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Highly Branched Polydimethylacrylamide Copolymers as Functional Biomaterials

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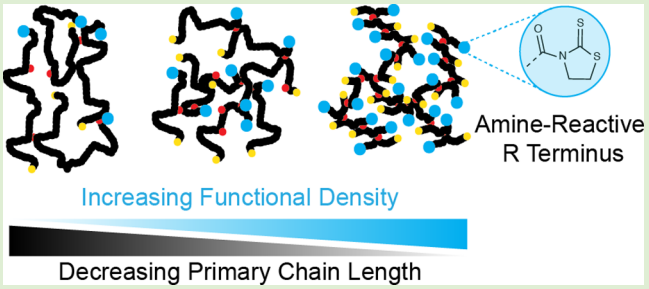
ABSTRACT: Controlled radical polymerization of vinyl monomers with multivinyl cross-linkers leads to the synthesis of highly branched polymers with controlled spatial density of functional chain ends. The resulting polymers synthesized in this manner have large dispersities resulting from a mixture of unreacted primary chains, low molecular weight branched species, and high molecular weight highly branched species. Through the use of fractional precipitation, we present a synthetic route to high molecular weight highly branched polymers that are absent of low molecular weight species and that contain reactivity toward amines for controlled postpolymerization modification. The controlled spatial density of functional moieties on these high molecular weight macromolecular constructs enable new functional biomaterials with the potential for application in regenerative medicine, immunoengineering, imaging, and controlled drug delivery.



INTRODUCTION

High molecular weight, water-soluble, polymeric species are crucially important to the field of biomaterials. When functionalized, these species alter the pharmacokinetics of small molecules and biologics,[1,2] increase the signaling output for imaging agents,[3] control the spatial density and therapeutic response of small molecules,[4,5] and generate the precursors to dynamic hydrogel platforms that are showing promise in the field of regenerative medicine and drug delivery.[6,7] For translational applications, it is important that these materials degrade into species with molecular weights below the threshold for glomereal filtration in the kidney to avoid undesirable accumulation in tissues.[8] Moreover, lower dispersity materials are desired to specifically probe structure−property relationships without undesired confounding variables.

Highly branched polymers are a class of high molecular weight macromolecules characterized by high-frequency main chain branching of linear polymers through randomly incorporated branch points and are promising materials for next generation biomaterials.[9−13] When the molecular weight between the branch points (M_x) of these polymers is lower than the entanglement molecular weight (M_e) of the linear polymer, these branched polymers do not entangle, thus significantly reducing the solution viscosity at a given concentration compared to a linear polymer of comparable molecular weight.[14] The reduced solution viscosity of highly branched polymers improves the utility of these materials in injectable formulations. Furthermore, the branch points of these materials can be engineered to biodegrade in the body, yielding primary chains with a molecular weight that is sufficiently low to be efficiently cleared renally. Due to the globular and highly branched nature of highly branched polymers, they exhibit significantly higher local concentrations of end groups than their linear counterparts. As such, a judicious choice of the polymerization technique provides opportunities for postpolymerization conjugation to these polymeric end groups. The simplicity of the material synthesis coupled with the controlled spacial density of functional moieties enables the use of highly branched polymers for diverse bioapplications, including drug delivery, imaging, immunoengineering, and regenerative medicine.

The first demonstrated routes to synthesize hyperbranched species involved the step growth polymerization of AB_x-type monomers. However, this approach does not include vinyl monomers polymerized through radical vinyl polymerization. The monomer scope and degree of synthetic control in the synthesis of branched polymers have been improved through the development of radical-based chain growth polymerization techniques involving AB* monomer-initiators and vinyl monomers (self-condensing vinyl polymerization),[15−17] thiol moiety chain transfer agents with vinyl and multivinyl monomers (MVMs),[18,19] and controlled radical polymerization (CRP) of vinyl monomers (VMs) and MVMs.[20−28]

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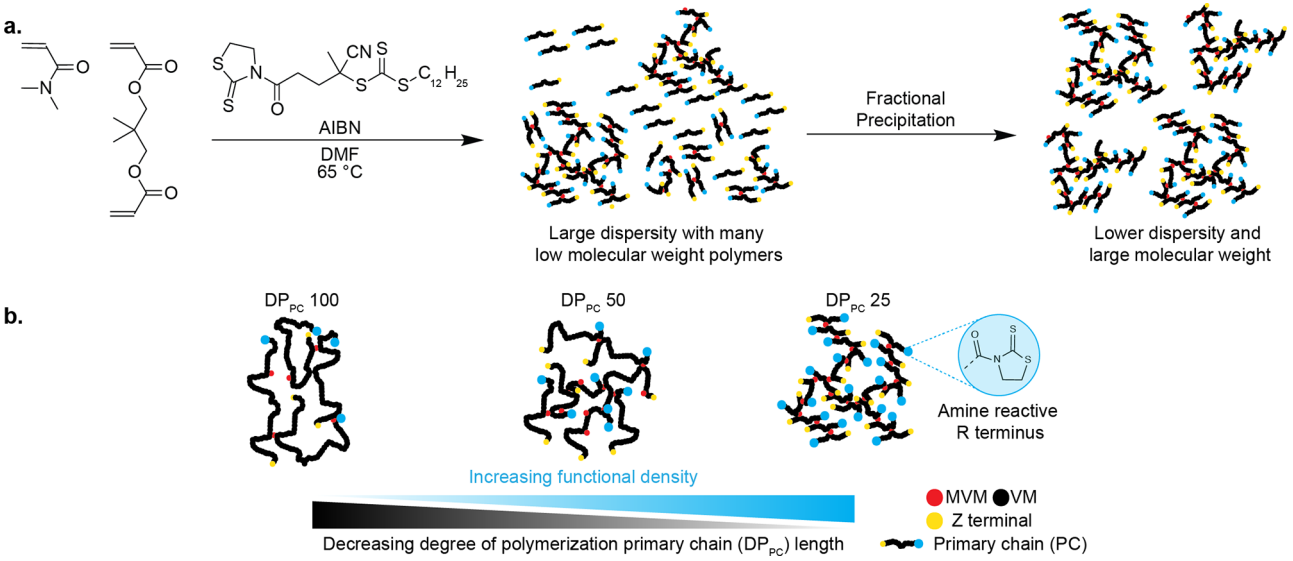

