Chao-Tsen Chen et al.
Tuning of hydrogen peroxide-responsive polymeric micelles of biodegradable triblock polycarbonates as a potential drug delivery platform with ratiometric fluorescence signaling
Tuning of hydrogen peroxide-responsive polymeric micelles of biodegradable triblock polycarbonates as a potential drug delivery platform with ratiometric fluorescence signaling†

Ying-Hua Fu, Chun-Yen Chen and Chao-Tsen Chen*

Dual-function theranostic micelles of amphiphilic triblock polycarbonates that allow the possible targeted drug release and simultaneous ratiometric fluorescence sensing in response to H₂O₂ are reported. The disassembly and fluorescence behaviors of micelles are highly influenced by the structural features of the H₂O₂-reactive core-forming hydrophobic blocks. DLS and TEM results indicate that the aliphatic boronate-bearing micelles swell to loosely-aggregated micro-sized nanostructures whereas the aromatic boronate-bearing micelles undergo a complete disassembly giving small fragments instead upon the treatment of H₂O₂. Correspondingly, two micelles exhibit different fluorescence response kinetics and magnitudes. Furthermore, cell uptake study, in vitro cytotoxicity analysis, and drug surrogate release experiment monitored by fluorescence resonance energy transfer confirm that the micelles are biocompatible and feasible for the targeted drug delivery application. Collectively, the study provides molecular insights into design of targeted dual-function theranostic nanocarriers with effective disassembly as well as ratiometric fluorescence signaling.

Introduction

Driven by the shortcomings of small-molecule anticancer drugs in the clinical application,1–3 a variety of biocompatible nanosystems, including liposomes,4–7 hydrogels,8–10 cross-linked polymeric nanoparticles,11–14 and dendrimers15–17 have been explored as drug delivery nanovectors to enhance the bioaccessibility and efficacy of anticancer drugs, and to reduce the effective dosage and systemic toxicity of therapeutic agents. Among these, stimuli-responsive nanocarriers can mediate targeted delivery of therapeutics to the lesion, largely minimizing the undesired side effects.18–21 Physiochemical abnormalities near or within the tumor cells enable stimuli-responsive nanocarriers to release payloads (drugs) in response to specific environmental stimuli at the targeted tumor sites; this enables precise delivery and improves pharmacokinetics.22–25 To date, many pH-sensitive26–32 and/or reduction-sensitive31,33–36 polymeric nanocarriers have been used to target the highly acidic extracellular pH and higher cytosolic GSH concentrations, respectively, of the tumor cells. In contrast, the nanocarriers that respond to the oxidative environment around the tumor sites with fluorescence signaling have been less frequently used for simultaneous targeted therapeutic delivery and biosensing/bioimaging.37–40

Due to an increased metabolic activity, cancerous and inflammatory cells are often considered to be surrounded by a higher concentration of reactive oxygen species (ROS)—mainly H₂O₂. The higher ROS concentration not only enhances the proliferation of cancerous cells but also modulates their angiogenesis and metastasis.41 In some cancers such as prostate cancers, the amount of intracellular ROS varies with the progression of cancer and has several pathological roles.42 Additionally, leukocytes discharge plenty of microbicidal ROS into phagolysosomes or in the extracellular environment to kill invading microorganisms. The generated ROS further activates cellular inflammatory pathways (e.g., NFκB/Rel pathway) by modulating cellular redox-sensitive subunits or precursors, and this leads to gene expression/amplification of pro-inflammatory mediators, resulting in a more aggravated inflammatory response.43–45 In addition, chronic inflammation, which often originates from failure to resolve acute inflammation, has also been proven to be highly relevant to cancer occurrence. Thus, the development of efficient ROS-sensitive nanocarriers is highly desirable for targeting cancer and for use as inflammatory therapeutics.

