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A "Mix-and-Click" Approach to Double Core–Shell Micelle Functionalization

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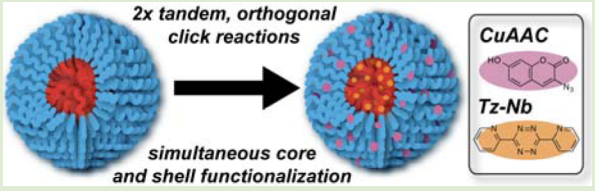
Supporting Information

ABSTRACT: A micellar scaffold formed by self-assembly of a reversible addition–fragmentation chain transfer (RAFT)-synthesized amphiphilic diblock copolymer has been prepared to contain two orthogonal click-compatible functionalities in the core and shell. These functionalities (norbornenes in the core and terminal alkynes in the shell) have been used as handles to modify the micellar assembly in the core using tetrazine–norbornene chemistry or the shell using the copper-catalyzed azide–alkyne reaction. Additionally, both core and shell modifications were carried out in a tandem, one-pot process using the orthogonal chemistries mentioned above. In all cases the reactions were found to be highly efficient, requiring little excess of the modifying small molecule and very simple to perform under ambient conditions.



The synthesis and application of polymeric nanoparticles and nanostructures of varying architectures has attracted significant attention, concurrent with efforts in synthetic chemistry to mimic nature's own nanoreactors[1] in their specificity of size, shape, architecture, and function. Other avenues of exploration have also opened up in a range of industrial and research settings, such as biomedical delivery applications.[2]

Contemporaneous with the growing sophistication of synthetic self-assembled nanostructures has been the widespread use of "click" chemistries[3] to modify these structures.[4] Advantages of postassembly modification over synthesis of new, specifically designed amphiphilic block copolymers are evident when considering the relative time scale and simplicity of modifying one micelle with several different functionalities, compared to synthesizing novel monomers, optimizing polymerization conditions, and only then self-assembling and purifying the resulting micellar solution. Of particular note is the use of the copper-catalyzed azide–alkyne (CuAAC) reaction, which has been exploited to modify the surface,[5] core,[6] shell,[7] and even core–shell interface[8] of micellar structures, although other click reactions have also been explored for micelle functionalization, for example the Diels–Alder[9] and thiol–ene[10] reactions.

Multiple orthogonal reactions on a polymeric scaffold[11] have already been demonstrated using CuAAC and other chemistries, and while a micelle is arguably a more complex framework for functionalization, phase segregation of hydrophobic and -philic reagents can also assist in functionalizing the core and shell domains separately. Surface functionalization is also possible, often by using an end-functional amphiphile as the micelle precursor, as has been done to provide saccharide-,[12] peptide-,[13] and antigen-decorated nanoparticles.[14] Other surface functionalities introduced postmicellization have included chelating ligands for radionucleide imaging agents,[15] nucleic acids,[16] proteins,[17] and cancer cell targeting ligands.[18] While a variety of chemical methods have been used for micelle and nanoparticle functionalization,[19] amidation chemistries are very frequently chosen, often for cross-linking purposes[20] and sometimes in tandem with another reaction for dual functionalization. Regardless of the purpose of the reaction, amidation chemistries are not as modular as click reactions, and therefore we sought to create a single micelle scaffold containing two orthogonal click handles, segregated into the core and shell, respectively, for easy modification in both domains. Such modifications could introduce cargos into the core or targeting ligands in the shell[21] in a manner that opens up the possibilities for synthesis of micellar libraries in a combinatorial manner.

The shell click reaction was chosen to be the CuAAC reaction, as it has been shown to work efficiently in aqueous media. The core click reaction was chosen to be the reaction between tetrazines (Tz) and norbornenes (Nb). The use of tetrazines as a bioorthogonal reaction partner with strained alkenes[22] has recently gained a lot of attention. Several recent reports[23] highlight the highly specific and efficient nature of the reaction, which requires no extra catalyst, additive, or stimulus to proceed. Among these are approaches modifying polymer chains using Tz-Nb click, lending promise to the supposition that it could perform equally well within a polymeric assembly.[24]

The Tz-Nb reaction has been shown to be a very fast, high-yielding click reaction, but tetrazines can also react with other alkenes and alkynes,[25] albeit at vastly reduced rates and often requiring forcing conditions. Likewise, norbornenes can also react with azides; however examples in the literature predominantly require heating to reflux temperatures and

