

Synthesis and MALDI-ToF characterization of dendronized poly(ethylene glycol)s

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Scott M. Grayson completed his PhD in Chemistry from the University of California, Berkeley (2002), studying the role of polymer architecture for drug delivery under Jean M. J. Fréchet. Following post-doctoral studies in the laboratories of C. Grant Willson at the University of Texas, he was appointed as a professor at the department of chemistry at Tulane University in New Orleans, Louisiana, USA. As an associate professor at Tulane, his present research explores the synthesis and applications of well-defined, yet complex macromolecular structures. His research group has recently focused on amphiphilic and cyclic polymers.

Well-defined hybrids of linear poly(ethylene glycol)s (PEGs) and dendritic polyesters were prepared via the dendronization of the alcohol end groups of the mono and difunctional linear PEGs. Though useful for rudimentary product characterization, GPC and NMR could not verify the overall structural purity of these linear-dendritic hybrids. On the other hand, the detailed data provided by MALDI-ToF mass spectrometry enabled confirmation of the high structural purity of the dendronized PEGs at each step of the dendronization procedure. The well-defined number of functionalities on these dendronized PEGs, renders them particularly useful for research in the biomedical sphere where functionality and purity are of the utmost importance. The MALDI-ToF mass spectrometric approach described herein represents a valuable technique for detailed monitoring of these dendronization reactions, as well as a variety of other polymer end group modifications.

Uniterms: Poly(ethylene glycol). Dendrons. Dendrimers. Dendronized polymers.

Híbridos bem definidos de poli(etilenoglicol) lineares (PEGs) e poliésteres dendriméricos foram preparados via “dendronização” de álcool e grupos de PEGs lineares mono e bifuncionais. Embora úteis para a caracterização rudimentar de produtos, Cromatografia por Permeação em Gel e RMN podem não demonstrar a pureza estrutural global desses híbridos lineares dendríticos. Por outro lado, informações detalhadas provenientes de espectrometria de massas MALDI-ToF permitiram a confirmação de elevada pureza estrutural de PEGs “dendronizados” em cada passo do processo de “dendronização”. O número de funcionalidades bem definidas destes PEGs “dendronizados”, torna-os particularmente úteis para pesquisa na área biomédica, na qual funcionalidade e pureza são de grande importância. A abordagem de espectrometria de massas MALDI-ToF descrita aqui representa uma técnica valiosa para o monitoramento detalhado destas reações de “dendronização”, bem como diversas modificações de outros polímeros e grupos.

Unitermos: Poli(etilenoglicol). Dendrons. Dendrímeros. Polímeros “dendronizados”.

INTRODUCTION

The search for functional, biocompatible polymeric materials has intensified in recent years and one class of macromolecules that has garnered particular interest is linear-dendritic hybrids (Gitsov, Fréchet, 1993; Gitsov, 2008; Lundberg *et al.*, 2011). These hybrid materials are of particular interest because they address some of the long-standing shortcomings of the more studied linear polymers, namely, limited chain end functionality. Among traditional linear polymers, poly(ethylene glycol) has seen the most widespread use for biomedical applications due to its high water solubility,

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biocompatibility, low-toxicity, non-immunogenicity, and non-antigenic nature (Veronese, Pasut, 2005). As a result of this unique set of properties, PEG has been used for applications as diverse as improving the water solubility of drugs (Pasut, Veronese, 2010) and increasing the molecular weight and therefore blood circulation of proteins (Abuchowski *et al.*, 1977). However, the applications of PEGs are limited by the simple fact that they bear only one or two modifiable functionalities at the polymer chain ends. In this context, the ability to incorporate an increased multiplicity of addressable functional groups through dendronization is invaluable. However, as purity remains a critical issue for biomedical materials applications, it is necessary to establish the reproducible synthesis of well-defined linear-dendritic hybrids as well as develop rigorous characterization methodologies to confirm their purity.

Dendrimers are highly branched, monodisperse macromolecules with an exact multiplicity of functional groups that increase exponentially in number as the generation of the dendrimer increases (Frechet, 1994). In this investigation, the dendritic component of linear dendritic hybrids is a divergently grown dendron, a “wedge-like” dendritic segment. Although numerous dendritic syntheses have been reported (Frechet, Tomalia, 2001), the polyester dendrimers and dendrons based on 2,2-bis(hydroxymethyl)propionic acid (bis-MPA (Ihre *et al.*, 1996; Ihre *et al.*, 2001) are the most promising given their ease of synthesis, exceptional structural purity, and biocompatible nature (Padilla de Jesus *et al.*, 2002; Lundberg *et al.*, 2011).

The key advantage of coupling dendrons to linear polymers is the multivalent nature of the resulting macromolecule which allows for increased functionalization and can impart both increased solubility (Gitsov, Frechet, 1993) and increased strength of intermolecular interactions (Gitsov *et al.*, 2000). These characteristics are particularly important when considering polymers for biomedical applications. One example of the useful modularity of the linear-dendritic hybrid system is that, while the linear PEG provides increased plasma half-life, low toxicity, and overall stability from enzymatic or hydrolytic degradation, the dendrons provide a multiplicity of functional groups to increase the drug payload or targeting groups (Padilla de Jesus *et al.*, 2002).