Our group recently reported a crosslinked polyboronate nanoprobe, which was covalently labeled with environmentally
sensitive 3-hydroxyflavone (3-HF) fluorophores to selectively detect H$_2$O$_2$ with the synergistic modality of fluorescence change and particle swelling. These features suggest that the nanoprobe has potential as a dual-function drug delivery nanocarrier with stimuli-responsive sensing and drug release. However, the limited swelling of the crosslinked nanoparticles prevents efficient drug release from the expanding interstices of the nanoparticles, especially when larger molecules are used as therapeutic agents. It is also noted that the crosslinked nanoparticle formulation, although robust, is still restricted by the difficulty encountered when anchoring targeting ligands to the particle surface, inconvenient drug loading, and the lack of metabolic biodegradation pathways, which reduce its feasibility for further biomedical applications. To alleviate the limitations of the crosslinked polymeric nanoparticles as an effective targeting delivery as well as fluorescent imaging probe, synthetic amphiphilic copolymers with covalently embedded fluorophores that can programatically self-assemble into polymeric micelles or vesicles (polymersomes) in aqueous media are promising as a multifunctional theranostic drug delivery system due to their considerable mechanical/chemical stability, high drug-loading capability, and flexibility. Herein, we describe a new bio-compatible theranostic drug delivery micellar nanosystem bearing different H$_2$O$_2$-reactive pendant groups and environmentally sensitive 3-hydroxyflavone (3-HF) fluorophores. The system is capable of simultaneous ratiometric sensing and drug release in response to H$_2$O$_2$ in the biologically relevant concentration range. The working principle of the ensemble is shown in Fig. 1. The dual-functional micellar nanoparticles are fabricated from the self-assembly of ABA-type triblock amphiphilic polycarbonates, generally consisting of a hydrophobic H$_2$O$_2$-reactive block covalently linked with two lateral hydrophilic blocks. Small hydrophobic drugs are thus encapsulated into the hydrophobic interior of the micelles to afford drug-loaded micellar nanoparticles. Upon exposure to H$_2$O$_2$, the H$_2$O$_2$-reactive hydrophobic moieties at the cores of micelles are oxidatively cleaved to give the corresponding hydrophilic residues, which leads to the disassembly of micelles with a concomitant release of the encapsulated drug molecules. Covalently labeled 3-HF fluorophores are programmed to report the drug release event with a ratiometric green-to-blue fluorescence response, signaling the liberation of buried 3-HFs from the hydrophobic micellar core (prefer to undergo excited state intramolecular proton transfer (ESIPT) emission) to the fully hydrated medium (prefer to undergo excited-state intramolecular charge transfer (ESICT) emission).

The chemical composition of the ABA-type triblock amphiphilic polymers HP-3HF-Alk$_n$PC and HP-3HF-ArPC is shown in Fig. 2. Polycarbonate was chosen to be the backbone of the triblock copolymers due to its excellent biocompatibility, non-toxic degradation products, and tunable mechanical properties. It can be synthesized from cationic, anionic, enzymatic, and organo-catalyzed ring-opening polymerization (ROP) of cyclic carbonate monomers. Furthermore, the living/controlled characteristics of the ROP process allow precise control of the polymer molar mass with narrow polydispersity, a well-defined composition/architecture, and complete end-group fidelity, rendering the construction of sophisticated multiblock copolymers equipped with diverse functionalities feasible. The poly(TMC-BE) block serves as the H$_2$O$_2$-reactive site and endows the required hydrophobicity for the core formation of micelles. To achieve the disassembly of micelles upon H$_2$O$_2$ treatment, it is critical that the polarity transition should be large enough to conform to the polarity reversal, and allow sufficient water influx to solvate the resulting hydrophilic residue and overcome the noncovalent interactions among the polymer chains. Accordingly, aliphatic and aromatic boronic esters, which are hydrolyzed via different demasking mechanisms, were used to investigate the effects of boronate structures on cleavage kinetics and disintegration behaviors of polycarbonate micelles (Fig. 2a). Triblock polycarbonates, namely HP-3HF-Alk$_n$PC and HP-3HF-Ar$_n$PC, appended with sterically hindered alkylboronic esters with a methylene and a trimethylene, respectively, are devised to afford hydrophilic alcohols after reacting with H$_2$O$_2$. The mechanism of transformation is illustrated in Fig. 2b. HP-3HF-Alk$_n$PC with a shorter linker (C$_3$) is expected to create a larger polarity reversal than its C$_3$ counterpart upon H$_2$O$_2$ treatment. The aryloboronic pinacol ester-bearing copolymer HP-3HF-ArPC is also devised to generate a highly hydrophilic and negatively-charged poly(TMC-carboxylate) product upon treatment with H$_2$O$_2$, via a two-step cascade reaction of oxidative boronic ester cleavage followed by a rapid 1,6-type quinone-methide elimination. Intuitively, the cleavage kinetics for the two-step cascade reaction would be slower than for the direct demasking of aliphatic boronates. However, the resulting product (carboxylate) of the two-step reaction should be much more hydrophilic than the...
product of the aliphatic boronates (aliphatic alcohols) resulting in a greater driving force for disassembly. It can therefore be expected that the disassembly behaviors of these two different micelles might be quite different.