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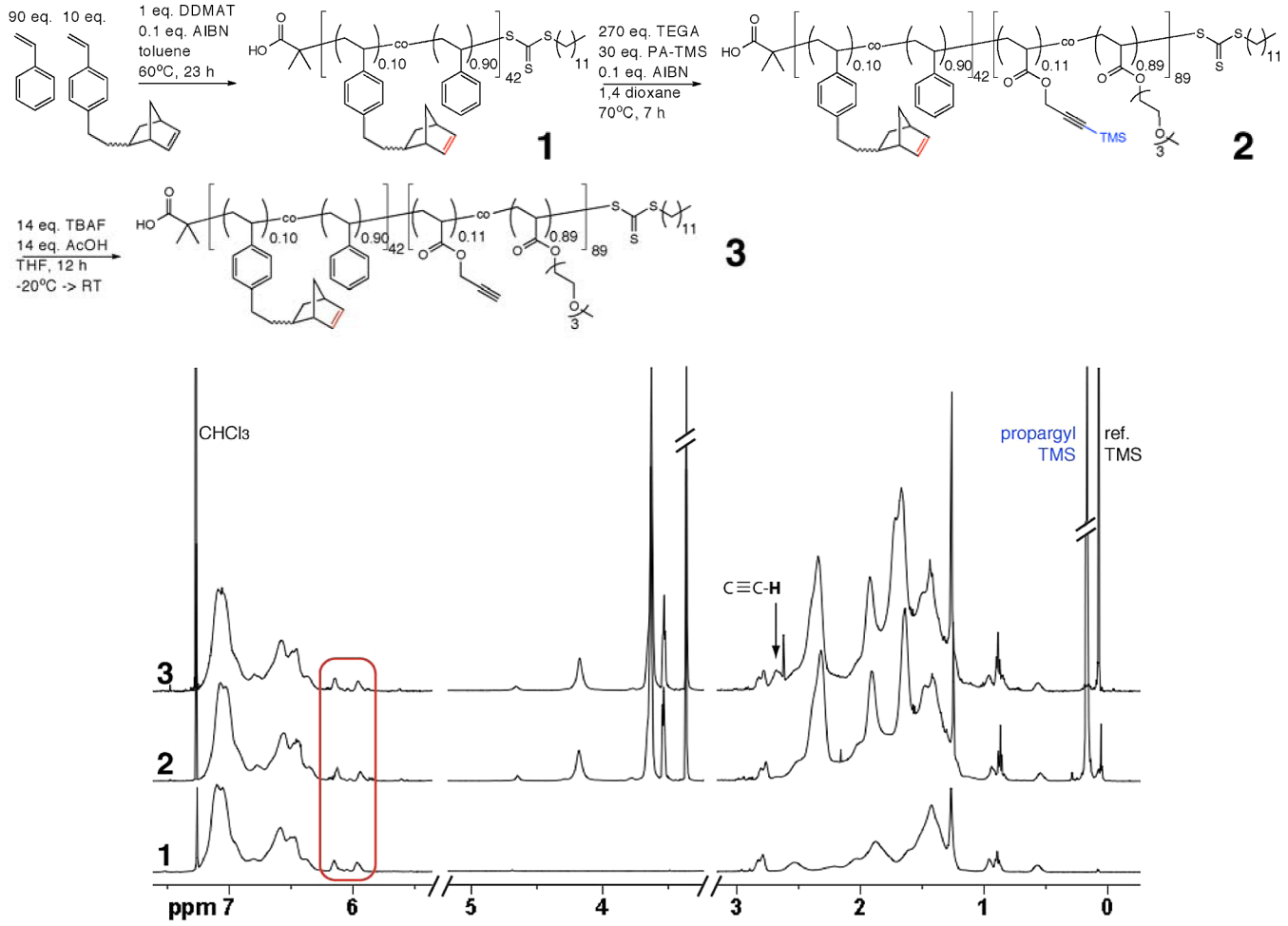

Figure 1. Synthetic approach to polymers **1**, **2**, and **3** (above) and ^{1}H NMR spectra (below) of polymers **1** (bottom), **2** (middle), and **3** (top). From ca. 3.2 ppm to ca. 5.3 ppm the *z*-axis scale is contracted due to the large amplitude signals arising from TEGA CH_2 protons, which otherwise swamp other peaks in the spectrum. Norbornenyl signals are highlighted in red, PA-TMS methyl signal in **2** highlighted in blue.

reaction times on the order of days.[26] Thus we considered that the mild reaction conditions employed would not result in any cross-coupling, although small molecule orthogonality testing was carried out to confirm this (see the Supporting Information).

