International Journal of
Translational Medicine

MDPI

Supplementary Materials

Influence of the Core Branching Density on Drug Release from Arborescent Poly(γ-benzyl L-glutamate) End-Grafted with Poly(ethylene oxide)

Mosa Alsehli [1,2] and Mario Gauthier [2,*]

[1] Department of Chemistry, Taibah University, P.O. Box 344, Medina, Saudi Arabia; mhsehli@taibahu.edu.sa
[2] Department of Chemistry, Institute for Polymer Research, University of Waterloo, 200 University Ave. W., Waterloo, ON N2L 3G1, Canada; mario.gauthier@uwaterloo.ca
* Correspondence: mario.gauthier@uwaterloo.ca

Synthesis of Glu(OtBu)$_2$-Poly(γ-benzyl L-glutamate) [(tBuO)$_2$-PBG)]

Linear (tBuO)$_2$-PBG serving as side chains for the last grafting cycle of the arborescent PBG core syntheses was obtained as described previously [1]. It was first pointed out by Knobler et al. in 1964 that primary amine salts are at equilibrium with their non-protonated form, which is capable of NCA initiation through the normal amine mechanism [2]. The propagating chains should also remain at equilibrium with their non-protonated (reactive) state. Since the equilibrium strongly favors the protonated (dormant) state of the primary amine, the chain-end is unreactive most of the time. When the hydrochloride salt dissociates to its non-protonated state, it can quickly react with the NCA monomer. For the reaction, γ-benzyl L-glutamic acid *N*-carboxyanhydride (Glu-NCA; 9.2 g, 35.0 mmol) was dissolved in dry DMF (23 mL) at 40 °C. The *tert*-butyl diester of glutamic acid H-Glu(OtBu)-OtBu·HCl (414 mg, 1.39 mmol, for a target X_n = 25) was dissolved in dry DMF (2 mL), added as initiator to the monomer, and the reaction was allowed to proceed for 7 days at 40 °C. The reaction was cooled to room temperature and triethylamine (0.25 mL, 1.84 mmol) was added. After 5 min the linear polymer was recovered by precipitation in cold methanol, centrifugation and drying under vacuum overnight before characterization. Yield = 68%, M_w/M_n = 1.17. ^{1}H NMR (300 MHz, DMSO-d_6): δ: 8.01-7.7 (b, 1H), 7.26–7.20 (s, 5H), 5.01–4.88 (s, 2H), 4.30–3.85 (b, 1H), 2.30–1.68 (b, 4H), 1.31 (s, 0.75H), X_n = 24.0.

Synthesis of Chain End-Functionalized G1 and G2 Arborescent PBG Substrates

The chain end-functionalized arborescent PBG substrates were obtained by grafting glutamic acid-functionalized PBG linear chains in the last grafting cycle as described previously [1]. For example, the G1 polymer was synthesized from the partially deprotected G0PBG substrate (0.27 g, 0.438 mmol -CO_2H) and (OtBu)$_2$-PBG side chains (2.22 g, 0.398 mmol chains), dissolved in 22 mL of dry DMSO. The peptide coupling reagents DIC (0.377 mL, 2.41 mmol) and HOBt (0.325 g, 2.41 mmol) were then added to the reaction with TEA (0.28 mL, 1.99 mmol) as a base. The reaction was allowed to proceed for 36 h at room temperature before adding *n*-hexylamine (0.29 mL, 2.89 mmol), to deactivate residual activated carboxylic acid sites. After 3 h the product was precipitated in cold methanol and recovered by suction filtration. The product was dissolved in DMF, purified by preparative SEC, precipitated in methanol, recovered by suction filtration, and dried under vacuum overnight before characterization. The G2 arborescent polymer was synthesized and purified as described for the G1 sample.

M_w/M_n = 1.07 (SEC-LS). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.01-7.7 (b, 1H), 7.26–7.20 (s, 5H), 5.01–4.88 (s, 2H), 4.30–3.85 (b, 1H), 2.30–1.68 (b, 4H), 1.31 (s, 18H).

Deprotection of Chain End-Functionalized Arborescent PBG Substrates

The *tert*-butyl ester protecting groups were removed selectively to generate coupling sites only at the chain ends for further grafting reactions. As an example, the arborescent G1PBG substrate (0.3 g) was dissolved in trifluoroacetic acid (3 mL). After 10 min the polymer was precipitated in methanol, recovered by suction filtration, and dried under vacuum overnight before characterization. Yield: 0.23 g (77%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.01-7.7 (b, 1H), 7.26–7.20 (s, 5H), 5.01–4.88 (s, 2H), 4.30–3.85 (b, 1H), 2.30–1.68 (b, 4H).

