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

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We report molecular design, synthesis and characterization of multifunctional unimolecular micelles formed by amphiphilic bottle-brush-like grafted block copolymers with theranostic functions of bright far red/near infrared (FR/NIR) fluorescence and anticancer drug delivery. Specifically, conjugated polymer poly(fluorene-*alt*-(4,7-bis(hexylthien)-2,1,3-benzothiadiazole)) (PFTB) was utilized as fluorescent backbone and macroinitiator for grafting with polycaprolactone (PCL) and poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA) blocks via ring opening polymerization (ROP) and atom transfer radical polymerization (ATRP) successively, resulting in highly fluorescent polymer bottlebrushes, PFTB-g-(PCL-*b*-POEGMA). The amphiphilic and branched nature of these grafted block copolymers enabled formation of unimolecular micelles (UMs) that were well dispersible in aqueous media. A series of PFTB-g-(PCL-*b*-POEGMA) polymers with the same conjugated backbone but different lengths of PCL and POEGMA side chains form UMs with different sizes and morphologies. A morphological transition of UMs from spherical to flower-like structure was observed with the increase of POEGMA chain length. Compared to polymer PFTB-g-POEGMA, the introduction of hydrophobic and biodegradable PCL segments improved the fluorescence quantum yield from 0.16 to 0.25, accompanied by an obvious enhancement of the photostability of the UMs. These optical properties and good biocompatibility of these UMs make them attractive as bioimaging probes for visualization of biological systems. Moreover, the hydrophobic PCL domains in these fluorescent UMs provide a reservoir for loading hydrophobic anticancer drugs such as doxorubicin (DOX). The effect of PCL and POEGMA chain lengths on the drug loading and release was studied. These DOX-loaded UMs could be well uptaken by HeLa and L929 cells and showed selectively higher cytotoxicity against HeLa cells than L929 cells, thereby representing a promising candidate for cancer theranostics.

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 biomedical/pharmaceutical industries.^{1,2} 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.³⁻⁷ 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, the capability

for long-term tracking^{8,9} and relatively low toxicity compared to inorganic materials.¹⁰⁻¹² For instance, indocyanine green (ICG), a FDA-approved near infrared (NIR) dye, 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.^{13,14} 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.¹⁴⁻¹⁸ Particularly, far red/near infrared (FR/NIR) fluorescent conjugated polymers with premium optical properties such as their high absorption coefficient, bright fluorescence and robust photostability have attracted considerable attention for bioimaging applications.¹⁹⁻²⁴ Nevertheless, the integration of conjugated polymers as FR/NIR fluorescence imaging modality into a theranostic platform remains rarely reported.²⁵

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.^{26,27} Although polymeric micelles often exhibit better stability with

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Electronic Supplementary Information (ESI) available: Experiment details about the synthesis of PFTB-g-(PCL-*b*-POEGMA); additional ¹H-NMR spectra; DLS results of UMs and DOX@UMs; cytotoxicity of UMs and DOX@UMs against L929 cells; additional confocal fluorescence microscope images. See DOI: 10.1039/x0xx00000x

CMC values in the μM range compared to the micelles formed by small molecular surfactants²⁸, after intravenous administration, the external factors such as temperature, pH, ionic strength, large shear forces during circulation in physiological systems will affect structural stability of micelles, resulting in the dissociation of micellar aggregates.²⁹ 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.^{30–36} 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.³⁵ 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.^{34, 37}

Herein, 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 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 1b).

The present strategy of unimolecular micelles based on amphiphilic diblock copolymer bottlebrushes shows the following advantages over the previously reported nanoREDs³⁸ 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 can 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 1(b-c)).

Scheme 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.

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.

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

Scheme 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), synthesized according to a similar method reported previously,³⁸ was used to induce ring opening polymerization (ROP) of ϵ -caprolactone in the presence of Sn(Oct)₂ as the catalyst at 100 °C. The following esterification reaction with 2-bromoisobutyl 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 Tab. 1.

Table 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 (g/mol)	M _{n,GPC} ^c (g/mol)	PDI ^d	$\lambda_{\max,abs}$ (nm)	$\lambda_{\max,em}$ (nm)	D _{UM} ^e (nm)	Φ_{UM} ^f (%)
I0	PFTB ₁₀ -OH	--	9,100	2.54	516 ^g	616 ^g	--	30.0 ^g
I1	(PFTB-g-(PCL ₈ -Br) ₂) ₁₀	28,900	35,800	1.91	--	--	--	--
I2	(PFTB-g-(PCL ₃₅ -Br) ₂) ₁₀	90,500	70,700	1.61	--	--	--	--
I3	(PFTB-g-(PCL ₅₉ -Br) ₂) ₁₀	145,200	111,900	1.56	--	--	--	--
P0	(PFTB-g-(POEGMA ₂₇) ₂) ₁₀	280,700	64,600	1.56	528	639	20.2	16.7
P1	(PFTB-g-(PCL ₈ -b-POEGMA ₁₆) ₂) ₁₀	188,900	115,100	1.35	518	635	26.8	22.0
P2	(PFTB-g-(PCL ₃₅ -b-POEGMA ₂₉) ₂) ₁₀	380,500	143,700	1.60	518	629	30.6	22.7
P3	(PFTB-g-(PCL ₃₅ -b-POEGMA ₁₁₈) ₂) ₁₀	1270,500	234,600	2.22	518	639	54.2	19.8
P4	(PFTB-g-(PCL ₅₉ -b-POEGMA ₃₆) ₂) ₁₀	505,200	193,200	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 (Φ_F = 0.95); ^g Measured in toluene.

