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Synthesis and characterization of a triazine dendrimer that sequesters iron(III) using 12 desferrioxamine B groups

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ABSTRACT

The synthesis of a third generation triazine dendrimer, **1**, containing multiple, iron-sequestering desferrioxamine B (DFO) groups is described. Benzoylation of the hydroxamic acid groups of DFO and formation of a reactive dichlorotriazine provide the intermediate for reaction with the second generation dendrimer displaying twelve amines. This strategy further generalizes the 'functional monomer' approach to generate biologically active triazine dendrimers. Dendrimer **1** is prepared in seven steps in 35% overall yield and displays 12 DFO groups making it 56% drug by weight. Spectrophotometric titrations (UV–vis) show that **1** sequesters iron(III) atoms with neither cooperativity nor significant interference from the dendrimer backbone. Evidence from NMR spectroscopy and mass spectrometry reveals a limitation to this functional monomer approach: trace amounts of *O*-to-*N* acyl migration from the protected hydroxamic acids to the amine-terminated dendrimer occurs during the coupling step leading to N-benzoylated dendrimers displaying fewer than 12 DFO groups.

1. Introduction

While an essential element for normal cellular metabolism and respiration, excess levels of iron in the body are cytotoxic and fatal if left untreated.[1] Iron absorption from the gut and transport throughout the body involves a complex pathway of proteins which is not fully understood.[2] The body has no active iron excretion system, but desquamation, menstruation, and other mechanisms for blood loss aid in maintaining iron homeostasis.[1a,3] The accumulation of excess iron in tissues occurs in patients with hereditary hemochromatosis, a condition that derives from a genetic mutation leading to abnormally increased iron absorption in the intestines. Diseases requiring frequent blood transfusions also result in excess iron including β-thalassemia major, sickle cell disease, myelodysplastic syndrome, and Fanconi anemia.[4] Iron overload is the most common, chronic metal toxicity condition worldwide with the highest morbidity and mortality rate.[5] Accordingly, chelating agents have been employed successfully to treat iron overload disorders.

These iron-sequestering agents actively remove excess iron from the blood through renal filtration to urine and through biliary drainage from hepatocytes into fecal matter.[4b] Several iron(III) specific chelation therapies have been explored to treat iron overload. However, only three agents are approved by the FDA for preclinical or clinical use: desferrioxamine B (also known as DFO, DFOB, deferoxamine, or Desferal®), deferiprone (Ferriprox®), and deferasirox (Exjade®).[6] Desferrioxamine B (DFO) is the most studied and successful iron(III) chelating agent since its isolation from *Streptomyces pilosus* in the early 1960s.[7] DFO is orally ineffective and has short plasma half-life (5–10 min for DFO and 90 min for its iron complex), thus requiring slow intravenous or subcutaneous infusion over 8–12 h at dose of 25–50 mg/kg/d for 5–7 days/week during treatment.[7a] Poor patient compliance has generated interest in the development of iron sequestration agents that are either orally active or show extended circulation times.

Accordingly, macromolecular constructs capable of iron sequestration have been investigated to prolong the plasma half-life and reduce the toxicity associated with some small molecule chelates. Such architectures include polysaccharide polymers such as dextran and hydroxyethyl starch containing 10–30 wt % DFO.[8] These constructs show promising in vivo data in mice with acute iron poisoning.[9] However, the poorly defined polymeric structure leads to ambiguous evaluation of pharmacokinetic and pharmacodynamic data. Other macromolecular iron-sequestering agents using poly(ethylene glycol), salicylate, catecholate, and hydroxypyridinoate have been proposed to improve these drawbacks.[10]

Here, we prepare **1** by incorporating twelve DFO groups into a second generation dendrimer **2** (R′ = H) that is available on kilogram scale (Fig. 1).[11] In addition to deriving from readily available and inexpensive building blocks, these materials are amenable to the rigorous characterization typically reserved for small molecules and not normally suited to macromolecules. These materials show surprising biocompatibility in acute dosing regimes.[12] Here,

