

Solution size variation of linear and dendritic bis-MPA analogues using DOSY-¹H NMR

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Dendrimers are globular, multi-functional, monodisperse macromolecules with perfect structure fidelity. Their architecture is composed of a series of branched polymeric arms, composed within “wedges,” that emanate from a central core. Their structure contains a high density of functional groups located at their periphery, referred to as the “outer shell.” Due to their globular structure, it is assumed that the relative “size” of a dendrimer does not fluctuate greatly between solvents. This may be due to the inability of the branched arms, or wedges, to significantly expand or collapse (comparative to analogous linear polymers) owing to steric barriers from branching, especially at higher generations. It is expected that a linear polymer, of similar molecular weight to a dendrimer analog, would have a greater degree of size fluctuation relative to the quality of the solvent. This stems from its innate flexibility and access to a wider range of conformations. For this investigation, analogous dendritic and linear bis-MPA polyesters as well as poly(caprolactone) (PCL) were analyzed using size-measuring techniques including gel permeation chromatography (GPC) and diffusion ordered spectroscopy-nuclear magnetic resonance (DOSY-¹H NMR).

Introduction

Dendrimers are perfect, globular, monodisperse macromolecules with a highly branched three-dimensional architecture.^{1,2} These branched “arms” are often divided into wedges that are made up of an AB₂-monomer. This bifunctionality allows for growth of the dendrimer through successive iterative steps, increasing functionality exponentially. Each of these growth steps increase branching and are referred to as generations using the [GX] naming scheme (Fig. 1). These iterative steps lead to a well-defined macromolecular structure, and as a result, a higher correlation of structure-property relationships compared to linear polymers. These aforementioned properties have led to the use of this class of polymeric material as drug delivery vectors, as imaging-contrast agents, as well-defined dendrimer films, as encapsulation agents, and as mass standards for mass analysis techniques such as matrix-assisted laser desorption ionization mass spectrometry (MALDI-ToF MS).^{1–9} One attractive family of branched polymers that has seen increasing intrigue are those based upon 2,2-bis(hydroxymethyl)propionic acid (bis-MPA). First reported by Hult *et al.*, the synthesis of bis-MPA dendritic materials has not only improved, but examples of their utility have also flourished over the last three decades.^{1,8–15} This improved synthesis allows for an unprecedented level of purity, rivaling convergently-grown dendrimers. This level of purity is

not seen in other divergently grown dendrimers and has the added benefit of both benign reaction conditions and efficient workups.

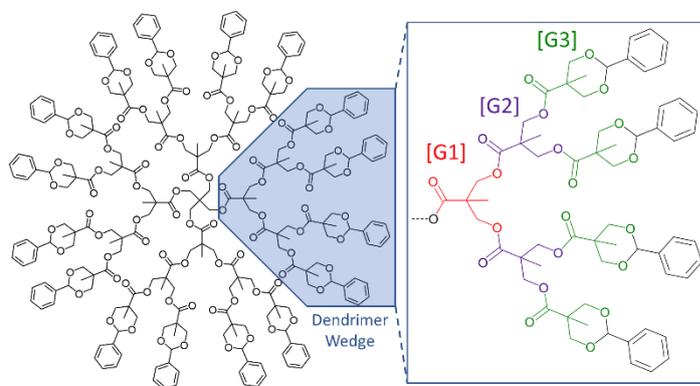


Fig. 1 Drawing of a benzylidene-protected pentaerythritol-core [G3] bis-MPA dendrimer (Tetra[G3]Bnz₁₆).

Examples of utility include work by Giesen *et al.* using triiodophenol-core bis-MPA dendrons as mass calibrants for proteomic analysis by MALDI-ToF MS. These bis-MPA dendrons exhibit the least amount of overlap with any possible peptide sequence below 2.5 kDa, pivotal for proteomics.¹⁶ Additionally, Vestburg *et al.* used azide and alkyne terminated bis-MPA dendrimers to make discrete multilayer thin films.¹⁷ This increase in the use of bis-MPA dendritic systems can be attributed to their relatively inexpensive monomer, ease of synthesis, high purity, and biocompatibility due to the presence

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of their polyester linkages.^{2,10,12,13,18,19} Their utilization by other researchers has led to a growing desire to understand how these dendrimers behave, especially in solution. There have been previous computational and experimental studies on how other dendrimers generally behave in solution, but many of these lack analogs for points of comparison. The understanding of how dendritic materials (or segments of hybrids) behave in solution has a significant impact on their use in linear-dendritic hybrids for drug delivery or in making discrete polymer films to name a couple of examples.

Conventional teaching often likens a linear polymer to a length of rope since its mass is spread out in a linear fashion resulting in a high level of flexibility. This means as solvent quality changes, a linear polymer may exist in a more elongated or more condensed conformation. This fluctuation in conformation is dependent upon several factors, not limited to, polymer length, rotational freedom of the polymer, and rigidity of the monomer. Each of these size influencing factors contribute to the overall apparent solution size of a polymer. The magnitude of this fluctuation varies among different types of polymers as well as the length of the polymer. Shorter, or more "rigid" linear polymers should see less variation compared to longer, or less "rigid" linear polymers. Regardless, conventional thinking believes that, given the same molecular weight, a linear polymer exhibits more size variation than a dendrimer.

In 1983, de Gennes *et al.* used a self-consistent field model to investigate the relationship between generation limit and spacer length.²⁰ This is one of the earliest studies investigating this relationship as dendrimers were only discovered a few years prior in 1978. De Gennes *et al.* found that every dendrimer has a theoretical growth limit where the polymer cannot fully achieve the next generation. This is driven by the density of functional groups at the periphery and the length of the spacer molecules within each arm, assuming a fully extended structure. Generally, the longer the spacer length between branch points (e.g. poly(propylene imine) (PPI) dendrimers $-(\text{CH}_2)_3-$ spacer) vs. poly(amido amine) PAMAM dendrimers $-(\text{CH}_2)_2\text{C}(\text{O})\text{NH}(\text{CH}_2)_2-$ spacer), the slower the peripheral density increases thus allowing for higher achievable generations. Plainly, a dendrimer with a longer spacer length such as PAMAM dendrimers should be able to reach higher generations than dendrimers with a shorter spacer length such as PPI dendrimers. This argument means the generation limit is driven by monomer (spacer) length and not the swelling behavior of the dendrimer in any given solvent.

When drawn on paper, dendrimers appear planar in nature, with each dendritic wedge occupying its own section of the total dendrimer volume. As generation increases, each of these drawn wedges grow closer together as the dendrimer volume does not scale with dendrimer functional density.^{20–22} Though this is a conventional example, it shows exactly what de Gennes and coworker describe as the "dendrimer growth limit" (also known as the "deGennes limit") where terminal functional group density reaches a point where quantitative functionalization is no longer possible. However, it should be noted this was one of the earliest investigations into dendrimer

shape and it is well understood that dendrimers are not planar but have three-dimensional architecture. As such, there a number of factors that contribute to the overall size of dendrimers, especially in solution.

