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¹⁹F- and Fluorescently-labeled Micelles as Nanoscopic Assemblies for Chemotherapeutic Delivery

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Abstract

Micelles from amphiphilic star-block copolymers, having a hydrophobic hyperbranched core and amphiphilic fluoropolymer arms, were constructed as drug delivery-agent assemblies. A series of polymer structures was constructed from consecutive copolymerizations of 4-chloromethylstyrene with dodecyl acrylate and then 1,1,1-trifluoroethyl methacrylate with *tert*-butyl acrylate, followed by acidolysis to release the hydrophilic acrylic acid residues. These structures were labeled with cascade blue as a fluorescence reporter. The series of materials differed primarily in the ratio of 1,1,1-trifluoroethyl methacrylate to acrylic acid units, to give differences in fluorine loading and hydrophobicity/hydrophilicity balance. Doxorubicin (DOX) was used as a therapeutic to study the loading, release and cytotoxicity of these micellar constructs on an U87-MG-EGFRvIII-CBR cell line. The micelles, with TEM-measured diameters ranging from 5–9 nm and DLS-measured hydrodynamic diameters from 20–30 nm, had loading capacities of *ca.* 4 wt% of DOX. The DOX-loaded micelles exhibited potent cytotoxicity with cell viabilities of 60–25% at 1.0 µg/mL effective DOX concentrations, depending upon the polymer composition, as determined by MTT assays. These cell viability values are comparable to that of free DOX, suggesting an effective release of the cargo and delivery to the cell nuclei, which was further confirmed by fluorescence microscopy of the cells. ¹⁹F-NMR spectroscopy indicated a partial degradation of the surface-available trifluoroethyl ester linkages of the micelles, which may have accelerated the release of DOX. ¹⁹F-NMR spectroscopy was also employed to confirm and to quantify the cell uptake of the micelles. These dual fluorescent- and ¹⁹F-labeled and chemically-functional micelles may be used potentially in a variety of applications, such as cell labeling, imaging, and therapeutic delivery.

INTRODUCTION

Nanoscope systems that can incorporate combined imaging and therapeutic capabilities are the focus of intensive research for applications in the growing field of nanomedicine.(1–7) Such systems offer the possibility to increase diagnostic accuracy and therapeutic effectiveness, while minimizing side effects from treatment. Nanoscopic polymeric assemblies from dendrimers,(8,9) polymer micelles,(5,10,11) shell crosslinked knedel-like (SCKs) nanoparticles(12–14) and other polymer assemblies(15,16) are promising candidates for such

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Supporting Information Available: ¹H and ¹⁹F NMR spectra of polymers, UV-vis spectra of DOX-loaded micelles, DLS data of the micelles, and data regarding the trifluoroethyl ester hydrolysis. This information is available free of charge via the Internet at <http://pubs.acs.org>.

multifunctional therapeutic platforms, since these materials are conveniently synthesized, variably functionalized, and can be assembled to a range of sizes and compositions.

Such polymeric assemblies usually have an amphiphilic composition, in which the hydrophobic component serves as a reservoir for therapeutic cargo and/or imaging agents, and the hydrophilic component provides stability in solution and can be tailored for attaching imaging agents and/or targeting ligands. Therapeutic cargos, usually packed through hydrophobic-hydrophobic interactions, when not covalently attached, can be released through passive diffusion(12) or as a response to external stimuli such as pH,(8–10,17) temperature, (17–19) and light.(19) In order to utilize polymer assemblies for systemic delivery of therapeutics, it is important to maximize the loading capacity to minimize injection volume for *intravenous* delivery, and also to achieve sustained and controlled release.