Figure 1. Synthesis of low dispersity and degradable highly branched polymers amendable to postpolymerization modifications. (a) Synthetic scheme for the copolymerization of the vinyl monomer (VM) dimethylacrylamide (DMA) with the multivinyl monomer (MVM) neopentyl glycol diacrylate (NPGDA), using an amine-reactive RAFT polymerization chain transfer agent (TA-CTA). RAFT polymerization using VM and MVM yields highly disperse reaction mixtures of primary chains, low molecular weight branched species, and high molecular weight highly branched species. Depending on the reaction conditions, these mixtures contain between 20 and 40 wt % unreacted primary chains. Unreacted primary chains and low molecular weight branched species are subsequently removed via fractional precipitation, yielding high molecular weight highly branched species with lower dispersities. (b) Alteration of the primary chain degree of polymerization (DP$_{PC}$) affords control over the spatial density of the amine-reactive R-terminus and thus the density of functional moieties via postpolymerization modification.

Specifically, atom transfer radical polymerization (ATRP) and reverse addition−fragmentation transfer (RAFT) CRP techniques have enabled precise control over the primary chain degree of polymerization (DP$_{PC}$), or the length between the initiating species and dormant terminal on the linear portion of the branched network. These techniques lead to branched macromolecules with end groups distributed more uniformly throughout the polymer.[29] Moreover, these polymerization techniques enable control over the number and weight-average primary chains per molecule.[30] Despite this control, ATRP and RAFT synthesis of branched polymers from VMs and MVMs yields a high dispersity mixture of molecules ranging from highly branched high molecular weight macromolecules to unincorporated primary chains (Figure 1).[30] Unincorporated primary chains and low molecular weight branched species increase the polymerization dispersity and lower the average molecular weight, confounding the effects of topological structure while presenting an uncertainty about the composition of the reaction mixture, potentially complicating biomedical translation. The usage of inimers (RAFT chain transfer agents bearing a vinyl group on either the R- or Z-terminus) lowers the dispersity and number of unincorporated primary chains, yet this approach severely limits the ability of postpolymerization conjugation of functional moieties.[31−34]

An alternative approach to low dispersity highly branched polymers, with no unincorporated primary chains, involves RAFT polymerization of VMs and MVMs with a subsequent fractional precipitation step (Figure 1a). Fractional precipitation of synthetic polymers involves the slow addition of antisolvent to the dissolved polymer until turbidity is observed. Since high molecular weight polymers are subjected to a higher enthalpic penalty than low molecular weight polymers with the addition of an antisolvent, higher molecular weight polymers precipitate first and can thus be isolated from the lower molecular weight polymers in the mixture.[35] Analogous fractional precipitation strategies have been implemented to remove unreacted arms in the arm-first synthesis of star polymers.[36,37] Implementation of fractional precipitation with an amine-reactive R-terminus on the chain transfer agent (CTA), coupled with the ability to control DP$_{PC}$ by altering $\frac{[VM]}{[CTA]}$, affords a synthetic route to high molecular weight highly branched polymers with controlled spacial density of functional moieties (Figure 1b).

MATERIALS AND METHODS

Materials. HPLC grade *N*,*N*-dimethylformamide (DMF, Alfa Aeser, > 99.7%), diethyl ether (Acros, 99%+, ACS reagent, stabilized with BHT), dioxane (Sigma, 99.8%, anhydrous), lauroyl peroxide (LPO, Luperox, 97%), sodium hydroxide (NaOH, Sigma-Aldrich, 97%), hydrochloric acid (HCl, Sigma ALdrich, 12M), and deuterated dimethyl sulfoxide (DMSO−D$_6$, EMD Milipore, 99.8%+) were used as received. *N*,*N*-Dimethylacrylamide (DMA, Sigma-Aldrich, 99%) and neopentyl glycol diacrylate (NPGDA, Sigma-Aldrich) were filtered with basic alumina before use. 2,2′-Azobis(2-methylpropionitrile) (AIBN, Sigma, > 98%) was recrystallized from methanol (MeOH, Fisher, HPLC grade >99.9%) and dried under vacuum before use. TA-CTA synthesis was adapted from literature protocols (see Supporting Information).[38]

