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A Facile Method for the Stain-Free Visualization of Hierarchical Structures with Electron Microscopy

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Within the last decade supramolecular self-assembly of diblock copolymers has become of great interest within the scientific community. This is largely on account of the introduction of controlled radical polymerization techniques such as atom transfer radical polymerization,[1–5] nitroxide mediated polymerization,[6–9] and reversible addition-fragmentation chain-transfer (RAFT) polymerization,[10,11] which allow for the facile synthesis of diblock copolymers with predictable molecular weights (MW) and narrow polydispersities (PDI) to mimic the multitude of amphiphilic structures present in nature. The ability to finely control block lengths in diblock copolymers allows for self-assembly in selected solvents into various hierarchical structures, which generally include spherical micelles, vesicles, cylinders, discs, rods, toroids, "Janus" micelles and "worm-like" micelles.[12–19] Access to such well defined assembled nanostructures has led to the advancement of a wide range of novel nanotechnological applications, in areas such as drug and gene delivery, nanoelectronics, and nanoreactors.[20–27]

Transmission electron microscopy (TEM) has proven to be a powerful and invaluable technique in polymer research for the visualization of such hierarchical polymeric structures. However, most of these materials traditionally consist of carbon and other light elements, which are weak electron scatterers, therefore, giving rise to low scattering constants and making them difficult (or impossible) to image.[28–30] Enhancement in scattering contrast can be achieved in a number of ways and has most often been achieved by decreasing acceleration voltages or staining the sample with a high atomic number element, both of which have inherent disadvantages.[28] Decreasing acceleration voltages can lead to the degradation of precious sample and also of the TEM grid. It is well known that the use of staining agents

influences the structural organization of the specimen and introduces artifacts, making images difficult to interpret.[31] Screening for an appropriate staining agent for a particular system is not only time consuming and expensive, but it can also be an impractical option, especially if there is only a limited amount of precious material. The major disadvantage of using staining agents, which typically include osmium tetroxide, uranyl acetate, and lead citrate, is the inherent health risks associated with them often requiring specialist handling and training.[30,32] Osmium tetroxide is poisonous at very low levels of exposure and inhalation at concentration levels below those at which a smell can be perceived can lead to blindness, pulmonary edema, and subsequent death. Lead citrate is also toxic and a carcinogenic, and uranyl acetate is both toxic and radioactive. Cryogenic-TEM (cryoTEM) has been utilized previously for the visualization of hierarchical structures without staining, yet this is a specialized technique and many researchers do not have access to the necessary equipment or expertise to successfully utilize it. Moreover, even with cryoTEM, few materials can be visualized effectively without the use of staining techniques. Therefore, development of a simple and safe method utilizing standard TEM, which is a much more prolific and accessible imaging technique, is highly desirable.

It has been reported that the stain free TEM imaging of block copolymer assemblies can be achieved using graphene oxide (GO) supports on carbon grids.[33] Although this method of analyses does not require TEM sample preparation, it does, however, involve further synthetic procedures for the synthesis of the GO solutions followed by subsequent functionalization of the carbon TEM grid. It has also been reported that facile imaging with TEM can be achieved with the incorporation of high atomic number

Additional Supporting Information may be found in the online version of this article.
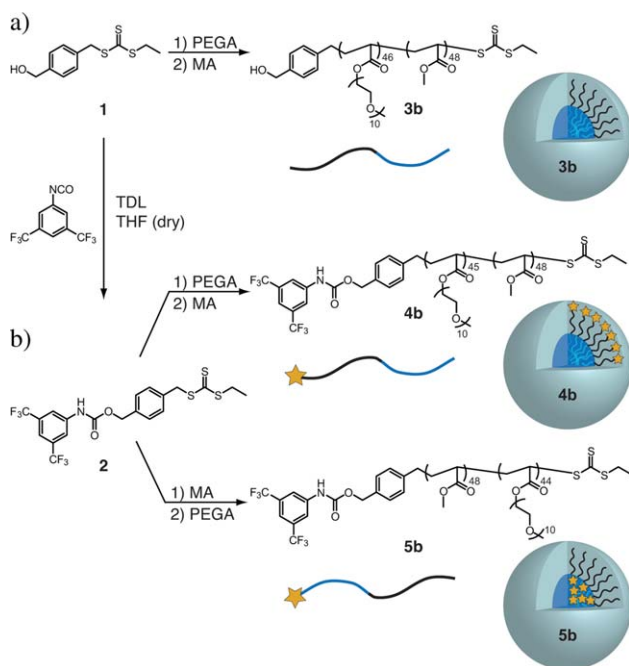
FIGURE 1 Schematic representation of the synthesis of (a) control block copolymer **3b** and (b) the fluorinated CTA **2** and subsequent preparation of end-group functional amphiphilic diblock copolymers **4b** and **5b** via two sequential polymerizations. Schematic representations of the corresponding "externally" and "internally" fluorinated micelles resulting from assembly of amphiphilic diblock copolymers are shown. PEGA = poly(ethylene glycol)acrylate, MA = methyl acrylate, TDL = dibutyltin dilaurate.