Our group has long interest in developing polymers for the construction of multi-functional nanoscopic assemblies for imaging and drug delivery purposes.(12,14,16,20,21) One such system is the 1,4,7,10-tetraazacyclododecane-N,N'N''N'''-tetraacetic acid (DOTA)-conjugated SCKs that can chelate ^{64}Cu for labeling and *in vivo* PET imaging.(14,22) Extension of the SCKs to serve as a therapeutic delivery system has also been reported.(12) Recently, we developed fluorinated hyperbranched star-like polymers that can serve as ^{19}F magnetic resonance imaging (MRI) agents.(23) The multi-compartmental amphiphilicity of these structures gave rise to the hypothesis that they are capable of therapeutic agent packaging while also providing for detectable imaging or labeling for tracking of the nanoparticle locations. In this study, utilization of these materials as ^{19}F and fluorescence dual-labeled imaging assemblies as well as nanocarriers for chemotherapeutic cargos was explored. ^{19}F NMR/MRI techniques were successfully applied to track and quantify cell migration and uptake. Due to near-zero background, the technique is highly sensitive. In this study, ^{19}F NMR was applied to quantify the number of micelles taken up by the cells and to determine the micelle stability. Doxorubicin (DOX) was selected the therapeutic agent and the loading, release, and cytotoxicity of the nanocarrier constructs was investigated in depth, and compared against the behaviors of the host nanomaterial and the small molecule drug, each individually and together. DOX has been studied extensively in micellar delivery strategies(5,8,24,25) and is a common therapeutic for the treatment of a range of cancers.(26) It also exhibits antibiotic properties, and may have benefit toward other diseases that involve a need for reduction in cell growth, such as to interfere with restenosis by using drug eluting stents.(27) Most importantly, for these fundamental studies, DOX is a viable model drug, due to its inherent UV-vis/fluorescence characteristics. We found that these polymer micelle assemblies were capable of packaging DOX and that the DOX-micelle constructs exhibited cytotoxicity against U87-MG-EGFRvIII-CBR cancer cells that nearly matched that of the free DOX. The successful construction of dual-labeled micelles may find applications in the area of drug delivery, wherein monitoring their migration and uptake activity require careful monitoring.

EXPERIMENTAL PROCEDURES

Materials and methods

All chemicals were purchased from Sigma–Aldrich Chemical Company, unless otherwise noted. Cascade Blue® (CB) ethylenediamine tri-sodium salt and MTT assay kit were purchased from Invitrogen Co. The amphiphilic hyperbranched polymer materials, **1a–c**, were prepared as previously reported.(23)

U87-MG-EGFRvIII-CBR cells (a human glioblastoma cell line modified to express the mutant EGFRvIII receptor and click beetle red luciferase) have been previously reported.(12) ^1H -NMR spectra were recorded on a 500 MHz Varian Inova 500 spectrometer, with the solvent proton signal as standard. ^{19}F -NMR spectra were recorded at 470 MHz on a Varian Inova 500

spectrometer equipped with a fluorine-observe 5 mm probe (Nalorac Co. CA) with trifluoroacetic acid (TFA) as an internal standard.

Dynamic light scattering (DLS) measurements were acquired using a Brookhaven Instruments (Worcestershire, U.K.) system, including a model BI-200SM goniometer, a model BI-9000AT digital correlator, a model EMI-9865 photomultiplier, and a model 95-2 Ar ion laser (Lexel, Corp.; Farmindale, NY) operated at 514.5 nm. Measurements were made at 25 ± 1 °C. Prior to analysis, solutions were filtered through a 0.22 μm Gelman Nylon Acrodisc® 13 membrane filter to remove dust particles. Scattered light was collected at a fixed angle of 90°. The digital correlator was operated with 522 ratio spaced channels, and initial delay of 5 μs , a final delay of 100 ms, and a duration of 10 minutes. A photomultiplier aperture of 400 μm was used, and the incident laser intensity was adjusted to obtain a photon counting of between, 200 and 300 kcps. The calculations of the particle size distributions and distribution averages were performed with the ISDA software package (Brookhaven Instruments Co.), which employed single-exponential fitting, cumulants analysis, non-negatively constrained least-squares (NNLS) and CONTIN particle size distribution analysis routines. The data is presented as the average values from four measurements.