General Synthesis of Highly Branched Polymers. The procedure to synthesize DMA-*co*-NPGDA branched copolymer targeting a CTA:MVM ratio of 1.45:1 and a CTA:VM of 50 is as follows. The protocol for other DP$_{PC}$ only varies in stoichiometry. DMA (10 g, 100.9 mmol, 50 equiv, filtered through basic alumina), NPGDA (621 mg, 2.92 mmol, 1.45 equiv), TA-CTA (1.02 g, 2.01 mmol, 1 equiv), and AIBN (66 mg, 0.4 mmol, 0.2 equiv) were diluted with DMF to a total volume of 25 mL ([DMA] = 4 M). The solution was divided into two 20 mL scintillation vials equipped with a PTFE septa. The reaction mixture was sparged with N$_2$ for 15 min and heated for 36 h at 65 °C. AIBN (33 mg, 0.2 mmol, 0.1 equiv) was added to each scintillation vial, which was purged with N$_2$ for 15 min and heated for 24 h at 65 °C. Monomer conversion was determined

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by ^{1}H NMR spectroscopy through disappearance of vinyl proton (1H, δ = 5.6 ppm) with DMF (δ = 8.0) as an internal standard.

General Fractional Precipitation Method. The general procedure for a fractional precipitation is as follows. Detailed accounts of the process are provided in the Supporting Information. A 10% (w/v) solution of a branched copolymer dissolved in dioxane was prepared in a 1 L round-bottom flask. With turbulent stirring, a 60:40 (by volume) mixture of ether:dioxane was added until the solution turned cloudy. The resulting solution was centrifuged. The pale yellow and transparent supernatant was set aside for a second fractional precipitation. The deep yellow, viscous, high molecular weight fraction was diluted in dioxane and precipitated into ether to generate the first fraction. The previously collected supernatant was stirred vigerously. A volume of a 60:40 (by volume) mixture of ether:dioxane was added to the supernatant such that the volume of antisolvent added was double the amount of solvent required to turn the supernatant opaque. The solution settled overnight into two phases. The upper, pale yellow phase was aspirated. The bottom, high-molecular weight, viscous, dark-yellow phase was diluted in dioxane and precipitated into ether. This procedure yields between 5 to 10 wt % and 30 to 40 wt %, respectively, for the first and second collection.

General Protocol for α-CD Conjugation. The protocol for the conjugation of α-CD to a primary chain-free (DP_{PC} 25) branched copolymer is as follows. The protocol for other DP_{PC} only varies in stoichiometry. First, 75 mg (0.03 mmol end group, 1 equiv) of the branched copolymer isolated from fractional precipitation was dissolved in 0.5 mL of DMSO. Then 29 mg of α-CD (0.026 mmol, 0.85 equiv) was dissolved in 0.5 mL of DMSO, added to the branched copolymer solution, agitated, and left at room temperature for 10 min. The solution was washed with ether 3 times, extracting residual DMSO. The resulting copolymer was dissolved in water, dialyzed for 48 h (Slide-A-Lyzer 3500 MWCO), and lyopholized. Conjugation was confirmed through ^{1}H NMR in deuterated DMSO by the presence of carbohydrate protons (δ = 4.4−6.0 ppm).

General Protocol for Degradation Assay. The protocol for basic degredation for a primary chain-free (DP_{PC} 100) branched copolymer is as follows. The protocol for other DP_{PC} only varies in copolymer usage. To remove the trithiocarbonate CTA z-terminus from the branched polymer, 100 mg of branched copolymer (0.01 mmol CTA, 1 equiv), 8 mg of lauroyl peroxide (0.02 mmol, 2 equiv), and 33 mg of AIBN (0.2 mmol, 20 equiv) were dissolved in 1 mL of DMF and heated to 90 °C for 12 h and precipitated into ether.[39] 25 mg of the resulting copolymer was dissolved in 2 mL of 0.1 M NaOH for 24 h, neutralized with acetic acid, and lyophilized. The degradation into primary chains was analyzed by size-exclusion chromatography (SEC) in DMF.

The protocol for acidic degradation for a primary chain-free (DP_{PC} 100) branched copolymer is as follows. The protocol for other DP_{PC} only varies in copolymer usage. To remove the trithiocarbonate CTA, 100 mg of branched copolymer (0.01 mmol CTA, 1 equiv), 8 mg of lauroyl peroxide (0.02 mmol, 2 equiv), and 33 mg of AIBN (0.2 mmol, 20 equiv) were dissolved in 1 mL of DMF and heated to 90 °C for 12 h, and precipitated into ether. Then, 25 mg of the resulting copolymer was dissolved in 2 mL of 12 M HCl for 48 h (degradation into primary chains was also successful in 1 M HCl). Aliquots were taken at 24 and 48 h, neutralized with sodium carbonate, filtered, and analyzed by aqueous SEC.

Mammalian Cell Viability Measurements. To remove the trithiocarbonate CTA z-terminus from the second fractional precipitation collection of DMA-*co*-NPGDA targeting a NPGDA:CTA ratio of 1.45:1 and DP_{PC} 100 (right), 100 mg of copolymer with 20 equiv azobis(isobutyronitrile) (compared to Z-group) and 2 equiv lauroyl peroxide were dissolved in DMF at 90 °C for 12 h.[39] The resulting copolymer was precipitated twice into ether, dialyzed (Slide-A-Lyzer 3500 MWCO) for 48 h, lyophilized, and resuspended in DMEM.