However, the use of linear-dendritic hybrids in the biomedical realm necessitates rapid and reliable methods of characterization to confirm their structural purity. Typically, nuclear magnetic resonance spectroscopy (NMR) and gel permeation chromatography (GPC) are employed to confirm the purity of macromolecules. However, these methods can provide vague data regarding the purity of

linear-dendritic hybrids. If flaws exist that truncate one or more of the expected branching points, this will yield a polymer with a different number of attachment points and, therefore, different biophysical properties. Additionally, in NMR, the multiplicity of both linear and branched repeat units makes it difficult to confirm the structural purity, while GPC measures the hydrodynamic radius which is not likely to be affected substantially by minor structural impurities. Though important, these traditional characterization methods must be complemented by a technique like matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) that offers a more complete picture in terms of composition, structure, number of end groups, and molecular weight distributions (Polce, Wesdemiotis, 2002).

Herein we report the synthesis and characterization of linear-dendritic hybrids, and dendritic-linear-dendritic “dumbbells” composed of a linear PEG block, and dendritic blocks based on the branched bis-MPA monomer. While the synthesis of such hybrids has already been reported (Ihre, Padilla de Jesus, Frechet, 2001), improvements in the resolution of MALDI mass spectrometers enable a more detailed characterization, which will be described in this investigation. These compounds have been fully characterized using the standard methods employed (NMR and GPC) but were also characterized extensively by MALDI-ToF MS to verify their structural integrity.

MATERIAL AND METHODS

Material

All reagents were purchased from Aldrich and used without further purification, unless otherwise noted.

Characterization

Mass spectral data was acquired using a Bruker Autoflex III matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-ToF MS) with delayed extraction using both positive ion and reflector detection modes. THF stock solutions of α -cyano-4-hydroxycinnamic acid or *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile were used as the matrix (10 mg mL⁻¹) and sodium trifluoroacetate was used to provide a cation for ionization (1 mg mL⁻¹). The dendronized PEG stock solutions were prepared at a 2 mg mL⁻¹ concentration in THF. MALDI samples were prepared by combining 4 μ L of the dendronized PEG solution, 2 μ L of the cation solution, and 2 μ L of the matrix solution. For data acquisition, the pulsed ion extraction delay was set to 40 ns, with an ion source 1 voltage of 19.00 kV, an

ion source 2 voltage of 16.30 kV, a lens voltage of 9.1 kV, a low mass gate at 300 AMU, and a laser power set to 65%. In order to provide highly accurate mass measurements, the mass scale for each spectrum was calibrated against Spheri-Cal dendritic mass standards (Polymer Factory, Sweden).

Gel permeation chromatography (GPC) was carried out on a Waters model 1515 series pump (Milford, MA) with three column series from Polymer Laboratories, Inc. (Amherst, MA) consisting of PLgel 5 μ M Mixed D (300 mm x 7.5 mm, molecular weight range 200-400,000), PLgel 5 μ M 500 Å (300 mm x 7.5 mm, molecular weight range 500-30,000), and PLgel 5 μ M 50 Å (300 mm x 7.5 mm, molecular weight range up to 2000) columns. The system was fitted with a Model 2487 differential refractometer detector and anhydrous tetrahydrofuran was used as the mobile phase (1 mL min⁻¹ flow rate). Data were collected and processed using Precision Acquire software and calibrated against polystyrene standards.

Methods

The benzylidene-protected *bis*-(hydroxymethyl) propionic anhydride was synthesized using a previously reported technique (Ihre, Padilla de Jesus, Frechet, 2001). Two different low-polydispersity PEGs were used as starting materials to prepare the linear-dendritic PEG hybrids: a linear monomethyl ether PEG alcohol (5000 Da) and a linear PEG diol (4600 Da). The linear-dendritic hybrids were then synthesized (Figures 1 and 2) by repetition of an acid anhydride esterification “dendron growth” reaction and a palladium-catalyzed hydrogenolysis “deprotection” reaction (Ihre, Padilla de Jesus, Frechet, 2001).

RESULTS AND DISCUSSION

Characterization techniques

For the end group analysis required to confirm the preparation of these linear-dendritic hybrids, the most reliable characterization method was MALDI-ToF MS. Neither GPC (Figure 3a and b) nor NMR (Figure 4) provided enough structural information to determine the purity of the compounds due to the methods’ lack of sensitivity to the subtle changes in overall molecular structure, and the possibility of a low percentage of incomplete reactions or byproducts. For example, ¹H NMR of the dendronization of dihydroxy PEG can be used to confirm the addition of the aromatic hydrogens (7.25-7.45 ppm) and the benzylidene methyne (5.42 ppm). However, the low proportion of these end group signals relative to the PEG backbone makes the completion of the reaction difficult to determine

via ¹H NMR integration alone. Likewise, the subtle shifts observed in the GPC chromatograms were insufficient to draw meaningful conclusions about the extent of dendronization. However, GPC was valuable in confirming that the narrow molecular weight distribution of the PEG starting materials were maintained throughout the dendronization process, and that low molecular weight impurities which might not be apparent in MALDI-ToF MS were absent from the samples. MALDI-ToF MS provided the necessary data for end group analysis and assurance of complete coupling and deprotection. While one can observe the expected shift in the entire mass distribution throughout the synthesis, it is perhaps most intuitive to select an oligomer of a specific number of repeat units (e.g. 109-mer or 110-mer) and follow it through the expected transformations during dendronization (Figures 6 and 7).

