Arborescent Polypeptides from γ-Benzyl L-Glutamic Acid

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ABSTRACT: The synthesis of arborescent polymers with poly(γ-benzyl L-glutamate) (PBG) side chains was achieved through successive grafting reactions. The linear PBG building blocks were produced by the ring-opening polymerization of γ-benzyl L-glutamic acid *N*-carboxyanhydride initiated with *n*-hexylamine. The polymerization conditions were optimized to minimize the loss of amino chain termini in the reaction. Acidolysis of a fraction of the benzyl groups on a linear PBG substrate and coupling with linear PBG using a carbodiimide/hydroxybenzotriazole promoter system yielded a comb-branched or generation zero (G0) arborescent PBG. Further partial deprotection and grafting cycles led to arborescent PBG of generations G1 to G3. The solvent used in the coupling reaction had a dramatic influence on the yield of graft polymers of generations G1 and above, dimethylsulfoxide being preferable to *N*,*N*-dimethylformamide. This grafting onto scheme yielded well-defined ($M_w/M_n \leq 1.06$), high molecular weight arborescent PBG in a few reaction cycles, with number-average molecular weights and branching functionalities reaching over 10^6 and 290, respectively, for the G3 polymer. α-Helix to coiled conformation transitions were observed from *N*,*N*-dimethylformamide to dimethyl sulfoxide solutions, even for the highly branched polymers. © 2013 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2013**, *51*, 5270–5279

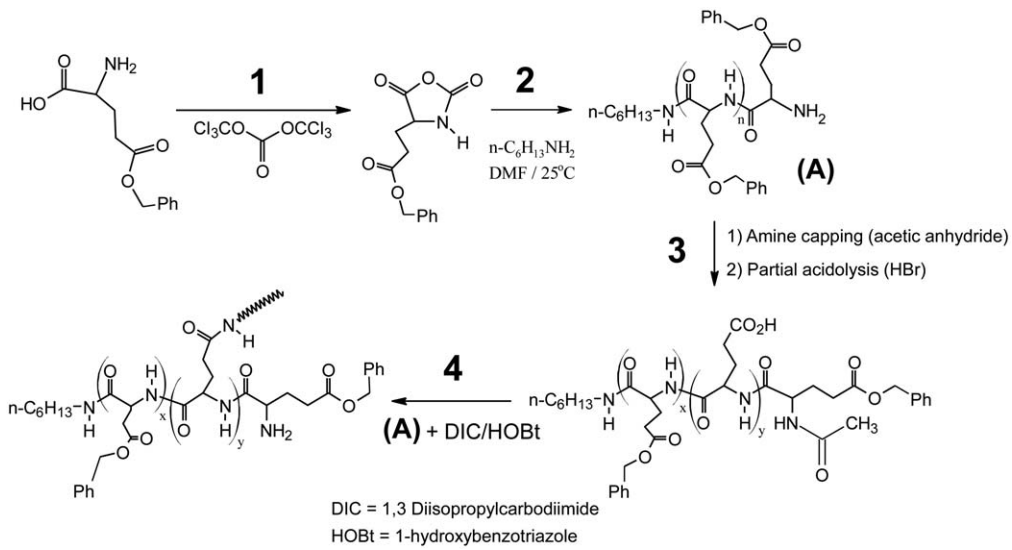
KEYWORDS: arborescent polymers; branched; chain conformation; conformational analysis; dendrigraft polymers; dynamic light scattering; polypeptides; ring-opening polymerization

INTRODUCTION Dendritic polymers have attracted much attention due to their intriguing structure and unusual properties. Many methods have been suggested to synthesize different families of dendritic macromolecules including dendrimers, hyperbranched polymers, and dendrigraft (arborescent) polymers from a wide range of monomers.[1–5] This allows tailoring of the properties of these materials to optimize their performance in different applications, among which the biomedical field certainly represents a major area of interest.[6–8] A primary concern for most biomedical uses is biocompatibility, typically requiring a lack of toxicity, immunological response, or other physiological reactions. For certain applications such as the intravenous delivery of drugs, the carriers ideally should be monodispersed and in the 10 to 100 nm size range, to enhance their ability to cross epithelial cell membranes and to avoid rapid clearance from the body through the liver or the spleen.[9] A narrow size distribution should also lead to more reproducible pharmacokinetic behavior.[10]

The earliest examples of dendritic structures included dendrimers analogous to globular polypeptides, derived from L-lysine building blocks.[11–13] The concept of dendrimers with amide-like structures was considerably expanded by Tomalia with the synthesis of polyamidoamine (PAMAM) dendrimers, now commercially available and being investigated in a number of biomedical applications.[8] Other examples of potentially biocompatible dendrimers reported in the literature include triazine (melamine) dendrimers,[14] oligosaccharide–polypeptide dendrimers,[15] glycodendrimers,[16] and PAMAM-poly(L-glutamic acid) dendrimers.[17] Hyperbranched dendritic systems in that category have also been reported, including polyglycerols[18] and polyesters.[19]

Apart from dendritic architectures, star-branched polypeptides with a wide range of branching functionalities, high molecular weights, and (in some cases) narrow MWDs have been synthesized by different methods. Hadjichristidis[20] thus obtained 3-arm star-branched polypeptides, with molecular weights reaching 1.8×10^5 and $M_w/M_n = 1.08$, by coupling polypeptides derived from γ-benzyl L-glutamic acid *N*-carboxyanhydride (Glu-NCA) and ε-benzyloxycarbonyl L-lysine NCA with a triphenylmethane-4,4′,4″-triisocyanate linker. The synthesis of peptide-based star polymers with branching functionalities $f = 3$–349 and $M_w/M_n = 1.20$–1.83, obtained mainly by an arms-first microgel-like coupling methodology,

Additional Supporting Information may be found in the online version of this article.