Transmission electron microscopy (TEM) measurements were conducted on a Hitachi H600 microscope. Micrographs were collected at 100,000 $\times$ magnification and calibrated using a 41 nm polyacrylamide bead from NIST. Carbon-coated copper grids were treated with oxygen plasma prior to deposition of the micellar samples. The samples were stained with 1% phosphotungstic acid (PTA) for 1 minute, and then the solution was wicked away and the samples were allowed to dry under ambient conditions. The number-average particle diameters (D_{av}) and standard deviations were generated from the analysis of a minimum of 100 particles from at least three different micrographs.

UV-vis spectroscopy was acquired on Varian Cary 1E UV-vis system (Varian Inc., Palo Alto, CA) using PMMA cuvettes.

Synthesis

Preparation of cascade blue-conjugated micelles [2a–2c]—To three solutions of polymers **1a–c** (20 mg each of **1a–1c**, 0.20, 0.22 and 0.21 μmol for **1a–1c**) in DMF (5 mL) was added HBTU (3.8 mg, 1×10^{-2} mmol) and HOBT (1.4 mg, 1×10^{-2} mmol). The solutions were allowed to stir under argon for 1 h. DIPEA (4 mg, 4×10^{-2} mmol) and Cascade Blue (0.63 mg, 1×10^{-3} mmol) were added. The reactions were stirred under argon overnight. DMF (15 mL) was added and the solutions were dialyzed (MWCO 30 kDa) against nanopure water for 84 h. Micellar solutions were thereby obtained and the amounts of Cascade Blue (CB) were determined by UV-Vis (400 nm, $\epsilon = 28000 \text{ M}^{-1} \text{ cm}^{-1}$ from manufacturer's manual). A portion of the micelle solution was lyophilized to dryness for ^1H and ^{19}F NMR analyses.

2a: 3.4 CB/polymer (68%), micelle concentration, 0.33 mg mL^{-1} . ^1H -NMR (500 MHz, DMSO- d_6): δ 0.8–2.3 (br, m, CH_2 and CH of backbone), 4.3–4.7 (s, br, CH_2CF_3 , PhCH_2), 6.4–7.5 (m, br, ArH), 7.6–8.0 (m, br, ArH of CB), 8.2 (ArH of CB), 8.6 (s, ArH of CB), 9.0 (s, ArH of CB), 9.9 (s, ArH of CB), 12.0–12.5 (s, br, COOH) ppm. ^{19}F -NMR (470 MHz, DMSO- d_6): δ 2.7 (s) ppm. TEM: D_{av} : 9 ± 2 nm, DLS: $D_{\text{h(n)}}$ 21 ± 2 nm, $D_{\text{h(v)}}$: 29 ± 4 nm, $D_{\text{h(i)}}$: 129 ± 20 nm.

2b: 4.2 CB/polymer (84%), micelle concentration 0.35 mg mL^{-1} . ^1H -NMR (500 MHz, DMSO- d_6): δ 0.8–2.3 (br, m, CH_2 and CH of backbone), 4.3–4.7 (s, br, CH_2CF_3 , PhCH_2), 6.4–7.5 (m, br, ArH), 7.6–8.0 (m, br, ArH of CB), 8.2 (ArH of CB), 8.6 (s, ArH of CB), 9.0 (s, ArH of CB), 9.9 (s, ArH of CB), 12.0–12.5 (s, br, COOH) ppm. ^{19}F -NMR (470 MHz, DMSO- d_6): δ 2.7 (s) ppm. TEM: D_{av} : 8 ± 2 nm; DLS: $D_{\text{h(n)}}$ 26 ± 4 nm, $D_{\text{h(v)}}$: 29 ± 6 nm, $D_{\text{h(i)}}$: 135 ± 25 nm.

2c: 4.0 CB/polymer (80%), micelle concentration 0.32 mg mL^{-1} . $^1\text{H-NMR}$ (500 MHz, $\text{DMSO-}d_6$): δ 0.8–2.3 (br, m, CH_2 and CH of backbone), 4.3–4.7 (s, br, CH_2CF_3 , PhCH_2), 6.4–7.5 (m, br, ArH), 7.6–8.0 (m, br, ArH of CB), 8.2 (ArH of CB), 8.6 (s, ArH of CB), 9.0 (s, ArH of CB), 9.9 (s, ArH of CB), 12.0–12.5 (s, br, COOH) ppm. $^{19}\text{F-NMR}$ (470 MHz, $\text{DMSO-}d_6$): δ 2.7 (s) ppm. TEM: D_{av} : $5 \pm 1 \text{ nm}$; DLS: $D_{\text{h(n)}}$ $29 \pm 3 \text{ nm}$, $D_{\text{h(h)}}$: $33 \pm 6 \text{ nm}$, $D_{\text{h(n)}}$: $135 \pm 20 \text{ nm}$.

