Using EPR To Compare PEG-branch-nitroxide “Bivalent-Brush Polymers” and Traditional PEG Bottle–Brush Polymers: Branching Makes a Difference

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S Supporting Information

ABSTRACT: Attachment of poly(ethylene glycol) (PEG) to polymeric nanostructures is a general strategy for sterically shielding and imparting water solubility to hydrophobic payloads. In this report, we describe direct graft-through polymerization of branched, multifunctional macromonomers that possess a PEG domain and a hydrophobic nitroxide domain. Electron paramagnetic resonance (EPR) spectroscopy was used to characterize microenvironments within these novel nanostructures. Comparisons were made to nitroxide-labeled, traditional bottle-brush random and block copolymers. Our results demonstrate that bivalent bottle-brush polymers have greater microstructural homogeneity compared to random copolymers of similar composition. Furthermore, we found that compared to a traditional brush polymer, the branched-brush, “pseudo-alternating” microstructure provided more rotational freedom to core-bound nitroxides, and greater steric shielding from external reagents. The results will impact further development of multivalent bottle-brush materials as nanoscaffolds for biological applications.

INTRODUCTION

The function of biological systems emerges from Nature's ability to produce monodisperse, sequence-defined polymers from a relatively small set of monomers.¹ If synthetic polymers could be prepared with this same level of control, the possibilities for development of new functional materials would be boundless.^{2,3} Biomimetic, template-controlled polymerization is a promising strategy;^{4–10} however, to date, the production of monodisperse, sequence-defined polymers is still a major outstanding challenge for macromolecular synthetic chemistry.

Living polymerization methodologies developed over the past half-century provide access to homo, block, and random copolymers with very narrow molecular weight distributions.^{11–14} True sequence control at the monomer level, however, is not possible for most combinations of monomers.¹⁵ Even the simplest sequence control, monomer alternation, is difficult to achieve. Typical copolymerization of two monomers leads to statistical copolymers with microstructure heterogeneity (Figure 1A); only when the monomer reactivity ratios for both monomers are zero can true alternation occur.¹⁶ While

some combinations of monomers—e.g., styrene and maleimide^{17–19}—approach the latter criterion, this phenomenon is rare.^{17,20–26} In general, preparation of alternating copolymers via chain polymerization requires design of unique polymerization catalysts.^{27–29} To date, there is no general methodology for the synthesis of alternating copolymers for any arbitrary set of two monomers.

Nature teaches us that the three-dimensional presentation of polymer side chains is more important than the polymer backbone and side chain identities. This lesson can be applied in the context of alternating copolymers: though monomer sequence control at the backbone level is an important goal for certain applications,³⁰ for many other applications, like self-assembly, side-chain sequence is crucial.^{31–41} In these cases, it is often possible to capture the properties of alternating side-chain functionality via simple polymerization of monomers that carry two or more functionalities (Figure 1B).^{42–45} In other words, rather than search for catalysts/monomers that produce

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Figure 1. (A) The microstructure of a copolymer is dependent on the reactivity ratios of monomers. (B) Polymerization of branched monomers generates homogeneous "pseudo-alternating" copolymers.

alternating copolymers from different monomers, synthetic efforts can be invested in the design of branched or cyclic monomers that carry multiple functional domains.^{45–49}

To demonstrate the utility of branched monomers for the direct synthesis of "pseudo-alternating" copolymers, we have focused on the design of branched macromonomers (BMMs) where one domain contains a linear polymer and the second a functional moiety of interest (Figure 1B). For example, we previously reported the graft-through synthesis of poly(ethylene glycol) (PEG)-*branch*-azide⁴⁸ and PEG-*branch*-chemotherapeutic drug⁴⁹ bivalent brush polymers (BBPs) via ring-opening metathesis polymerization (ROMP) of branched norbornene macromonomers (MMs) using third generation Grubbs catalyst (**1**, Figure 2A).⁵⁰ These systems represent the first examples of pseudoalternating BBPs designed specifically for drug delivery applications. Further development of these materials requires a deeper understanding of their solution-state structure and reactivity properties. In this work, we are interested in comparisons between BBPs and traditional brush polymers.^{51,52}

