) copolymerization of POEGMA and acrylate decorated camptothecin (CPTA) monomers. A series of polymers PFTB-g-(POEGMA-co-PCPTA) with different side chain lengths and drug loading contents were synthesized by adjusting the feed

monomer ratio for polymerization. The presence of disulfide bond in CPTA units enables the controllable triggered-release of CPT in the redox microenvironment such as high GSH level in tumor. The triggered-release of CPT was evidenced by the disappearance of FRET manner between donor CPT and acceptor PFTB upon the addition of DTT ($\sim 10\ \mu\text{M}$). After intravenous injection into HepG2 tumor-bearing nude mice, **UMPD1** exhibited an excellent tumor targeting monitoring performance with bright far red fluorescence and a high tumor inhibitory rate of 50.35%. The *ex vivo* biodistribution results indicated the specific accumulation and long retention of **UMPDs** in tumor.

In summary, this thesis has developed several effective strategies to overcome the challenges in the field of R/NIR fluorescence imaging through precise control of molecular packing structure, functionalization of fluorophores with biodegradable polymers and design of novel unimolecular micelles for diagnosis and imaging guided chemotherapy. Future work on R/NIR fluorescent imaging materials could be directed towards the following aspects.

First, more exploration and optimization in controlling molecular packing structures of R/NIR emissive fluorophores in the nanoconfined space are needed. To date, many fluorophores in bulk have been reported to undergo the variation of the molecular-packing structure in response to different stimuli such as temperature and mechanical stress, resulting in tunable wavelength and intensity. However, the limitations of these fluorophores such as unfavorable short wavelength and the poor accessibility of spectral tailoring in confined space hamper their imaging applications at an *in vitro* or *in vivo* level.

Second, the leading role of biodegradable polymer such as PCL, PLA and PLGA in previous organic probes is to improve the quantum yield through preventing fluorophores from approaching each other. Beyond this, it should be possible to tune optical properties by regulating the chemical structure, glass-transition temperature, melting points of incorporated polymers and even controlling the morphology of phase separation between fluorophore and polymer in the interior of nanoparticles. For example, the special interaction such as Van Der Waals force

and hydrogen bonding between fluorophores and polymers may direct the various molecular packing structures.

Third, our strategies by conjugating biodegradable polymers to fluorophores also have the potential to be applied in organic NIR-II systems. Dai and coworkers have reported that probes emitting in the NIR-II window (1000-1700 nm) exhibited deeper tissue penetration and signal-to-background ratios with respect to NIR-I ones.¹ However, an ideal clinical candidate of NIR-II probe requires further improvement in fluorescence quantum yield.

Finally, nowadays, near-infrared fluorophores for biomedical bioimaging have attracted increasing attention and research efforts with many publications. However, the translation into clinical diagnostics is hindered by the lack of FDA-approved NIR probes. Therefore, it is imperative to increase the clinical applicability of currently available probes, which requires further preclinical studies such as evaluation of toxicology and pharmacokinetics.

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List of Publications

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