General procedure for DOX loading—A solution of DOX (2.7 mg mL^{-1} in CH_2Cl_2 and 3 eq. of triethylamine, 30% w/w with respect to the micelle) was added to a vial containing a magnetic stir bar and the micelle (4 mL, polymer concentration $\sim 0.30 \text{ mg mL}^{-1}$). The solution were protected from light and stirred without a cap in a well-ventilated fume hood for 16 h. Insoluble DOX was removed by centrifugation ($3000 \text{ rpm} \times 10 \text{ min}$) and the supernatants were transferred to a centrifugal filter device (Amicon Ultra 4, 30 kDa MWCO, Millipore Corp., Billerica MA, USA) and washed extensively with 5 mM PBS pH 7.4 buffer at 37°C to remove all unincorporated DOX. After eight washing cycles, the filtrate was analyzed by UV-vis (488 nm) to confirm the successful removal of the free DOX. The DOX-loaded micelle solutions were then reconstituted to a final volume of 4 mL. The amount of incorporated DOX was determined by UV-vis (480 nm, $\epsilon = 12500 \text{ M}^{-1}\text{cm}^{-1}$ determined by a calibration curve in DMF/PBS 4:1) in a 4:1 v/v mixture of DMF and DOX-micelle solution.

General procedure for the release experiments—DOX-micelle solutions (2 mL) were transferred to a presoaked dialysis cassette (Slide-A-Lyzer, 10 kDa MWCO, Pierce Biotechnology, Rockford IL, USA). The cassettes were then stirred in a beaker containing 4 L of 5 mM PBS at pH 7.4 and 37°C for a period of 48 h. Samples ($\sim 100 \mu\text{L}$) were removed from the cassette at 0, 1, 2, 3, 4, 8, 16, 24, and 48 h and analyzed by UV-vis (488 nm, $\epsilon = 12500 \text{ M}^{-1}\text{cm}^{-1}$ determined by a calibration curve in PBS). All release experiments were conducted in triplicate.

Cell cytotoxicity assay on U87-MG-EGFRvIII-CBR cells—Cell cytotoxicity assay was conducted using MTT assay kit and followed the manufacturer's protocol. Briefly, 5000 cells/well of the U87-MG-EGFRvIII-CBR cells were plated in triplicate in 96-well plate 24 h before treatment. Cells were then washed twice with sterile PBS and exposed to different doses of either free DOX or DOX-micelles at 37°C for 2 h in 10% FBS α -MEM. After 2 h of incubation, the cells were washed again twice with sterile PBS and re-fed with fresh media and further incubated at 37°C for 72 h before the MTT assay was performed. To assess cell viability, $10 \mu\text{L}$ of MTT (5 mg mL^{-1}) solution was added into each well and incubated at 37°C for 4 h. The reaction was terminated with 10% SDS stop solution. Absorbance was read at 570 nm using μQuant plate reader (Bio-Tek Instruments Inc.) and viability was calculated as the percentage of control (cells receiving no treatment).

In vitro fluorescent microscopy study—U87-MG-EGFRvIII-CBR cells were treated for 24 h with either $1 \mu\text{g mL}^{-1}$ of DOX or DOX-loaded micelles. Afterwards, cells were washed twice with PBS buffer and fixed with 4% paraformaldehyde. Fluorescence was observed using Zeiss fluorescence microscope equipped with a appropriate filter set (Axioskop plus, Carl Zeiss, Germany) and pictures were taken using $63\times$ oil lenses.