Reaction monitoring by MALDI-ToF MS

For the dihydroxy PEG, an excess (up to 3 eq./hydroxyl) of the anhydride monomer, **1**, was used in order to ensure that the esterification reaction was carried to completion. The workup when using an excess of esterification reagent was technically simple, as the product could be isolated via precipitation. The coupling reaction could be easily monitored by MALDI-ToF MS, to determine when the reaction was complete (Figure 5). While interpretation of the MALDI mass spectra was complicated by the polydisperse nature of PEG, rigorous calibration enabled the identification of the reactant and product distributions with a high degree of certainty. Within the first minute of the reaction, the reaction mixture distribution exhibited a 204.1 mass increase, corresponding to a single esterification (A to B). By 16 minutes, a second distribution (C), which corresponded to the diester and an additional 204.12 mass increase, became the predominant distribution. By 256 minutes, only the diester product was apparent, and no signal could be observed for the previous distributions, suggesting the esterification had been carried out to completion.

Product characterization

When comparing the reactant and product, in the case of the esterification of monomethyl ether PEG alcohol, reaction with the acid anhydride monomer, **1**, yields the corresponding ester, **2**. This product is expected to exhibit a mass shift of 204.08 amu in its MALDI-ToF mass spectrum, and a shift of 204.07 is actually observed. The GPC chromatogram confirmed the narrow polydispersity of product, and the absence of low molecular weight con-

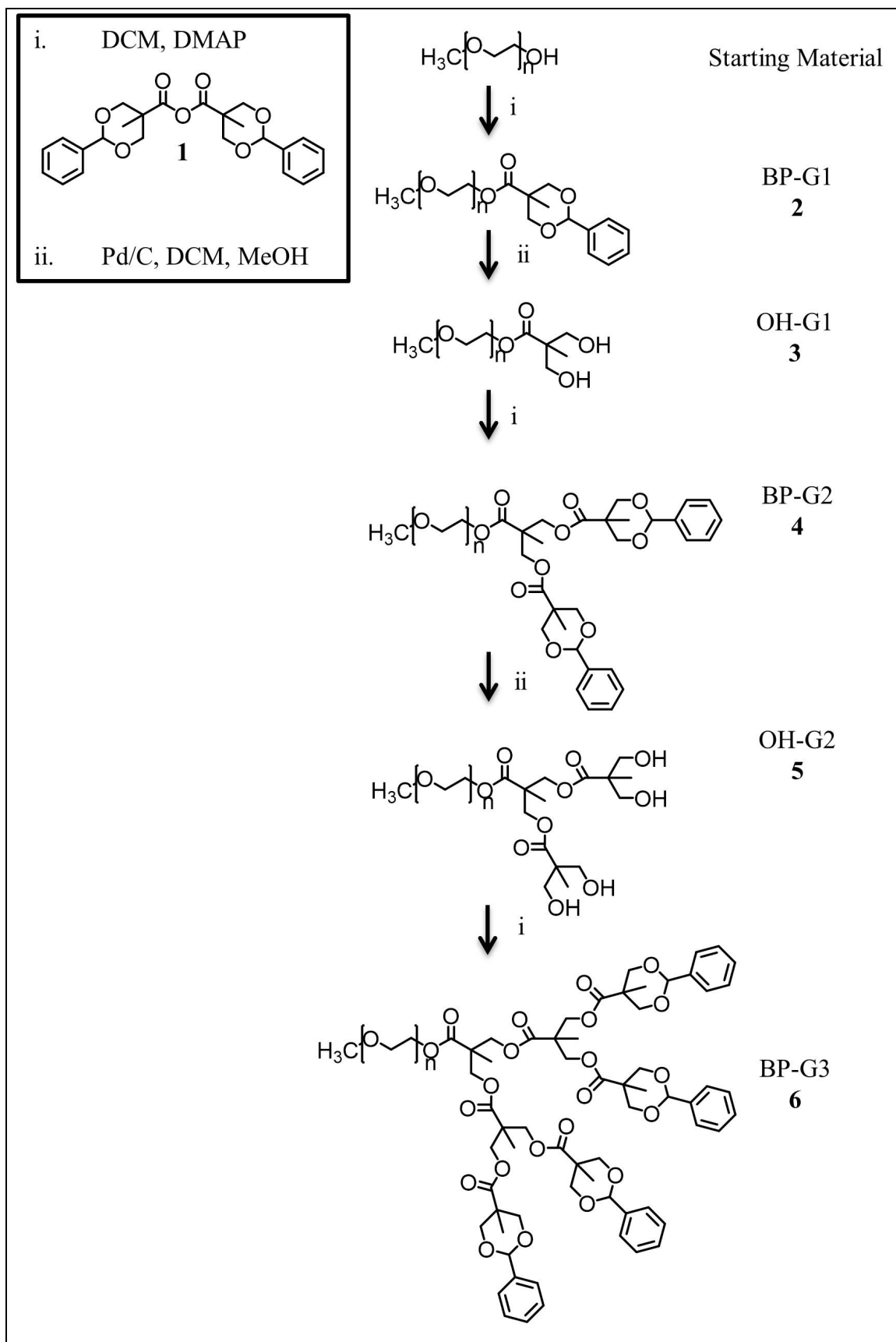


FIGURE 1 - Synthesis of linear-dendritic diblock via divergent dendronization of monomethyl ether PEG alcohol.