Polymer Chemistry

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Fig. 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 (PFTB-g-(PCL₃₅)₂)₁₀ completely shifted to 4.19 ppm (labeled by Δ in Fig. 1a&b) while a new methyl signal (peak f) at 1.9 ppm appeared in the ^1H NMR spectra of the (PFTB-g-(PCL₃₅-Br)₂)₁₀. Moreover, in Fig. 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. 1b. As shown in ^1H NMR spectra of (PFTB-g-(PCL₃₅-b-POEGMA₂₉)₂)₁₀ (Fig. 1c), the signals at 3.40 and 3.66 ppm could be assigned to methylene protons adjacent to oxygen and the protons of the methoxy end groups on OEGMA units, respectively, while the signals at 2.32 and 4.07 ppm from the PCL are still present. Similarly, the DP of POEGMA block ($\text{DP}_{\text{POEGMA}}$) can be determined by the integral ratio of peak a to peak f in Fig. 1c. In addition, the ^1H NMR spectra of PFTB-Br and PFTB-g-POEGMA shown in Fig. S1 confirm the complete conversion from PFTB-OH to PFTB-Br and successful synthesis of hydrophilic polymer bottlebrushes PFTB-g-POEGMA.

Fig. 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)).

Fig. 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 (wavelength: 500 nm).

The gel permeation chromatography (GPC) traces of all diblock polymer bottlebrushes in Fig. 2 depicted monomodal molar mass distribution with moderate polydispersity indices ($PDI=1.35\sim2.20$). The number-average molecular weight and PDI of the obtained polymers measured by GPC using linear polystyrenes as standards are shown in Tab. 1. Chromatogram peaks of PFTB-g-POEGMA (P_0) and PFTB-g-(PCL-b-POEGMA) (P_1 - P_4) from the refractive index (RI) detector and UV-vis detector all shifted to lower elution volume due to the chain extension (Fig. 2), compared with the elution peaks of their respective macroinitiators (I_0 - I_3).

Size and Morphological Characterization of Unimolecular Micelles

The solubility of the PFTB-g-(PCL-b-POEGMA) polymers (i.e. P_1 - P_4) is determined by the relative chain lengths of PCL and POEGMA. First of all, P_0 with the long hydrophilic POEGMA brushes and the absence of hydrophobic PCL is well soluble in aqueous media. Such good solubility of P_0 in water enables convenient preparation of bioimaging probes without use of any organic solvent. Among P_1 - P_4 , only P_1 with the shortest PCL side chains could be directly dissolved in water under stirring or shaking. For polymers P_2 - P_4 , the longer PCL chains limited their solubility in water. As a consequence, in order to obtain unimolecular micelles (UMs) of P_2 - P_4 , it was necessary to dissolve them first in a common solvent such as THF before addition of water as the selective solvent for POEGMA.³⁹ 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 P_0 - P_4 in water and in THF were measured with dynamic light scattering (DLS). While P_0 in water and THF both

exhibit the similar micellar size of 20 nm as shown in Fig. 3b and Fig. S2a, the particle size of P_1 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. 3) of the amphiphilic polymer bottlebrushes P_2 - P_4 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 P_2 to P_3 is consistent with the increase of the chain length of POEGMA corona, while the chain length of PCL is maintained the same. The number-average hydrodynamic diameter for P_2 , P_3 and P_4 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. S2a-e and Tab. S2), which should also be attributed to the swelling of inner PCL cores in THF. Based on the results above, we conclude that P_{1-4} all existed as unimolecular micelles (denoted as UM-P1, UM-P2, UM-P3 and UM-P4, respectively) in aqueous media.^{40, 41} 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 colloidal stability, evidenced by little change of particle size after being stored at 5 °C over three months (as shown by the DLS results in Fig. S2f-j).

The morphologies of UM-(P_0 - P_4) 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- P_0 and UM- P_1 (Fig. 3a and Fig. 3c). The average diameter of both UM- P_0 and UM- P_1 from TEM images was determined to be 10.8 ± 2.5 and 18.9 ± 4.2 nm, respectively (Fig. 3b and Fig. 3d). Evidently, in the dry state, the shrinkage of POEGMA corona with high density

Fig. 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 schematic structures of the unimolecular micelles of UM-P0, UM-P1, UM-P2, UM-P3 and UM-P4, respectively, in the dry states, in which the light-blue part corresponds to PEOGMA domain, the orange part corresponds to PCL domain, and the red rod in the center corresponds to single PFTB backbone.