SCHEME 1 Synthesis of G0 arborescent PBG, with a comb-branched structure. G1–G3 dendritic structures are obtained by repetition of the partial acidolysis and grafting steps.

was reviewed recently.[21,22] Another strategy relied upon the initiation of Glu-NCA polymerization with poly(propylene imine) dendrimers carrying up to 64 primary amine functionalities, depending on the dendrimer generation number used.[23] Variations in the dendrimer to NCA ratio led to molecular weights of up to 5×10^5 and polydispersities quoted as "less than 1.2" for all the samples. A similar approach used silica nanoparticles functionalized with primary amine groups as initiator for Glu-NCA to generate star polypeptides with a silica core.[24,25]

The arborescent (or dendrigraft) systems are graft polymers incorporating polymeric chain segment building blocks similar to star-branched molecules, but they have a multilevel (dendritic) branched architecture arising from successive grafting reactions, in analogy to dendrimer generations. This graft-upon-graft strategy can yield relatively large (10–100 nm) macromolecules in a few synthetic cycles (generations), while maintaining low polydispersity indices ($M_w/M_n \sim 1.1$ typically).[26] A convenient grafting from technique was reported by Klok et al. using L-lysine derivatives to synthesize dendrigraft polypeptides.[27] This synthetic scheme involved the polymerization of a protected L-lysine NCA to produce a linear substrate, which was subsequently deprotected to generate primary amine moieties serving as initiating sites for the next generation of side chains. The method of Klok et al. yielded large dendrigraft structures (up to generation G2) but suffered from significant molecular weight distribution (MWD) broadening over successive cycles due to side reactions. A variation of this technique used the copolymerization of a protected L-lysine NCA with another amino acid NCA.[27,28] Selective deprotection of the L-lysine units provided control over the branching density of the molecules in this scheme, but the MWD broadening issue remained unsolved. The grafting from technique developed by Klok was subsequently modified by Collet et al., using trifluoroacetyl-protected L-lysine NCA.[29] In this case the polymerization was carried out in mildly acidic (pH 6.5) water, and the polypeptide was deprotected with ammonia to afford short linear poly(L-lysine trifluoroacetate) segments with a number-average degree of polymerization $X_n = 8$. The fully deprotected linear substrate served as polyfunctional initiator for the growth of protected poly(L-lysine) side chains, in analogy to the Klok procedure. Subsequent cycles of deprotection and side chain growth led to dendrigraft polymer structures of generations up to G3, with $M_n \leq 1.72 \times 10^5$ and $M_w/M_n = 1.36$–1.46. Apart from the examples provided above, the number of potentially biocompatible dendrigraft polymers currently available, and particularly those with peptide-like structures, remains limited.

We now report the synthesis of well-defined ($M_w/M_n < 1.1$) arborescent polymers from poly(γ-benzyl L-glutamate) (PBG) building blocks. This method is distinct from the ones described above, in that in relies upon a generation-based grafting onto methodology analogous to those reported for the synthesis of arborescent polymers from different vinyl monomers.[1,26] This approach enables the full structural characterization of the branched polymers (side chain and overall molecular weight, and branching functionality), while maintaining a narrow MWD over successive grafting cycles. The approach used is summarized in Scheme 1. Linear PBG S1
chains are obtained by ring-opening polymerization of γ-benzyl L-glutamic acid *N*-carboxyanhydride (Glu-NCA) with a primary amine initiator. Partial deprotection of linear PBG provides a grafting substrate with carboxylic acid moieties, that can be reacted with the primary amine terminus of PBG by standard peptide coupling techniques to create a generation zero (G0) or comb-branched polypeptide. Subsequent generations of arborescent polypeptides are obtained by successive cycles of partial deprotection and grafting reactions. The arborescent PBG molecules obtained should be

interesting as model compounds for globular proteins, and can serve as intermediates in the preparation of unimolecular micelles potentially useful for controlled drug delivery applications.

EXPERIMENTAL

Materials

N,*N*′-dimethylformamide (DMF; Aldrich, peptide synthesis grade) was purified by distillation under reduced pressure and was stored in the dark to prevent degradation due to photochemical reactions. Dimethyl sulfoxide and *n*-hexylamine were purified by stirring overnight with CaH_2 and distillation under reduced pressure. Ethyl acetate (Fisher, 99.9%) was distilled from $LiAlH_4$ under nitrogen. The purified compounds were stored under nitrogen in round-bottomed flasks (RBF) over 3A molecular sieves (EMD). γ-Benzyl L-glutamic acid (Bz-Glu; Bachem, >99%), HBr solution (Aldrich, 33% in acetic acid), *N*,*N*′-diisopropylcarbodiimide (DIC; Aldrich, 99%), 1-hydroxybenzotriazole (HOBt; Fluka, water content ca. 15% w/w), trifluoroacetic acid (TFA, Caledon), methanol (Omnisolv), diethyl ether (Omnisolv), triethylamine (TEA, EMD), acetic anhydride (Caledon), tetrahydrofuran (THF, Omnisolv), triphosgene (Aldrich, 98%), and $LiAlH_4$ (Aldrich, 95%) were used as received from the suppliers.

Characterization

Analytical SEC measurements were done on a system with a Waters 510 HPLC pump, a 50 μL injection loop, and a Waters 2410 differential refractometer (DRI) detector. A Wyatt MiniDAWN laser light scattering detector operating at a wavelength of 690 nm served to determine the absolute molecular weight of the graft polymers. The column used was a 500 mm × 10 mm Jordi Gel DVB Mixed Bed model with a linear polystyrene molecular weight range of 10^2 to 10^7. *N*,*N*-dimethylacetamide (DMA) with LiCl (1 g/L, added to minimize adsorption of the polymer on the column) at a flow rate of 1.0 mL/min served as the mobile phase at room temperature.

Preparative SEC was carried out on a system consisting of a Waters M45 HPLC pump, a 2 mL sample injection loop, a Waters R401 differential refractometer detector, and a Jordi Gel DVB 1000 Å 250 mm × 22 mm preparative SEC column. *N*,*N*-Dimethylacetamide with 0.2 g/L LiCl served as the mobile phase. The crude polymer was injected as a 30 mg/mL solution and the SEC system was operated at room temperature at a flow rate of 3.0 mL/min.

^{1}H NMR spectroscopy served to determine the degree of polymerization of the linear polymers, to monitor the deprotection level of the substrates, and to analyze the conformation of the polypeptide chains. The instruments used were Bruker 300 MHz and 500 MHz spectrometers; the latter was employed only in conformation analysis. The concentration of all the samples was 20 mg/mL and 16 scans were averaged on both instruments. ^{19}F NMR spectroscopy on a Bruker 300 MHz instrument was used to determine the chain end primary amine functionality, f_{NH2}, of the polymers used in the grafting reactions. The concentration of the samples was 30 to 35 mg/mL and 64 scans were averaged.