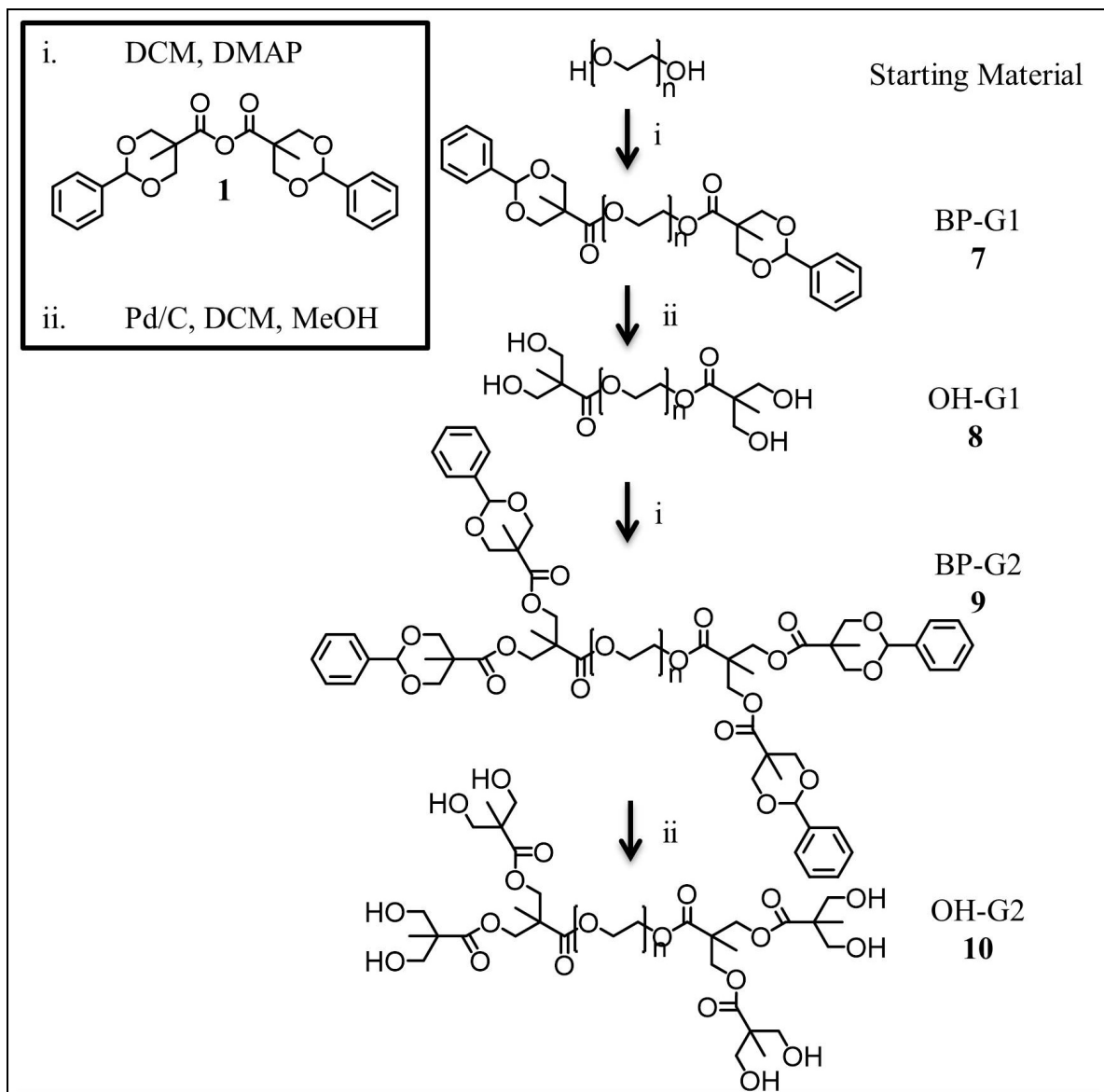


FIGURE 2 - Synthesis of dendritic-linear-dendritic triblock via divergent dendronization of dihydroxy PEG.