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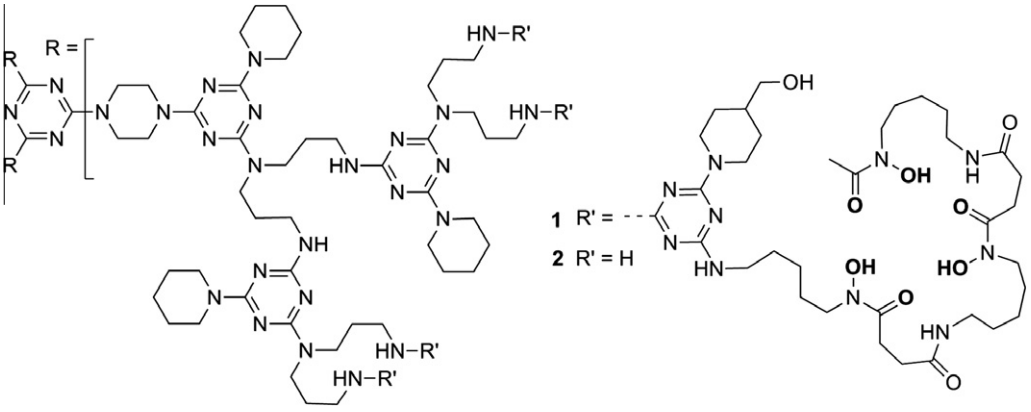


Figure 1. Dendrimer **1** derives from **2** and displays 12 DFO groups that form hexadentate complexes with Fe(III) using the groups shown in bold.

we pursue DFO constructs based on (i) interests in their intrinsic biological activity and (ii) the opportunity they present to probe the scope and limits of synthetic methods aimed at these targets. DFO appears (and proves) to be markedly more complex from a reactivity standpoint than either paclitaxel[14] or camptothecin. Accordingly, synthesis and evaluation of a proof-of-concept target was undertaken.

2. Results and discussion

We recently described an iterative route to dendrimer synthesis wherein three reactions were required to complete a generation.[13] To functionalize these dendrimers, two different strategies have been pursued. The 'functional monomer' approach relies on the incorporation of the drug of interest into a reactive dichlorotriazine. This strategy has been used successfully to introduce paclitaxel onto triazine dendrimers.[14] Alternatively, the 'functional cap' strategy relies on elaboration of the dendrimer to yield a reactive poly(chlorotriazine) to which the drug with pendant nucleophile is incorporated. We recently showed that an isonipecotic ester of camptothecin could be incorporated into dendrimers in this way.[15]

Our initial efforts to directly react the amine nucleophile of DFO with cyanuric chloride failed due to the nucleophilicity of the hydroxamic acid groups. The result of these reactions are macrocycles and insoluble material presumed to be polymeric. Protection of the hydroxamic acids was first attempted using iron(III). DFO shows high binding affinity for a variety of metals including Fe(III), Al(III), and Ga(III) through the carbonyls and nucleophilic hydroxamic acids.[16] Reaction of DFO-Fe(III) complex with cyanuric chloride generated a mixture of the desired dichlorotriazine product and macrocyclic monochlorotriazines. The necessity to remove iron in later steps—especially in light of its high iron-binding constant ($\sim 10^{30}$) over a wide range of pH values[17]—combined with solubility challenges led us to pursue alternative strategies including a 'functional cap' strategy using an isonipecotic amide of DFO that also failed. Ultimately, we adopted more conventional protection strategies of the hydroxamic acid groups.