In addition to these H2O2-reactive groups, the environmentally sensitive 3-HF dye is incorporated into the middle of the hydrophobic block to serve as a dual-colored fluorescent indicator to signal the polarity of the microenvironment. In polar and aprotic media, coupled ESICT/ESIPT processes in 3-HF fluorophores give rise to dominant green fluorescence emission whereas suppression of the ESIPT pathway in highly polar and protic media results in blue fluorescence emission.63–65 It is noteworthy that 3-HF fluorophores have a relatively large Stokes shift of approximately 200 nm between the absorption and ESIPT bands, and thus effectively avoid self-quenching of fluorescence, even when all the 3-HF dyes are confined within the micellar core; this provides a low dye-loading nanosystem with enough fluorescence intensity for sensing. Poly-(TMC-OmDEG) blocks at both sides of the core-forming block form the hydrophilic corona of the micelles and share similar stealth characteristics with standard PEG coatings. This stealth behavior can effectively reduce renal filtration and prevent clearance by the reticuloendothelial (RES) system, thus prolonging circulation in blood vessels and enhancing bioavailability.66,67 In combination, all of these chemical design features are expected to make HP-3HF-AlkₙPC and HP-3HF-ArPC promising multifunctional drug delivery nanovectors.

Results and discussion

Synthesis of TMC monomers and fluorescent initiator

In order to construct these designed functional polycarbonate materials, a series of trimethylene carbonate (TMC) monomers were used for ring-opening polymerization (Scheme 1). One hydrophilic monomer, three H2O2-reactive hydrophobic monomers, and two H2O2-inert hydrophobic monomers were designed and synthesized, and the chemical structures are shown in Scheme 1a. All of the monomers were derived from trimethylene carbonate 5-methyl-2-oxo-1,3-dioxane-5-carboxylic acid (TMC-COOH) via a variety of activated esterification methods (Scheme 1b). Briefly, the hydrophilic TMC monomer TMC-OmDEG was obtained from the nucleophilic addition–elimination reaction between diethylene glycol monomethyl ether and acyl chloride equivalent of TMC-COOH (path a), whereas the hydrophobic, H2O2-reactive TMC monomers TMC-O3dCHBE, TMC-OArBE,69 and TMC-O1dCHBE, all consisting of acid-labile boronic ester functionality, were derived from the corresponding alcohols by using the much milder pentafluorophenyl (Pfp) ester activation (path b), Mitsunobu-type esterification (path c), and SN2 substitution (path d), respectively. In addition, the control hydrophobic monomers TMC-OBn and TMC-O3CH, which are inherently H2O2-inert, were prepared in a similar manner.

To ensure the incorporation of “one” 3-HF dye molecule to “one” polymer chain and warrant 3-HF fluorophores at the core of the micelles once the micelles formed in aqueous media, the 3-HF fluorophore derivative bis-MPA-3HF was designed featuring a two-armed α,γ-diol handle as the initiator for ring-opening polymerization of boronic ester monomers (Scheme 1c). It was synthesized from a simple EDCI/HOBt amide coupling of the commercially available precursor 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) and the amino-terminal 3-hydroxyflavone derivative 3-HFNH₂, which was readily obtained from a four-step sequence as previously reported by our group.51 The fluorescent initiator bis-MPA-3HF displays similar polarity-sensitive fluorescence behaviors with the previously reported 3-HF and its derivative, indicating that the
structural modification doesn’t alter the desired fluorescence properties. Detailed synthetic procedures and structural elucidation of all TMC monomers and the fluorescent initiator can be found in the ESI.†

Synthesis and characterization of amphiphilic triblock copolymers

Co-administration of the electrophile-activating 1-(3,5-bis(trifluoromethyl)-phenyl)-3-cyclohexyl-2-thiourea (TU) and nucleophile-activating 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) has been reported to effectively enhance polymerization rates as well as to improve the selectivity of ROP for cyclic carbonate monomers over linear polycarbonates, which leads to minimal transesterification of the polymer chains.70,71 We thus explored the ring-opening polymerization of these TMC monomers using a combination of Lewis acid TU and Lewis base DBU as a bifunctional activation system.72,73 The applicability of the TU/DBU organocatalyst system on the designed monomers was then examined and the results are summarized in Table S1 of the ESI.†

Most of the less hindered TMC monomers, such as TMC-OmDEG, TMC-O3CH, and TMC-OBn, underwent ROP effectively to form the corresponding homopolymers with conversions of >90% in 1.5 h. In contrast, the sterically hindered monomers TMC-O3dCHBE and TMC-OArBE took a longer time (3 h) to achieve the comparable conversions. The severely hindered cyclic carbonate TMC-O1dCHBE did not polymerize using TU/DBU-catalyzed ROP, even after an extended reaction time (24 h). Further catalyst screening indicates that TMC-O1dCHBE failed to undergo ROP regardless of the use of acidic (CH₃SO₃H), basic (DMAP, TBD) or metallic (Sn(Oct)₂) catalysts. Presumably, the shorter linker (C₁) of TMC-O1dCHBE could not effectively relieve the congestion around the six-membered cyclic carbonate so the entry of the initiators or propagating alcohols into cyclic carbonate cores was severely blocked. The preparation of the homopolymer of TMC-O1dCHBE using post-polymerization modification of activated pentafluorophenyl ester-equipped polycarbonates with the corresponding alcohol also failed. Our results strongly indicated that TU/DBU activation for ROP is highly susceptible to the steric nature of monomers, which is in accordance with previous reports.74–76 More discussion about the monomer scope in the TU/DBU catalysis system can be found in the ESI.†

Despite our best efforts, the sterically bulk of the C₁-bridged aliphatic boronic ester monomer TMC-O1dCHBE made it impossible to prepare the corresponding triblock copolymer HP-3HF-Alk₁PC. Thus, only HP-3HF-Alk₃PC and HP-3HF-ArPC were pursued.