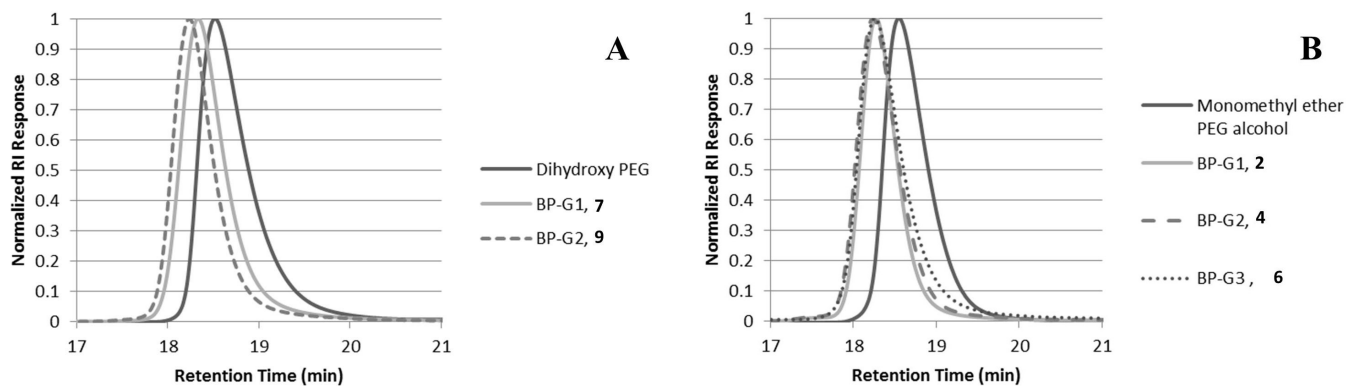


FIGURE 3 - a) GPC chromatogram of benzylidene-protected dendritic-linear-dendritic triblocks. b) GPC chromatogram of benzylidene-protected linear-dendritic diblocks.

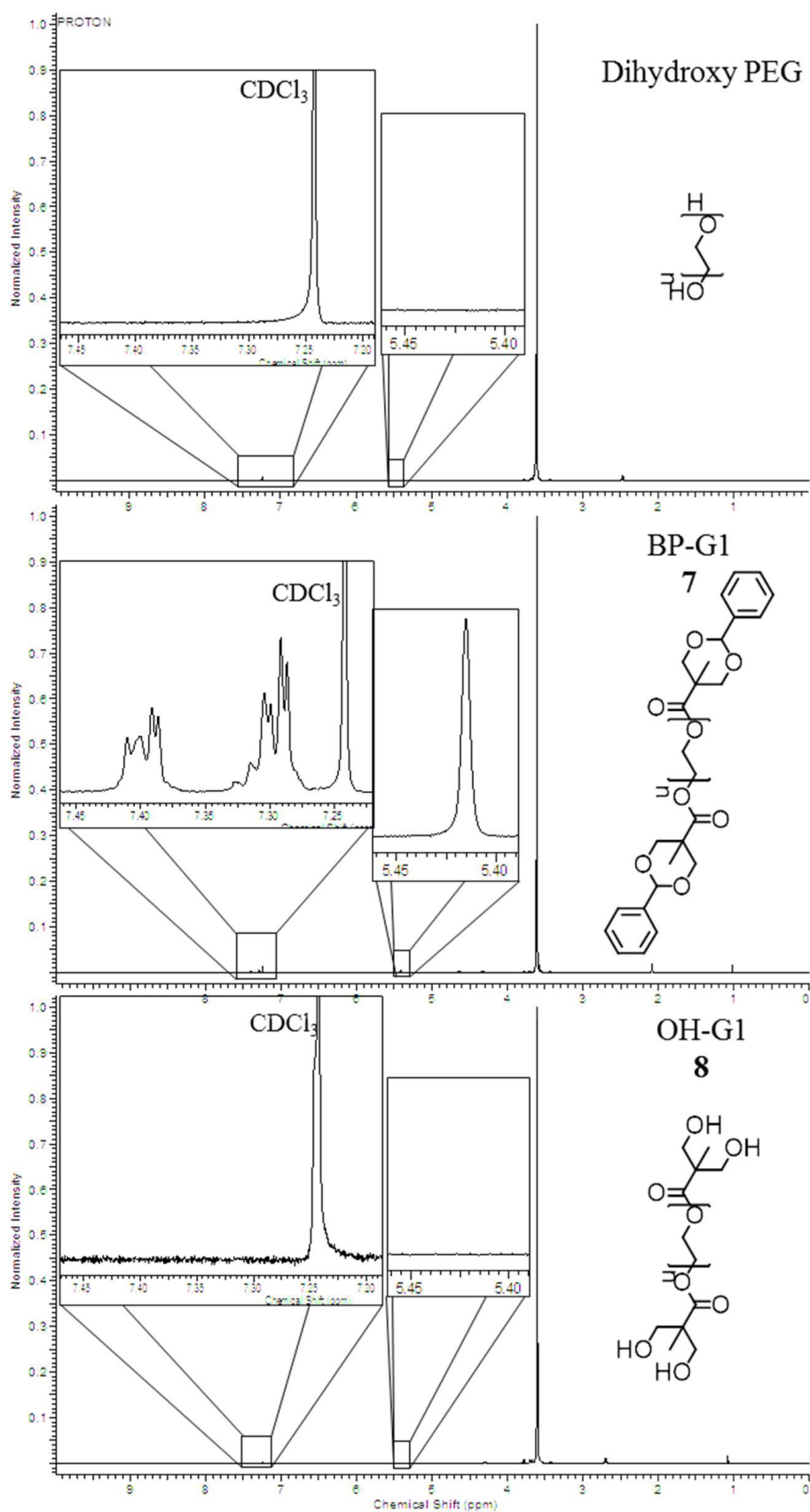


FIGURE 4 - ^1H NMR spectra of dihydroxy PEG starting material, BP-G1 (7), and OH-G1 (8).