2.1. Synthesis

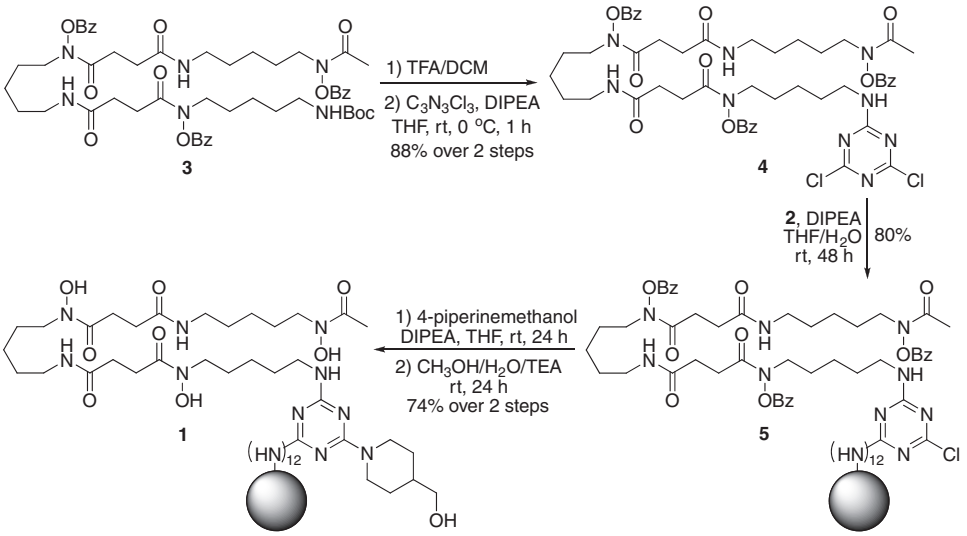
The successful synthesis of the DFO-displaying dendrimer, **1**, is shown in Scheme 1. In order to improve the poor solubility of DFO in organic solvents and suppress unwanted side reactions, the chelating agent is first masked with Boc and benzoyl groups. DFO mesylate salt is reacted first with BOC anhydride in dioxane/water. After subsequent installation of the benzoyl groups, the amine is liberated with trifluoroacetic acid. This strategy was initially avoided for fear of rapid *O*-to-*N* acyl migration. This reaction is not observed and accordingly, the free amine can be reacted with cyanuric chloride, a step that is central to the synthesis of these dendrimers.[18] The resulting dichlorotriazine **4**—our functional monomer—is reacted with **2** to yield the desired product **5**. We expected this reaction to proceed without migration of benzoyl groups from DFO to the dendrimer. However, S_NAr reaction rates are reduced by steric hindrance and evidence of benzoyl migration is seen in trace amounts (vide infra). The synthesis concludes with reaction of poly(monochlorotriazine) **5** with 4-piperidinemethanol and hydrolysis of the *O*-acyl groups using a solution of methanol, water, and triethylamine to yield **1**. In summary, **1** is accessed from **2** in seven steps in 35% overall yield and contains 12 DFO groups predominantly. Dendrimer **1** can be described as having high drug loading: it is 56 wt % DFO, but not water soluble.

2.2. Characterization

^1H and ^{13}C NMR spectroscopy as well as MALDI-TOF MS are used to follow all the reactions. For the low molecular weight intermediates, **3** and **4**, thin layer chromatography (TLC) is also useful to monitor the spot-to-spot conversions of the compounds. The complexity increases with dendrimers **5** and **1**. MALDI-TOF MS of **5** shows the product ion at m/z 14,762 and lines corresponding to species missing approximately one to three dichlorotriazine groups that could result either from incomplete S_NAr reaction of functional monomer **4** with **2**, or from benzoyl migration.

As shown in Figure 2, the ^1H NMR spectrum of **5** shows no clear sign of incomplete reaction and/or the migration, but this is far from definitive due to both spectral broadness and the limit of detection of the technique. For example, the theoretical H number of **5** is very close to the integration values. That is, the recorded integral areas observed throughout the spectra match expectations (indicated in parentheses) in the aromatic region (*o*-72H-Ph, *p*-36H-Ph, and *m*-72H-Ph of benzoyl groups at δ 8.05, 7.63, and 7.48 ppm, respectively), the aliphatic region populated by the dendrimer (276H of G2 dendrimer and DFO at δ 3.77–3.10 ppm), regions unique to DFO (48Hs for both $N(OBz)COCH_2CH_2$ and $N(OBz)COCH_2CH_2$ at δ 2.64 and 2.47 ppm, respectively; 36H of $COCH_3$ at δ 2.02 ppm), as well as regions associated with DFO and dendrimer (306H of G2 dendrimer and DFO at δ 1.75–1.20 ppm). Details including specific assignments are available in the Supplementary data.

The ^1H NMR spectrum of the target, **1**, shows lines in the aromatic region that integrate for less than 1 benzoyl group (Fig. 2). An observed value of 2.3H (theoretically 3H for one benzoyl migration) of *p*- and *m*-H-Ph at δ 7.58–7.38 ppm represents 0.77 benzoyl groups. Accordingly, we approximate that each dendrimer displays



Scheme 1. Synthesis of **1** using the functional monomer approach with the dichlorotriazine intermediate **4**.