The three-dimensional architecture of dendrimers was first highlighted by a kinetic growth model by Lescanec and Muthukumar in 1990.²¹ Their study suggested that the dendrimer terminal functional groups do not strictly populate the periphery of the dendrimer space, at any generation, but are rather distributed throughout the dendrimer volume.²¹ Further computational work done by Mansfield and coworker with PAMAM dendrimers also supports this viewpoint.²² Since these early computational studies, there have been numerous experimental investigations into how dendrimers behave in solution, some with differing results.

One of the earliest experimental studies on dendrimer solution size was conducted by Ihre *et al.* using pulsed field spin echo proton nuclear magnetic resonance (PGSE- ¹H NMR) looking at acetyl terminated bis-MPA dendrimers.²³ They found that the estimated hydrodynamic radii did increase as generation size increased (from [G1] to [G4]) in chloroform (CHCl_3), though this was not strictly linear. Lyulin *et al.* used small-angle neutron scattering (SANS) in their studies of [G5] and [G8] PAMAM dendrimers $-(\text{CH}_2)_2\text{C}(\text{O})\text{NH}(\text{CH}_2)_2-$ spacer) to find that the overall solution size of each dendrimer did not change significantly as solvent quality was reduced. This suggest a relatively "rigid" structure where each of the arms must be restricted in its movement, by some barrier, at least for the [G5] and [G8] size range.²⁴ However, when looking at poly(propylene imine) (PPI or DAB) $-(\text{CH}_2)_3-$ spacer) dendrimers, Chai *et al.* found using 2-D nuclear Overhauser effect spectroscopy (NOESY) NMR that the arms of a [G3] sample seem to have increased interaction between the terminal groups and interior methylenes in benzene (poor solvent quality) versus increased interaction between the terminal groups and solvent in CHCl_3 (good solvent quality).²⁵ These findings suggests that the apparent size of PPI dendrimers may be impacted by the extent of backfolding depending on the solvent used, despite still being soluble in a solvent such as benzene.

More recent examples within the last decade are largely focused on charged dendrimer systems to evaluate the effect positive charges and charge repulsion have on the overall size of dendrimers. By varying the protonation level of [G7] and [G8] PAMAM dendrimers in D_2O , Liu *et al.* found that PAMAM dendrimers exhibit segmental backfolding even at high generations despite expected charge repulsion.²⁶ Additionally, work done by Filipe *et al.* used both experimental diffusion NMR and molecular dynamics to conclude that in the case of [G3] charged peptide dendrimers, increased positive charge results in larger dendritic conformations.²⁷ At this point, the extent of backfolding of dendrimers seems to be dependent on the analytical approach used and the dendritic family one is studying. It is clear that individual dendrimer family investigations are necessary to improve application design where dendrimers may be useful. Additionally, studies that include linear or branched analogs as points of comparison can provide context as to the significance of this backfolding.

Unfortunately, this type of work has proven difficult since not all dendrimer families have linear equivalents to conduct such a study. Thus far, most experimental studies of dendrimers have been limited by examination in one or two solvents, or by looking solely at the dendrimer and no additional analog. PAMAM dendrimers are arguably one of the most widely used commercially available dendrimers. However, a linear equivalent for these dendrimers does not yet exist for such a comparison. In contrast, poly(benzyl ether) dendrimers have been compared to other polymeric analogs^{28–30}, including their linear poly(benzyl ether) variants. These dendrimers have shown they can expand and contract quite readily depending on solvent but not to the same degree as their linear variants.^{28,29} Though bis-MPA dendrimers have seen increased use, they have not had a true linear equivalent until Kareem *et al.* recently published a benzoyl-protected bis-MPA linear polyester, poly(3-(benzoyloxy)-2-(bromomethyl)-2-methylpropanoic acid) (PBBM).³¹ Though a linear bis-MPA polycarbonate has existed for some time^{32–36}, it is not the best linear analog to traditional bis-MPA dendrimers due to the lack of polyester linkages.³¹ With the advent of PBBM, it should now be possible to investigate the contribution the branched architecture a dendrimer provides to the overall “rigidness” of a bis-MPA dendrimer.

One recent application inquiry is in using dendrimers as small molecular weight calibrants for size measuring techniques such as GPC, also known as size exclusion chromatography (SEC). GPC is a very powerful tool for dispersity and molecular weight analysis of polymer samples. It can provide useful information for polymeric materials, especially for the evaluation of broad or poorly ionizing samples not easily analyzed by other means such as NMR (cannot determine dispersity) or MALDI-ToF MS (low-mass ionization bias). However, GPC systems typically use narrowly disperse linear polymer samples, such as poly(styrene) (PS), for calibration. As a result, GPC values are typically reported relative to the mass standard used. Therefore, GPC values can be misleading depending on the quality of polymer and solvent system used. For example, a 5 kDa polymer sample may be reported as 7 kDa in one solvent and as 4 kDa in another. These fluctuations are a product of both the changes in hydrodynamic volume of the polymer and the changes in hydrodynamic volume of the polymer calibrant. This requires careful calibration to ensure accurate reporting of data. These issues may be circumvented by using multi-angle light scattering (MALS) detectors that are capable of absolute molecular weight determination. However, this is not as useful for looking at structures other than linear polymers due to the way these systems are calibrated with linear standards.

In order to lessen this potential variance, dendrimers have drawn interest since, theoretically, their hydrodynamic size variation may be more limited than a linear counterpart as well as they have increased solubility across a wider range of solvents than linear polymers.^{37,38} However, as discussed earlier, the conclusions of previous studies are difficult to rely on when generalized for all other dendrimer families. Ultimately, dendrimer solution size is dependent on several

factors including, but not limited to, favorable intramolecular interactions, generation number, size of subunits, core molecule used, etc.³⁰ Therefore, it is more appropriate to study each dendritic system individually. Additional comparison to a linear analog would provide a clearer picture of how they behave. With bis-MPA dendrimers already showing their worth as mass calibrants for MALDI-ToF MS^{2,8,9,16,19} and now having a true linear analog³¹, an apparent size investigation on this dendrimer family would be of great importance.

As such, a study exploring solution size variation of bis-MPA dendrimers was performed. To provide even more context to this investigation, poly(caprolactone) (PCL) was included as it is a common polyester that has a similar atom composition (C(O)(CH₂)₅O) and repeat unit mass (~ 114 Da) to the bis-MPA subunit of bis-MPA dendrimers (C(O)C(CH₃)(CH₂)₂O₂) (~ 115 Da). Additionally, PBBM has a similar atom composition and repeat unit mass (OC(O)C(CH₃)(CH₂OC(O)C₆H₅)(CH₂Br)) (~ 220 Da) to the terminal benzylidene-protected bis-MPA groups (C(O)C(CH₃)(CH₂O)₂CHC₆H₅) (~ 205 Da) at the dendrimer periphery. These two linear analogues should be good comparative points for evaluating the size fluctuation of these dendrimers. Herein, benzylidene-protected pentaerythritol (tetra)-core [G1-G4] dendrimers, PBBM, and PCL are analyzed using an observed mass versus apparent mass comparison using MALDI-ToF MS and GPC, respectively. Furthermore, diffusion ordered spectroscopy-nuclear magnetic resonance (DOSY-¹H NMR) is used to measure rates of diffusion, and subsequently the van der Waals radii of each polymer in five deuterated solvents: tetrahydrofuran (THF-d₈), chloroform (CDCl₃), acetone (Ace-d₆), dimethyl formamide (DMF-d₇), and dimethyl sulfoxide (DMSO-d₆).