NIH/3T3 mouse fibroblasts from ATCC were cultured in DMEM containing 10 wt % FBS and 1 wt % penicillin−streptomycin in a 37 °C, 5% CO_2 incubator. 3T3s at passage 4 were seeded with 5000 cells per well in a 96-well plate and cultured for 24 h in 100 L of media. Media were subsequently replaced with 100 L of media containing DP_{PC} 100 copolymer at various concentrations and incubated for 24 h. The polymer-containing media were then aspirated from each well. Each well was then washed with 100 L of PBS and charged with both 100 L of new media and 10 L of WST reagent. After 3 h of incubation in the WST solution, the absorbance was read using a plate reader at λ = 450 nm. The cell viability was calculated using eq 1, where A_{well}, $A_{control}$, and A_{WST} are the absorbance measurements for the cells cultured with the polymer, the cells cultured without polymer, and WST in media, respectively. All experiments were conducted in triplicate.

$$\text{Viability} = \frac{A_{well} - A_{WST}}{A_{control} - A_{WST}} \quad (1)$$

DMF−SEC Measurements. SEC traces were determined after passing through two size-exclusion chromatography columns (resolve mixed bed low divinylbenzene (DVB), inner diameter (ID) of 7.8 mm, weight-average molecular weight (M_w) range of 200−600000 g mol^{-1} (Jordi Laboratories)) in a mobile phase of *N*,*N*-dimethylformamide (DMF) with 0.1 M LiBr at 35 °C and a flow rate of 1.0 mL min^{-1} (Dionex Ultimate 3000 pump, degasser, and autosampler (Thermo Fisher Scientific). Molar percentage of unincorporated primary chains were determined using the differential refractive index output of the SEC traces. The area under the curve (AUC) of the primary chain (AUC_{PC}) was determined by measuring the AUC of the right-most peak (primary chain) from the baseline to its apex (1/2 of the peak) and multiplying this value by 2. The molar percentage of unincorporated primary chains is calculated by dividing the AUC_{PC} by the AUC of the entire spectra.

THF−SEC−MALLS Measurements. Apparent molecular weight and dispersity were determined with the ASTRA software package (Wyatt Technology Corporation) after passing through two size-exclusion chromatography columns (resolve 1000 Å DVB, ID of 7.8 mm, M_w range of 100−50000 g mol^{-1} (Jordi Laboratories); resolve mixed bed low DVB, ID of 7.8 mm, M_w of range 200−600000 g mol^{-1} (Jordi Laboratories)) in a mobile phase of tetrahydrofuran (THF) at 40 °C and a flow rate of 1.0 mL min^{-1}. Detection consisted of a Optilab T-rEX (Wyatt Technology Corporation) refractive index detector operating at 658 nm and a TREOS II light scattering detector (Wyatt Technology Corporation) operating at 659 nm. A d*n*/d*c* value of 0.11 for DMA in THF was determined in the ASTRA software package by batch injection of 4 samples of known concentrations into an Optilab T-rEX refractive index detector. While we do not have the d*n*/d*c* for poly(NPGDA), the d*n*/d*c* for poly(ethyl acrylate) in THF is 0.061.[40] If this is used as a substitute, the resulting d*n*/d*c* calculated is 0.105, 0.107, and 0.109. The molecular weight calculated during multi-angle laser light scattering (MALLS) is a function of $(dn/dc)^2$. Using the d*n*/d*c* from poly(ethyl acrylate), the maximum reported error in molecular weight would be smaller than 10%.

Aqueous−SEC Measurements. Aqueous SEC-RI (PBS buffer, 300 ppm sodium azide) traces were obtained on a Optilab rEX refractive index detector (Wyatt) after passing through a column (Superose 6 Increase 10/300 GL column, M_w range of 5000−5000000 g mol^{-1} (GE healthcare)).

RESULTS AND DISCUSSION

For this work, we selected dimethyl acrylamide (DMA) as the VM on account of its hydrophilicity and due to the demonstrated biocompatibility of poly(DMA).[41] Neopentylglycoldiacrylate (NPGDA) was selected as the MVM to ensure the cross-link junctions would be composed of biodegradable ester units; however, it is important to note that this synthesis is amenable to alternative MVM moieties with different degradability profiles. We selected a trithiocarbonyl chain transfer agent for RAFT polymerization bearing a thiazolidinethione (TA)-activated ester on the R-terminus for its