Synthesis of Linear Amine-Terminated Poly(ethylene oxide)

Poly(ethylene oxide) (PEO) with a primary amine end group was synthesized using a vacuum manifold with a 5-neck round-bottom flask (RBF) containing a magnetic stirring bar and a sealed ampoule containing the purified EO monomer (17.4 g, 0.395 mol) [3]. After the RBF was flame-dried under vacuum and purged with nitrogen, dry THF (200 mL) was added. A diphenylmethylpotassium (DPMK) solution was introduced dropwise through one neck until a faint yellow color persisted, followed by 3-aminopropanol (0.138 mL, 1.75 mmol). Additional DPMK solution (4.7 mL, 2.3 mmol) was then added to deprotonate the alcohol. The EO monomer (17.4 g, 0.395 mol, for a target X_n = 226, M_n = 10000 g/mol) was added to the reaction, transferred to the ampoule and sealed carefully. The reaction was allowed to proceed for 9 days at 45 °C in an oil bath, after which acidified methanol (0.5 mL, 1/10 v/v HCl/methanol) was added to the dark brown solution to terminate the reaction. The solution was concentrated by rotary evaporation and precipitated in diethyl ether. The brownish powder recovered by filtration was redissolved in methanol and precipitated again in diethyl ether. The polymer, recovered by suction filtration and drying under vacuum, was obtained as a white powder. Yield: 12.8 g (73%). SEC (THF): M_n^{app} = 10,100 g/mol, M_w/M_n = 1.12. ^{1}H NMR: (300 MHz, $CDCl_3$): δ: 3.87-3.35 (m, 912H), 3.1 (br, 2H), 1.96 (br, -OH), X_n = 228 (M_n = 10,100 g/mol).

Synthesis of Arborescent Copolymers

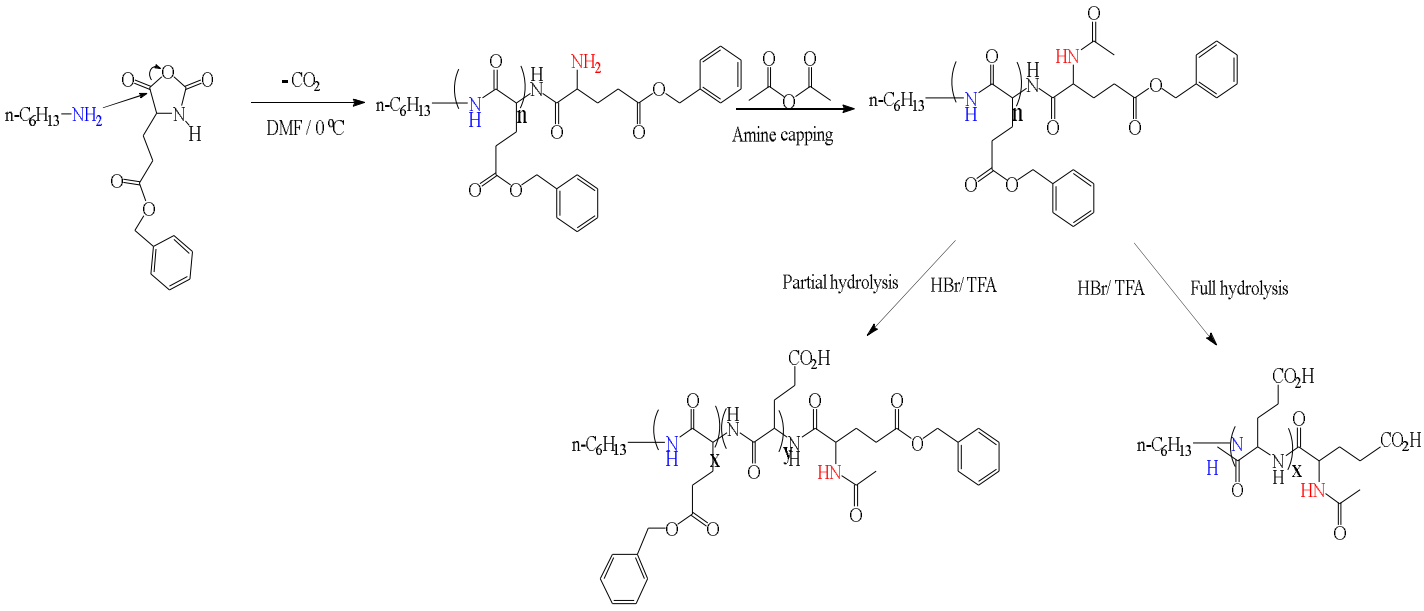
All the arborescent copolymers were synthesized and purified similarly to the reaction described for the arborescent PBG samples. The synthesis of G1PBG$_{15}$-*eg*-PEO is described below as an example. The deprotected chain end-functionalized arborescent G1PBG$_{15}$ substrate (0.120 g, 0.051 mmol -CO_2H) and the PEO serving as side chains (0.46 g, 0.046 mmol chains) were dissolved in 5 mL of dry DMSO. The peptide coupling reagents *N*,*N*′-diisopropylcarbodiimide (DIC, 0.044 mL, 0.281 mmol) and 1-hydroxybenzotriazole (HOBt, 0.038 g, 0.281 mmol) were then added to the reaction with trimethylamine (TEA, 0.030 mL, 0.233 mmol) as a base. The reaction was allowed to proceed for 36 h at room temperature before adding *n*-hexylamine (0.034 mL, 0.338 mmol) to deactivate residual activated carboxylic acid sites. After 3 h the product was precipitated in cold methanol and recovered by suction filtration. Unreacted PEO was removed from the G1PBG$_{15}$-*eg*-PEO crude polymer by preparative size exclusion chromatography (SEC) in DMF and the sample was recovered by precipitation in cold diethyl ether, suction filtration, and drying under vacuum. Grafting yield: 70%, M_n = 890,000, M_w/M_n = 1.06 (SEC-LS).