Synthesis of the amphiphilic triblock polycarbonates HP-3HF-Alk₃PC and HP-3HF-ArPC was commenced by mixing the H₂O₂-sensitive boronic ester monomer TMC-O3dCHBE or TMC-OArBE with the fluorescent initiator bis-MPA-3HF at a molar ratio of 50:1 using 5% TU/DBU as a catalyst to afford hydrophobic homopolymers P(TMC-O3dCHBE) and P(TMC-OArBE), respectively (Scheme 2). Subsequently, the single-block homopolymers as double-headed macroinitiators, featuring hydroxyl functionalities at both ends of the polymer chain, were extended bilaterally by adding the hydrophilic monomer TMC-OmDEG at a molar ratio of 150:1 (monomer : initiator) under the same catalytic conditions to furnish fully functionalized triblock polycarbonates HP-3HF-Alk₃PC and HP-3HF-ArPC. The 3-hydroxyl group of the bis-MPA-3HF molecule may also serve as an initiator for ROP resulting in a broad molecular weight distribution of polymers accompanied by the destruction of fluorescence emission of 3-HF. However,
the electron-deficient “phenolic-like” 3-hydroxyl group of the bis-MPA-3HF molecule is intrinsically less nucleophilic than its primary diol handle, and thus the possibility of the 3-hydroxyl group as a ROP initiator is substantially limited. The observation that HP-3HF-Alk₃PC and HP-3HF-ArPC emit the ESIPT-dominated green fluorescence at their initial states also provides the convincing evidence of preserving the 3-hydroxyl functionality. In addition, two inherently H₂O₂-inert triblock polycarbonates HP-3HF-Alk₃PC-ctrl and HP-3HF-ArPC-ctrl were prepared as negative controls in a parallel manner by replacing TMC-O₃dCHBE and TMC-OArBE monomers with TMC-O₃CH and TMC-OBn monomers, respectively (see Scheme S3† for chemical structures of both the control polymers). Detailed synthetic procedures and structural characterization of the four triblock polymers can be found in the ESI.†

Based on the fact that each polymer chain contains only one 3-HF molecule, the number-averaged degrees of polymerization (DP) and molecular weights of all four polymers could be determined using ¹H NMR spectroscopy by comparing the signal intensity of the phenyl protons of the initiator bis-MPA-3HF at 8.2 ppm with that of the characteristic protons on the hydrophobic or/and hydrophilic pendent groups of the polymers (e.g., benzylic protons of the TMC-OArBE block at 5.2 ppm). The results are summarized in Table S2 of the ESI.† Unexpectedly, the molecular weights (Mₙ, NMR) and compositions (DP) of the four purified polymers obtained from ¹H NMR spectra were significantly lower than the corresponding theoretical values, especially for those copolymers containing sterically hindered boronic ester moieties. It is believed that the steric bulk of these boronic ester monomers may retard the ROP process and make side reactions more likely resulting in non-polymeric side products, as evidenced by the presence of considerable amounts of low-molecular-weight side products during the purification of crude polymers using size-exclusion chromatography. In addition, MALDI-TOF/TOF mass spectrometry was used to determine the molecular weights of the individual polymers, as shown by traces in Fig. S2† and data displayed in Table S2.† Notably, the molecular weights measured by MALDI-TOF/TOF spectroscopy are smaller than that derived from ¹H NMR spectroscopy, presumably due to the incomplete ionization of high-mass polymer chains.

The gel permeation chromatography (GPC) traces of the four copolymers show relatively broad molecular weight distributions with minor shoulders on the high molar mass side, as traces shown in Fig. S1† and data displayed in Table S2.† The shoulder or multimodal peaks of higher molar mass could be explained by the existence of a higher degree of aggregates. Notably, the Mₙ values of the four copolymers determined by GPC are much higher than those calculated from ¹H NMR spectra. The disparity originated from the fact that GPC characterization of these samples was performed using polyethylene oxide (PEO) standards in an aqueous chromatography system, where these amphiphilic polycarbonates may assemble into micelles with much larger exclusion sizes.⁷⁷ The micellar aggregation numbers of polymers in aqueous solution were also calculated and ranged from 10 to 32, depending on the stacking of amphiphilic polymers in aqueous media (Table S2†).