Results and discussion

Due to its radial distribution of molecular weight, it is expected that a dendrimer should not only appear smaller than a linear analog of similar mass, but also be more restricted in its apparent size variation. At higher generations, the distance between each dendritic wedge is reduced leading to possible steric strain. To relieve this strain, it is possible that each wedge may back fold or twist out of plane with higher generations exhibiting a more globular conformation. This may also occur in lower generations in lower quality solvents.

Though dendrimers exhibit much higher solubility than linear polymers^{1,30,37,39}, it is possible that, in lower quality solvents, dendritic wedges may back fold to a more condensed conformation. However, this backfolding should be limited by steric hinderance, rigidity of the dendrimer subunits, and the rotational freedom between branch point just to name a few factors.

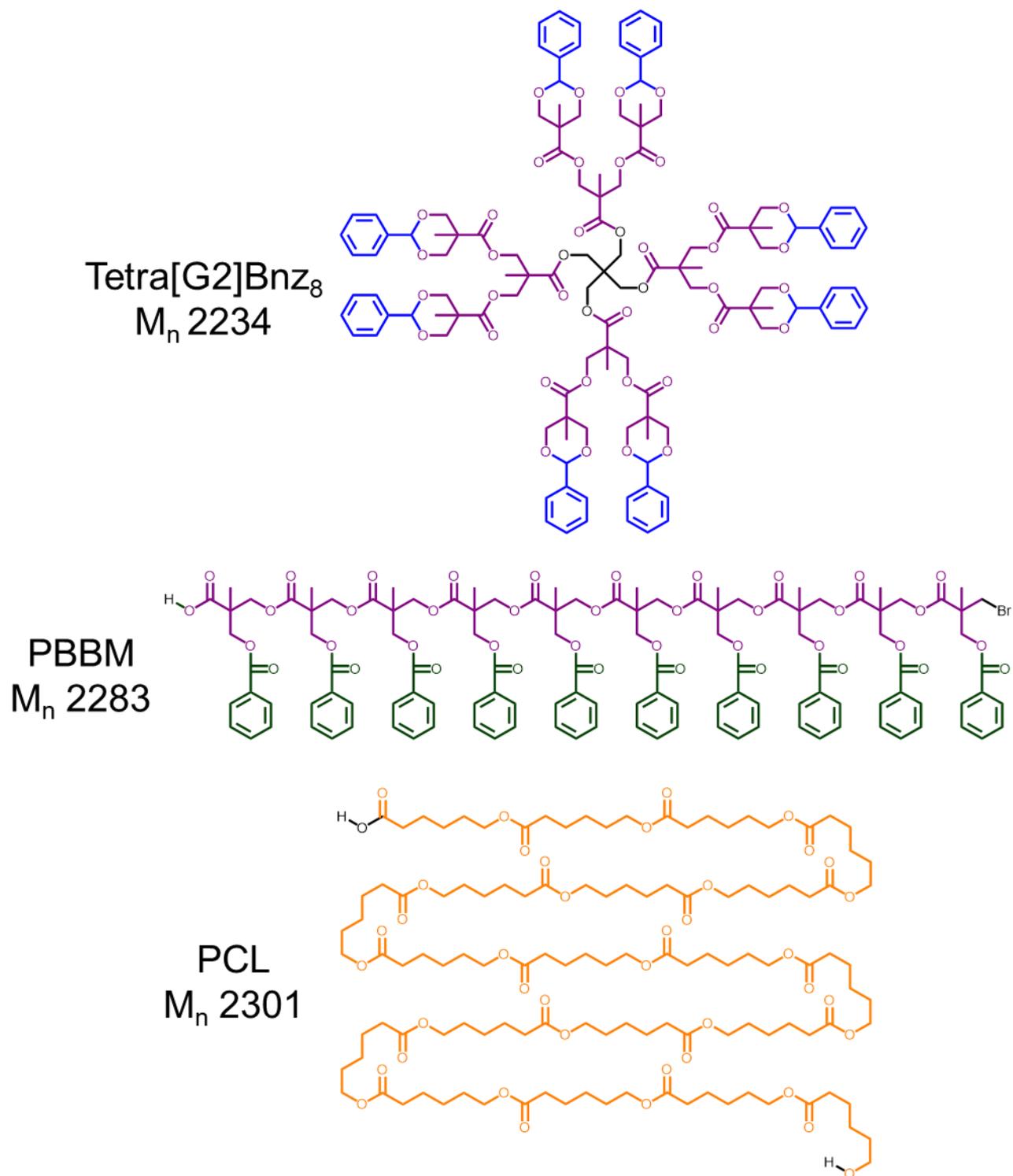


Fig. 2 Structures of Tetra[G2]Bnz₈ (Exact M_n = 2232.9) and analogous linear polymers PBBM (10-mer Exact M_n = 2280.6) and PCL (20-mer Exact M_n = 2299.3). Repeat units of bis-MPA are highlighted in purple. Benzylidene protecting groups are highlighted in blue and benzoyl protecting groups are highlighted in green. Repeat units of caprolactone are highlighted in orange.

MALDI-ToF MS observed mass vs. GPC apparent mass

While each of these factors, and possibly others, should impact the conformational flexibility of dendrimers, dendrimers should also have a smaller apparent size compared to a linear polymer of equivalent molecular weight. To test this concept, tetra-core [G1-G4] benzylidene-protected bis-MPA dendrimers were analyzed using MALDI-ToF MS and GPC (Figs. S5-10). Each generation of tetra-core bis-MPA dendrimer was used as the base point for molecular weight ranges for the linear polymers tested in this study. As a result, PBBM and PCL samples were purified using preparative GPC to isolate molecular weight fractions close to the observed mass of a corresponding dendrimer generation (Table 1). These molecular weight (M_n) ranges are henceforth referred to as 1 kDa, 2.2 kDa, 4.8 kDa, and 10 kDa. After isolation, these molecular weight analogs were also analyzed using MALDI-ToF MS and GPC (Fig 3).