Titrations were performed for selected linear and arborescent partially deprotected substrates, to confirm the deprotection levels determined by ^{1}H NMR analysis. The substrate (50 mg) was added to a mixture of DMF (10 mL) and water (5 mL) with 3 drops of phenolphthalein indicator (0.5% w/v in methanol). The solution was quickly titrated to a pink coloration (stable over 30 s) with a 0.1 N NaOH solution in methanol, to minimize interference from atmospheric CO_2.

Dynamic laser light scattering (DLS) measurements were carried out to determine the hydrodynamic diameter of the arborescent PBG molecules in DMF and in DMSO. The concentration of the samples ranged from 0.5–2% w/v (depending on the generation number) and LiCl (0.05% w/v) was added to prevent aggregation. The measurements were done on a Brookhaven BI-200 SM instrument at a temperature of 25 °C and a scattering angle of 90°. The 256-channel correlator was operated in the exponential sampling mode, the last four data acquisition channels being used for the baseline measurements. The *z*-average translational diffusion coefficients used for the hydrodynamic diameter calculations were determined from first- and second-order analysis of the normalized electric field correlation function.

Synthesis of γ-Benzyl L-Glutamic Acid N-Carboxyanhydride (Glu-NCA)

The procedure used was adapted from Poché et al.[30] Bz-Glu (10.0 g; 42.0 mmol) was suspended in 300 mL of dry ethyl acetate in a 1 L RBF fitted with a refluxing condenser and a gas bubbler. The flask was purged with N_2 and heated to reflux. Triphosgene (4.8 g, 16 mmol) was then added and refluxing was continued for 3 h. Caution: triphosgene is highly toxic, so the whole procedure should be carried out in a well-vented fume hood. The flask was then removed, stoppered, and cooled in a freezer (−10 °C) for 1 h. The solution was transferred to a cold separatory funnel and quickly washed successively with 100 mL of ice-cold water and 100 mL of chilled 0.5% aqueous $NaHCO_3$ solution. The organic phase was dried over anhydrous $MgSO_4$, filtered, and concentrated to 100–120 mL on a rotary evaporator. An equal volume of cold hexane was then added to induce crystallization of the product. The mixture was left in the freezer overnight and the solid product was recovered by filtration in a Schlenk funnel under N_2. It was then dried overnight under vacuum, and stored under N_2 in a refrigerator (4 °C).

Yield = 10.2 g (92%); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.58–7.24 (s, 5H), 6.75 (s, 1H), 5.11 (s, 2H), 4.38–4.33 (t, 1H), 2.59–2.53 (t, 2H), 2.35–2.21 (m, 1H), 2.21–2.02 (m, 1H).

Synthesis of Poly(γ-benzyl L-glutamate)

A linear polymer serving as side chain material (sample PBG-41) was synthesized by dissolving Glu-NCA (8.00 g, 30.4 mmol) in dry DMF (20 mL) in a 50 mL RBF at 0 °C. After the monomer was dissolved *n*-hexylamine (200 μL, 1.52 mmol, for a target $X_n = 20$) was added with rapid stirring.

The evolution of CO_2 was noticeable at the beginning, and the reaction was allowed to proceed for 2.5 to 3 days at 0 °C. The linear polymer was recovered by precipitation in methanol and suction filtration, and dried under vacuum overnight.

Yield = 80%, $M_w/M_n = 1.10$. ^{1}H NMR (300 MHz, d_6-DMSO): $X_n = 26.0$, δ: 8.04 (b, 26H), 7.48–7.20 (s, 130H), 5.02–4.89 (s, 52H), 4.10–3.88 (b, 26H), 2.34–1.91 (b, 104H), 1.33–1.18 (b, 10H), 0.78–0.76 (s, 3H).

Quantification of Primary Amines by ^{19}F NMR Analysis

The linear polymers were analyzed for their terminal primary amine content by ^{19}F NMR spectroscopy. For example, a linear PBG sample (PBG-A; 0.119 g, $M_n = 5300$, 2.25×10^{-5} mol chains) was dissolved in 3 mL of deuterated DMSO (d_6-DMSO). A solution of trifluorobenzaldehyde (TFBA, 0.1191 g, 6.84×10^{-4} mol) and benzotrifluoride (BTF, 0.1014 g, 8.15×10^{-4} mol; internal standard) in 2 g of d_6-DMSO was prepared. The reagent solution (0.2306 g, 7.07×10^{-5} mol TFBA, 7.18×10^{-5} mol BTF) was added to the polymer solution and stirred for 2 h; a 0.5 mL sample was then transferred to an NMR tube for analysis. The peak areas from the ^{19}F NMR spectra were used to determine the f_{NH2} values as described in the Results and Discussion section.

Synthesis of PBG Precursor for Grafting Substrate

A linear PBG sample (PBG-34) serving as substrate for the preparation of a G0 (comb-branched) polypeptide was synthesized as described above with minor modifications: The target X_n was 50, the reaction was performed at room temperature, and it was quenched with acetic anhydride to deactivate the terminal amine moiety. The sample was synthesized from Glu-NCA (2.0 g, 7.6 mmol) in 5.0 mL of DMF and *n*-hexylamine (20 μL, 0.15 mmol) at room temperature. After 4 h acetic anhydride (290 μL, 3.1 mmol) was added and the reaction was stirred for 1 h before sample recovery.

Yield = 1.5 g (90%), ^{1}H NMR (300 MHz, d_6-DMSO): $X_n = 51$, δ: 8.04 (b, 51H), 7.48–7.20 (s, 255H), 5.02–4.89 (s, 102H), 4.10–3.88 (b, 51H), 2.34–1.91 (b, 204H), 1.33–1.18 (b, 10H), 0.78–0.76 (s, 3H), SEC: $M_w/M_n = 1.19$.