The electron paramagnetic resonance (EPR) spectrum of a nitroxide free radical is highly sensitive to environmental variables such as local polarity, steric hindrance, viscosity, and nitroxide spin–spin interactions. Since most organic molecules lack free radicals, the EPR spectrum for a nitroxide-labeled molecule is typically clean and allows straightforward access to quantitative information regarding the above variables via established spectral simulation methods. Because of this unique combination of features, nitroxides have found extensive use as environmental probes for macromolecular structures such as micelles,⁵³ dendronized polymers,⁵⁴ dendrimers,^{55–59} synthetic

polymers,^{60–62} and biological polymers.^{63,64} We recently reported the first EPR study of nitroxide-labeled bottle-brush polymers prepared via block copolymerization of a norbornene-poly(lactide) (PLA) MM and a norbornene-nitroxide small monomer.⁶⁵ This work demonstrated that brush polymers possess distinct periphery and backbone environments in solution; reactions between a polymeric phenylhydrazine derivative and nitroxide-labeled brush polymers were much faster for peripherally-labeled samples. Here we utilize ROMP to prepare a PEG-*branch*-nitroxide pseudoalternating homopolymer, a series of PEG BBP copolymers with nitroxide labels in specific locations (periphery, backbone end, and backbone middle), and a similar series of traditional PEG bottle-brush copolymers with nitroxide labels. EPR was then used to compare environmental features of these novel nanostructures in water.

■ PSEUDOALTERNATING HOMOPOLYMER SYNTHESIS

In order to probe the core environment of BBPs via EPR, PEG-*branch*-nitroxide BMM, **2**, was prepared via copper-catalyzed azide–alkyne cycloaddition (CuAAC) coupling of a previously reported PEG-*branch*-alkyne BMM and an azido-nitroxide (Figure 2A, see Supporting Information for synthetic procedures). MM **2** features a 3 kDa PEG chain (blue, Figure 2A), a nitroxide for EPR analysis (green, Figure 2A), and a norbornene group for ROMP (red, Figure 2A). To ensure that **2** could be efficiently polymerized via ROMP, a solution of **2** in CH₂Cl₂ (0.05 M) was treated with **1** (0.01 equiv to **1**) to generate a PEG-*branch*-nitroxide BBP homopolymer with a target degree of polymerization (DP) of 100 (Figure 2A). After

Figure 2. (A) Synthesis of PEG-branch-TEMPO BBP homopolymer, poly(2), via ROMP of **1**. (B) GPC analysis of **2** and crude ROMP reaction mixture containing poly(2). (C) Overlaid EPR spectra of **2**, poly(2), and a 50:50 random copolymer of small nitroxide-labeled monomer **3** and PEG-MM **4**.

1 h, the polymerization was quenched and analyzed directly by gel permeation chromatography (GPC). The GPC trace (Figure 2B) indicated that polymerization of **1** proceeded in a controlled fashion with >90% conversion. The number-average molecular weight (MW) of the BBP product was 598 kDa with a polydispersity index (PDI) of 1.11.

The EPR spectrum of this homopolymer in water showed broad nitroxide radical peaks due to the hindered BBP core environment and extensive spin–spin interaction between adjacent nitroxide labels (Figure 2C). We previously reported ROMP of a small-molecule TEMPO–norbornene monomer (**3**, Scheme 1) to generate a poly nitroxide that also displayed highly broadened EPR resonances. These previous materials were only soluble in organic solvents. Poly(**2**) carries a 3 kDa PEG chain and a nitroxide label on each repeat unit. Such a structure would be impossible to synthesize via a combination of two monomers; polymerization of a 50–50 mixture of a nitroxide-labeled small monomer and a PEG MM—e.g., **3** and **4** from Scheme 1—would yield a polymer with overall DP = 100 but only 50 PEG chains and 50 nitroxide labels. Furthermore, in this random copolymer case there would inevitably be domains of nitroxide blocks and PEG blocks,

which would lead to microstructural heterogeneity. The key strength of the pseudoalternating polymerization approach is the ability to capture two distinct properties in a single homogeneous nanostructure where the number of both functionalities equals the DP (Figure 1B). Here, the aqueous solubility of the BBP is imparted by the PEG chains while the EPR function of the nitroxide label is maintained at every backbone repeat unit. The unimolecular micelle structure, which is accessible in a single polymerization step, is structurally analogous to other PEGylated nanostructures that have demonstrated success in drug delivery applications.^{66–68}