Preparation and characterization of HP-3HF-Alk₃PC and HP-3HF-ArPC micelles
HP-3HF-Alk₃PC and HP-3HF-ArPC micelles were fabricated by the addition of the corresponding amphiphilic polycarbonates dissolved in a small amount of DMSO into aqueous HEPES buffer solution with gentle stirring. The resulting micelles were subsequently analyzed by dynamic light scattering (DLS) and transmission electron microscopy (TEM), and the results are shown in Fig. 3. DLS measurements show that both HP-3HF-Alk₃PC and HP-3HF-ArPC form monodisperse nanostructures with hydrodynamic diameters of ca. 72 and 74 nm, respectively, and have narrow size distributions (PDI < 0.3) in aqueous 10 mM HEPES (pH 7.4) buffer (Fig. 3a). The corresponding TEM images also reveal a homogeneous distribution of spherical micelles with sizes in accordance with those determined by DLS (Fig. 3b and c). The cryo-TEM image of the HP-3HF-ArPC copolymer further confirms that the HP-3HF-ArPC copolymer self-assembles in water as a micellar nanostructure with a slightly larger particle size of ca. 90–100 nm instead of multilayer vesicles (Fig. 3d).
H$_2$O$_2$-responsiveness of micelles probed by NMR

The H$_2$O$_2$-responsive nature of HP-3HF-Alk$_3$PC and HP-3HF-ArPC micelles was first validated by comparative $^1$H NMR spectroscopic experiments, as shown in Fig. 4, to provide the molecular level insights. Upon treatment with H$_2$O$_2$, the characteristic $\alpha$-proton signal of the boronic ester in HP-3HF-Alk$_3$PC (Hd in trace i of Fig. 4a) at 0.8 ppm completely disappears, accompanied by the appearance of the corresponding signal of the aliphatic alcohol (He in trace ii of Fig. 4a) at 3.7 ppm, which exactly overlaps with the signals from the methyldiethylene glycol segment with two more proton integrals, as marked in trace ii of Fig. 4a. In order to confirm the identity of the resulting product polymer, a control triblock polycarbonate P(TMC-OmDEG)-b-P(TMC-O3OH)-b-P(TMC-OmDEG) with a central block bearing hydroxypropyl pendent groups was independently obtained from sequential ROP of the TBDMS-masked hydroxypropyl TMC monomer TMC-OTBDMS and hydrophilic TMC-OmDEG followed by TBAF-mediated desilylation (see Scheme S4 in the ESI† for detailed chemical structures and synthetic procedures). The $^1$H NMR spectrum of P(TMC-OmDEG)-b-P(TMC-O3OH)-b-P(TMC-OmDEG) in trace iii of Fig. 4a matches well with that of the H$_2$O$_2$-treated HP-3HF-Alk$_3$PC (trace ii), validating the H$_2$O$_2$-sensitive mechanism of HP-3HF-Alk$_3$PC micelles as designed. On the other hand, the H$_2$O$_2$-sensitive mechanism of HP-3HF-ArPC, involving the cascade cleavage of pendent arylboronic esters, is rather intriguing. Upon H$_2$O$_2$ treatment, the signal of methylene protons (Hf in trace i of Fig. 4b) at 5.2 ppm is up-shifted to 4.6 ppm (Hf' in trace ii of Fig. 4b), indicating that the cleavage of carboxylic ester linkages affords free benzylic alcohols. Correspondingly, the signals that appeared at 2.7 and 5.5 ppm (Hi and Hh, respectively, in trace ii of Fig. 4b) were assigned as free phenolic and alcoholic protons, respectively, further confirming the generation of 4-(hydroxymethyl)phenol molecules via the oxidative demasking of boronic esters followed by quinone-methide elimination and trapping with water. Moreover, the broad signals of phenyl protons (Hd and He in trace i of Fig. 4b) up-shift to 6.8 and 7.2 ppm, respectively (Hd' and He' in trace ii of Fig. 4b) with monomeric splitting patterns, again indicating the release of the pendent groups from the polycarbonate backbone. The carboxylic proton (Hg in trace ii of Fig. 4b) at 8.5 ppm also confirms that the resulting polymers are carboxylate-bearing polycarbonates. Given all these pieces of NMR
evidence, the full molecular profile for the H₂O₂-responsive behavior of HP-3HF-ArPC is thus elucidated.