Table 1 MALDI-ToF MS and GPC M_n and D values used for observed mass vs. apparent mass comparison

Sample	M_n^a	D^a	M_n^b	D^b
Tetra[G1]Bnz ₄	975	1.00	900	1.00
Tetra[G2]Bnz ₈	2257	1.00	1600	1.00
Tetra[G3]Bnz ₁₆	4820	1.00	2900	1.01
Tetra[G4]Bnz ₃₂	9941	1.00	4800	1.04
1 kDa PBBM	1200	1.03	1200	1.05
2.2 kDa PBBM	2190	1.03	1900	1.05
4.8 kDa PBBM	4830	1.01	4000	1.02
10 kDa PBBM	10560	1.01	9400	1.04
1 kDa PCL	1150	1.01	1500	1.03
2.2 kDa PCL	2110	1.02	4400	1.08
4.8 kDa PCL	4420	1.02	10600	1.04
10 kDa PCL	10430	1.02	23400	1.12

^a M_n and D calculated by MALDI-ToF MS using DCTB with Na⁺ counterion calibrated against SpheriCal[®] standards. ^b M_n and D calculated by GPC analysis calibrated against poly(styrene) (PS) standards.

As seen in Table 1 and Fig. 3, despite being of similar molecular weights according to MALDI-ToF MS, bis-MPA dendrimers consistently report lower GPC M_n values in THF than PBBM and PCL. This makes sense conceptually, since in the case of the [G1-G4] dendrimers, molecular weight is spread out radially. Additionally, the terminal bis-MPA units are protected with benzylidene groups which add significantly to their observed mass (terminal group mass \sim 205 Da). PBBM contains a pendant benzoyl group that also adds significant mass to each repeat unit (repeat unit mass \sim 220 Da). As a result, Tetra[G1]Bnz₄ contains four bis-MPA units and an equivalent PBBM chain also contains four bis-MPA units despite the dendrimer having the additional “core” molecule. In contrast, though the PCL unit has a similar atom composition to the bis-MPA repeat unit, it lacks a heavy protecting group. Therefore, at the 1 kDa molecular weight range, PCL contains eight repeat units compared to the four of the [G1] dendrimer. To provide a clearer example, in Fig. 2, Tetra[G2]Bnz₈ is drawn along with the nearest molecular weight analog for PCL and PBBM. As shown

in Fig. 2, the dendrimer contains twelve bis-MPA units while PBBM and PCL contain ten and twenty repeat units, respectively. These differences increase when looking at higher molecular weights and will be discussed later in this study.



Fig. 3 Observed m/z by MALDI-ToF MS versus apparent M_n by GPC (calibrated against PS standards) using the M_n values from Table 1.

Despite the [G1] dendrimer containing a “core” molecule, it still appears to be very similar in apparent mass to PBBM in THF. This similarity seems to continue even up to 2 kDa when following the linear trendline fits. Since both contain roughly the same number of bis-MPA units up to this range, the observed overlap is not surprising. 1 kDa PCL also seems to be only marginally larger than the [G1] dendrimer despite having approximately double the number of repeat units. Thus far, it appears that at the 1 kDa range, there is not much difference in the apparent size of these analogs.

However, at the 10 kDa range significant differences can be observed. Tetra[G4]Bnz₃₂ contains sixty bis-MPA units while an equivalent PBBM chain contains approximately 45 bis-MPA units. Despite containing less bis-MPA units in this 10 kDa mass range, PBBM still has a higher apparent mass by GPC suggesting a larger conformation in THF. 10 kDa PCL contains approximately 87 repeat units. Though this is \sim 1.5 times as many repeat units as the [G4] dendrimer, this results in an almost five-fold higher apparent mass. At this mass range, linear polymers can appear much larger than dendrimers.

Although they have similar molecular weights, in order to reach the apparent mass of linear polymers, dendrimers of much higher molecular weights would need to be used. However, this is not feasible, since traditional dendrimers all have a growth limit. In this example, the tetra-core bis-MPA dendrimers appear to have a generation limit of [G5]. With these differences in apparent mass shown in this preliminary size comparison, it leads some credence to the potential of apparent size fluctuation being greater for the linear analogs, though more data outside of THF would be needed to confirm this.

DOSY-¹H NMR

The molecular weight versus apparent mass comparison suggests that the dendrimers have a smaller hydrodynamic volume than both linear analogs. However, that experiment was limited to THF as a solvent and does not consider the fluctuations that may occur in other solvents of different polarities. To investigate this, DOSY-¹H NMR was used to analyze the selected analog samples in five solvents: THF, CHCl₃, acetone, DMF, and DMSO. These five solvents were chosen to balance range of polarity, solubility of each polymeric material across the molecular weights analyzed, boiling point to reduce convection, and cost effectiveness.

DOSY-¹H NMR measures the decay of ¹H signal intensity with decreasing gradient strength. This decay was used to calculate a self-diffusion constant (D) using Equation 1 for each sample. DOSY-¹H NMR spectra of each these samples in each solvent are shown in Figs. S11-69). The calculated D values were then used in the Stokes-Einstein equation (Equation 2) to calculate an

estimated hydrodynamic radius (R_h) which corresponds to the van der Waals radius. Though these calculations assume an ideal spherical shape for each sample and are not a direct measurement of actual molecular size, they should still be able to yield good insight into how each polymer is behaving in each solvent. As solvent quality changes for each molecular weight and sample, samples that are less “rigid” should see greater size “fluctuation”.

Table 2 shows the calculated D and R_h of each sample in the respective solvent. Additionally, average R_h across all five solvents and calculated “fluctuation” value are shown in Fig. 4. The “fluctuation” value was calculated by taking the mean value of R_h across all five solvents for each sample and molecular weight range and then calculating a deviation value for each experiment from this average. These deviation values were then averaged to attain the fluctuation value.

Table 2 Average D and R_h for all tested samples in THF, CHCl₃, acetone, DMF, and DMSO.^a