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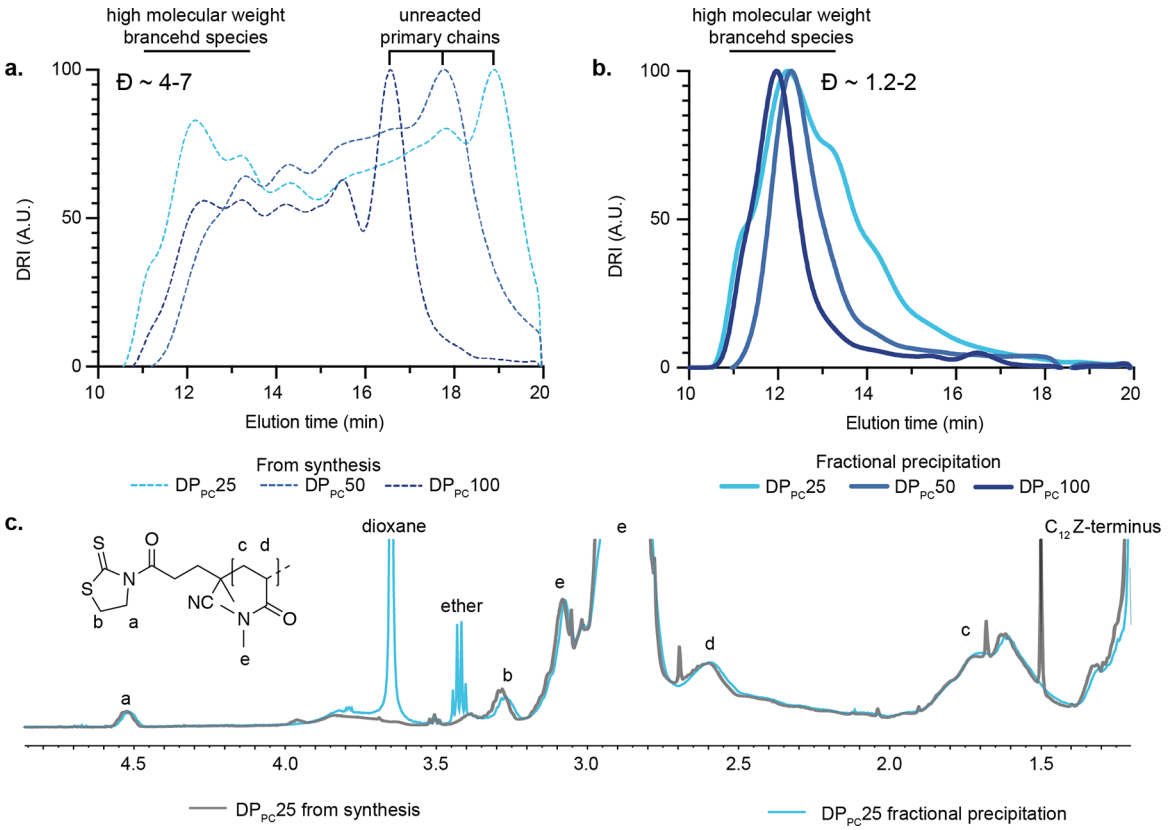

Figure 2. Fractional precipitation lowers dispersity, removes primary chains, and maintains R-group reactivity. (a) SEC traces for highly branched DMA-*co*-NPGDA at a NPGDA:CTA ratio of 1.45:1 for DP_{PC} 25 (light blue), 50 (blue), and 100 (navy) after synthesis. (b) SEC traces for highly branched polymers after a single fractional precipitation. The differential refractive index (DRI) for the copolymers are normalized such that the maximum peak intensity is identical for each chromatogram. Disperisty ($Đ$) was determined from multi-angle laser light scattering in THF. (c) ^{1}H NMR spectroscopy of a DP_{PC} 25 copolymer from synthesis (gray) and after fractional precipitation (light blue) demonstrating no decrease in the thiazolidinethione protons (a, δ = 4.5 ppm) when compared to the polymeric backbone of DMA (b and c δ = 2.6, 1.6−1.7).

Table 1. Network Information for Synthesized Polymers Pre- and Post-Fractional Precipitations

fractional precipitation no.	VM:MVM:CTA	M_w (kDa)[a]	$Đ$[a]	primary chain (molar %)[b]
0	25:1.45:50	520	7.5	23[c]
1	25:1.45:50	1300	1.25	minimal
0	50:1.45:50	120	7.5	36.5
1	50:1.45:50	720	2.1	minimal
2	50:1.45:50	210	2.0	minimal
0	100:1.45:50	120	3.9	37.5
1	100:1.45:50	630	1.8	3.1
2	100:1.45:50	250	2.2	6.8

[a]Determined using SEC equipped with multi-angle laser light scattering (SEC-MALLS) in THF (dn/dc = 0.11). [b]The molar fraction of primary chains was determined by integrating the area under the curve of the primary chain peak from the right-most baseline to the apex, multiplying this value by two, and dividing the resulting value by the area under the curve of the entire chromatogram. [c]The measured response is artificially low due to the overlap of the primary chain peak and injection peak.

previously reported efficacy for postpolymerization conjugation.[38]

We synthesized three separate branched species (DP_{PC} ($\frac{[VM]}{[CTA]}$) = 25, 50, and 100) on a multigram scale (4 g, 10 g, and 10 g, respectively). Highly branched DMA polymers at DP_{PC} 25, 50, and 100 correspond to 0.4, 0.2, and 0.1 mmol of functional end group/g of polymer. Polymerization was conducted with a DMA concentration of 4 M, as high concentrations favor intermolecular cross-link formation over intramolecular loop formation.[30] We first determined the critical number of NPGDA moieties per primary chain required to reach gelation at full conversion: $\frac{[NPGDA]_{GP}}{[CTA]}$ = 1.5. We selected a $\frac{[NPGDA]}{[CTA]}$ value of 1.45 for each polymerization to maximize the concentration of high molecular weight highly branched species. While the DP_{PC} 100 polymerization reached ≥99.9% conversion after 24 h, DP_{PC} 25 and DP_{PC} 50 polymerization reached 95% conversion after 24 h. To improve the conversion at lower DP_{PC} values, an extra initiator was added into the DP_{PC} 25 and DP_{PC} 50 reaction mixtures, and these reactions were left to react for a further 12 h until ≥99.9% conversion was reached.