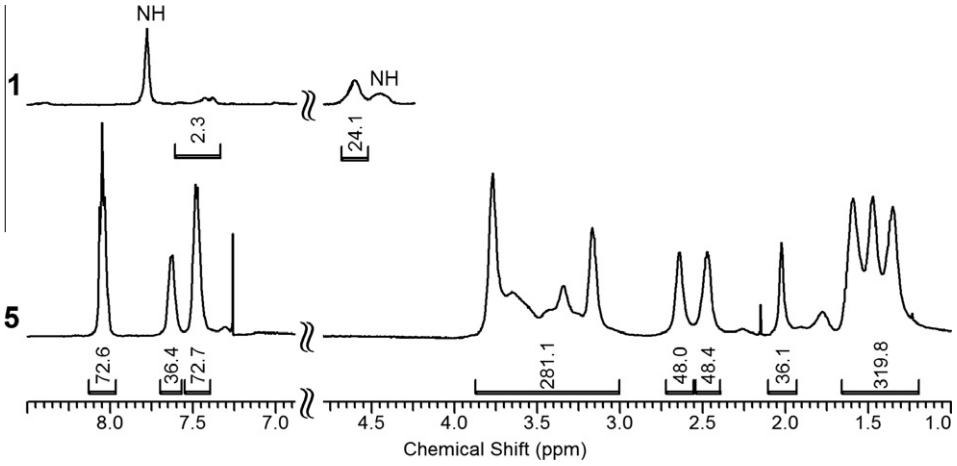


Figure 2. ^{1}H NMR spectra of **1** (500 MHz, DMSO-d_6) and **5** (500 MHz, $CDCl_3$).

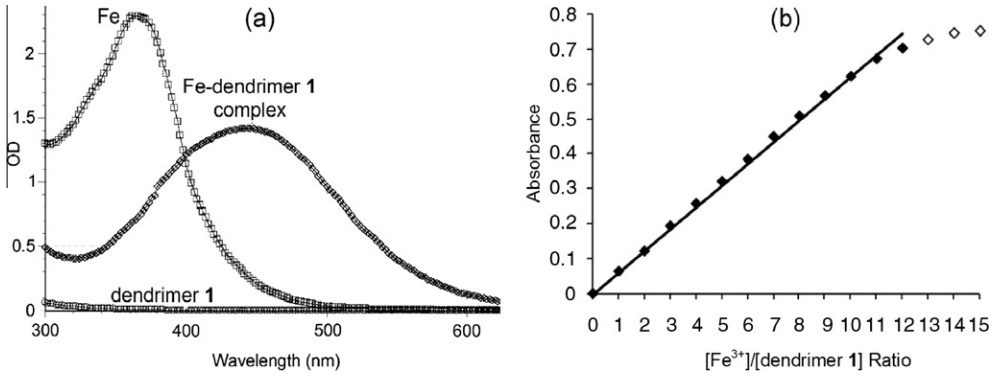


Figure 3. UV–vis absorbance spectra of (a) the species of interest and (b) a titration plot of absorbance (λ_{max} 442 nm) against [Fe^{3+}]/[**1**] that is linear up to almost 12 mole equivalents (black diamonds) before excess iron leads to no significant change (open diamonds).

on average 11¼ DFO groups. At this time, quantifying the exact distribution of these species is outside the scope of this work. Although mass spectrometry suggests species that have undergone up to three acyl transfer reactions, these materials clearly exist in small amounts relative to **1**.

2.3. Chelation

Spectrophotometric titrations corroborate both this migration and the ability of the dendrimer to behave as intended. Dendrimer **1**, $FeCl_3$ and the complex are dissolved in methanol and absorbance

is measured at 25 °C (Fig. 3a). Stoichiometry is established by titrating 27.8 μM of **1** with twelve portions of 27.8 μM of $FeCl_3$ as plotted with black diamonds in Figure 3b. The absorbance of Fe(III)-dendrimer complex is linear up to 11 mole equivalents of Fe(III): **1**. After 12 equiv of Fe(III), the absorbance does not change significantly (open diamonds). The extinction coefficient of the complexed iron (III) is calculated as $\varepsilon = 2.23 \times 10^3\ M^{-1}\ cm^{-1}$ at λ_{max} 442 nm. The linear relationship suggests that iron chelation by this multivalent host is not cooperative.