**Monitoring of H₂O₂-triggered micelle disassembly using DLS**

To probe the H₂O₂-triggered disintegration behaviors of HP-3HF-Alk₃PC and HP-3HF-ArPC micelles, time-dependent DLS measurements were conducted and the results are shown in Fig. 5. In the case of HP-3HF-Alk₃PC, the micelles shrunk slightly from 72 nm (black line in Fig. 5a) to 41 nm (red line in Fig. 5a) at initial 1 h treatment, and expanded to nearly 700 nm after 72 h treatment (violet line in Fig. 5a). The expulsion of bulky dicyclohexyl groups at the outer layer of cores leading to more compact nanostructures can be accounted for the shrinkage at the early stage, as illustrated in the middle diagram of Fig. 5b. However, the HP-3HF-Alk₃PC micelles did not disassemble as expected; instead, with the cleavage of boronic esters and the diffusion of exterior water into the core region of micelles, the micelles gradually swelled as a result of the extensive hydration of polymer chains. It may be rationalized that the structural change from poly(TMC-O₃CHBE) to poly(TMC-O₃OH) of the core-forming block in HP-3HF-Alk₃PC does not provide a sufficiently stark polarity variation to drive the complete disassembly of micelles. The poly(TMC-O₃OH) core block with little hydrophobicity facilitates the reorganization of the architectures of demasked HP-3HF-Alk₃PC poly-carbonates to form micro-sized polymeric micelles with loosely associated but fully swollen cores, as illustrated in the right diagram of Fig. 5b. The swollen nanostructure of H₂O₂-treated HP-3HF-Alk₃PC was also clearly observed by TEM (see Fig. S3a in the ESI†).

In the case of HP-3HF-ArPC, the micelles swelled slightly from 74 nm (black line in Fig. 5c) to 139 nm (green line in Fig. 5c) at initial 8 h treatment, and later gradually collapsed into small fragments with an average size of <1 nm after 48 h (violet line in Fig. 5c). The swelling behavior at the early stage can be regarded as the hydration of the intermediate polycarbonate micelles, which are possibly composed of mostly phenol moieties and small amounts of unreacted boronic ester groups at the innermost part of the core as well as carbonate residues at outer cores, as shown in the middle diagram of Fig. 5d. Poly(TMC-phenol), the intermediate product of the cascade cleavage reaction of the poly-(TMC-OArBE) block in HP-3HF-ArPC micelles, provides modest hydrophilicity compared with its hydrophobic parent block, and thus leads to slight swelling of the intermediate micelles. The pi–pi stacking interactions between the aromatic rings of phenol moieties, on the other hand, stabilize the assembly of micelles to some extent. Once the subsequent quinone-methide elimination has proceeded to completion, the cores of micelles are no longer stably maintained and collapse into pieces due to the loss of pi–pi stacking and the generation of highly hydrophilic and electrostatic repulsive carboxylate poly-

![Fig. 5](link-to-image) **Fig. 5** H₂O₂-triggered disaggregation behaviors of micelles. Time-dependent DLS analysis of (a) HP-3HF-Alk₃PC and (c) HP-3HF-ArPC micelles upon exposure to H₂O₂ at various reaction times. Rationalization of (b) particle swelling of HP-3HF-Alk₃PC micelles and (d) disassembly of HP-3HF-ArPC micelles upon H₂O₂ treatment.
anions, as illustrated in the right diagram of Fig. 5d. The disassembly of HP-3HF-ArPC micelles was also observed by TEM with images from spherical nanostructures to clusters of debris (see Fig. S3b in the ESI†). The disassembly behavior of HP-3HF-ArPC micelles echoes to the corresponding NMR spectroscopic result, which provides molecular-level evidence for fragmentation of the polymer pendent groups. These results strongly indicate that the structural features of boronic ester pendent groups play a critical role in determining the $\text{H}_2\text{O}_2$-responsive behaviors. Only a sufficiently large polarity transition in the cores of micelles can cause disassembly of the micelles instead of swelling. The same time-dependent experiments were also conducted and observed by DLS and TEM, respectively, using $\text{H}_2\text{O}_2$-inert HP-3HF-Alk$_3$PC-ctrl and HP-3HF-ArPC-ctrl micelles (Fig. S4f). No significant differences in either sizes or shapes were observed after $\text{H}_2\text{O}_2$ treatment for both control micelles.