Partial Deprotection of Linear PBG Substrate

PBG-34 ($X_n = 51$, 1.46 g, 6.66 mmol Bz-Glu units) was dissolved in TFA (15 mL) and 0.35 mL of 33% (w/w) HBr solution in acetic acid (0.14 g HBr, 0.25 equiv HBr per Bz-Glu residue) with stirring. After 3 h the polymer was precipitated in diethyl ether and recovered by suction filtration to give an orange solid. After drying the polymer was dispersed in 10 to 12 mL of THF and enough DMF (ca. 1–2 mL) was added to obtain a clear solution. The polymer was precipitated again in diethyl ether to obtain a white product. Yield = 0.92 g (72%), 31 mol % of free glutamic acid moieties.

Synthesis of G0 Arborescent PBG

The partially deprotected polymer serving as substrate [PBG-34-CO_2H, 0.141 g, 0.220 mmol –CO_2H] and the polymer serving as side chains [PBG-64, 1.10 g, 0.275 mmol chains] were dissolved in 6 mL of dry DMF in a 25-mL RBF. The peptide coupling reagents DIC (1.72 mL of 10% v/v solution in DMF, 1.10 mmol) and HOBt (0.149 g, 1.10 mmol) were then added to the reaction with TEA (0.8 mL, 5.5 mmol). The reaction was allowed to proceed for 24 h at room temperature before adding *n*-hexylamine (0.50 mL, 4.94 mmol), to deactivate residual activated carboxylic acid sites. After 1 h the product was precipitated in methanol and recovered by suction filtration. Linear PBG contaminant was removed from the G0 crude polymer by preparative size exclusion chromatography (SEC). The purified G0 polymer was recovered by evaporation to dryness under high vacuum, dissolution in TFA, and precipitation in methanol.

Synthesis of Upper Generation (G1-G3) Arborescent PBG

The purified G0 polymer (G0–52, 0.400 g, 1.82 mmol Bz-Glu units) was first partially deprotected by dissolution in TFA (4 mL), and 0.13 mL of 33% (w/w) HBr solution in acetic acid (0.044 g HBr, 0.30 equiv HBr per Bz-Glu residue) was added with stirring. After 3 h the polymer was recovered and further purified as described for the linear sample. Yield = 0.240 g, (68%), 32 mol % glutamic acid moieties. The deprotected G0 polymer (G0–52-CO_2H, 0.212 g, 0.356 mmol –CO_2H) was coupled with linear side chains [PBG-64, 1.90 g, 0.445 mmol chains] by the same method described for the G0 reaction, but using DMSO (8 mL) rather than DMF as solvent. The crude G1 polypeptide was purified by preparative SEC as described for the G0 polymer. The G2 and G3 arborescent PBG samples were synthesized and purified as described for the G1 sample.

RESULTS AND DISCUSSION

Several methods reported in the literature for the synthesis of PBG have led to different results in terms of yield and their ability to minimize side reactions. The PBG building blocks serving in the synthesis of arborescent polypeptides should ideally be obtained in high yield, have a narrow MWD and a predictable X_n, and preserve their primary amine terminus. Beyond the influence of monomer purity and the activated monomer polymerization mechanism, Mitchell et al. proposed as early as 1957 that cyclization into a lactam structure (Scheme S1, Supporting Information) during the polymerization or sample storage was the dominant side reaction affecting the primary amine terminus of linear PBG.[31] Preservation of the amino group is essential for the grafting reaction: Deactivation of this moiety by side reactions yields linear PBG chains incapable of coupling with the substrate.

One approach suggested to minimize side reactions in the polymerization of NCA monomers was to use the hydrochloride salt form of amine initiators, to decrease the reactivity of the primary amine propagating center.[32,34] For example, block copolymers with poly(ε-benzyl carbamate L-lysine) contents of 66–70% by weight were synthesized at 40 to 80 °C from an amine-terminated polystyrene macroinitiator in the hydrochloride form (PS-NH_2·HCl, $X_n = 52$), $M_w/M_n < 1.03$

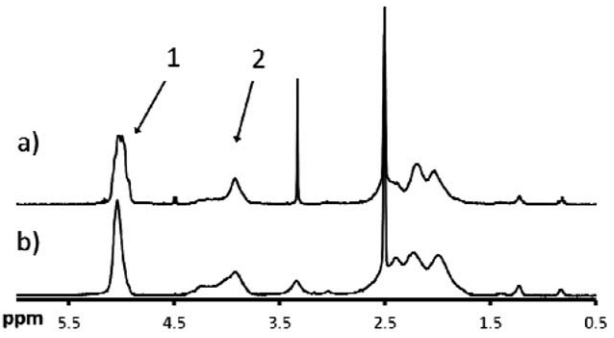


FIGURE 1 ^{1}H NMR spectra for PBG-34 (a) before and (b) after partial deprotection with HBr. The peaks labelled as 1 and 2 correspond to the benzylic methylene and the backbone methine protons, respectively.

being reported for the product.[33] Short PBG segments ($X_n \sim 8$, $M_n = 2000$) were also grown from a poly(ethylene oxide) macroinitiator ($X_n \sim 110$, $M_n = 5000$) terminated with a primary amine in the hydrochloride form in DMF at 40 to 60 °C. Values of $M_w/M_n < 1.05$ were likewise reported,[34] but it is clear that the short PBG segment extending the PEO chain had a limited influence on the overall polydispersity of the block copolymers obtained under these conditions. Aliferis et al. rather relied on the high vacuum break-seal technique to create a strictly anhydrous environment and eliminate impurities causing side reactions.[35] This approach yielded high molecular weight PBG ($M_n \sim 10^5$) with $M_w/M_n < 1.20$.