Sample	Solvent	Avg D (m ² s ⁻¹) ^b	R_h (nm) ^c	Sample	Solvent	Avg D (m ² s ⁻¹) ^b	R_h (nm) ^c	Sample	Solvent	Avg D (m ² s ⁻¹) ^b	R_h (nm) ^c
Tetra[G1]Bnz ₄	THF	8.44 x10 ⁻¹⁰	0.54 ± 0.03	1 kDa PBBM	THF	7.53 x10 ⁻¹⁰	0.60 ± 0.00	1 kDa PCL	THF	4.45 x10 ⁻¹⁰	1.02 ± 0.04
	CHCl ₃	6.95 x10 ⁻¹⁰	0.59 ± 0.08		CHCl ₃	7.20 x10 ⁻¹⁰	0.56 ± 0.00		CHCl ₃	5.31 x10 ⁻¹⁰	0.76 ± 0.06
	Ace	1.17 x10 ⁻⁰⁹	0.59 ± 0.05		Ace	1.13 x10 ⁻⁰⁹	0.61 ± 0.01		Ace	9.49 x10 ⁻¹⁰	0.74 ± 0.10
	DMF	4.80 x10 ⁻¹⁰	0.57 ± 0.02		DMF	4.12 x10 ⁻¹⁰	0.67 ± 0.01		DMF	2.93 x10 ⁻¹⁰	0.94 ± 0.01
	DMSO	1.65 x10 ⁻¹⁰	0.67 ± 0.03		DMSO	1.84 x10 ⁻¹⁰	0.60 ± 0.02		DMSO	1.15 x10 ⁻¹⁰	0.96 ± 0.03
Tetra[G2]Bnz ₉	THF	5.39 x10 ⁻¹⁰	0.84 ± 0.02	2.2 kDa PBBM	THF	5.36 x10 ⁻¹⁰	0.85 ± 0.03	2.2 kDa PCL	THF	3.92 x10 ⁻¹⁰	1.16 ± 0.06
	CHCl ₃	5.07 x10 ⁻¹⁰	0.80 ± 0.07		CHCl ₃	5.51 x10 ⁻¹⁰	0.73 ± 0.01		CHCl ₃	2.67 x10 ⁻¹⁰	1.51 ± 0.02
	Ace	8.46 x10 ⁻¹⁰	0.82 ± 0.02		Ace	8.99 x10 ⁻¹⁰	0.77 ± 0.08		Ace	5.55 x10 ⁻¹⁰	1.25 ± 0.02
	DMF	3.22 x10 ⁻¹⁰	0.85 ± 0.02		DMF	3.12 x10 ⁻¹⁰	0.88 ± 0.01		DMF	2.30 x10 ⁻¹⁰	1.19 ± 0.00
	DMSO	1.20 x10 ⁻¹⁰	0.92 ± 0.04		DMSO	1.21 x10 ⁻¹⁰	0.91 ± 0.03		DMSO	8.40 x10 ⁻¹¹	1.31 ± 0.05
Tetra[G3]Bnz ₁₆	THF	3.91 x10 ⁻¹⁰	1.17 ± 0.10	4.8 kDa PBBM	THF	2.98 x10 ⁻¹⁰	1.53 ± 0.01	4.8 kDa PCL	THF	2.68 x10 ⁻¹⁰	1.70 ± 0.06
	CHCl ₃	3.61 x10 ⁻¹⁰	1.12 ± 0.04		CHCl ₃	3.06 x10 ⁻¹⁰	1.32 ± 0.10		CHCl ₃	2.12 x10 ⁻¹⁰	1.90 ± 0.05
	Ace	6.34 x10 ⁻¹⁰	1.09 ± 0.03		Ace	5.56 x10 ⁻¹⁰	1.24 ± 0.01		Ace	4.06 x10 ⁻¹⁰	1.71 ± 0.03
	DMF	2.76 x10 ⁻¹⁰	1.00 ± 0.10		DMF	1.96 x10 ⁻¹⁰	1.40 ± 0.07		DMF	1.57 x10 ⁻¹⁰	1.75 ± 0.05
	DMSO	9.21 x10 ⁻¹¹	1.20 ± 0.02		DMSO	8.05 x10 ⁻¹¹	1.37 ± 0.10		DMSO	8.85 x10 ⁻¹¹	1.25 ± 0.06
Tetra[G4]Bnz ₃₂	THF	3.37 x10 ⁻¹⁰	1.35 ± 0.01	10 kDa PBBM	THF	2.51 x10 ⁻¹⁰	1.81 ± 0.08	10 kDa PCL	THF	1.75 x10 ⁻¹⁰	2.60 ± 0.06
	CHCl ₃	2.94 x10 ⁻¹⁰	1.37 ± 0.09		CHCl ₃	2.55 x10 ⁻¹⁰	1.58 ± 0.03		CHCl ₃	1.55 x10 ⁻¹⁰	2.61 ± 0.11
	Ace	5.44 x10 ⁻¹⁰	1.28 ± 0.09		Ace	3.73 x10 ⁻¹⁰	1.86 ± 0.08		Ace	2.70 x10 ⁻¹⁰	2.56 ± 0.08
	DMF	2.65 x10 ⁻¹⁰	1.39 ± 0.06		DMF	1.47 x10 ⁻¹⁰	1.87 ± 0.01		DMF	1.12 x10 ⁻¹⁰	2.46 ± 0.04
	DMSO	1.98 x10 ⁻¹⁰	1.40 ± 0.09		DMSO	5.67x10 ⁻¹¹	1.95 ± 0.05		DMSO ^d	-	-

^aEach sample was analyzed in duplicate using DOSY-¹H NMR at 5 mg ml⁻¹ at 298 K in all five solvents. ^bWithin each DOSY-¹H NMR spectrum, D for four separate peaks were calculated using Equation 1. This was done for each of the duplicate spectra. All eight of these calculated D values were then averaged to give the D values shown in the above table. Peaks used for this analysis are shown in Figs. S71-S73. ^c R_h value was calculated from the average D from two separate runs of each sample using Equation 2. ^d10 kDa PCL was not sufficiently soluble in DMSO at 298 K to complete the experiment.

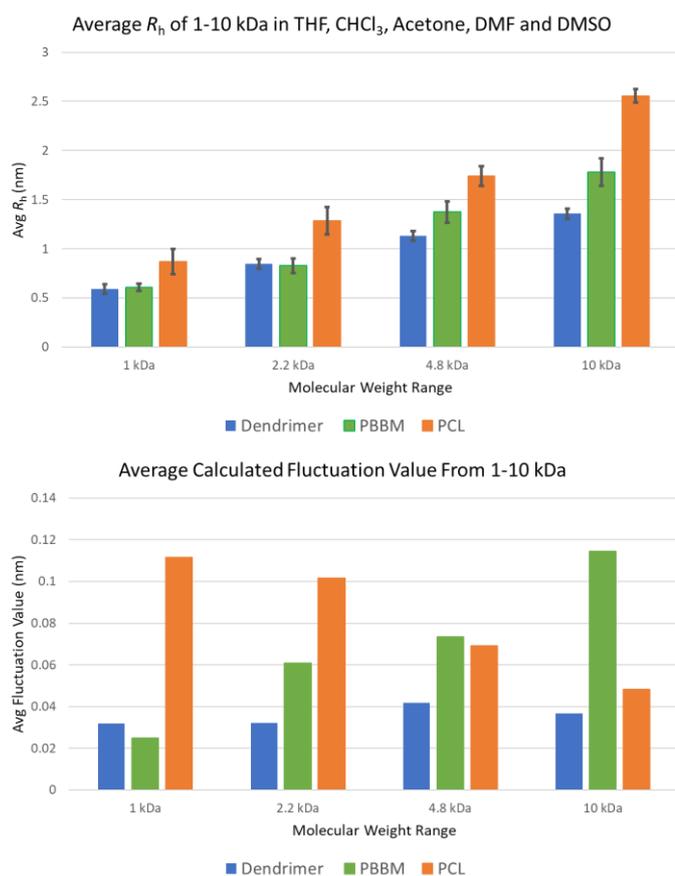


Fig. 4 Average R_h and “fluctuation” value for each polymer across all tested molecular weight ranges using data from DOSY- ^1H NMR.