Table S1. Characteristics of linear PBG substrates.

Sample	Target X_n	X_n(NMR)	M_n(NMR)	M_n^{app}(SEC)	PDI
PBG_{15}	15	15	3,600	3,880	1.11
PBG_{29}	30	29	6,600	6,900	1.10
PBG_{65}	68	65	14,500	15,700	1.12

Table S2. Characteristics of arborescent G0PBG with different branching densities (b_d).

Sample	Deprotection level (%) of substrate	Molar ratio CO_2H : NH_2	M_n(g/mol)	PDI	f_n	b_d
$G0PBG_{15}$	100	1.1 : 1	65400	1.04	12	0.80
$G0PBG_{29}$	31	1.7 : 1	35300	1.08	5.4	0.19
$G0PBG_{65}$	31	1.7 : 1	51000	1.06	7.1	0.11



Scheme S1. Polymerization of γ-benzyl L-glutamic acid *N*-carboxyanhydride (Glu-NCA) and deactivation of the terminal amine moiety on the linear PBG substrate, followed by partial or complete deprotection of the PBG substrate.

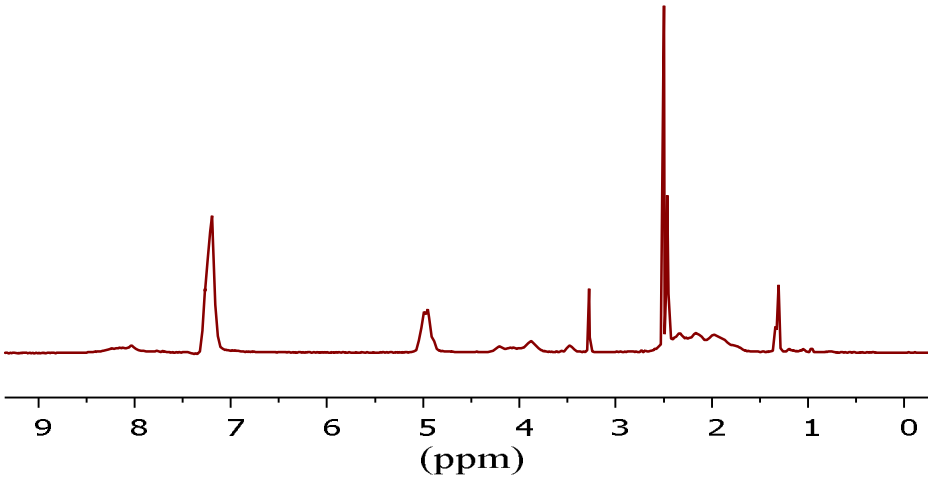


Figure S1. ^{1}H NMR spectrum for linear PBG with two *tert*-butyl ester protecting groups at one chain end $(tBuO)_2$-PBG.