The preferred method to generate the linear PBG building blocks in the current investigation was the polymerization of Glu-NCA at 0 °C, as suggested by Vayaboury et al.[36] Using MALDI-TOF analysis, Habraken et al. indeed confirmed recently that this approach was beneficial for the polymerization of various amino acid N-carboxyanhydrides, in terms of avoiding cyclization and other side reactions, since these occurred mainly after complete monomer conversion was achieved.[37,38] Cyclization should also be minimized by maintaining PBG at lower temperatures during sample isolation and storage. This approach was deemed to yield a satisfactory fraction of primary amine-terminated chains on the basis of ^{19}F NMR analysis results discussed in the grafting reaction section. The results obtained for the polymerization of Glu-NCA at either 0 or 25 °C are compared in Table S1 (Supporting Information). A monomer to initiator ratio $M/I = 50$ was used for the polymer serving as linear substrate ($M_n \sim 11{,}000$), and M/I = 23 ($M_n \sim 5000$) for the polymers used as side chains in the subsequent grafting reactions. Side chains synthesized under different conditions were initially used in the grafting reactions. A sample synthesized at 0 °C gave a grafting yield (defined as the fraction of side chains becoming attached to the substrate) of 65%, as compared with 58% for a sample synthesized at room temperature. The side chains obtained at 0 °C also had somewhat lower polydispersities (Supporting Information Table S1). These findings are in agreement with the MALDI-TOF analysis results of Habraken et al., who found that side reactions were minimized and the fraction of chains carrying a reactive primary amine terminus was highest for PBG chains synthesized at 0 °C.[37,38] Consequently, all subsequent polymerizations were carried out under these conditions.

The treatment of PBG with HBr allowed the cleavage of a desired fraction (ca. 25–35%) of benzyl ester protecting groups to generate coupling sites, in analogy to other arborescent polymer syntheses.[1,26] The extent of deprotection was monitored by ^{1}H NMR spectroscopy. The most reliable peaks for the analysis were the benzylic protons (2H at 4.9–5.0 ppm), present only on repeating units with the benzyl ester protecting group, and the methine protons (1H at 3.7–4.4 ppm) present in each repeating unit, unaffected by the deprotection reaction. An example is provided in Figure 1 for PBG-34-CO_2H, where the integration of the benzylic protons (1.38/2H, protected units only) divided by the integration of the methine protons (1.00/1H in each repeating unit) yielded the fraction of protected repeating units (0.69, or 69%). The corresponding deprotection level was therefore 0.31 or 31%.

For G0–G2 arborescent polypeptides the ratio of benzylic to methine protons was not exactly 2:1 before deprotection but rather 1.8:1, due to the acidolysis steps carried out in the previous reaction cycles. This was taken into account when comparing spectra before and after deprotection for the higher generation substrates. Typical results for the partial deprotection of linear and branched substrates by acidolysis are provided in Table 1. Analysis of the deprotection level by ^{1}H NMR spectroscopy and by titration yielded comparable results for the linear polypeptides. The graft polymers were more difficult to titrate due to their decreased solubility leading to precipitation during titration. The results obtained for sample G0–62 nevertheless demonstrate that ^{1}H NMR analysis and the titration procedure yielded consistent deprotection levels even for the branched substrates. It has been reported that deprotection with HBr/HOAc-TFA may lead to peptide chain cleavage and is especially pronounced if the reactions are not performed under strictly anhydrous conditions.[39] In the case of partial deprotection (0.30 equivalents HBr added with respect to the benzyl ester moieties), all the HBr is consumed over the 3 h reaction period while maintaining anhydrous conditions. This should limit the

TABLE 1 Partial Deprotection of PBG Substrates

Sample	Mole Ratio HBr: Benzyl Ester Units	Mol % Deprotection	
		^{1}H NMR	Titration
PBG-19-CO_2H	0.25:1	34	31
PBG-34-CO_2H	0.25:1	31	30
G0-52-CO_2H	0.3:1	32	–
G0-53-CO_2H	0.3:1	32	–
G0-62-CO_2H	0.3:1	41	38
G1-2-CO_2H	0.2:1	16	–
G2-3-CO_2H	0.3:1	26	–

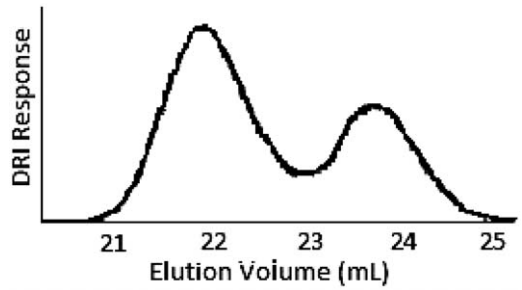


FIGURE 2 SEC Analysis of crude sample G0PBG in Table 2 (62% grafting yield).

occurrence of chain cleavage during deprotection. To confirm this for the branched polypeptides, SEC analysis was performed before and after deprotection to detect the occurrence of any degradation or chain cleavage. For example, a deprotection reaction monitored for G0–52 (Table 1) led to a decrease in molecular weight from $M_n = 54{,}000$ to 47,000, which corresponds to 32% deprotection of the benzyl ester moieties, as determined by ^{1}H NMR analysis. The SEC traces for G0–52 and G0–52-CO_2H are compared in Supporting Information Figure S1. The M_w/M_n values for G0–52 and partially deprotected G0–52-CO_2H were 1.04 and 1.05, respectively. It is therefore clear that no significant degradation occurred during deprotection of the arborescent PBG.