elements such as silicon in poly(dimethylsiloxane),[34,35] sulphur in monomers[36] and fluorinated groups incorporated into the side chain of polymers[37] without the need for staining. All of these options require special monomers and certain considerations must be taken into account when attempting to copolymerize such monomers into a polymer backbone. There is, therefore, a need for complementary methodologies for imaging with electron microscopy techniques. Additionally, small angle scattering techniques utilizing X-rays[38] and/or neutrons[39] are extremely powerful and provide a wealth of morphological information for soft hierarchically assembled structures, yet require appropriate specialized facilities and extensive expertise for their implementation. Moreover, these techniques oftentimes require prior structural knowledge to determine appropriate mathematical models for fitting analysis and in these instances, a direct visualization technique such as TEM can provide a valuable first step in obtaining such detailed morphological data.

As the advent of RAFT polymerization it has been shown to be highly advantageous on account of the ability to polymerize a wide range of monomeric species with predictable MW and narrow PDIs, but also more importantly, the near infinite ability to functionalize both R and Z group positions either pre- or postpolymerization.[11,40–42] Herein, we report the

design and synthesis of a RAFT chain-transfer agent (CTA), which incorporates 3,5-bis(trifluoromethyl)phenyl functionality into the R position through facile and near quantitative isocyanate chemistry (Fig. 1). The CTA has been shown to polymerize both hydrophilic and hydrophobic monomers to produce amphiphilic diblock copolymers with good control over MW and PDI, which was determined by gel permeation chromatography (GPC) and nuclear magnetic resonance (NMR) techniques. Self-assembly of the amphiphilic diblock copolymers in aqueous media was achieved and verified by dynamic light scattering (DLS) and the hierarchical assemblies were visualized by TEM without the need for any further preparation of either the polymer assemblies or the TEM grids.

Trithiocarbonate functionality has been shown to offer exceptional control over the RAFT polymerization of a broad range of monomers and can be easily synthesized by facile, rapid, "one-pot" procedures.[10] Moreover, benzyl R-group functionality in combination with simple mercapto-alkyl Z-group functionality have been demonstrated to impart control over the polymerization of several monomer classes (including styrenics, acrylates, and acrylamides) and polymerization conditions (i.e., bulk, solution, emulsion, and suspension).[43] For this work, it was desirable for the CTA to contain a functional group readily available for chemical conjugation. For these reasons, CTA **1** (Fig. 1) was chosen as it contains a hydroxyl group, which has been used previously for the preparation of semitelechelic polymers that are easily functionalized either pre- or postpolymerization using isocyanate coupling reactions.[41] Moreover, 3,5-bis(trifluoromethyl)phenyl isocyanate is a commercially available isocyanate that contains a high number of fluorine atoms, highlighting an important opportunity to utilize a simple isocyanate coupling reaction to prepare a highly fluorinated CTA. The successful synthesis of CTA **2** was confirmed with NMR and infrared spectroscopy.

Amphiphilic diblock copolymers were then synthesized on account of their ability to readily self assemble in water to form compartmentalized structures. Poly(ethylene glycol)acrylate (PEGA; $M_n \sim 480$ g/mol) and methyl acrylate (MA) were chosen as representative hydrophilic and hydrophobic monomers, respectively. As both monomers bear acrylate functionality, it was possible to synthesize the diblock copolymers with either the PEGA block first as well as the MA block first, forming diblock copolymers **4b** and **5b**, containing the fluorinated R-group of the CTA on either the hydrophilic block or hydrophobic block, respectively. Additionally, diblock copolymer **3b** was synthesized with non-fluorinated CTA **1** to be used as a control for TEM visualization studies (vide infra). Analysis of the extended ^{1}H NMR spectrum of the diblock copolymers confirms an almost 1:1 degree of polymerization for both blocks of poly(PEGA-b-MA) **4b**, poly(MA-b-PEGA) **5b**, and for the control polymer poly(PEGA-b-MA) **3b**. However, when considering the mass fraction of the polymers, the mass ratios are closer to 1:3 given the higher mass of the PEGA monomer relative to MA.
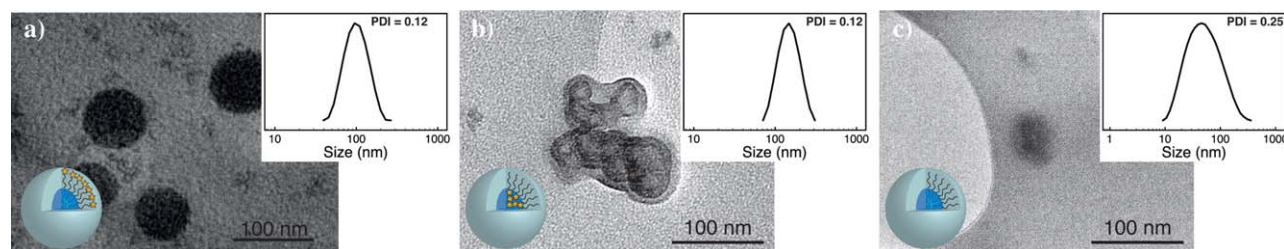
FIGURE 2 TEM images of unstained micelles formed from functional diblock copolymers (a) **4b** and (b) **5b**, and from the nonfunctional control diblock copolymer (c) **3b**. Inset: DLS measurements of the respective micelles.