3. Experimental section

3.1. General procedures

Dendrimer **2** was prepared as previously reported.[11] All other chemicals were purchased from Aldrich and Acros and used without further purification. All solvents were ACS grade and used without further purification. Diafiltration purification was performed with Amicon stirred ultrafiltration cell equipment (Model 8050, PLCC membrane, Millipore Corp.) at 35 psi of N_2. UV–vis absorption spectra were obtained with SPECTRAmax Plus384 (Molecular Devices) and analyzed using SoftMax Pro v. 4.7.1. NMR spectra were recorded on an Inova 300 or 500 MHz spectrometer in $CDCl_3$, CD_3OD, or DMSO-d_6. Mass spectrometry was carried out by the Laboratory for Biological Mass Spectrometry at Texas A&M University.

3.2. Experimental procedures

3.2.1. Dendrimer 1

A solution of 4-piperidinemethanol in THF (10 mL) was added to a solution of **5** (0.20 g, 0.0135 mmol) and DIPEA (0.3 mL, 1.71 mmol) in THF (20 mL). The solution was stirred for 24 h at room temperature and evaporated under vacuum. The residue was dissolve in a solution of triethylamine (1.2 mL), H_2O (2.4 mL), and methanol (8 mL). The solution was stirred for 24 h at room temperature and evaporated under vacuum. The residue was dissolved in deionized water and filtered. The resulting solution was subjected to diafiltration to remove low molecular weight impurities using an Amicon stirred ultrafiltration cell with a PLCC membrane (MWCO: 5 kDa) in deionized water over 3 days. The purified solution was evaporated under vacuum to afford **1** (0.12 g, 74% over two steps) as a colorless solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.60 (br, 24H), 3.80–3.05 (br m, 252H), 2.99 (br m, 48H), 2.65–2.57 (br m, 72H), 2.26 (m, 48H), 1.96 (s, 36H), 1.80–1.20 (br m, 366H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.8, 174.4, 174.3, 173.4, 167.3, 166.7, 165.9, 67.9, 48.7, 45.3, 44.3, 41.4, 40.3, 31.5, 30.6, 30.0, 28.9, 27.3, 25.1, 24.9, 20.4. MS (MALDI-TOF) calcd for $C_{549}H_{948}N_{192}O_{108}$ 11959.46, found 11964.65 $(M+H)^+$.

3.2.2. Intermediate 3

A solution of Boc anhydride (0.50 g, 2.29 mmol) in dioxane (5 mL) was added to a suspension of deferoxamine mesylate (1.20 g, 1.83 mmol) and triethylamine (0.6 mL, 4.30 mmol) in dioxane (7 mL) and H_2O (7 mL). The solution was stirred for 16 h at 40 °C and evaporated in vacuum. The residue was suspended in methanol (5 mL) and precipitated by adding diethyl ether (70 mL). The precipitate was filtered, washed with diethyl ether, and dried to give the *N*-Boc DFO derivative (1.0 g, 83%) that was used without further purification. Benzoyl chloride (0.694 mL, 5.98 mmol) was slowly added to a solution of the protected intermediate (0.66 g, 1.0 mmol) followed by pyridine (0.51 mL, 6.32 mmol) in DMF (8 mL) at 0 °C. The solution was warmed to room temperature, stirred under nitrogen for 16 h, and evaporated under vacuum. The residue was dissolved in dichloromethane, washed with brine, dried over $MgSO_4$, filtered, and evaporated under vacuum. The crude product was purified by silica gel chromatography (from hexane/EA = 1:1 to acetone/hexane = 1:1) to give **3** (0.80 g, 82%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.03 (m, 6H), 7.60 (m, 3H), 7.46 (m, 6H), 3.75 (br m, 6H), 3.14 (br m, 4H), 3.03 (br m, 2H), 2.62 (br, 4H), 2.45 (br m, 4H), 2.00 (s, 3H), 1.53 (br, 6H), 1.44 (br m, 6H), 1.36 (br, 15H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 171.7, 164.4, 156.0, 134.5, 130.0, 129.9, 128.9, 126.6, 126.5, 78.8, 47.7, 40.3, 39.2, 30.3, 29.5, 28.9, 28.4, 27.9, 27.7, 27.5, 26.6, 23.8, 23.5, 20.4; MS (MALDI-TOF) calcd for $C_{51}H_{68}Cl_{12}N_6O_{13}$ 972.4844, found 973.3748 $(M+H)^+$.