To install norbornene functionalities in the hydrophobic core of the micelle and alkyne functionalities in the hydrophilic shell, sequential reversible addition−fragmentation chain transfer (RAFT) copolymerizations were carried out to afford an amphiphilic block copolymer. RAFT[27] was chosen as it is highly tolerant to functional monomers, and access to amphiphilic block copolymers is simple and well-documented.[28] The loading of the norbornene and alkyne functionalities can also be tailored by varying the monomer feed composition during synthesis.

Styrene was copolymerized with 10 mol % styrenic norbornene (Nb-St) monomer in toluene.[29] Conversions were kept low (ca. 30−40%) to avoid side reactions involving the norbornene; this is an oft-observed phenomenon when polymerizing Nb-containing monomers.[30] The M_n of polymer **1** was determined by size exclusion chromatography (SEC), relative to PS standards, to be 4.1 kDa (PDI 1.13). A comparison of the integrals of the methylene adjacent to the trithiocarbonate (3.25 ppm) with the Nb signals at 6.0 ppm and the aromatic peaks from 6.2 to 7.2 ppm gave the degree of polymerization as St_{38}-Nb-St_4 (M_n = 5.2 kDa). The reactivity ratios of the monomers in solution are $r_{Nb\text{-}St}$ = 0.56 and r_{St} = 0.94,[29] although since the conversion was deliberately kept low compositional drift was minimized and the norbornene moieties spaced reasonably evenly along the polymer chain. This macroCTA **1** was used to chain extend with a copolymerization of hydrophilic triethylene glycol acrylate (TEGA) and 10 mol % trimethylsilane protected propargyl acrylate (PA-TMS).[31]

Kinetic studies showed that the conversions of PA-TMS and TEGA were approximately equal during polymerization, suggesting, in the absence of reactivity ratios for the monomers, that the resulting copolymer segment is statistically random. Polymer **2** was characterized by ^{1}H NMR to determine the block ratios, using the prominent TEGA CH_2 signal at 4.2 ppm and the PA-TMS CH_2 signal at 4.6 ppm in comparison to the aromatic protons of the styrenic block. This gave a calculated M_n by NMR of 24.3 kDa. The M_n by SEC was much lower (14.5 kDa), although as TEGA-*co*-PA-TMS is vastly different to the PS standards used this is perhaps not surprising. The TMS protecting groups were easily removed following the method of Haddleton et al.;[32] the complete deprotection was confirmed by the disappearance of the TMS methyl signals at ca. 0.2 ppm in the ^{1}H NMR ($CDCl_3$) and the appearance of the alkyne proton signal at 2.65 ppm, as highlighted in Figure 1. Fourier