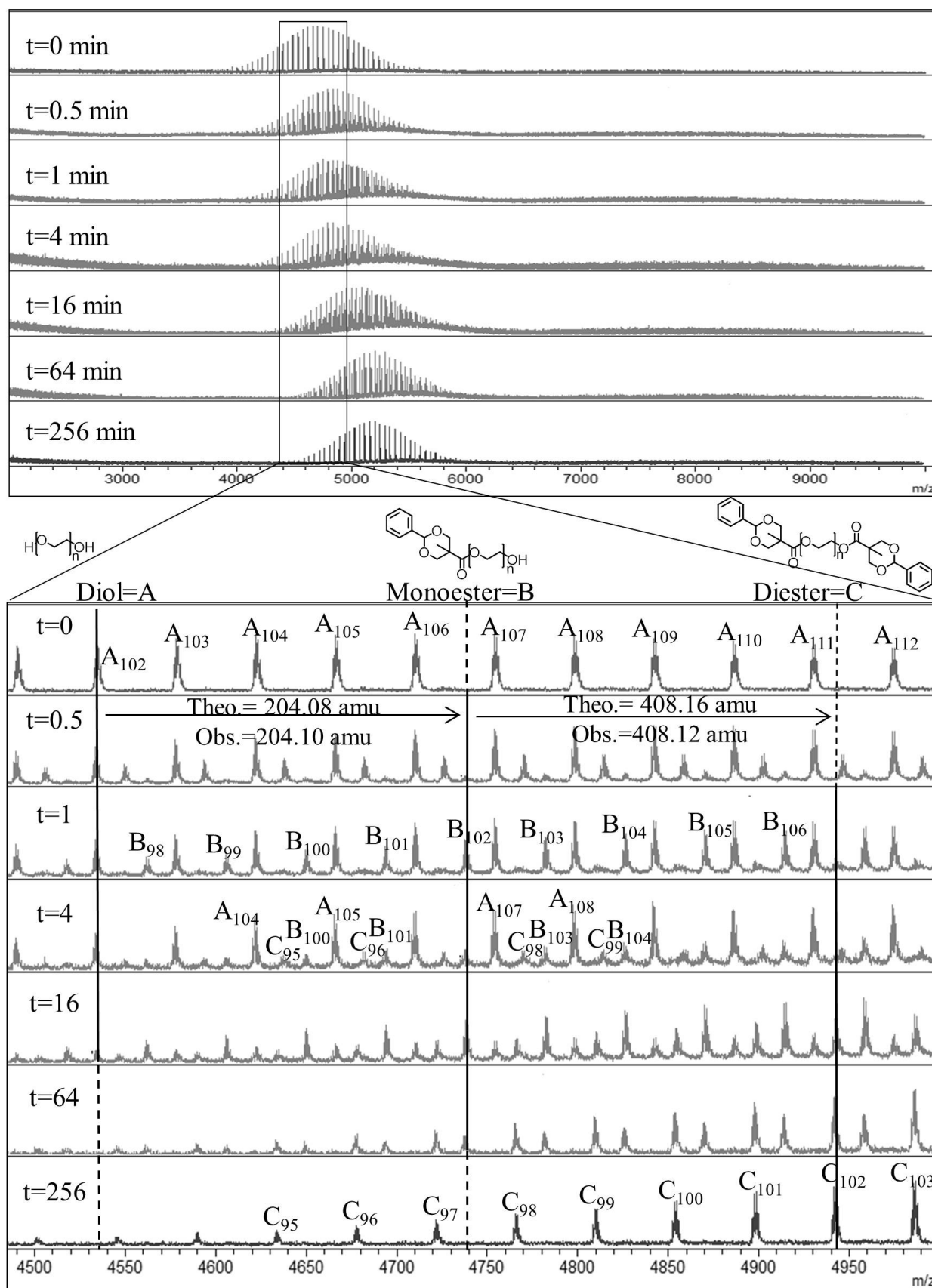


FIGURE 5 - Reaction monitoring via MALDI-ToF MS: divergent dendronization of dihydroxy PEG to afford compound 7.