1 kDa Molecular Weight Range

As seen in the GPC apparent size comparison (Fig. 3), Tetra[G1]Bnz₄ and 1 kDa PBBM have similar apparent masses with 1 kDa PCL being slightly larger. This holds true when looking at just THF according to the DOSY- ^1H NMR data. However, measurements of 1 kDa PCL in other solvents, such as acetone and chloroform, show it may not be significantly different from PBBM or the [G1] dendrimer in these solvents (Fig. 5 and Table 1). On average, 1 kDa PCL has a R_h that is ~ 43% (2.6 Å difference) larger than 1 kDa PBBM and ~ 47% (2.8 Å difference) larger than the [G1] dendrimer (Fig. 4). Though by GPC, 1 kDa PCL seemed much closer to the other analogs, DOSY- ^1H NMR reveals there is a larger difference between PCL and the other analogs even at this weight range.

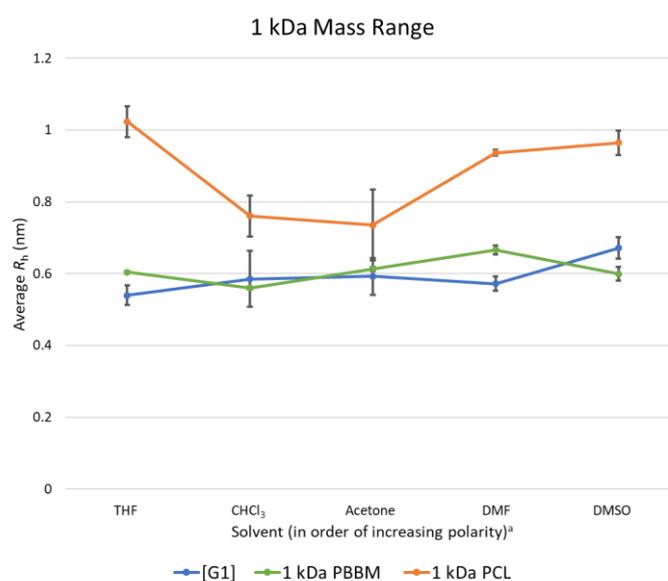


Fig. 5 Calculated average R_h values for Tetra[G1]Bnz₄, 1 kDa PCL, and 1 kDa PBBM in all tested solvents.

^aSolvent polarity values from Reichardt were used to determine order of polarity.⁴⁰

When comparing the “fluctuation” values of the [G1] dendrimer and equivalent PBBM (Fig. 4), it appears that the dendrimer may exhibit slightly more size variation (~ 0.07 Å more), though when looking at R_h calculations they are quite similar (Fig. 4 and 5). The lower fluctuation value of PBBM may be due in part to the pendent benzoyl group which could aid in its rigidity as there are possible pi bond interactions between repeat units. This, along with the low number of repeat units (four bis-MPA units), may be aiding in its size stability across these measured solvents. Regardless, this result was unexpected and leads credence to the idea that PBBM is a good analog for these benzylidene-protected dendrimers. The size variability of 1 kDa PCL on the other hand is much higher than the other analogs (~ 4.5 times more than PBBM and ~ 3.5 times more than Tetra[G1]Bnz₄) (Fig. 4). This may be due to the 1 kDa PCL containing 10 repeat units compared to the four of the [G1] dendrimer and 1 kDa PBBM, thus being larger. A lack of stabilizing intramolecular interactions may also play a role. Though all three analogs appeared to be similar in our GPC comparison at 1 kDa, differences already exist when looking at multiple solvents though these differences are on the Angstrom scale.

Table 3 Average R_h and calculated "size fluctuation" value of 1-10 kDa bis-MPA dendrimers, PBBM, and PCL.

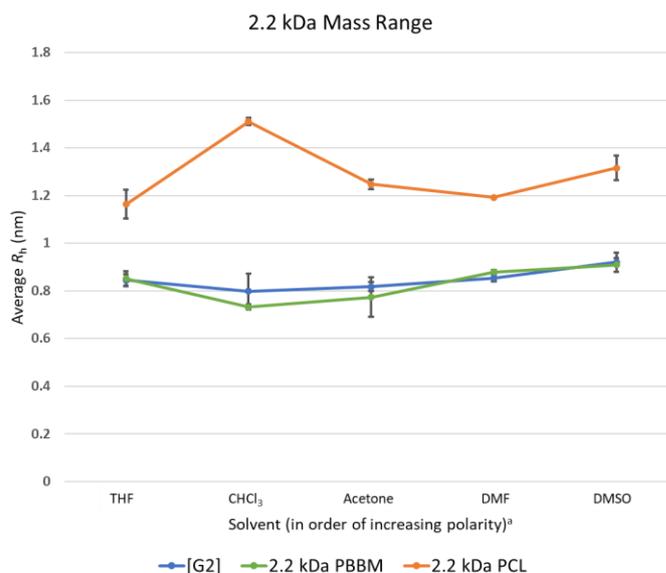
Sample	Avg R_h^a (nm)	Size Fluctuation Value ^b (Å)
Tetra[G1]Bnz ₄	0.59 ± (0.05)	0.32
Tetra[G2]Bnz ₈	0.87 ± (0.05)	0.32
Tetra[G3]Bnz ₁₆	1.13 ± (0.05)	0.42
Tetra[G4]Bnz ₃₂	1.36 ± (0.05)	0.37
1 kDa PBBM	0.61 ± (0.04)	0.25
2.2 kDa PBBM	0.83 ± (0.07)	0.61
4.8 kDa PBBM	1.37 ± (0.11)	0.73
10 kDa PBBM	1.78 ± (0.14)	1.14
1 kDa PCL	0.87 ± (0.13)	1.11
2.2 kDa PCL	1.29 ± (0.14)	1.01
4.8 kDa PCL	1.74 ± (0.10)	0.69
10 kDa PCL	2.56 ± (0.07)	0.48

^aEach of the calculated R_h values from each DOSY-¹H NMR spectrum was used to calculate the average R_h of each sample across all five tested solvents.

^bSize fluctuation value was calculated by taking the average R_h values and calculating the deviation of each R_h value, in each respective solvent, from the average R_h in all five solvents. These deviations were then averaged to give a "size fluctuation" value.