Block and Pseudoalternating Brush Polymer Synthesis. We next prepared a series of BBPs with nitroxides specifically located at defined locations within the BBP nanostructure. Unlabeled PEG MM **4** was used as the primary component of these systems. Labels were introduced via graft-through ROMP of nitroxide-labeled monomers: telechelic nitroxide MM **5**, BMM **2** or small monomer **3** (Scheme 1). The peripherally-labeled sample, **4**₉₅-**5**₅, was prepared via random copolymerization of a 95:5 mixture of **4** and **5**, respectively. The end-labeled BBP, **4**₉₅-**2**₅, was prepared via addition of **2** (5 equiv. to cat) to living bottle-brush polymers

Scheme 1. Structures of Macromonomers Used for This Study^a

^aSequential or random polymerization of these monomers generates either BBPs or traditional bottle-brush polymers that possess nitroxide labels at defined positions throughout the periphery, backbone end, or backbone middle.

generated from ROMP of **4** (95 equiv. to **cat**). Similarly, for the mid-labeled BBP, **4**₄₅-**2**₅-**4**₄₅, **4** (45 equiv) was polymerized followed by **2** (5 equiv), followed by **4** (45 equiv) once again. Identical procedures were employed for the synthesis of end- and mid-labeled brush polymers from small monomer **3**.

GPC was used to characterize the MW and PDI for each polymer sample depicted in Scheme 1. All of the polymerization reactions reached >90% conversion and provided polymer samples with low PDIs (<1.15) and MWs in close agreement with theoretical values (Table 1). To our knowledge, the mid-labeled BBP (**4**₄₅-**2**₅-**4**₄₅) is the first reported A–B–A triblock brush polymer; the efficiency of **cat** for propagation of sterically demanding MMs and BMs is remarkable. Furthermore, the functional group tolerance of **cat** is highlighted by the presence of free radical agents throughout these systems. Other controlled polymerization methods based on radical chemistry would not enable access to these types of materials. Dynamic light scattering (DLS) was used to obtain

the hydrodynamic radius for each sample (Table 1). The particles ranged from 24 to 32 nm; no appreciable aggregation was observed. The latter observation is important, as it suggests that spin–spin interactions observed in EPR studies are due to intramolecular nitroxide interactions.

EPR Studies. Samples of each of the above polymers were dissolved in water at 0.01 mM concentration and subjected to EPR analysis. EPR spectra of degassed and nondegassed samples were identical. As a result, all of the studies discussed below pertain to samples that were not degassed. The EPR spectra were simulated using the well-established computation procedure of Budil and Freed.⁶⁹ The g_{ii} components of the $\mathbf{g}$ tensor for the Zeeman coupling between the electron spin and the magnetic field were taken as constants ($g_{ii} = 2.009, 2.006, 2.003$). The main environmental parameters that can be extracted from the EPR line shape analysis and simulation are as follows. (a) The correlation time for rotational diffusional motion of the nitroxide (τ), which provides a measure of steric

Table 1. GPC and DLS Data for Nitroxide-Labeled Macromonomers, BBPs, and Traditional Brush Polymers

description	sample name	$M_{n,theor}/$ kDa ^a	$M_{n,GPC}/$ kDa ^b	PDI	R_h/nm^c
BMM	2	3.64	3.54	1.05	—
telechelic MM	5	3.47	3.43	1.07	—
peripherally-labeled brush	4 ₉₅ -5 ₅	330	324	1.09	14 (1)
end-labeled BBP	4 ₉₅ -2 ₅	331	373	1.08	16 (1)
end-labeled, small monomer	4 ₉₅ -3 ₅	314	324	1.07	15 (1)
mid-labeled BBP	4 ₄₅ -2 ₅ -4 ₄₅	314	309	1.14	14 (1)
mid-labeled, small monomer	4 ₉₅ -3 ₅ -4 ₄₅	298	264	1.11	12 (1)