Coupling of the HOBt-activated substrate with the side chains is illustrated in Supporting Information Scheme S2; the initial activation of the carboxylic acid groups with DIC is not shown. The diisopropylurea by-product formed in the reaction is relatively soluble and easily eliminated by precipitation of the graft polymers. The success of the grafting reaction can be quantified in terms of grafting yield and coupling efficiency. The grafting yield, defined as the fraction of the side chains in the reaction becoming attached to the substrate, can be estimated from the relative areas of the peaks for the graft polymer and the side chains in the SEC analysis of the crude product. Taking as an example sample G0PBG (Fig. 2), generated from substrate PBG-34-CO_2H ($M_n \sim$ 11,000, 31 mol% –CO_2H functionalities) and side chains PBG-38 ($M_n \sim$ 6600, not shown in Supporting Information Table S1), the peaks on the left (graft polymer) and the right (linear contaminant) have areas (in arbitrary units) of 71,500 and 38,800, respectively. To take into account the weight fraction of the substrate in the graft polymer (10.7%), the peak area for the graft polymer can be corrected as $71{,}500 \times 0.893 = 63{,}800$. A grafting yield of $63{,}800/(63{,}800 + 38{,}800) = 0.622$ (62%) is thus calculated. The coupling efficiency, defined as the fraction of active sites on the substrate consumed in the grafting reaction, corresponds to the ratio of the number of side chains grafted to the number of coupling sites available on the substrate. This calculation requires the knowledge of the absolute molecular weight of both components. Consequently the absolute M_n of the graft polymers was determined on a SEC system equipped with a MALLS detector, while the M_n of the side chains was determined by ^{1}H NMR analysis (since the SEC-MALLS signal was too noisy). Thus sample G0PBG had $M_n = 53{,}000$, while $M_n = 11{,}000$ and 6600 for the substrate and the side chains, respectively. This gives a number-average branching functionality $f_n = (53{,}000–11{,}000)/6600 = 6.4$ chains per graft polymer. Since the linear substrate had $X_n = 51$ and a deprotection level of 31 mol %, corresponding to $51 \times 0.31 = 15.8$ coupling sites on average, a coupling efficiency of $6.4/15.8 = 0.405$ (41%) was achieved for sample G0PBG. The grafting yield observed in the reactions is obviously determined in part by the loss of the primary amine termini on the side chains. To compensate for the presence of unreactive chain ends (*vide infra*), a 25% excess of side chains was used in all the reactions. This excess also contributed to decreasing the grafting yield. The coupling efficiency (and indirectly also the grafting yield) depend on the accessibility of the coupling sites on the substrate during the grafting reaction. Any coupling sites remaining on the substrate necessarily become more hindered as the grafting reaction proceeds, making further grafting reactions more difficult. To ensure that the grafting yield was maximized, SEC analysis was performed after 2, 4, 12, and 24 h. The corresponding grafting yields were 37, 50, 62, and 62%. Consequently the reaction was already completed after 12 h for the G0 polymer synthesis, but it was nonetheless allowed to proceed for 24 h as a precaution.

The grafting procedures purposely used a 25% molar excess of side chains in the reactions, to maximize the coupling efficiency at the expense of the grafting yield. For the same reason the coupling agents (DIC and HOBt) were used in fivefold excess to activate all the carboxylic acid sites, and triethylamine was added to ensure that the amino termini of the side chains remained in their primary amine (nonprotonated) form throughout the reaction. The addition of *n*-hexylamine at the end of the grafting reaction converted all the remaining activated coupling sites to amides, so that they would not be available in future grafting reactions and lead to structural imperfections.

The synthesis of the upper generation (G1–G3) arborescent polymers was carried out by the same method as the G0 materials, but DMSO was substituted for DMF as the solvent in the reaction. DMSO was used initially because it is known that the formation of α-helices occurs for PBG in both its protected[40] and fully deprotected [poly(glutamic acid)] forms in DMF.[41,42] It was felt that the formation of α-helices by the G0 arborescent polymer substrate and/or the side chain material could potentially hinder the diffusion of the side chains to the coupling sites, and thus limit the grafting yield attained. Conversely a non-helicogenic solvent such as DMSO, by favoring a randomly coiled conformation for the substrate and the side chains, might enhance the accessibility of the coupling sites.

The conformation of the G0 substrate and the side chains was investigated by ^{1}H NMR spectroscopy in both solvents, since distinct signals are expected for the methine proton in the benzyl glutamate units when the chains adopt either α-helical or random coil conformations.[40,43] A study of PBG by Maeda

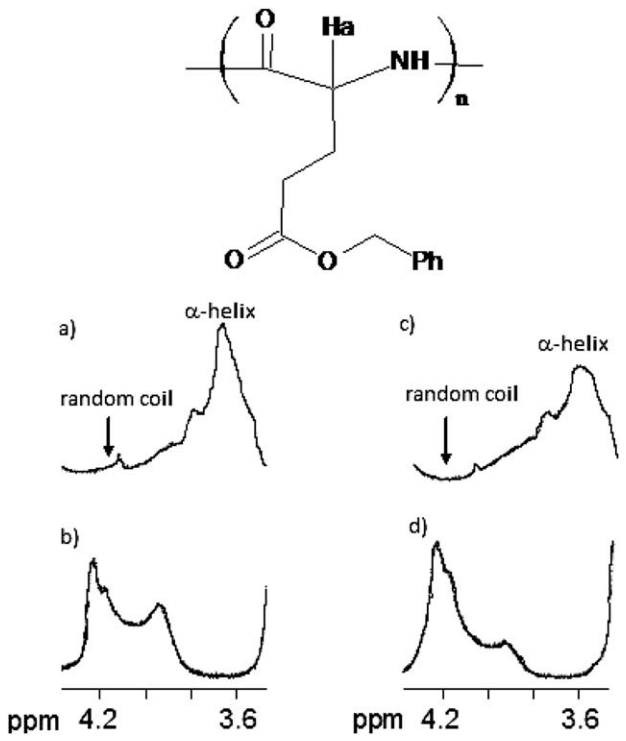


FIGURE 3 ^{1}H NMR spectra for the methine protons of G0 and G3 arborescent PBG in d_7-DMF (a, c) and in d_6-DMSO (b, d).

et al. confirmed the formation of random coils in DMSO for linear chains with $X_n = 11$ and 26,[40] a size range comparable to the polymers serving as side chains in the current investigation. ^{1}H NMR spectra for the G0 and G3 arborescent PBG polymers, recorded in DMF and in DMSO, are compared in Figure 3; the G1 and G2 polymers yielded similar results (not shown). Surprisingly, in spite of their highly branched structure, the arborescent PBG molecules behave the same way as the linear samples, α-helices being formed predominantly in DMF while random coils are observed in DMSO.

Beyond the potential influence of the chain conformation of PBG, it was brought to our attention that DMF is susceptible to contamination by traces of cyanide ions forming quickly after its purification.[44–46] Careful purification without exposure to light was determined to be the best method to avoid this problem,[44] and when using this solvent the grafting reactions indeed proceeded with a yield comparable to the G1 synthesis performed in DMSO. The lack of influence of DMF contamination in the synthesis of the G0 polymers could be explained by a faster coupling rate for the linear than for the G0 substrate, leading to insignificant competition between the rate of photogeneration of the impurities and the coupling reaction. While purified DMF and DMSO appear equally suitable as solvents for the coupling reaction, the purification of DMSO is less problematic than DMF from a practical viewpoint. For that reason, subsequent reactions were carried out exclusively in DMSO. To ensure that the grafting yield was maximized for the reactions involving the G0 substrates, SEC analysis was performed on samples removed from the G1 PBG synthesis in DMSO. After reaction times of 2, 4, 12, 24, and 30 h, grafting yields of 32, 46, 51, 53 and 53% were achieved, respectively. The grafting yield was therefore maximized after 24 h.