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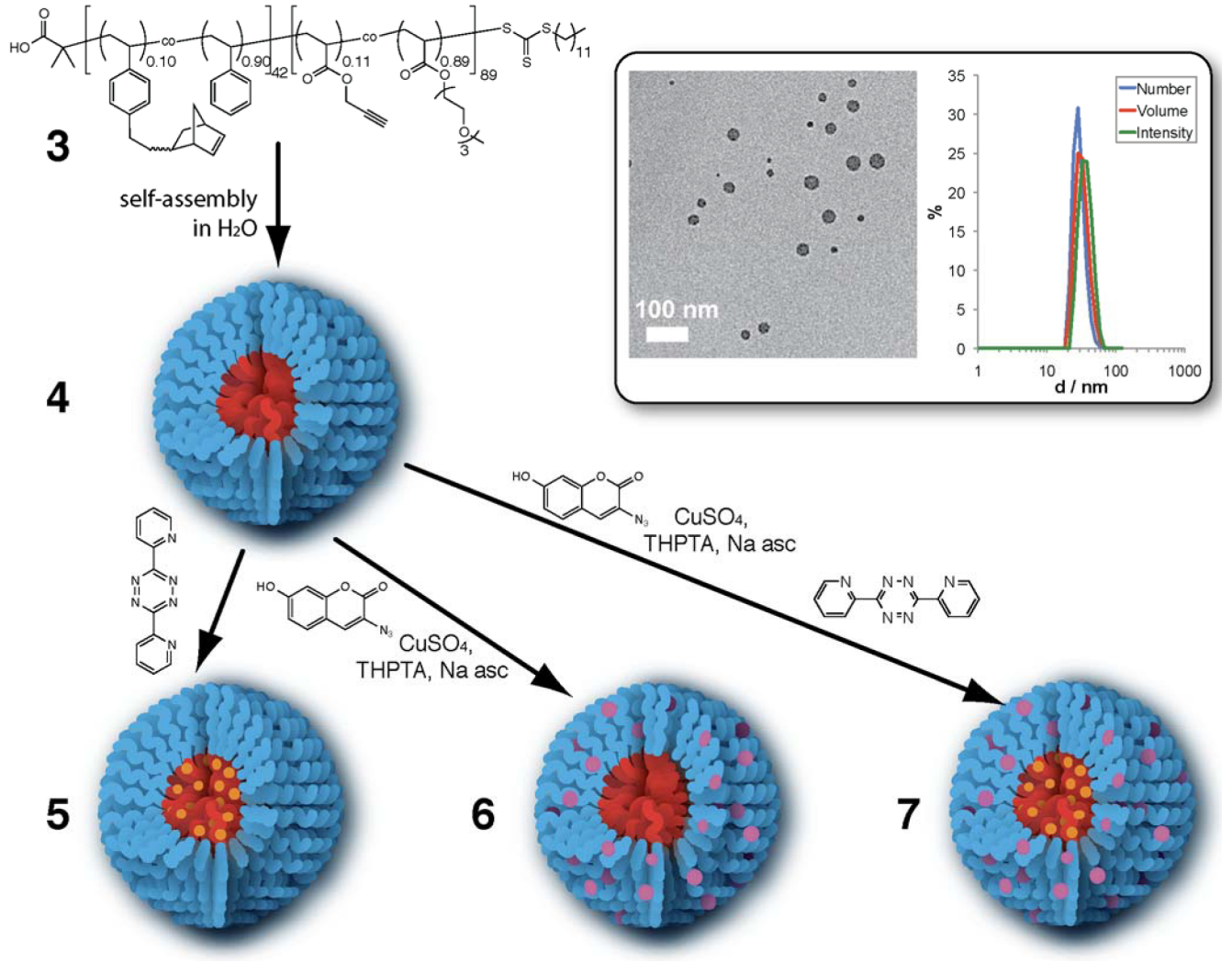
Scheme 1. Synthesis of Micellar Scaffold 4 and Subsequent Double Click Reactions to Afford Functionalized Micelles 5, 6, and 7



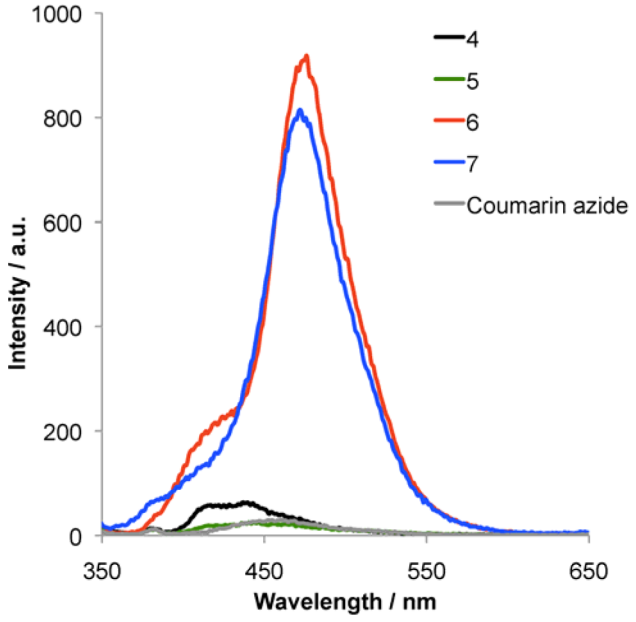


Figure 2. Fluorescence spectra for micelles **4**–**7** and the free coumarin azide.

transform infrared (FT-IR) analysis of polymer **3** also showed the appearance of a characteristic alkyne C–H vibration at 3257 cm^{-1}. The M_n and M_w of **3** by SEC were virtually unchanged from polymer **2**, showing that the deprotection had no adverse effect on the other functionalities on the polymer. Crucially, the integrations of the norbornenyl alkene signal (6 ppm) relative to the aromatic protons remained constant from polymers **1**–**3** showing that the norbornene functionalities were not affected by the chain extension or deprotection steps (Figure 1). The amphiphilic block copolymer **3** was dissolved in tetrahydrofuran (THF), followed by slow addition of water at ca. 2 mL/h to form micellar structures **4**. Exhaustive dialysis against 18.2 MΩ/cm water was carried out, after which the spherical nature and size of the structures was confirmed by dry-state transmission electron microscopy (TEM) imaging on graphene oxide[33] (D_{av} = 30 nm) and dynamic light scattering (DLS; D_h = 33 nm), shown in the inset of Scheme 1.