**$\text{H}_2\text{O}_2$-responsive fluorescence response**

The ability of HP-3HF-Alk$_3$PC and HP-3HF-ArPC micelles to fluoresce sense $\text{H}_2\text{O}_2$ was gauged by measuring the time-dependent fluorescence response of the two micelles in buffered HEPES solutions upon exposure to 50 mM of $\text{H}_2\text{O}_2$, as shown in Fig. 6a and b, respectively. As confirmed by $^1$H NMR experiments, treatment with $\text{H}_2\text{O}_2$ leads to enormous structural changes, mainly involving the removal of bulky and hydrophobic masking groups concomitant with the generation of hydrophilic hydroxyl or carboxylate functionalities for HP-3HF-Alk$_3$PC and HP-3HF-ArPC micelles, respectively. Consequently, a drastic hydrophobic-to-hydrophilic transition occurs in the cores of the micelles and results in a decrease in the intensity of the ESIPT band accompanied by an increase in the intensity of the ESICT band with time. As expected, visual identification of fluorescent colors changing from green to blue is feasible. The same experiments were also conducted using two $\text{H}_2\text{O}_2$-inert HP-3HF-Alk$_3$PC-ctrl and HP-3HF-ArPC-ctrl micelles. No significant fluorescence change was observed upon addition of $\text{H}_2\text{O}_2$, as shown in Fig. S6 of the ESI.† To further analyze the ratiometric fluorescence performance of the two micelles in probing $\text{H}_2\text{O}_2$, the normalized fluorescence ratios of the ESICT and ESIPT bands for HP-3HF-Alk$_3$PC and HP-3HF-ArPC micelles in the absence or presence of $\text{H}_2\text{O}_2$ were plotted against the reaction time (Fig. 6c). HP-3HF-ArPC exhibited a faster fluorescence response as well as a larger ratiometric transition than HP-3HF-Alk$_3$PC, in which HP-3HF-ArPC reaches its plateau of 27-fold ratiometric fluorescence enhancement at 10 h whereas HP-3HF-Alk$_3$PC takes 22 h to achieve the saturation with 20-fold enhancement. The significantly slower reaction rate of HP-3HF-Alk$_3$PC micelles toward $\text{H}_2\text{O}_2$ could be attributed to the crowded hydrophobic core structure of the micelle that impedes the diffusion of aqueous solution containing the reactive hydrophilic $\text{H}_2\text{O}_2$ molecules leading to the slow demasking reaction. Beyond our expectations, the steric nature of the boronate structures of the two micelles is the principle determining factor of the relative response rates toward $\text{H}_2\text{O}_2$ even though the $\text{H}_2\text{O}_2$-triggered cleavage reaction of HP-3HF-ArPC, involving a two-step cascade reaction, is considered to be more time-consuming. The polarity difference in the core of HP-3HF-Alk$_3$PC before and after $\text{H}_2\text{O}_2$ treatment, from aliphatic boronic esters to the corresponding alcohols can be attributed to the smaller ratiometric fluorescence changes compared to that of HP-3HF-ArPC, where π–π aromatic stacking is possible before the disassembly and the electronic repulsion among poly-(TMC-carboxylate) is pervading afterwards, resulting in a rather large ratiometric fluorescence transition. As a negative control, the normalized ratios of the ESICT and ESIPT are generally less than 2 over the entire reaction time for the two micelles in the absence of $\text{H}_2\text{O}_2$. The slow hydrolysis (degradation) of the polycarbonate backbone in aqueous media leading to the partial soaking of 3-HF-containing micellar cores in water is accounted for the slight increase in the ratios of the ESICT and ESIPT with time in the absence of $\text{H}_2\text{O}_2$. However, the ratiometric fluorescence change incurred by the spontaneous degradation of polycarbonates is relatively...
insignificant compared to that generated by H₂O₂-induced disassembly or swelling.

Given the rather different reactivities toward H₂O₂, the ratiometric fluorescence responses of the two micelles were further characterized over a range of H₂O₂ concentrations from 0.05 to 250 mM to deduce the detection limit. As shown in Fig. 6d, HP-3HF-AlkPC displays an approximately linear correlation over the H₂O₂ concentration from 20 to 60 mM, whereas HP-3HF-ArPC shows its detection range from 0.1 to 10 mM, which is applicable to the biologically relevant H₂O₂ concentrations (0.05–0.1 mM). Although there is still room to improve the detection limit of the system. The sigmoidal correlation between the ESICT/ESIPT ratios and H₂O₂ concentration indicates the response of the micelles to H₂O₂ seems cooperative, which is rather different from the reaction-based small-molecular probes. The corresponding fluorescence spectra and photoimages of the two micelles with various concentrations of H₂O₂ at a given time can be found in Fig. S7 of the ESI.

### Cell uptake and cytotoxicity

Given the advantages of HP-3HF-ArPC in H₂O₂-triggered sensing and disassembly, HP-3HF-ArPC micelles were chosen to demonstrate the feasibility as a theranostic drug delivery nanosystem. Firstly, the cellular internalization and biocompatibility of HP-3HF-ArPC micelles were studied. RAW 264.7 macrophage cells have been widely utilized as a model system for assessing ROS-responsive therapeutics because they produce higher levels of endogenous ROS upon stimulation with specific biological mediators. Accordingly, RAW 264.7 cells were pre-incubated with HP-3HF-ArPC micelles for 24 h at 37 °C and subjected to confocal laser scanning microscopy (CLSM) imaging. Dual-band emissions of HP-3HF-ArPC were observed by acquiring different fluorescence channels (435–465 nm for ESICT and 535–565 nm for ESIPT, respectively), showing the internalization of bright 3-HF-decorated fluorescent micelles—mainly in the narrow cytosol region of RAW 264.7 cells (Fig. 7a). The cellular uptake of HP-3HF-ArPC micelles was proven successfully in RAW 264.7 cells. To further confirm the uptake of the fluorescent HP-3HF-ArPC micelles by RAW 264.7 cells, the flow cytometry analysis of RAW 264.7 cells was conducted after incubating with various concentrations (0–320 μg mL⁻¹) of HP-3HF-ArPC micelles for 12 h and the result is shown in Fig. S11. The fluorescence histograms of ICT and IPT channels in HP-3HF-ArPC-treated cells showed a significant rightward shift compared with the untreated cells (controls), which proved that HP-3HF-ArPC micelles were indeed taken up by RAW 264.7 cells.