Micelle stability studies by $^{19}\text{F-NMR}$ —U87-MG-EGFRvIII-CBR cells (1 million) suspended in $200 \mu\text{L}$ PBS buffer were mixed with micelles in PBS buffer and this cell solution was then placed in a 5 mm NMR tube. TFA (0.050 mg/mL) was used as an internal reference. The pH was measured to be 7.4 before the kinetics studies. The degree of hydrolysis was calculated based on the relative integration value of the trifluoroethyl ester at 2.7 ppm and the integration value of trifluoroethanol at -1.2 ppm . In a control study, PBS buffer, instead of the

cell solution was used and the protocol was identical. The parameters used were: $d1 = 10$ s, $sw = 19189.3$ Hz, $nt = 378$, $lb = 5$ Hz, $temp = 37$ °C.

Cell uptake studies by ^{19}F -NMR—U87-MG-EGFRvIII-CBR cells (4 million) were plated on a 6-well plate and then treated with 500 μL of micelle solution for at 37 °C 2 h. Afterwards, the cells were washed 3 times with PBS buffer and lifted by trypsin. Cells were washed again with PBS buffer and centrifuged 3 times (3000 rpm $\times$ 5 min). Cell pellet was collected and fixed with 4% paraformaldehyde and re-suspended into 250 μL PBS buffer. The cells were sonicated for 1 minute, 100 μL of D_2O and 10 μL of 0.050 mg/mL TFA solution were added. The solution was transferred into a water compatible BMS-005TJ Advanced NMR microtube (Shigemi Inc., PA) and subjected to ^{19}F -NMR spectroscopic analysis. In a control study, PBS buffer instead of micelle solution was used and the protocol was identical. The parameters used were: $d1 = 10$ s, $sw = 19189.3$ Hz, $nt = 8620$, $lb = 5$ Hz.

RESULTS AND DISCUSSION

Synthesis and micellization of the cascade blue-conjugated fluoropolymers

The polymers **1a–1c**(23) were functionalized with Cascade Blue (CB) ethylene diamine to give CB-functionalized polymers (Scheme 1). Coupling of CB ethylene diamine to the pre-established macromolecules, having a hyperbranched hydrophobic core and amphiphilic copolymer arms comprised of trifluoroethyl methacrylate and acrylic acid residues, was facilitated by the addition of standard peptide coupling reagents, N-hydroxybenzotriazole (HOBt) and O—benzotriazol-1-yl-N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) in N,N-dimethylformamide (DMF), to give an amide linkage between the CB and the macromolecule acrylic acid sites. Initially, polymers with *ca.* two CB units per polymer chain were prepared, but the corresponding micelles failed to produce adequate fluorescence microscopy images with our instrumental resolutions. Therefore, the theoretical number of CBs per polymer was increased to *ca.* five during the coupling reactions, which afforded CB/polymer values of 3.3, 4.2, and 4.0 per polymer for samples **2a**, **2b**, and **2c**, corresponding to conjugation efficiencies of 69%, 82% and 79%, respectively, as determined by UV-vis spectroscopy.

Recently, we reported that purification and micellization can be performed simultaneously, (28) thus, after the conjugations of CB, additional DMF was added to the reaction mixtures and the solutions were dialyzed subsequently against nanopure water for 84 h to induce self assembly into polymeric micelles while removing non-covalently-linked CB. The polymers readily formed micelles, as determined by transmission electron microscopy (TEM) and dynamic light scattering (DLS) studies (Figure 1 and Table 1). Compared to the original micelles of the precursors, **1a–1c**(23), conjugation of CB did not change the TEM size of the micelles significantly (Table 1). The DLS data showed slight increases in number-average hydrodynamic diameter (D_h) values.