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. S3. 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. 3e). Such morphological transition from UM-P1 (Fig. 3c) to UM-P2 (Fig. 3e) could be attributed to the larger PCL core, leading to the decreased PCL/POEGMA interfacial curvature and lower density of POEGMA corona.⁴²⁻⁴⁵ 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. 3g, h). Due to the relatively broad molar-mass distribution of PFTB backbones, those amphiphilic bottlebrushes with longer PFTB backbones formed larger micelles with more petals as shown in the inset of Fig. 3g. The strong steric effect of ultra long POEGMA chains in P_3 may force the stretching and branching of both PCL and POEGMA arms, resulting in the flower-like morphology of UM-P3 with longer petals.²⁸ 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. 3h). P_4 containing longer PCL blocks ($DP_n = 59$) and relatively short POEGMA ($DP_n = 36$) formed irregular butterfly-like micelles in water, as shown in Fig. 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. S3).

The DLS and TEM results described above prove the formation of unimolecular micelles for P_0 - P_4 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.

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.⁴⁶⁻⁴⁹ We expected that the polymers (P_0 - P_4) 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 the hypothesis above, 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- $(P_0$ - $P_4)$ are presented in Fig. 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 diluted toluene ($\lambda_{max,abs} = 512$ nm, $\lambda_{max,em} = 616$ nm) (Fig. 4a & 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 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.⁵⁰⁻⁵² Unimolecular micelles formed by **P** with the shortest PCL block ($DP_n = 8$) showed a moderately

increased FL quantum yield to 0.22, with the absorption and emission bands centered at 518 and 635 nm, respectively (Fig. 4c & 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.^{38, 50}

Fig. 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 Fig. S5 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. 4c & 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 overstretching 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. 3g).

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 $\sim 40\%$ (Fig. 5), which was obviously lower than that (by $\sim 76\%$) 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. S4), 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.⁹ 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.

Biocompatibility of PFTB-g-(PCL-b-POEGMA) 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. Fig. S6 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 Fig. S6. 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.

Fig. 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.

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 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 Tab. S3. 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 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.⁵³ After drug encapsulation, the hydrodynamic diameter of UM-P1 slightly

increased from 26.8 to 29.4 nm (Fig. S7). A similar size variation of UM-P2 and UM-P4 was observed upon loading DOX.

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 Fig. 6a, 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.^{54, 55} 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 (as shown in Fig. 6b). 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.

Fig. 6 (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 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 Fig. 6b. And the cytotoxicity was independent on the concentration of DOX (from 5 to 25 µg/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. 6b). 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 µg/mL (Fig. 6c), but not at a lower concentration of DOX (e.g. in the range of 5 to 15 µg/mL). In addition, the cytotoxicities 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. 6d) under all the tested concentrations of DOX. In particular, the unimolecular micelles containing 25 µg/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 Fig. 7a.

In order 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 Fig. S8, 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 µg/mL of DOX, the relatively low cytotoxicity was reflected from a relatively high cell viability of ~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,⁵⁶ 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 Fig. 3(c,e)) and a relatively loose hydrophobic core of the unimolecular micelles (e.g. UM-P2, Fig. 6b) that released the drugs faster. A further increase of the POEGMA chain length from $DP_n = 29$ to 36 retarded the release of drugs, presumably due to the bulkiness of the hydrophilic corona that induces a diffusion barrier for the drugs. Overall, the chain lengths of both PCL ($DP_n = 8$) and POEGMA ($DP_n = 16$) blocks in UM-P1 appears optimal regarding its single-micelle FL brightness and drug release kinetics, despite its moderate drug-loading capacity (ca. 7wt%).

Cellular Imaging Using Fluorescent Unimolecular Micelles as Contrast Agents

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.

Fig. 7 and S9 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 cytoplasm, which implies that UMs with the sub-30 nm size were well dispersed in 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.⁵⁷ 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.⁵⁸

In addition, to avoid the interference of fluorescence from DOX and PFTB for future in vivo theranostic application, the backbone of PFTB could be modified or replaced with other π -conjugated units to extend the emission to longer wavelength (e.g. > 750 nm). In addition, other hydrophobic drugs such as paclitaxel and camptothecin with shorter emission wavelengths than DOX could also be encapsulated using the present unimolecular micelles as carriers.

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 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 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.

Fig. 7 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 .

Experimental

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), Na_2HPO_4 (10 mM) and KH_2PO_4 (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.^{38, 59}

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 1mg/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 a 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 488nm. 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 as follows:

$$QY_{UM} = QY_{ref} \times \frac{slp_{UM}}{slp_{ref}} \times \frac{n_{UM}^2}{n_{ref}^2}$$

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 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°. 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 -Bromoisobutyl 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), N,N,N',N'',N'' -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 taken away from glove box. After three freeze-and-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 ($Sn(Oct)_2$) (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. BIB (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 PCL macroinitiator. 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 N_2 protected glove box, 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 the cases, ATRP was proceeded by choosing 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 Tab. S1. The mixture was immediately frozen in liquid nitrogen after taken away from glove box. After three freeze-and-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 μ L 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 predetermined 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 provided by American Type Culture Collection (ATCC, USA). They 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-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, 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.

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Table of Content

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