The grafting yields achieved for the arborescent PBG system remain quite low as compared to other arborescent systems synthesized by the anionic grafting onto method.[1,26] To investigate this further, a grafting reaction was performed in which the unreacted side chains were isolated by preparative SEC and analyzed to determine their fraction of amine-terminated chains. If the limiting factor in the grafting reaction were the instability of the amine termini, the unreacted side chains should not contain any residual primary amine groups. After isolation of the linear contaminant by preparative SEC, a technique outlined by Ji et al.[47] was used to quantify the fraction of amine-terminated (active) chains in the PBG sample. In this method either 3,5-*bis*-(trifluoromethyl)benzaldehyde (BTFBA) or 4-trifluorobenzaldehyde (TFBA) can be reacted to produce imine chain ends, and ^{19}F NMR spectroscopy serves to quantify the amount of imine formed with respect to benzotrifluoride (BTF) added as an internal standard. A threefold excess of aldehyde is used to ensure complete reaction of the chain ends in less than 2 h. TFBA was preferred for the analysis of PBG in practice, since there was overlap of the signals for BTFBA and the PBG-imine. An example of analysis for a linear sample (PBG-A in Supporting Information Table S1), synthesized at 0 °C, is provided in Figure 4(a). An amine functionality level $f_{NH2} > 98\%$ was obtained. The ^{19}F NMR analysis procedure was repeated for two samples of the same polymer 4 weeks later: one stored in the refrigerator (5 °C), and the other one at room temperature, both in powder form and under nitrogen. The f_{NH2} of these samples decreased to 90 and 78% respectively (^{19}F NMR spectra not shown). These analysis results confirm the hypothesis by Mitchell et al. that chain end cyclization could occur even after the polymerization was completed.[31] The sample with $f_{NH2} = 90\%$ was then used in a grafting reaction with a G0 substrate with a deprotection level of 30%, so that isolation of the side chains could be achieved easily from the G1 graft polymer. The ^{19}F NMR spectrum obtained for the unreacted side chains isolated from the crude grafting product [Fig. 4(b)], after 24 h of reaction (to maximize the grafting yield), corresponds to $f_{NH2} = 16\%$. Since a fraction of the chains recovered was still active after the grafting reaction, the loss of amine chain ends clearly does not suffice to explain the low grafting yields observed.

Aliferis et al. performed coupling reactions to generate 3-arm star copolypeptides from linear PBG. Their coupling technique used isocyanate groups, that are highly reactive towards primary amines, but it was still determined that a 30% excess of linear PBG and 4 weeks of reaction were necessary to complete the reaction.[20] This suggests that, even in the synthesis of these simple 3-arm star copolypeptides, steric hindrance was a factor to be reckoned with. The arborescent PBG substrates used in this work are far more congested than the star polypeptides, due to their highly

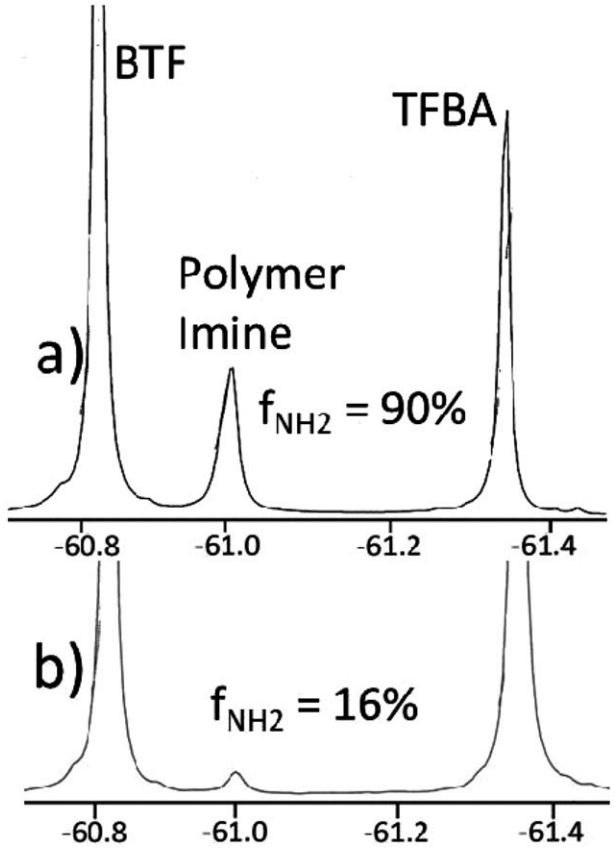


FIGURE 4 ^{19}F NMR analysis of (a) PBG-A polymerized at 0 °C, (b) unreacted PBG-A side chains isolated from the grafting reaction.

branched architecture, so the influence of steric congestion should be that much more significant. Consequently, steric congestion (as opposed to chain end cyclization) is likely at the origin of the relatively low grafting yields observed in the arborescent PBG syntheses.

The synthesis of a series of arborescent PBG samples of generations G0–G3 is illustrated in Figure 5 with their SEC elution curves. It is clear that the elution volume of the graft polymers decreases over successive generations as expected, while the breadth of the peaks remains relatively constant.

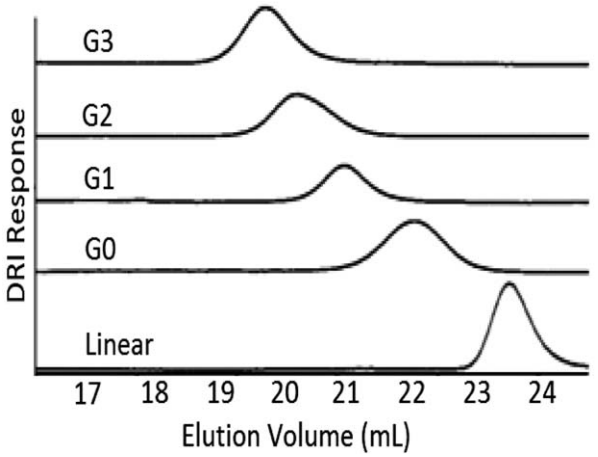


FIGURE 5 SEC traces for purified arborescent PBG samples up to G3.