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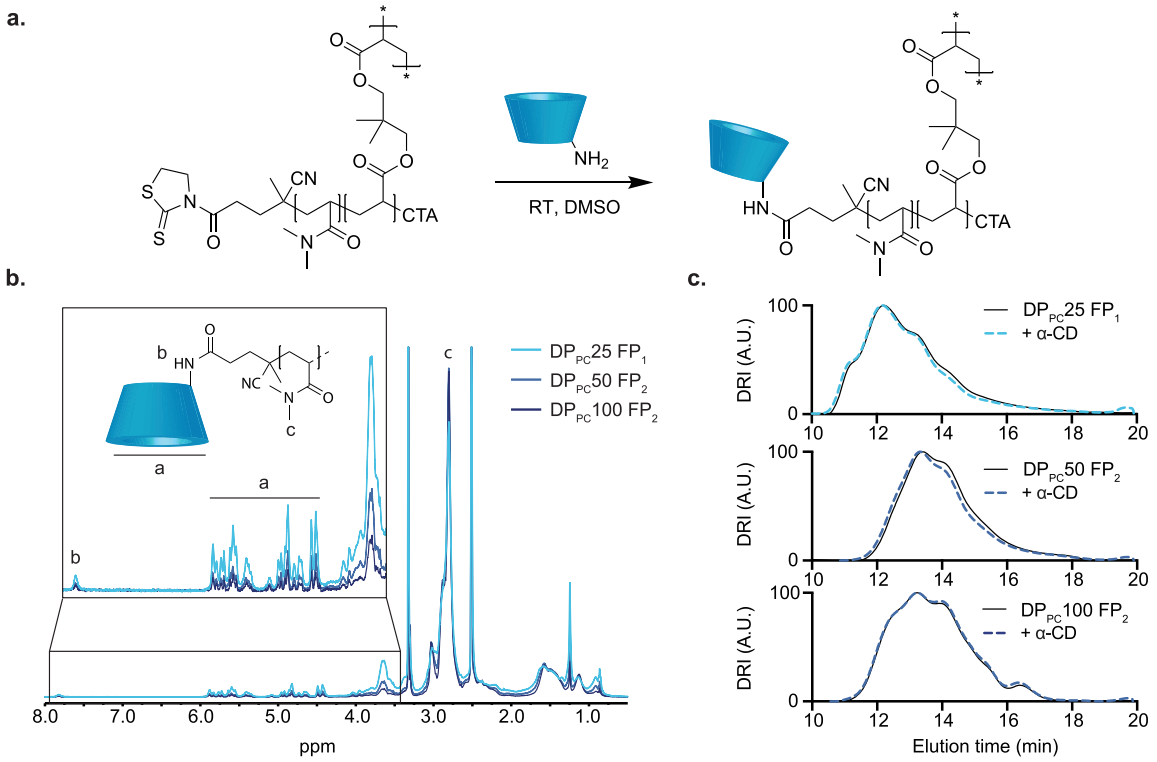

Figure 3. Controlled density of amine-bearing functional moieties through simple conjugation without altering the molecular architecture. (a) Scheme for the synthesis of α-cycldoextrin (α-CD) functionalized primary chain-free branched polymers. (b) ^{1}H NMR spectrum in DMSO for primary chain-free highly branched DMA-*co*-NPGDA at a NPGDA:CTA ratio of 1.45:1 for DP$_{PC}$ 25, 50, and 100, functionalized with α-CD (peaks b and c used for quantification.). (c) SEC traces for branched polymers after fractional precipitation (black line) and subsequent conjugation with α-CD (colored line) .

These polymerization reactions were found to contain approximately molar percentages of unreacted primary chains of 25% for DP$_{PC}$ = 25 and 35% for DP$_{PC}$ 50 and 100. Molar percentage refers to the proportion of unincorporated primary chains versus the proportion that are incorporated in branched molecules and is determined from size exclusion chromatography under the assumption that unincorporated and incorporated primary chains have similar, low dispersity mass distributions. The SEC traces for the unincorporated primary chains of highly branched copolymers are the peaks at approximately 16.5, 18, and 19 min for DP$_{PC}$ 100, 50, and 25 in Figure 2a. Previous experiments regarding copolymerizations of VM and MVM at different DP$_{PC}$ values but identical [MVM]:[CTA] ratios reported that the molar fraction of unincorporated primary chains is independent of DP$_{PC}$.[30] The scale of this synthesis was over a magnitude larger than both the experiments reported in previous literature and the experiment described above to determine [MVM]$_{GP}$, potentially giving rise to this observed discrepancy. At a high VM concentration and conversion, the viscosity of the synthesized highly branched copolymers increased dramatically such that issues of mass transfer in larger solution volumes may require increased temperatures, vigorous stirring, or longer reaction times to validate experimental observations from previous studies conducted on smaller scales.