^a $M_{n,theor}$ was calculated based on the target DP for each sample and the mass of the corresponding monomers. ^b $M_{n,GPC}$ was obtained via GPC with refractive index and multiangle laser light scattering detection. ^cHydrodynamic radii (R_h) were obtained via DLS in water. The DLS correlation function was fitted using the CONTIN algorithm. Volume-average radii are reported.

Figure 3. GPC traces for nitroxide labeled macromonomers, BBPs, and traditional brush polymers.

interactions experienced by the nitroxide. On the basis of the τ value for a given sample, we may define the spectrum or the spectral components as “fast,” “slow,” or “very slow.” For the analyses here, “fast” is defined as $\tau < 0.5$ ns, “slow” is 0.5 ns $\leq \tau < 0.8$ ns, and “very slow” is $\tau \geq 0.8$ ns. (b) The intrinsic line width, which increases when dipolar–dipolar interactions occur between nitroxides in close positions. (c) The spin–spin exchange frequency, ω_{ex} , for the Heisenberg exchange interaction, which occurs due to collisions between nitroxides at high local concentration (the electron spin is exchanged between the nitroxides). (d) The percentage of the different spectral components constituting the EPR spectrum, which provides the distributions of nitroxides in different environments. (e) The intensity of the spectrum (measured in arbitrary units by double integration of the EPR spectrum), which is mainly important in a comparative way to verify the solubility of the different compounds. Intensity may be considered proportional to solubility/concentration. EPR spectra for the polymers prepared in Scheme 1 are shown in Figure 4A–D. The simulated parameters outlined above are listed in Figure 4E.

To demonstrate that pseudoalternating polymers are different from random copolymers, we first compared the EPR spectrum of poly(2) to that of a random copolymer (rand)

comprised of 50 3 and 50 4 (Spectra overlaid in Figure 2C). Qualitative differences between the two spectra are apparent. For example, the spectrum for **rand** possesses distinct broad and sharp regions; the spectrum for poly(2) is uniformly broad. Because of its microstructural homogeneity, we expect that the EPR spectrum of poly(2) consists primarily of a single “slow” contribution. The simulated data agree with this expectation. As highlighted in green in Figure 4E, the spectrum for poly(2) consists primarily (98%) of a single slow component; nearly all of the nitroxide labels are equivalent. The same result would be expected for a perfectly alternating copolymer of 3 and 4 if such a structure could be synthesized.

The spectrum for the random copolymer **rand** is comprised of a mixture of three distinct signals: 8% of a “fast” signal, 80% of a “slow” signal with significant exchange interactions, and 12% of a second “slow” signal absent of spin–spin interactions (Figure 4E, highlighted in red). These disparate signals likely arise from nitroxide labels near the polymer chains ends that are exposed to solvent (fast relaxation), blocks of nitroxide labels buried in the hydrophobic poly(norbornene) core (slow relaxation with spin–spin interaction), and single nitroxide labels buried in a PEG domain (slow relaxation without spin–spin interactions). All nitroxide labels in this sample are not equivalent; the sample is heterogeneous. These data provide the first direct evidence that pseudoalternating BBPs possess more well-defined microstructures than similar random copolymers.