The cytotoxicity of HP-3HF-ArPC micelles towards RAW 264.7 cells was also evaluated using the WST-1 cell viability assay. RAW 264.7 cells were incubated with various amounts of HP-3HF-ArPC micelles and the cytotoxicity was determined at 24 h after the treatment, by comparing the absorbance of reduced formazan at 450 nm in each well with that of the control well (Fig. 7b). No significant cytotoxicity was observed in RAW 264.7 cells incubated with up to 500 μg mL⁻¹ of HP-3HF-ArPC micelles, suggesting that the micelles are indeed biocompatible and non-toxic for biomedical applications.

### Drug release experiment utilizing FRET efficiency

Next, Nile red (NR) was utilized as a model of a hydrophobic drug to evaluate the drug-loading and H₂O₂-triggered drug-releasing behavior of HP-3HF-ArPC micelles. NR is highly solvofluorochromic; it absorbs at 549 nm and emits strongly at 628 nm in a hydrophobic environment, but only a weak-to-dim fluorescence is observed in a hydrophilic milieu (see Fig. S8a in the ESI† for the corresponding fluorescence spectra). Furthermore, the absorption profile of NR overlaps with the ESIPT green fluorescence of 3-HF dyes (λ_em = 550 nm) in the cores of HP-3HF-ArPC micelles but not the ESICT blue fluorescence of 3-HF dyes (Fig. S8b†). This suggests that Förster resonance energy transfer (FRET) from HP-3HF-ArPC to NR is feasible when it is encapsulated in the micelles. Upon excitation at 356 nm for 3-HF, strong NR fluorescence at 628 nm should be observed. Nevertheless, Förster resonance energy transfer is strictly prohibited when the micelles disassemble accompanied by the release of the encapsulated NR. Upon irradiation of the disassembly with 356 nm light, only
ESICT blue fluorescence of 3-HF dyes should be observed. Utilizing these photo characteristics to assess the encapsulation and release of small hydrophobic drugs with fluorescence responses is thus conceptually feasible (see Fig. S9 in the ESI† for schematic illustration of the drug surrogate release experiment). NR-loaded micelles \( \text{NR@HP-3HF-ArPC} \) were prepared by adding a DMSO solution of NR and \( \text{HP-3HF-ArPC} \) triblock copolymer into aqueous HEPES buffer solution with stirring followed by homogenization and dialysis purification. The drug release study of \( \text{NR@HP-3HF-ArPC} \) was carried out following the addition of 2 mM \( \text{H}_2\text{O}_2 \) at room temperature, and the corresponding fluorescence response was recorded as a function of time and shown in Fig. 8. At the beginning (\( t = 0 \), the fluorescence at \( \sim 630 \) nm from the NR appeared when 356 nm light was used to excite 3-HF located at the core of \( \text{HP-3HF-ArPC} \) indicating that NR was firmly encapsulated within the \( \text{HP-3HF-ArPC} \) micelles and FRET occurred between 3-HF and NR in the core of \( \text{NR@HP-3HF-ArPC} \) micelles. The fluorescence excitation spectrum of \( \text{NR@HP-3HF-ArPC} \) (Fig. S10†) showing a small and broad excitation band at 330–400 nm, which exactly overlapped with the absorption band of 3-HF (FRET donor), repeatedly proved the FRET relationship between the 3-HF dye and NR. After treating with \( \text{H}_2\text{O}_2 \), the micelle \( \text{NR@HP-3HF-ArPC} \) disassembled, accompanied by the release of the encapsulated NR over time and the fluorescence emission switching from NR color to the blue fluorescence of ESICT slowly when irradiated with the same wavelength. This indicated mismatched spectral overlap of ESICT and Nile red as well as the efflux of Nile red resulted in the reduced FRET efficiency. The corresponding fluorescence photoimages of \( \text{NR@HP-3HF-ArPC} \) taken at various time points (\( t = 0, 48, 96, \) and \( 144 \) h) also displayed an orange-to-blue transition, as shown in the inset of Fig. 8. The results indeed demonstrated that the origin of the color change is genuinely through the assembly-disassembly of \( \text{NR@HP-3HF-ArPC} \) in response to \( \text{H}_2\text{O}_2 \) acting on the demask-