Self-assembly of amphiphilic polymers into compartmentalized structures is highly dependent on the hydrophobic/hydrophilic ratio of the diblock copolymers, and for the polymers used in this study, micelles were expected to form. Slow addition of water to a solution of the diblock copolymers in tetrahydrofuran (THF; 20 mg/mL) followed by removal of the THF under vacuum, led to self-assembled, three-dimensional structures that where then characterized by DLS. Self-assembly of **4b** and **5b** resulted in almost identically sized structures (Fig. 2- inset), which was expected as the two polymers had almost identical MW and PDIs (Table 1). Moreover, the structures were investigated with static light scattering (SLS) to investigate their morphology (see supporting information). The ratio of the radius of gyration over the hydrodynamic radius (R_g /R_h) can identify whether a structure is a hard sphere (i.e., a micelle) or not and is well known to be $\sqrt{3/5} \sim 0.77$.[44] According to these measurements, these structures are indeed micelles whereby R_g/R_h = 57/77 nm = 0.74 (see Supporting Information), corroborating our expectations based on the relative volume fractions of the hydrophilic and hydrophobic domains of the diblock copolymers and the hydrodynamic radii observed using independent DLS measurements.

With the self-assembled structures in hand, TEM was used to image their morphology. The as prepared solutions of **3b**, **4b**, and **5b** were drop-cast onto holey carbon copper grids with no further preparation steps required, avoiding the need for any harmful, toxic, or radioactive staining techniques as well as ruling out the need for time consuming grid preparations. All samples were imaged with an acceleration voltage of 80 kV. When the control polymer **3b** was used, it was possible to observe only slight interactions with the electron beam on account of concentrated regions of polymer, presumably the PEG-based corona, possibly indicating micellar structures. However, no accurate information about the three-dimensional architecture could be obtained (Fig. 2c).

When the "externally" flourinated micelle **4b** was imaged via TEM, the three-dimensional architecture was readily observed on account of the presence of the highly flourinated R-groups within the corona of the micelle interacting with the electron beam (Fig. 2a). Moreover, Figure 2b clearly demonstrated the "internally" flourinated core–shell morphology expected for amphiphilic diblock copolymer **5b**. The tightly packed core containing the multitude of flourinated groups interacts highly with the electron beam and is clearly visible. In this case, a slight corona can also be seen on account of the poly(PEGA) outer shell having a slight interaction with the electron beam. These hierarchical polymer architectures were readily imaged without any need for staining or specialized grid preparations.

In conclusion, a RAFT CTA has been synthesized that incorporates 3,5-bis(trifluoromethyl)phenyl functionality into the R position using a facile, quantitative isocyanate coupling reaction using commercially a available isocyanate. The ability of this CTA to polymerize amphiphilic diblock copolymers with good control over the MW and PDI was demonstrated. Self-assembly of the amphiphilic diblock copolymers was demonstrated with DLS and SLS and it was shown that the resulting micellar structures could easily be visualized using TEM without the need for

TABLE 1 Polymer Characterization[a]

Polymer	Monomer	Time (min)	Conversion[b] (%)	M_n theo[c] (kDa)	M_n[b] (kDa)	DP_n[b]	M_n[d] (kDa)	PDI[d]
3a	PEGA	90	90	21.6	22.6	46	12.1	1.19
3b	MA	960	98[e]	25.8	26.7	48	20.6	1.16
4a	PEGA	90	88	21.1	21.6	45	9.0	1.19
4b	MA	960	98[e]	25.3	26.2	48	18.1	1.19
5a	MA	90	94	4.1	4.6	48	4.3	1.12
5b	PEGA	960	90[e]	25.6	25.9	44	18.5	1.21

[a] All polymerizations were conducted at 90 °C and in dimethylformamide with targeted DP_n of 50.
[b] Determined by ^{1}H NMR.
[c] Determined from the % conversion.
[d] Determined by THF GPC.
[e] Determined for the chain extension polymerization.

further preparation of either the sample or the grid. These findings demonstrate the facile nature of the incorporation of flourinated groups into such a amphiphilic diblock copolymers for visualization of their hierarchical structures. Future work is currently underway to determine the applicability of this simple system to the visualization of other hierarchical self-assembled structures, such as vesicles and worm-like micelles, and will appear in a forthcoming publication.

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