A tetrazine–norbornene click reaction was carried out in the core of the micelle by simple addition of dipyridyl tetrazine, solubilized in THF due to its poor water solubility. The reaction was monitored by the reduction in intensity of the UV–vis signal arising from the tetrazine ring at 546 nm. The point at which the first derivative reached zero was used to determine the reaction time (approximately 8 h). Subsequently,

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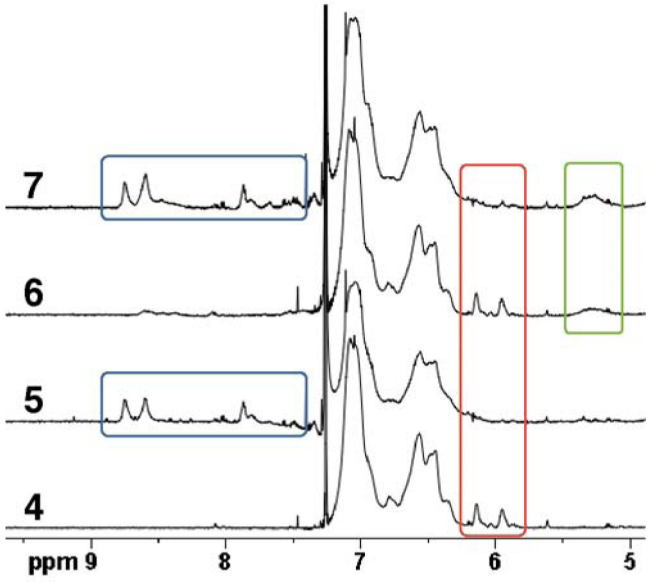

Figure 3. Section of ^{1}H NMR spectrum ($CDCl_3$) for polymers isolated by freeze-drying micelles **4**–**7**. The norbornene alkene signal at 6 ppm (red box) in the starting polymer is retained in micelles **6** (shell CuAAC) but consumed in both the single (**5**) and double (**7**) functionalized micelles. Signals from the clicked dipyridyl tetrazine (blue boxes) are evident in **5** and **7**, and coumarin hydroxyl proton signals (green box) are evident in **6** and **7**.

the micelles **5** were characterized by DLS (D_h = 31 nm) and TEM (D_{av} = 30 nm), and a portion was freeze-dried to isolate the constituent polymers. In the ^{1}H NMR spectrum, complete disappearance of the norbornene alkene at 6 ppm was observed, with new signals corresponding to the pendant pyridine groups of the clicked tetrazine, indicating complete functionalization of the core norbornene moieties in the micelle. SEC analysis indicated no change in the polymer distribution and a slight increase in M_n (14.9 to 15.6 kDa) due to the clicked dipyridyl tetrazines along the backbone.

To investigate the viability of the CuAAC click reaction in the micelle shell, a pro-fluorogenic, water-soluble 3-azido-7-hydroxy-coumarin was used for ligation. As previously described in the literature,[34] this coumarin is not fluorescent until clicked with a terminal alkyne, after which it fluoresces strongly with an emission wavelength between 400 and 490 nm. This provides an ideal method to confirm CuAAC functionalization in the micelle shell.

To a solution of micelles **4** was added coumarin azide (1.2 equiv relative to alkyne groups), copper sulfate pentahydrate, THPTA, and sodium ascorbate in aqueous solution. The mixture was allowed to stir for several hours, after which the copper was removed by adsorption onto CupriSorb beads, and any remaining small molecules were removed by extensive dialysis against water. The resulting micelles **6** were analyzed by fluorescence spectroscopy (λ_{ex} = 340 nm, λ_{em} = 473 nm). Interestingly, the excitation and emission maxima found were different to previously reported values.[34] The closest of these to our current approach is where the coumarin azide was clicked into the core of a micelle[6] (λ_{ex} = 496 nm, λ_{em} = 551 nm). We hypothesize that this disparity in wavelengths is due to the difference in coumarin environment between the hydrophobic, styrenic core reported previously and the hydrophilic micelle shell in this work. The difference in coumarin environment explains this rather than failure of the CuAAC reaction, which would result in no fluorescence rather than altered fluorescence properties.