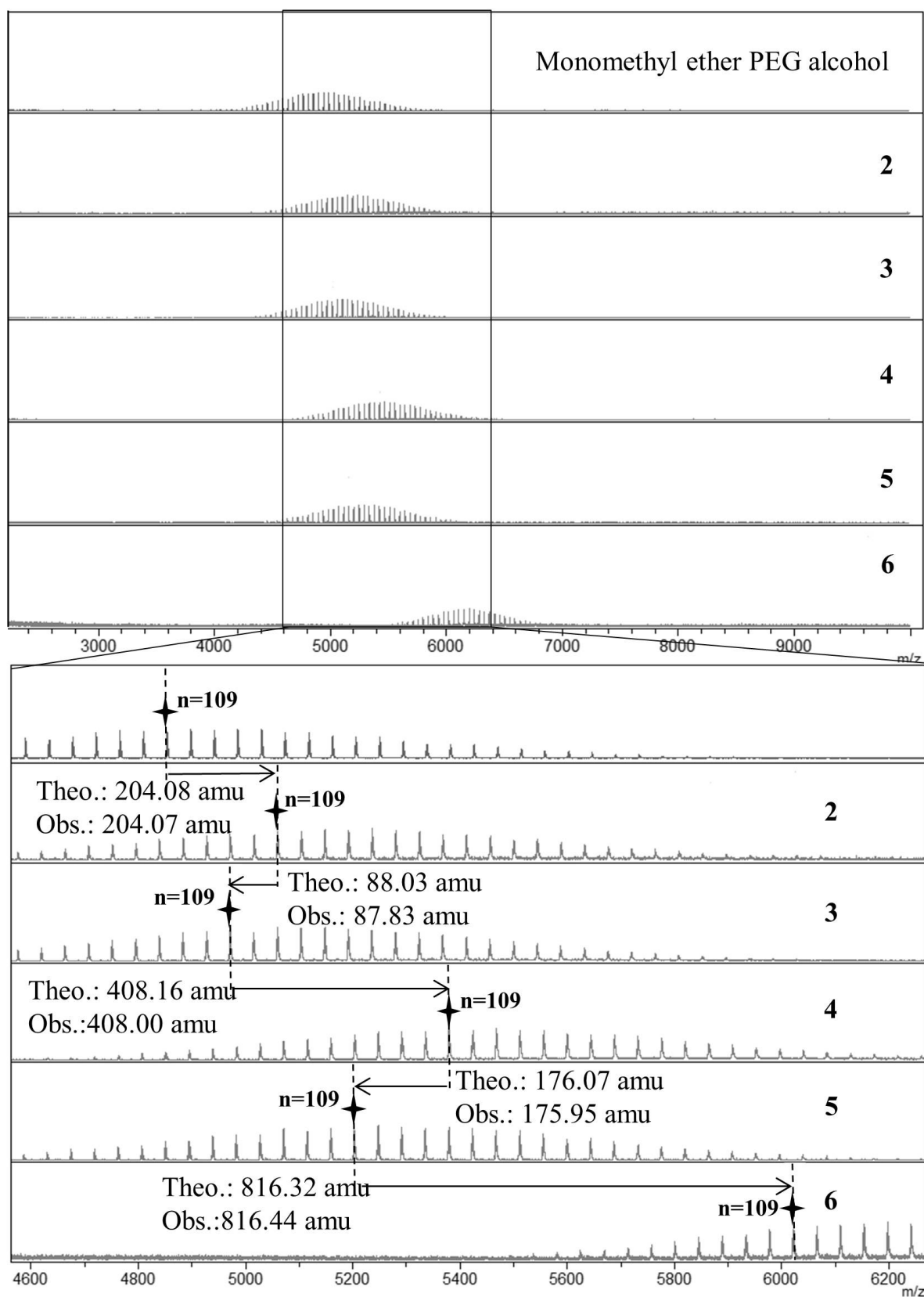


FIGURE 6 - MALDI-ToF mass spectra of the monomethyl ether PEG alcohol after each reaction (starting material and compounds 2-6). Monitoring the dendritic growth for a polymer with 109 repeat units is highlighted to demonstrate the accuracy and utility of MALDI-ToF MS as a characterization tool.