We next sought to compare the BMM 2 and telechelic MM 5. These samples are both effectively ~ 3 kDa PEG polymers with a single nitroxide label. We hypothesized that they would have similar EPR spectra. Figure 4A shows an overlaid comparison of the EPR spectra collected for 2 and 5. The observation of sharp resonances is in accordance with the hypothesis that nitroxide probes in these species are relatively freely moving. The EPR spectra for 2 and 5 are comprised of only a single component with a “fast” nitroxide mobility. The correlation times for rotational diffusion, τ_{fast} , for 2 and 5 were 0.07 and 0.03 ns, respectively. The τ value for a free TEMPO nitroxide in solution is about 0.005 ns. As expected from the complex structure and size of these MMs, the mobility decreases by about 1 order of magnitude with respect to TEMPO; it is slightly lower for 2 than for 5. The similarity between the two MMs suggests that comparisons between polymers comprised of 2 and 5 are valid; the EPR spectra are not significantly altered by the different nitroxide probe structure.

Figure 4B shows overlaid EPR spectra for peripherally-labeled, end-labeled, and mid-labeled samples. The latter two possess the nitroxide probe in a branched domain derived from BMM 2. Inspection of the EPR spectra shows a clear broadening as the probe is moved from the brush polymer surface to the backbone end and finally to the backbone middle. Placement of the nitroxides at the polymer surface allows for fast rotation and facile interaction with solvent; the hyperfine coupling constant for the peripherally-labeled sample was 17.1 versus 16.9 G for the backbone-labeled samples, which suggests that the peripheral nitroxides experience a slightly increased micropolarity. The spectrum for the peripherally-labeled sample is comprised entirely of a fast component with $\tau_{fast} = 0.03$ ns, which is identical to MM 5. The spectrum for the end-labeled BBP sample is comprised of 35% of a fast component ($\tau_{fast} = 0.05$ ns), 28% of a slow component ($\tau_{slow,1} = 0.53$ ns) with a corresponding spin–spin exchange interaction (ω_{ex}), and 37%

Figure 4. (A–D) Comparative EPR spectra for various samples generated in Scheme 1. (E) EPR simulation data table.

of another slow component ($\tau_{\text{slow},2} = 0.56$ ns). The spectrum for the mid-labeled BBP sample is comprised of the same three components. Their τ values are nearly the same as the end-labeled sample (Figure 4E), but the percent abundances are skewed toward slower nitroxide motions: 8% τ_{fast} , 49% $\tau_{\text{slow},1}$, and 43% $\tau_{\text{slow},2}$. The increased contribution of the “slow” component for the mid-labeled sample is expected as the nitroxide probes within these materials are buried in the center of the BBP backbone, but the EPR analysis provides more detailed information, indicating that the mid-labeled sample experiences a larger spin–spin interaction. The spectral intensity is comparable for the three cases, indicating that the solubility is almost the same, and the distribution of the nitroxides is homogeneous.

To study the differences between BBP architectures and traditional brush block copolymers, EPR spectra were collected for end- and mid-labeled polymers that varied only in the

structure of the labeled monomer: BMM 2 versus small monomer 3. Figure 4C compares the two end-labeled samples. The EPR spectrum for the sample with small monomer, 4₉₅-3₅, is noticeably broader; similar results were obtained for the mid-labeled samples (Figure 4D). This broadening is due to the shorter tether used to link the nitroxide of 3 to the brush backbone compared to the tether from 2, which impedes fast rotation for samples labeled with 3. This hypothesis is supported by the simulated data (Figure 4E), which show a much greater contribution of very slow and slow moving nitroxides that involve spin–spin exchange for samples labeled with 3. This increased spin–spin exchange results from intramolecular interaction between adjacent nitroxides, which would be expected for nitroxides in close proximity. The steric constraints in the mid-labeled sample forces a larger fraction of the nitroxides on the same BBP to approach each other leading to strong spin–spin interactions and decreased mobility.

Figure 5. (A) Scheme for nitroxide quenching with PEG-PhNHNH₂ (Q). “Quenching” refers to the reaction of polymer-bound nitroxides with Q, which leads to EPR inactive hydroxylamines. (B) Plots of percentage of the EPR signal quenched versus time throughout 100% conversion. (C) Log plot for the first 20 min of quenching.