DOX loading and release profiles

DOX was incorporated into the micelles using a similar procedure as published previously (8) Dichloromethane was used as the organic solvent to solvate the DOX and to swell the core of the micelle, facilitating the loading of the therapeutic. The amount of DOX in the micelle solutions were determined by UV-vis measurements. The loading capacities for micelles **2a–2c** were 3.6 ± 0.5 , 4.9 ± 0.6 and 4.0 ± 0.7 wt% based on polymer mass, respectively (Table 1). During the preparation of the micelles, we noticed that the micelle solution **2a** precipitated after *ca.* one week of storage, while micelles **2b** and **2c** were storage stable for months, indicating that a high fluorine content adversely affected the formation and stability of the micelles, which may be a reason for its low loading capacity. The balance of fluorine and

carboxylic acid content also affected the DOX release profiles. The release of the DOX was assessed by monitoring the decrease of UV-vis absorbance over time.(8,12) Samples were withdrawn from dialysis cassettes at progressive time intervals and analyzed by UV-vis spectroscopy. In this release model, DOX molecules are first released from the micelles, and then pass through the dialysis membrane. Since the rate-determining step is the rate that DOX is released from the micelles (Figure 2), the overall rate can be considered as the rate that DOX was released from the micelles. Using this release model, the DOX release behavior was similar for **2b** and **2c**, but **2a** exhibited a faster release rate (Figure 2), possibly due the fact that **2a** does not have a stable micelle structure. For **2b** and **2c**, a release of *ca.* 25% of the cargo was observed in 8 h, followed by a slow, sustained release over the course of 48 h. At the end of 48 h, 66%, 45% and 51% of DOX had released from micelles **2a**, **2b** and **2c**, respectively. The release profiles were slower than those observed for highly branched dendritic constructs(8) loaded with DOX, but similar to release rates from shell crosslinked nanoparticles.(12)

Cell cytotoxicity studies

The cytotoxicities of **2a–2c** were studied by incubating U87-MG-EGFRvIII-CBR cells with varying concentrations of the micelles. Cell viability was assessed using a Vibrant MTT cell proliferation assay kit. Cells were first incubated with DOX-loaded and non-loaded micelles for 2 h, subsequently, the cells were washed twice with PBS buffer and re-fed with fresh media. After 72 h of incubation, MTT assays were conducted. No significant toxicity was observed at concentrations up to 100,000 ng mL⁻¹ for the non-loaded micelles (Figure 3, top). In comparison, the DOX-loaded micelles exhibited potent cytotoxic effects (Figure 3, bottom). At 1,000 ng mL⁻¹, only 30% of the cells treated with DOX-loaded micelles survived (sample **2b**), which is comparable to free DOX at the same dose concentration (20% survived, Figure 3, bottom), indicating that this nanocarrier can achieve excellent cytotoxic effect on this glioma cell line. Interestingly, compared to glassy amorphous SCK nanoparticles derived from poly (acrylic acid)-*b*-poly(styrene) (PAA-*b*-PS),(12) the micelles in this study have lower loading capacity of DOX, but showed significantly greater cytotoxicity. The improvement of therapeutic efficacy is possibly due to a higher extent of release of DOX in these systems (45–66% after 48 h, compared to the SCK system that retains more cargo, with only 30–55% release after 48 h), as this new system was designed to contain an integrated mixing between the hydrophobic pockets and hydrophilic domains, giving a more open, multi-compartment structure, in contrast to the core-shell structure of SCK nanoparticles.

We also noticed that in the presence of cells, a partial degradation of the trifluoroethyl ester moiety occurred, which may further enhance the release of DOX. The substantial chemical shift difference between trifluoroethyl ester and trifluoroethanol, and the zero-background of ¹⁹F-NMR signal allows real-time monitoring of the hydrolysis of these fluorinated micelles, upon cellular incubation. As seen in Figure 4, the trifluoroethyl esters in the micelle exhibited a broad ¹⁹F resonance at 2.7 ppm, and the degradation product, trifluoroethanol, gave a sharp triplet at -1.2 ppm with a ³J_{H-F} = 8.5 Hz. While the micelle solutions were very stable in water and PBS (for months), as monitored by ¹⁹F-NMR spectroscopy, incubation with cells resulted in quick release of trifluoroethanol. Within 1 h, *ca.* 2% of trifluoroethanol was detected by ¹⁹F-NMR spectroscopy. The hydrolysis proceeded rapidly up to *ca.* 4 h, with a total of *ca.* 8% degradation. Afterwards, a sudden halt of degradation was observed, resulting in a non-linear degradation profile (see supporting information). A halt in degradation may be due to a loss of enzymatic activity. Alternatively, the hydrolysis may be limited to the surface-available trifluoroethyl ester groups, while others remain stable beneath the surface of the polymer or polymer micelle assembly. A thorough study of the hydrolysis of these micellar constructs using lipases and enzymatic inhibitors is currently underway. Nevertheless, the partial hydrolysis of trifluoroethyl ester during the cell incubation may result in a faster and more complete release of DOX *in vitro*, as compared to release data from the dialysis model.