Fractional precipitations were conducted in a dioxane solution at a 10 wt % copolymer concentration (Table 1). The antisolvent used was a mixture of ether and dioxane. The combination of a relatively dilute copolymer sample and weak antisolvent provided synthetic ease for collecting the fractionally precipitated high molecular weight copolymer fractions. SEC traces of polymers after the initial fractional precipitation collection are provided in Figure 2b. The fractional precipitation reduced the primary chain molar fraction to an unresolvable fraction for the DP$_{PC}$ 25 and 50 branched copolymers and approximately 3% mol/mol for DP$_{PC}$ 100. Because the DP$_{PC}$ 25 copolymer synthesis was smaller in scale than the DP$_{PC}$ 50 and 100 copolymer syntheses, we collected a larger fraction (approximately 30% w/w yield, compared to approximately 5% w/w for DP$_{PC}$ 50 and 100). The collection of a second fraction during the fractional precipitation increased the recovery (30 and 45% w/w for DP$_{PC}$ 50 and DP$_{PC}$ 100, respectively). The second fractional precipitation collection for the DP$_{PC}$ 50 copolymer did not contain a discernible primary chain peak but resulted in a lower molecular weight collection (Figure S4). This indicates that fractional precipitation affords a synthetic procedure to control the molecular weight of branched copolymers independent of the spatial density of functional moieties. The second fractional precipitation collection for DP$_{PC}$ 100 copolymers contained approximately 7% mol/mol of unincorporated primary chains (Figure S5). We expect that fractional precipitation for this copolymer was less effective for DP$_{PC}$ 100 primary chains due to the increased molecular weight of the unincorporated primary chains. Lastly, we verified that the fractional precipitation method did not alter the reactivity of the amine-reactive thiazolidinethione unit on the highly branched polymers (Figures 2c and S8). We similarly explored the implementation of ultrafiltration (spin filters) on the DP$_{PC}$ 100 highly branched copolymers, but it was not successful in removing a large fraction of primary chains (Figure S6).

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The fractional precipitation similarly increased the weight-average molecular weights (M_w) and lowered the dispersities for the branched copolymer regardless of the DP_{PC} value. The weight-average molecular weight was increased from 0.5 to 1.3 MDa for DP_{PC} 25 while reducing the dispersity from 7.5 to 1.25, respectively. The weight-average molecular weight was increased from 0.1 to 0.7 MDa (fractional precipitation collection 1) and 0.2 MDa (fractional precipitation collection 2) for DP_{PC} 50 while reducing the dispersity from 7.5 to approximately 2 for each collection, respectively. The weight average molecular weight was increased from 0.1 to 0.6 MDa (fractional precipitation collection 1) and 0.2 MDa (fractional precipitation collection 2) for DP_{PC} 100 while reducing the dispersity from 4 to approximately 2 for each collection, respectively. These M_w values correspond to 520, 144, and 63 functional primary chains per molecule (FP_1 DP_{PC} 25, 50, and 100, respectively) and 42 and 25 functional primary chains per molecule (FP_2 DP_{PC} 50 and 100, respectively).

With amine-reactive highly branched polymers in hand, we sought to demonstrate the utility of the primary chain-free branched copolymers for potential use as a biomaterial through the conjugation of α-cyclodextrin (α-CD) to the branched polymer, a facile room temperature conjugation (Figure 3a). α-CD is a cyclic oligosaccharide, which functions as a supramolecular host for a variety of hydrophobic guest moieties. Specifically, α-CD exhibits an equilibrium rate constant (K_{eq}) on the order of 10^4 with alkyl chains, making these promising candidates for supramolecular hydrogels or as supramolcular carriers for alkylated therapeutic molecules. β-CD has similarly been functionalized to hyperbranched poly(amido amine), poly(glycerol) for hydrophobic drug and gene delivery.[42−44] The control of the spatial density of cyclodextrin by altering the DP_{PC} has the ability to alter the cross-link density (and thus the resulting mechanical properties of the gel) or to enhance the drug solubility for a given polymer concentration.[2] While the amine reactivity of the thiazolidinethione moiety has been demonstrated elsewhere,[38] we first demonstrated the reactivity of this molecule with benzylamine and confirmed conjugation via the presence of the appropriate aromatic protons in the ^{1}H NMR spectrum (Figure S9). Similarly, we conjugate α-CD through reacting the branched copolymer with an amine-functionalized α-CD, demonstrating the emergence of carbohydrate protons (δ 4.4−6.0, Figure 3b) and amide protons (δ 7.8). Through integration of the protons in the ^{1}H NMR spectrum, we verify the molar ratio of DMA to the amide cyclodextrin peak for DP_{PC} 25, 50, and 100 as approximately 33, 63, and 116, respectively. These values correspond to 0.30, 0.16, and 0.086 mmol of α-CD/g of polymer. Moreover, we demonstrate that the conjugation of α-CD to the branched copolymer preferentially reacts with the thiazolidinethione moiety on the R-terminus without significantly altering the molecular weight distribution of highly branched species through SEC (Figure 3c). However, there is a slight left-shift in the elution time due to the increased molecular weight of the end group (Figure S11).

For use in translational biomedical applications, it is important that high molecular weight polymeric materials demonstrate sufficient degradation into lower molecular weight species such that they do not accumulate undesireably in tissues. Previous literature studies have demonstrated triggered degradability of branched architectures through the use of disulfide,[24,26,45] glucarodilactone,[27] and ester[46] cross-link junctions. We confirm the degradability of the neopentylglycoldiacrylate cross-link junctions through both acidic and alkaline exposure, where the conversion of the highly branched polymers into primary chains is monitored using SEC. The results of the acidic treatment, where the second fractional precipitation collection of the DP_{PC} 100 branched copolymer is digested for 48 h in 12 M HCl, are presented in Figure 4a.