Quenching Studies. We next sought to compare the reactivity of nitroxides placed at different locations throughout BBPs and brush polymers. Nitroxide free radicals react with phenylhydrazine derivatives to generate EPR inactive hydroxylamines.⁷⁰ We previously used this reaction to study the location-dependent reactivity of TEMPO labels in PLA brush polymers using a poly(styrene-*co*-phenylhydrazine) random copolymer. For this work, we designed water-soluble PEG-phenylhydrazine derivative Q (Figure 5A). Treatment of nitroxide-labeled polymers with Q leads to conversion of the EPR active nitroxide free radical to an EPR inactive hydroxylamine moiety. Quenching of the EPR signal can be readily monitored with time. The rate of quenching, k_Q , is linked to the steric environment of the nitroxide probe. In agreement with our prior results, peripherally bound nitroxides are quenched the fastest, followed by end-labeled and mid-labeled samples (Figure 5A).

We found that BBPs are quenched more slowly, especially at early time points, than traditional brush polymers labeled with 3. This behavior is expected because the PEG chain in 2 works as a physical barrier, which slows down the reaction of the nitroxide with Q. *Even though the 3-labeled polymers have longer EPR correlation times, and a higher fraction of spin–spin interactions, the absence of PEG side chains in these materials facilitates faster reaction with Q compared to BBPs labeled with 2.* In fact, over the first 20% of quenching, branched end-labeled 4₉₅-2₅ and traditional mid-labeled 4₄₅-3₅-4₄₅ react at very similar rates. This observation provides a clear distinction between the BBP architecture and traditional brush polymers: the BBP core is significantly shielded from interactions with external reagents when compared to traditional brush polymers.

Log plots of nitroxide quenching versus time over the first 20% quenching were linear for all samples (Figure 5C). These data clearly show the effects of BBP shielding. The k_Q for the mid-labeled BBP is 10X smaller than the peripherally-labeled sample, and ~2.7X slower than the 3-labeled traditional brush polymer. It should be noted that at longer reaction times differences in reactivity arise. In particular, the end-labeled samples display clean pseudo-first-order kinetics through 100%

conversion, whereas the mid-labeled samples deviate from pseudo-first-order kinetics after 20% conversion. For example, the mid-labeled BBP sample, 4₄₅-2₅-4₄₅, appears to quench more quickly after ~30% conversion (blue trace, Figure 5B), which ultimately leads to a crossover with the 3-labeled brush (cyan trace, Figure 5B) near 70% conversion. We hypothesize that this behavior again arises from the polymer properties revealed by the EPR analysis. The plots in Figure 5A for the mid-labeled polymers clearly show three different slopes; the EPR spectra show three different components. We expect that for the mid-labeled branched sample, 4₉₅-2₉₅-4₉₅, Q may slowly insert itself into the BBP structure to maximize PEG interactions and minimize exposure of the phenylhydrazine chain end to solvent. After embedding into the polymer, Q can then efficiently quench slow moving nitroxides located near the backbone. Conversely, Q may rapidly approach and quench the fast and slow moving nitroxides of 4₉₅-3₅-4₉₅, as they are less hindered due to the lack of PEG chains. In this case, however, the very slow moving nitroxides require longer quenching time.

CONCLUSIONS

The synthesis and EPR characterization of water-soluble PEG-branch-nitroxide pseudoalternating brush polymers (BBPs) and traditional brush copolymers is reported. ROMP of branched macromonomer 2 generated the first core-nitroxide-labeled PEG BBP. Such a structure would not be possible via polymerization of two monomers. Preparation of block copolymers using 2 allowed for detailed BBP microstructure analysis via EPR. We found that though the EPR spectra for traditional bottle-brush polymers suggested greater nitroxide interactions and increased steric hindrance around the nitroxides compared to BBPs, the greater PEG side chain density of BBPs more effectively shielded nitroxides from a polymeric nitroxide quencher. The combination of synthetic ease and unique microstructure suggests that core-functional PEG BBPs prepared via graft-through polymerization have great potential as novel nanocarriers for biological applications.

■ ASSOCIATED CONTENT

■ Supporting Information

Materials and methods, synthesis of novel compounds, and spectral data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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