3.2.3. Intermediate 4

A solution of **3** (0.65 g, 0.668 mmol) in trifluoroacetic acid (4 mL) and dichloromethane (8 mL) was stirred for 30 min at room temperature. The solution was diluted with dichloromethane, washed with brine, dried over $MgSO_4$, filtered, and evaporated under vacuum. The residue was dissolved in THF (30 mL). Cyanuric chloride (0.13 g, 0.705 mmol) and DIPEA (0.3 mL, 1.71 mmol) was added to the solution at 0 °C. The reaction solution was stirred for 1 h at 0 °C and evaporated under vacuum. The residue was dissolved with dichloromethane, washed with brine, dried over $MgSO_4$, filtered, and evaporated under vacuum. The crude product was purified by silica gel chromatography (acetone/hexane = 3:2) to give **4** (0.60 g, 88% over two steps) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.02 (m, 6H), 7.60 (br m, 3H), 7.45 (br m, 6H), 3.75 (m, 6H), 3.37 (br m, 2H), 3.14 (br m, 4H), 2.62 (br, 4H), 2.46 (br m, 4H), 2.00 (s, 3H), 1.59 (br, 8H), 1.43 (br, 6H), 1.33 (br, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 171.9, 171.8, 170.4, 169.3, 165.6, 164.4, 134.5, 129.9 (×2), 128.9, 126.5, 126.4, 47.7, 41.3, 39.2, 30.2, 28.9, 28.4, 27.7, 26.6, 26.4, 23.7, 23.5, 20.3; MS (MALDI-TOF) calcd for $C_{49}H_{59}Cl_2N_9O_{11}$ 1019.3711, found 1020.2622 $(M+H)^+$.

3.2.4. Intermediate 5

A solution of **2** (0.11 g, 0.0372 mmol) in THF (3 mL) and H_2O (2 mL) was slowly added to a solution of **4** (0.55 g, 0.539 mmol) and DIPEA (0.2 mL, 1.13 mmol) in THF (7 mL). The solution was stirred for 48 h at room temperature and evaporated under vacuum. The residue was dissolved with dichloromethane, washed with brine, dried over MgSO4, filtered, and evaporated under vacuum. The crude product was purified by silica gel chromatography (from acetone/hexane = 3:2 to DCM/MeOH = 7:1) to give **5** (0.44 g, 80%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (m, 72H), 7.63 (br, 36H), 7.48 (br m, 72H), 3.77–3.10 (br m, 276H), 2.64 (br, 48H), 2.47 (br, 48H), 2.02 (s, 36H), 1.59–1.35 (br m, 306H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 171.9, 165.7, 165.4, 164.6, 134.6, 130.1 (×2), 129.0, 126.8, 126.6, 47.9, 44.3, 43.1, 40.8, 39.3, 30.5, 29.1, 28.6, 27.9, 26.8, 25.9, 25.1, 23.9, 23.7, 20.5; MS (MALDI-TOF) calcd for $C_{729}H_{948}Cl_{12}N_{180}O_{132}$ 14754.93, found 14762.05 $(M+H)^+$.

4. Conclusion

Here, we add to the number of macromolecular agents that are able to chelate iron(III). Triazine dendrimer **1** is not only capable of sequestration, but comprises a significant amount of active agent by weight (56%). The material, while a multi-component mixture, is still well-defined for a macromolecule. Limitations of the functional monomer approach have emerged: protecting group migrations suggest that orthogonal conjugation/protecting group strategies will be needed if single-chemical entity materials are desired. The dispersity observed, however, is likely to be much narrower than previously described constructs wherein the polymer is

inherently more disperse prior to derivatization. While the literature provides numerous examples of small molecule sequestrants,[19] both the synthetic accessibility and opportunities that this and related macromolecular agents provide compelling motivation for future efforts in this area.

Acknowledgments

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Supplementary data

Supplementary data (assignments for ^{1}H and ^{13}C NMR spectra, and the original NMR and mass spectra) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.039.

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