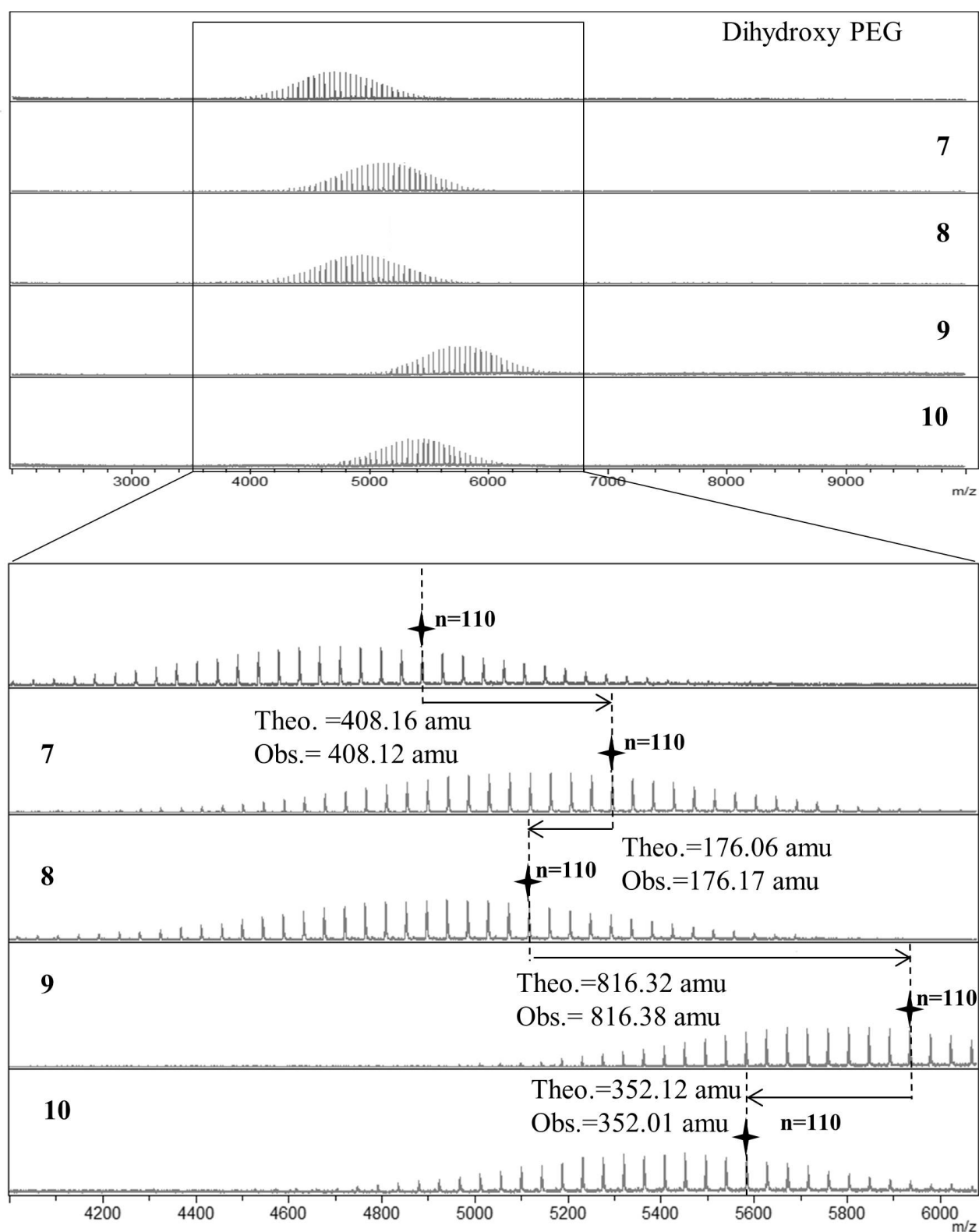


FIGURE 7 - MALDI-ToF mass spectra of the dihydroxy PEG after each reaction (starting material and compounds 7-10). Monitoring the dendritic growth for a polymer with 110 repeat units is highlighted to demonstrate the accuracy and utility of MALDI-ToF MS as a characterization tool.

taminants, but exhibited a larger than expected shift, likely because the hydroxyl-functionalized precursor can exhibit non-specific interactions with the separation media. The ^1H NMR spectrum exhibited the expected addition of aromatic (7.25-7.44 ppm) and benzylidene methyne (5.42 ppm) resonances, though these peaks were difficult to quantify due to the low signal relative to the PEG backbone. In the deprotection step, the benzylidene group of **2** could be removed to reveal a diol end group functionality in polymer **3**. The ^1H NMR data confirmed the loss of the benzylidene group, however the GPC exhibited only a negligible shift in retention time. The MALDI-ToF mass spectrum, however, clearly exhibited the removal of the benzylidene group through an observed 87.83 amu reduction in mass (theoretical 88.03 amu). Likewise, for the next esterification reaction to convert polymer **3** to the second generation dendronized PEG, **4**, the MALDI mass spectrum provided the most convincing data, with a mass increase that corresponded to the addition of two monomer units (theoretical: 408.16 amu, observed 408.12 amu). The next deprotection to yield polymer **5** could again be confirmed by a uniform loss of 175.95 amu in the mass spectrum (theoretical 176.07 amu). And finally, the purity of the third generation dendronized polymer, **6**, could be confirmed by the addition of 816.44 amu (theoretical 816.31 amu) with respect to the mass of the tetra-ol precursor, **5**. The overall yield for this series of reactions was 68%.

For the dendronization of the diol, analogous results were obtained. For the dendronization to yield the first generation dendronized-PEG dumbbell, **7**, the product is expected to exhibit a positive mass shift of 408.16 amu in its MALDI-ToF mass spectrum, and a shift of 408.12 amu was observed. Characterization by GPC showed a small decrease in retention time, while ^1H NMR (Figure 2) could be used to confirm the presence of the benzylidene-protected G1 dendron (e.g. methyne: 5.42 ppm, aryl: 7.25-7.45 ppm). After hydrogenolysis with Pd catalysts, the deprotected tetra-ol, **8**, can be identified by an expected loss of 176.06 amu (observed 176.17). Again, the GPC retention time exhibits a negligible shift, but NMR studies verify that the benzylidene protecting group has been removed, via loss of the aromatic (7.25-7.45 ppm) and benzylidene methyne resonances (5.42 ppm). The next dendronization reaction yielded the protected second generation dendronized PEG dumbbell, **9** with a mass shift corresponding to the addition of four benzylidene protected monomers (theoretical: 816.32 amu, observed: 816.38 amu) Finally, the deprotection could be carried out on polymer **9**, to yield the dendronized PEG dumbbell, **10**, with four hydroxyl end groups on each end. Again, the MALDI mass spectra could be used to confirm the loss of

all four benzylidene groups via a mass reduction of 352.01 amu (theoretical 351.12 u). The overall yield for this set of reactions was 72%.

This relatively rapid and easily scalable synthetic approach provides a reliable method for preparing hyperfunctional PEGs. In addition, the rapid and straightforward confirmation of the exceptional structural purity of the dendronized PEGs via MALDI-ToF MS makes these biocompatible materials highly attractive for biomedical research.

CONCLUSIONS

Well-defined hybrid poly(ethylene glycol)-dendron polymers have been prepared via the dendronization of the alcohol end groups of the mono and difunctional linear polymers. While GPC and NMR exhibit the expected data, they cannot alone confirm the high purity of these linear-dendritic hybrids. MALDI-ToF mass spectrometry, on the other hand, provides tremendously detailed data which can enable verification of the high structural purity of the dendronized PEG analogs. Because biomedical applications require high purity, and a well-defined degree of functionalization, these high-purity, multifunctional PEGs represent important materials for future medical research. The MALDI-ToF mass spectrometric approach described herein demonstrates the value of this characterization technique in defining the purity of polymeric materials, particularly when structural purity is critical for the specific application.

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