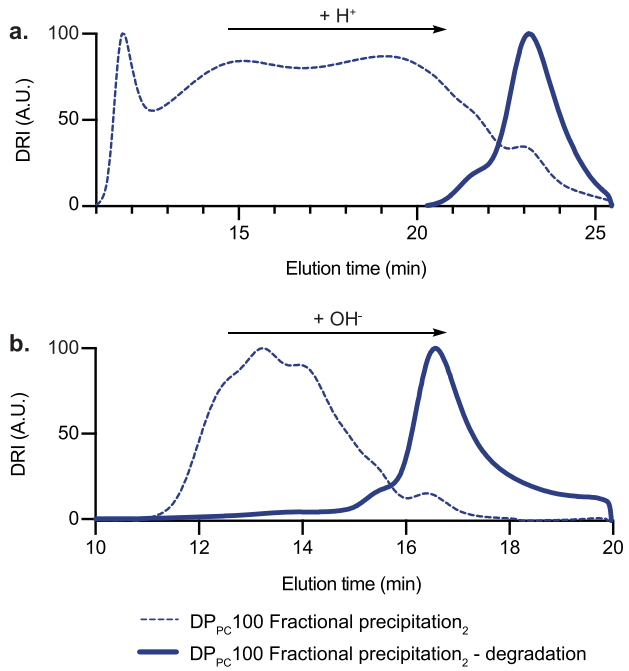

Figure 4. SEC traces demonstrating the degradation of highly branched DMA-*co*-NPGDA at a NPGDA:CTA ratio of 1.45:1 for DP_{PC} 100. (a) DRI response using an aqueous eluent for the degradation of the highly branched copolymer in acidic conditions (12 M HCl for 48 h). (b) DRI response using DMF as an eluent for the degradation of the highly branched copolymer in basic conditions (0.1 M NaOH for 24 h). SEC traces with dashed lines are representative of the highly branched copolymer after fractional precipitation (collection 2), while traces with solid lines are representative of the polymers after degradation.

The results of the basic treatment, where the second fractional precipitation collection of the DP_{PC} 100 branched copolymer is digested for 24 h in 0.1 M NaOH, are presented in Figure 4b. The results of both assays with other DP_{PC} branched polymers are presented in Figure S12 and S13. The resulting SEC traces for the linear primary chains from both acidic and basic degradation show a tailing to lower molecular weight species, likely resulting from chain scission during these accelerated degradation conditions. Despite the tailing of low molecular weight polymeric species, it is important to note that the degraded species are below the threshold for renal clearance (<30 kDa for poly(*n*-isopropylacrylamide)).[8]

We then probed the cytoxicity of these highly branched copolymer materials. Mammalian cell viability was investigated through a WST assay by culturing NIH/3T3 mouse fibroblasts for 24 h with highly branched DMA-*co*-NPGDA copolymers at a NPGDA:CTA ratio of 1.45:1 and DP_{PC} 100 following Z-group and R-group removal with excess lauroyl peroxide and azobisisobutyronitrile according to established protocols (Figures 5 and S7).[39] Highly branched copolymers at concentrations up to 1 mg/mL demonstrated no statistical

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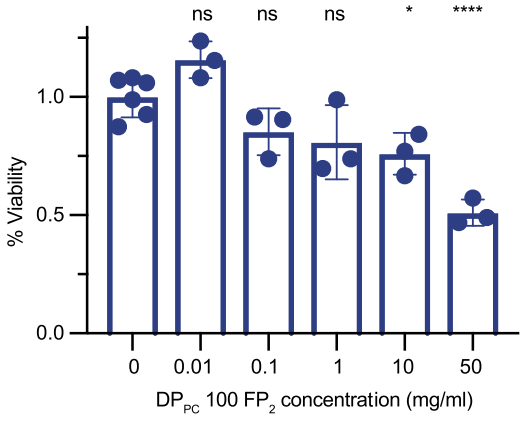

Figure 5. WST assay of NIH/3T3 mouse fibroblasts cultured for 24 h with highly branched DMA-*co*-NPGDA at a NPGDA:CTA ratio of 1.45:1 for DP_{PC} 100 after removal of the Z-terminus. The LC50 value for the material is approximately 50 mg/mL. Error bars indicate the standard deviation, where $n = 3$, $*p < 0.05$, $***p < 0.0001$ ($n = 6$ for the control).

difference in cytotoxicity compared to the control. Moreover, the LC50 (lethal concentration 50%) value of the highly branched copolymer was determined to be approximately 50 mg/mL, an order of magnitude larger than that of parenteral excipients used clinically in pharmaceutical formulations.[47] It is important to note that, upon injection, a formulated sample is rapidly diluted between 3 and 4 orders of magnitude (e.g., for an injection of 0.5−5 mL administered into a patient with 5 L of blood).

CONCLUSION

Altogether, this study presents a synthetic route to low dispersity, high molecular weight highly branched polymers with controlled end-group functionality utilizing RAFT copolymerization of a vinyl monomer and a multivinyl monomer, followed by a subsequent fractional precipitation step. Despite the high molecular weight, these highly branched polymers degrade under acidic and basic conditions to the molecular weight of their primary chains, which can be tuned to fall well below the threshold for renal clearance. While this study employs dimethylacrylamide as the vinyl monomer and neopentylglycol-diacrylate as a multivinyl cross-linker, this synthetic approach should be amenable to other combinations of vinyl and multivinyl monomers, as well as alternative controlled radical polymerization techniques.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.0c00539.

Detailed synthetic procedures, SEC traces of all fractional precipitations, NMR and SEC traces for conjugation to benzylamine, and full suite of degradation assay SEC traces (PDF)

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Notes

The authors declare no competing financial interest.

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