Although the hydrolysis product, trifluoroethanol, may be toxic to cells, we did not observe a significant negative effect on the viability of the cells incubated with the control micelle solutions (Figure 3, top).

Fluorescence microscopy studies

The cellular uptake of the polymer micelles were also studied by fluorescence microscopy. We took advantage of the blue fluorescence from CB and red autofluorescence from DOX to study the cellular uptake of the polymeric micelles and localization of DOX within the cells delivered by these polymers. As shown in Figure 5, cells treated with all three control micelles (**2a–2c**) demonstrated significant amounts of blue fluorescence, mostly in the cytoplasm. This intracellular trafficking is in contrast with that of free CB dye as a small molecule, which binds strongly to the nucleus (data not shown), confirming that the fluorescence observed arises from polymer-dye conjugates. At this moment, the mechanism of cellular uptake of these micelles is not clear and remains to be investigated. However, the blue fluorescence clearly indicated that cell uptake indeed occurred.

Because micelles **2b** showed the best cell viability reduction (Figure 3, bottom), we chose micelles **2b** for further microscopy studies on DOX localization. As seen in Figure 6, within 2 h treatment, DOX molecules were already delivered inside the cells, although mostly present in the cytoplasm surrounding the nucleus (Figure 6, a2). In contrast, after 24 h treatment, DOX was located within the nucleus (Figure 6, b2); the nuclei are brighter, indicating more DOX molecules have been incorporated. These images indicated efficient deliveries of DOX to the nucleus, which may be responsible for cell viability reductions.

¹⁹F-NMR studies on the cellular uptake of the micelles

Fluorine-containing nanoscopic assemblies have been used for cell uptake and cell trafficking studies using ¹⁹F-NMR spectroscopy or ¹⁹F-MRI.(7,29,30) Since there is no background signal, the ¹⁹F-NMR/MRI technique is highly sensitive, with detection limits in the ng scale. In this study, ¹⁹F-NMR spectroscopy was employed to confirm and to quantify cell uptake of **2b** in U87-MG-EGFRvIII-CBR cells. This micelle was selected because it had good loading capacity, desired release profile (Figure 2), and the best cytotoxicity result (Figure 3). Cells (4 million) were first incubated with the micelle solution for 2 h, followed by repeated washings with fresh PBS buffer and centrifugation to remove any free micelles. The cells were harvested and sonicated to lyse the cells. Trifluoroacetic acid (TFA) was used as an internal standard to quantify the fluorine concentration. Since the CF₃ groups on the micelles have much shorter T₁ relaxation times (*ca.* 500 ms) than that of TFA (*ca.* 2.5 s), it was necessary to adopt a long relaxation delay (10 s) to assure that both fluorine species were fully relaxed before the next acquisition scan. However, with such long delay times, *ca.* 24 h scanning time was required to acquire a spectrum with reasonable resolution (Figure 7). Using the same method as reported by Ahrens and coworkers,(30) it was possible to calculate that the total amount of fluorines in each cell was *ca.* 4.6 × 10⁸ fluorines, indicating about 0.1% cell uptake efficiency. This very low cell uptake is possibly due to the negatively-charged micelle that limits cell uptake compared to neutral nanoemulsions, wherein the cell uptake of fluorine species can reach concentrations of 10¹¹–10¹³.(30) Nevertheless, even with limited cell uptake, these nanostructures loaded with DOX exhibited a significant ability to reduce cell viability.