The corresponding characterization data are summarized in Table 2. The values of $M_w/M_n < 1.06$ obtained for all the samples highlight the success of the grafting onto scheme developed for the synthesis of arborescent polypeptides: Polymers with M_n values reaching over 10^6 were obtained in only four grafting cycles, while a narrow MWD was maintained over successive generations. This contrasts with the situation encountered when arborescent polylysines were synthesized from their NCA derivatives by a grafting from methodology (branches grown from the substrate):[27] M_w/M_n values of 1.3–1.5 were obtained for G0–G2 arborescent poly(Z-lysine), as well as for the analogous poly(TFA-lysine) systems. Even for the improved procedure of Collet et al.,[29] a polydispersity index of 1.46 was obtained for a G3 polymer with $M_n = 1.72 \times 10^5$. The molecular weight of the arborescent polylysines obtained in both cases was also lower than in the current investigation. A significant advantage of these grafting from procedures remains the minimized formation of linear polylysine contaminant in the reactions, which greatly simplifies sample purification. It is also clear that the NCA derived from the protected lysine and glutamic acid monomers are not sensitive to the same types of side reactions.

The number-average branching functionality of the arborescent polypeptides f_n, defined as the number of side chains

TABLE 2 Characteristics of Arborescent PBG Samples of Successive Generations

		DRI	MALLS				
Sample	M_n Side Chains[a]	M_n^{app} [b]	M_n	M_w/M_n	G_y (%)[c]	C_y (%)[d]	f_n [e]
G0PBG	6600	1.80×10^4	5.3×10^4	1.04	62	41	6.6
G1PBG	4000	3.93×10^4	1.3×10^5	1.06	38	30	21
G2PBG	3900	8.31×10^4	4.9×10^5	1.03	46	50	96
G3PBG	3900	1.34×10^5	1.1×10^6	1.03	32	21	165

[a] From ^{1}H NMR analysis.
[b] Apparent M_n from a linear polystyrene standards calibration curve.
[c] Grafting yield from SEC analysis using a DRI detector.
[d] Fraction of coupling sites on the substrate consumed in the reaction.
[e] Branching functionality: number of branches added in the last grafting cycle.

TABLE 3 Hydrodynamic Diameter of Arborescent PBG

	DMF[a]		DMSO[a]	
	d_{h1}	d_{h2}	d_{h1}	d_{h2}
G1PBG	10.7	8.4	15.7	14.1
G2PBG	13.1	12.1	21.3	20.1
G3PBG	24.5	23.5	34.5	32.5

[a]All values are in nm; 0.05% LiCl added to suppress aggregation.

added in the last grafting reaction, is also reported in Table 2. The branching functionality increases over successive generations, since more coupling sites are available after each grafting cycle. On the other hand, it also becomes difficult for the coupling sites to react in the more crowded upper generation substrates. The branching functionality thus increases 4.2-, 4.4-, and 2.3-fold when grafting onto the G0, G1, and G2 substrates, respectively. The modest increases in branching functionality and M_n observed in the synthesis of the G3 polymer, in particular, are attributed to the dense structure of the G2 substrate making it difficult for the linear side chains to diffuse to the coupling sites. Since the same batch of side chains was used to synthesize the G2 and G3 polymers, possible variations in coupling efficiency due to fluctuations in the fraction of active (primary amine-terminated) side chains can be excluded. Similar decreases in molecular growth rate were observed when grafting onto G2 substrates in the synthesis of other arborescent polymers by analogous schemes.[1,26] The coupling efficiency and the branching functionality should be correlated to some extent, as they both depend on the accessibility of the reactive sites.

Comparison of the apparent (polystyrene-equivalent) and absolute M_n values obtained for the samples (columns 3 and 4 of Table 2, respectively) shows that the apparent molecular weights are strongly underestimated for the dendritic polymers in all cases, similarly to all other arborescent systems.[1] While these results are consistent with a highly branched molecular structure, the usefulness of this comparison is somewhat limited in practice since, strictly speaking, the comparison should be done with linear PBG samples rather than with linear polystyrene. Unfortunately such calibration standards are not available for comparison, in contrast to the arborescent polystyrene systems investigated previously.[26]

Dynamic light scattering (DLS) measurements were performed on arborescent PBG samples of successive generations, to compare their hydrodynamic diameter (d_h) in DMF and in DMSO. The results obtained are summarized in Table 3. First- and second-order analysis of the correlation function provides information on the size dispersity of the system. For a strictly monodispersed sample the results from first- and second-order analysis of the DLS correlation function should be identical, since the correlation function can be represented by a monoexponential decay.[48] The relatively small differences between the numbers reported in Table 3 for the first- versus second-order analysis results are therefore indicative of a uniform molecular size distribution, in agreement with the low M_w/M_n values reported in Table 2. However it is clear that there is a significant difference in d_h between the DMF and DMSO solutions for all the generations. A low concentration of a salt (0.05% w/v LiCl) was added to both solvents used in the DLS measurements as aggregation was otherwise apparent, particularly in DMF. ^{1}H NMR spectra were also compared before and after the addition of salt at the same concentration, to ensure that it had no influence on the α-helix versus random coil conformations of the chains. The smaller d_h values obtained in DMF are attributed to the more compact α-helix conformation adopted by the PBG chains (as confirmed by ^{1}H NMR analysis, Fig. 3) in comparison to the randomly coiled chains in DMSO. This result is surprising since a conformation change is observed even for the highly crowded G3 polymer structure, containing 289 PBG side chains in all.

CONCLUSIONS

The results presented show that well-defined arborescent polypeptides can be synthesized in a controlled fashion over successive generations. Narrow MWD ($M_w/M_n \leq 1.06$) were maintained for molecular weights reaching about 10^6 over four grafting cycles. The low grafting yields (30–65%) and coupling efficiencies (20–60%) attained in these reactions are attributed to steric congestion due to the highly branched nature of the arborescent structures. ^{1}H NMR and dynamic light scattering confirmed α-helix to coiled conformation transitions from *N*,*N*-dimethylformamide to dimethyl sulfoxide solutions, even for the highly branched polypeptides.

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