2.2 kDa Molecular Weight Range

At the 2.2 kDa range, only PBBM sees an increase in fluctuation value with the PCL dropping and the dendrimer remaining the same as it was for the 1 kDa range (Fig. 4 and Table 3). It is important to remember the [G2] dendrimer has 12 repeat units, 2.2 kDa PBBM has ~ 8 repeat units, and 2.2 kDa PCL has ~ 20 repeat units (Fig. 2). Though the [G2] dendrimer has a higher average R_h value than the [G1] as expected, its fluctuation value stays roughly the same. In the case of 2.2 kDa PBBM, there is an expected increase in R_h but unlike the 1 kDa counterpart, its fluctuation is now almost double (~ 90% higher) (~ 0.3 Å higher) that of the equivalent [G2] dendrimer. Despite this, the average R_h of the [G2] and 2.2 kDa PBBM appear to be very close, much like it was at the 1 kDa range (Fig. 4). These R_h values seem to be consistent with the GPC data (Fig. 3) where PBBM and the bis-MPA dendrimers appear to be similar in apparent size up to ~ 2 kDa. When looking at all 5 solvents, there is a great amount of overlap between the [G2] dendrimer and 2.2 kDa PBBM according to DOSY-¹H NMR (Fig. 6). Again, this is likely due to the 2.2 kDa PBBM still having a relatively low number of repeat units.

**Fig. 6** Calculated average R_h values for Tetra[G2]Bnz₈, 2.2 kDa PCL, and 2.2 kDa PBBM in all tested solvents.

^aSolvent polarity values from Reichardt were used to determine order of polarity.⁴⁰

In the GPC analysis (Fig. 3), 2.2 kDa PCL appears to be significantly larger than the [G2] dendrimer and 2.2 kDa PBBM. This remains consistent in the DOSY-¹H NMR data as it is ~ 51% larger (~ 4.3 Å larger), on average, than the [G2] dendrimer (Fig. 4). Its fluctuation value is also ~ 1.0 Å which is ~ 3 times higher than the [G2]. This is likely related to the fact that 2.2 kDa PCL contains ~ 20 repeat units. As can be seen in Fig. 6, the apparent size of 2.2 kDa PCL is significantly larger than the other two analogs: a trend that will continue for the rest of this study.

4.8 kDa Molecular Weight Range

At 4.8 kDa, there is again an expected increase in the R_h of all three analogs (Fig. 4 and Table 3). Though R_h of the dendrimer has increased, its fluctuation value remains lower than both linear analogs. In fact, the fluctuation value of the [G3] dendrimer is only ~ 0.1 Å higher than the [G1] dendrimer suggesting a high level of size stability for the bis-MPA dendrimers. By comparison, the 4.8 kDa PBBM has a fluctuation value that is ~ 0.5 Å higher than its 1 kDa counterpart. It is at this mass range that the difference between linear and dendritic bis-MPA analogs can be seen (Fig. 7). The [G3] dendrimer contains 28 bis-MPA units while 4.8 kDa PBBM has ~ 21 bis-MPA repeat units. Despite having fewer repeat units, PBBM still has a higher apparent R_h (2.4 Å higher) and higher fluctuation value (~ 0.3 Å higher) than the [G3] dendrimer. However, this size variation difference is still on the sub-angstrom scale, so these differences are still minimal.



Fig. 7 Calculated average R_h values for Tetra[G3]Bnz₁₆, 4.8 kDa PCL, and 4.8 kDa PBBM in all tested solvents.

^aSolvent polarity values from Reichardt were used to determine order of polarity.⁴⁰

4.8 kDa PCL not only has a larger average R_h (~6.1 Å larger) than the [G3] dendrimer, its fluctuation value is also ~64% higher (higher by ~0.28 Å) (Fig. 4). Interestingly, despite being the largest analog thus far, its fluctuation value appears to be lower than it was at both the 1 kDa and 2.2 kDa range. This was not unexpected since at this mass range, PCL contains ~42 repeat units which is 1.5 times the number of repeat units for the [G3] dendrimer despite having similar molecular weights (Table 1). Thus, a higher size variability was expected. This phenomenon is possibly explained through intramolecular chain entanglement, but it is hard to know if this is the case. It should be noted that this trend is in opposition of the PBBM analog. As molecular weight is increasing the PBBM analogs seem to have a growing level of size variability whereas the PCL seems to become more size stable. This trend continues into the 10 kDa range which may suggest a more globular conformation across all solvents is being adopted for the PCL.

10 kDa Molecular Weight Range

At the 10 kDa range, the [G4] dendrimer contains 60 bis-MPA units, the 10 kDa PBBM contains ~45 bis-MPA units, and 10 kDa PCL has ~87 repeat units. An expected increase in the average R_h for the [G4] dendrimer is seen at the 10 kDa range. However, this increase in R_h also seems to lead to a slightly lower “fluctuation” value (0.04 Å lower) than the [G3] dendrimer. Though the R_h of the [G4] has increased by ~1.7 Å over the [G3], its fluctuation value has dropped marginally (Fig. 4 and Table 3). This is somewhat expected as one would assume the larger dendrimer would start to adopt a more “globular” structure, much like the PCL seems to be doing, especially as it gets closer to its generation limit, [G5]. Though the variability of the PCL seems to be dropping, the [G4] dendrimer still has a

lower fluctuation value (0.1 Å lower) (Fig. 4). The 10 kDa PBBM now appears to have the highest amount of fluctuation out of the three analogs (Fig. 4) and its fluctuation value is ~0.4 Å higher than it was in the 4.8 kDa range. It is interesting how PBBM is behaving less like the dendrimer above ~2 kDa, mirroring what was expected based upon the GPC comparison (Fig. 3). Though 10 kDa PBBM is smaller than the 10 kDa PCL, it is not as “rigid” (Fig. 8).

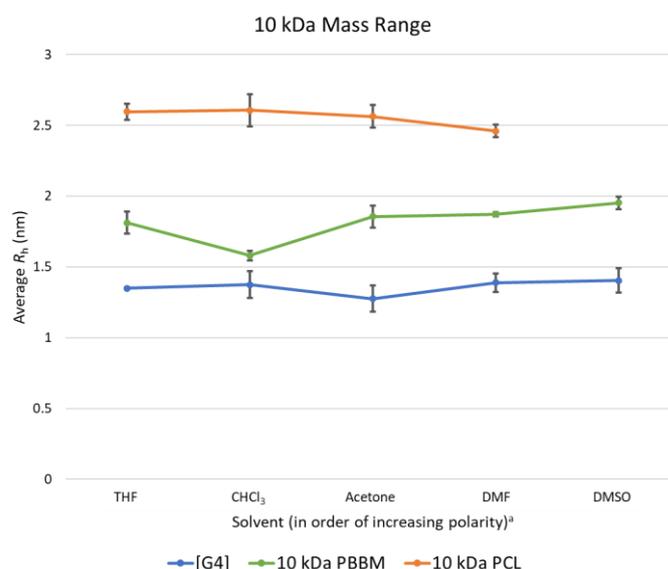


Fig. 8 Calculated average R_h values for Tetra[G4]Bnz₃₂, 10 kDa PCL, and 10 kDa PBBM in all tested solvents.