After functionalization and dialysis, the micelles **6** were analyzed by DLS (D_h = 33 nm) and TEM (D_{av} = 33 nm). The micelles were then freeze-dried to their constituent polymer chains. The complete disappearance of the alkyne signal in the ^{1}H NMR spectrum at ca. 2.5 ppm was difficult to confirm due to significant overlap with the polymer backbone and Nb-St signals; reduction in the signal was observed, but 100% conversion of alkyne to triazole was unable to be categorically ascertained using only the alkyne proton signal. However, the integral of the aromatic region in the ^{1}H NMR of freeze-dried **6** increased relative to the starting polymer **4** due to the aromatic coumarin protons. Importantly, the Nb alkene signals at 6 ppm were still clearly present, indicating that the CuAAC reaction in the shell left the core norbornene moieties unaffected. SEC analysis of the resulting polymer revealed that the polymer distribution remained unchanged, with a small change in molecular weight (14.9 kDa to 15.3 kDa), which was consistent with the addition of several coumarin units per chain.

We first attempted to carry out the tandem reaction by adding all of the CuAAC and Tz-Nb click reagents (1.2 eq. of the coumarin azide and dipyridyl tetrazine) at the same time; however this was discovered to lead to a reduced efficiency of both reactions (approximately 50%). Increasing the ratios of the Tz-Nb and CuAAC click reagents to 5 equiv did not result in any increase in conversion relative to using only 1.2 equiv. Since tetrazines and related pyradizines are known to form metal complexes,[35] we hypothesize that such a Cu-tetrazine complex was forming and inhibiting both the CuAAC and Tz-Nb reactions, possibly by reducing the phase segregation of the relevant reagents and catalysts between the hydrophobic core and the hydrophilic shell.

To overcome this, a one-pot, sequential addition strategy was employed as an alternative to an exactly simultaneous addition. The CuAAC reagents were added in aqueous solution to the micelles **4**, after which the mixture was stirred for 20 min before the addition of dipyridyl tetrazine in THF. The micelle solution was allowed to stir for 12 h before characterization by DLS and TEM and analysis of the constituent polymers by ^{1}H NMR, SEC, and UV–vis of the freeze-dried solution. Reversing the order of addition (i.e., dipyridyl tetrazine followed by CuAAC reagents) resulted in the same high efficiency for the Tz-Nb reaction but greatly reduced the CuAAC reaction efficiency.

As with separate core–shell functionalization, the micelle dimensions remained essentially unchanged after the dual core–shell functionalization: D_h (DLS) = 30 nm and D_{av} (TEM) = 30 nm. The fluorescence intensity of micelles **7** was almost the same as micelles **6** at the same dilution (Figure 2), thus showing that the CuAAC click reaction efficiency was not reduced by the subsequent addition of dipyridyl tetrazine for the core Tz-Nb reaction. The possible influence on the fluorescence intensity by the core Tz-Nb was ruled out as core-functionalized micelles **5** did not have any significant fluorescence emission. Freeze-drying the micelles to isolate the polymer forming **7** showed that the fundamental properties (M_n, M_w) of the polymer were changed very little from the parent polymer. Extracting the UV–vis spectrum using the PDA detector coupled to the SEC showed that the resulting UV–vis spectrum was made up of two peaks: a peak at 297 nm resulting from the Tz-Nb reaction and a smaller peak from the CuAAC clicked coumarin at 357 nm, showing that both reactions had taken place. Analyzing the ^{1}H NMR spectrum

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showed that all Nb functionalities in the core had reacted, by the loss of the alkene signal at 6 ppm (Figure 3).

We have shown that a single micellar scaffold can be both core- and shell-functionalized in a one-pot process using two orthogonal click reactions. This opens up the potential for functionalization with a large array of water-soluble azide-bearing compounds for the shell and hydrophobic tetrazine-bearing compounds for the core. Azides are undemanding to introduce during synthesis, and the increasing array of tetrazines containing functional handles[23a,36] means that potentially any target of interest could be azide- or tetrazine-functionalized and therefore introduced into the micelle in either the core or shell domains. The two reactions occur highly efficiently in a one-pot process with only a slight excess of small molecule reagent, thus significantly reducing the preparation and purification time of functionalized micelles.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, NMR, IR, and UV−vis spectra, DLS data, and TEM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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