CONCLUSIONS

In conclusion, we have successfully constructed fluorine and CB dual-labeled nanostructures, studied their loading and release characteristics for therapeutic guests, and applied ¹⁹F-NMR and fluorescence microscopy as tools to study their cellular uptake and cell viability reduction. The nanostructures were designed to possess an amphiphilic, multi-compartment morphology

for packaging of therapeutic guests, based upon a hyperbranched-linear hybrid star-like structure that assembled into uni- or multi-molecular micelles having optimum hydrodynamic diameters of 20–30 nm. Reasonable quantities of DOX could be loaded into the structures, and they exhibited differential release profiles, in terms of rates and ultimate amounts, depending upon their composition. These DOX-loaded nanostructures exhibited potent cytotoxicity on U87-MG-EGFRvIII-CBR cells. We anticipate that the incorporation of ligands for cell targeting will improve the DOX delivery even further. ¹⁹F-NMR spectroscopy showed a partial degradation of the surface available fluorinated polymer segments, which could accelerate the release of DOX from the micelles *in vitro* and *in vivo*. The successful construction of this dual-labeled, multifunctional nanocarrier may find promising applications in nanomedicine. The key unmet challenge is to now prepare such nanoscale constructs with cell-specific targeting groups so that therapeutics can be efficiently delivered with minimal side effects.(5,15,31)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. TEM images of micelles **2a–2c** (shown from left to right, respectively), on a carbon-coated copper grid with negative staining by 1% phosphotungstic acid.

Figure 2. Release profiles of DOX from a dialysis cassette (control labeled as free DOX in the legend) and from dialysis cassettes containing micelles of **2a–2c** over time in PBS buffer (5 mM, pH 7.4) at 37 °C.

Figure 3.

Cell viability studies using MTT assay. U87-MG-EGFRvIII-CBR cells were incubated with **2a–2c** without (top) and with DOX (bottom) loading at varied concentrations.

Figure 4.
 ^{19}F -NMR spectra demonstrating partial hydrolysis of the trifluoroethyl ester units of **2b** (470 MHz, $\text{H}_2\text{O}/\text{D}_2\text{O}$, with TFA as an internal standard, 4 h after incubation of micelles with cells at 37 °C).

Figure 5.

Cellular uptake of micelles by fluorescence microscopy study. U87-MG-EGFRvIII-CBR cells were incubated with micelle solutions of **2a**, **2b** and **2c**, respectively. 1: phase contrast. 2: blue fluorescence. The cells were treated with mice lle (0.1 mg/mL) for 2 h at 37 °C, followed by repeated washings and then fixed with 4% paraformaldehyde.

Figure 6. Localization of DOX upon treatment of DOX-loaded micelles by fluorescence microscopy. U87-MG-EGFRvIII-CBR cells were incubated with DOX-loaded micelle **2b**. 1: phase contrast. 2: red fluorescence. a: 2 h after treatment. b: 24 h after treatment, more DOX were incorporated into the nucleus.

Figure 7. ^{19}F -NMR spectrum of U87-MG-EGFRvIII-CBR cell suspensions. ^{19}F signal from micelles, engulfed by the cells, appears at 2.7 ppm.

Scheme 1.

Chemical structures and compositions for micelles **1a–1c** and **2a–2c**, and schematic illustrations of the amphiphilic star-like structure, assembled as uni- or multi-molecular aggregates and the loading of DOX into those nanostructures.

Table 1
Summary of characterization data and doxorubicin loading for micelles **2a–2c**.

Sample	TEM [nm]	$D_h(n)$ [nm] ^a	$D_h(v)$ [nm] ^a	$D_h(i)$ [nm] ^a	DOX [μ M] ^b	Loading capacity [wt %]	CB/polymer ^b
2a	9±2	21±2	29 ±4	129 ± 20	11	3.6 ± 0.5	3.4
2b	8±2	26 ±4	29 ±6	135 ±25	24	4.9 ± 0.6	4.2
2c	5±1	29 ±3	33 ±6	138 ±20	22	4.0 ± 0.7	4.0

^aby DLS

^bby UV-vis