^aSolvent polarity values from Reichardt were used to determine order of polarity.⁴⁰

Following the same trend from the 2.2 kDa range, 10 kDa PCL has the largest average R_h value across these tested solvents. Its average R_h is ~7.8 Å higher than the equivalent PBBM and ~12 Å higher than the equivalent [G4] (Fig. 8). Though the 10 kDa PCL is ~8.2 Å larger than the 4.8 kDa PCL, another drop in fluctuation value is observed: a reduction of ~69% (~0.2 Å reduction) following the trend starting from the 2.2 kDa range. This again supports the notion of the PCL adopting a more “globular” structure. 10 kDa PCL may not be as size stable as the [G4] dendrimer but its fluctuation value is only ~0.1 Å higher. This is much lower than was expected, especially considering that 10 kDa PCL contains ~27 more repeat units than the [G4] dendrimer.

Fluctuation comparison of 5 kDa apparent mass range

Though the previous comparisons were based on observed mass by MALDI-ToF MS, it is important to also compare analogs that have similar “apparent” mass by GPC in THF. When looking at the observed MALDI-ToF mass vs. apparent GPC mass comparison in THF (Table 1 and Fig. 3), the [G4] dendrimer has an apparent mass of ~5 kDa despite having a nominal mass of 10 kDa. This apparent mass is similar to the apparent mass of 4.8 kDa PBBM and 2.2 kDa PCL in THF. Therefore, it is more appropriate to compare the “fluctuation” differences of these analogs to one another (Fig. 9). While comparing nominal mass

is good, an apparent GPC mass comparison is what is needed to determine if dendrimers are a sufficient GPC calibrant.

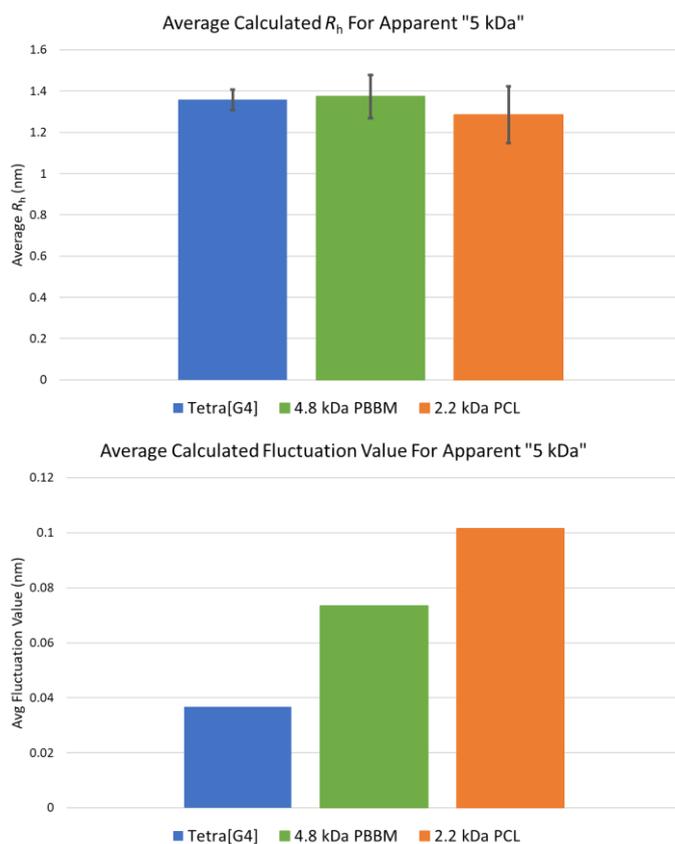


Fig. 9 Average R_h and fluctuation values for apparent 5 kDa Tetra[G4]Bnz₃₂, 4.8 kDa PBBM, and 2.2 kDa PCL.

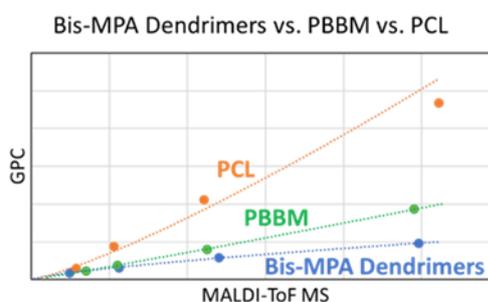
The similarities observed in THF also carry over when looking at the other solvents using DOSY-¹H NMR. Despite having ~ 39 more bis-MPA units than 4.8 kDa PBBM and ~ 40 more repeat units than 2.2 kDa PCL, the [G4] has a similar R_h value to these analogs. This adds credence to this comparison since GPC is an apparent size measuring technique. Though they are of similar sizes, the fluctuation behavior between these analogs is different. The [G4] dendrimer has a fluctuation value (0.37 Å) that is ~ 102% (~ 0.4 Å) less than the 4.8 kDa PBBM and ~ 183% (~ 0.7 Å) less than the 2.2 kDa PCL. This agrees with the notion of dendrimers being globular molecules that have limited size variability compared to linear polymers. When comparing the dendrimers and PCL by observed MALDI mass, the fluctuation value of 10 kDa PCL started to approach the level of the [G4] dendrimer. However, when comparing the [G4] to its PCL apparent mass analog, 2.2 kDa PCL, the difference is substantially more significant. The observed MALDI mass comparison reveals that PCL must reach a GPC M_n that is ~ five times that of an equivalent bis-MPA dendrimer to exhibit a similar level of size stability. Therefore, the bis-MPA dendrimers are indeed more size stable than both linear analogs when comparing both observed mass and apparent mass, as hypothesized.

Conclusions

The first investigation into the solution size fluctuation of bis-MPA dendrimers and their linear analog, PBBM, has been reported. With the advent of PBBM, it was now possible to evaluate how the branched architecture of bis-MPA dendrimers may affect solution size stability. Across the four tested molecular weight ranges, bis-MPA dendrimers exhibited less size fluctuation than the linear analogs except at the 1 kDa mass range with PBBM. However, it is important to note this (~ 0.07 Å) difference is minimal. According to GPC, despite being of similar mass by MALDI-ToF MS, bis-MPA dendrimers consistently report smaller GPC M_n values. This means the bis-MPA dendrimers are indeed "smaller" than their linear analog, PBBM. This is mirrored in the analysis by DOSY-¹H NMR. DOSY-¹H NMR also reveals the bis-MPA dendrimers are also more size stable than their observed MALDI mass analogs. When comparing the [G4] dendrimer to its apparent mass analogs, the difference in stability is even more evident.

According to this data, bis-MPA dendrimers do exhibit more solution size stability than their direct linear analog, PBBM, and potentially other linear polymers such as PCL given the same apparent mass or nominal mass. Though these differences exist on the Å scale, it is important to note that Å scale differences on R_h value can have a significant impact on the overall size and shape of a molecule. Additionally, the data here supports the idea of using dendrimers as small apparent mass calibrants for techniques like GPC from ~ 0-5 kDa. Though dendrimers cannot practically reach the same apparent size as, and will not replace, linear calibrants, they do have added benefits such as increased solubility in a wider range of solvents than linear polymers. This means that dendrimers may prove to be good calibrants for apparent size measuring techniques especially when looking at non-linear architectures or switching between solvents.

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Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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