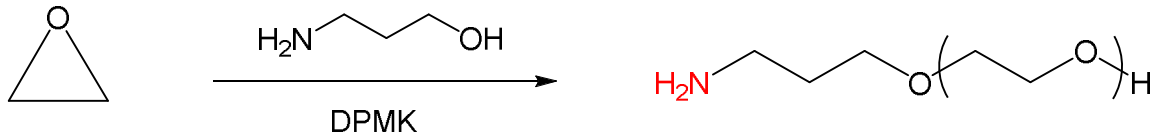
Table S3. Characteristics of chain end-functionalized generation G1 and G2 arborescent PBG substrates. The deprotection level of the PBG substrates was ca. 30%, and a 1 : 1.1 molar ratio of side chains $-NH_2$ chain ends to $-CO_2H$ groups on the substrate was used to maximize the grafting yield and the coupling efficiency [4].

Sample	M_n(g/mol)[a]	M_w/M_n[a]	G_y (%)[b]	C_e (%)[c]	f_n[d]
$G1PBG_{15}$	220,000	1.09	27	31	28
$G1PBG_{29}$	210,000	1.07	53	64	31
$G1PBG_{65}$	270,000	1.09	48	56	39
$G2PBG_{15}$	1.2×10^6	1.08	51	57	171
$G2PBG_{29}$	970,000	1.06	39	48	137
$G2PBG_{65}$	1.3×10^6	1.07	43	50	185

[a] Values from SEC-LS analysis in DMSO; [b] Grafting yield: fraction of side chains attached to the substrate; [c] Coupling efficiency: fraction of coupling sites on the substrate consumed in the reaction; [d] Branching functionality: number of branches added in the last grafting cycle.

Characterization of Linear Amine-Terminated Poly(ethylene oxide)

Poly(ethylene oxide) with a primary amine terminus was used as hydrophilic shell material to demonstrate the synthesis of water-dispersible arborescent copolymer micelles. The anionic polymerization of ethylene oxide with a bifunctional initiator, 3-aminopropanol deprotonated with DPMK, was used to obtain linear PEO with a primary amine chain end as shown in Scheme S2.



Scheme S2. Polymerization of ethylene oxide with 3-aminopropanol and DPMK.

The ^{1}H NMR spectrum obtained for the α-amino PEO sample synthesized is shown in Figure S2. The ratio of intensities for the -CH_2- protons next to the terminal amine (δ3.1 ppm) and the four protons in the repeat units (δ3.6 ppm) was used to determine the number-average degree of polymerization X_n = 228, which corresponds to M_n = 10,100 g/mol.

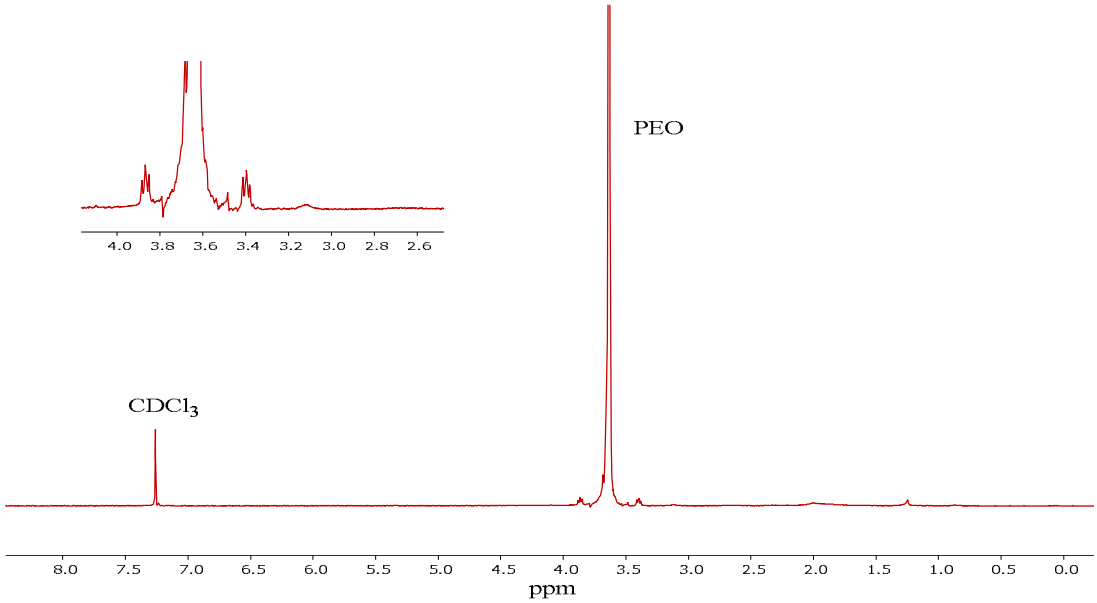


Figure S2. ^{1}H NMR spectrum for α-amino PEO in $CDCl_3$.

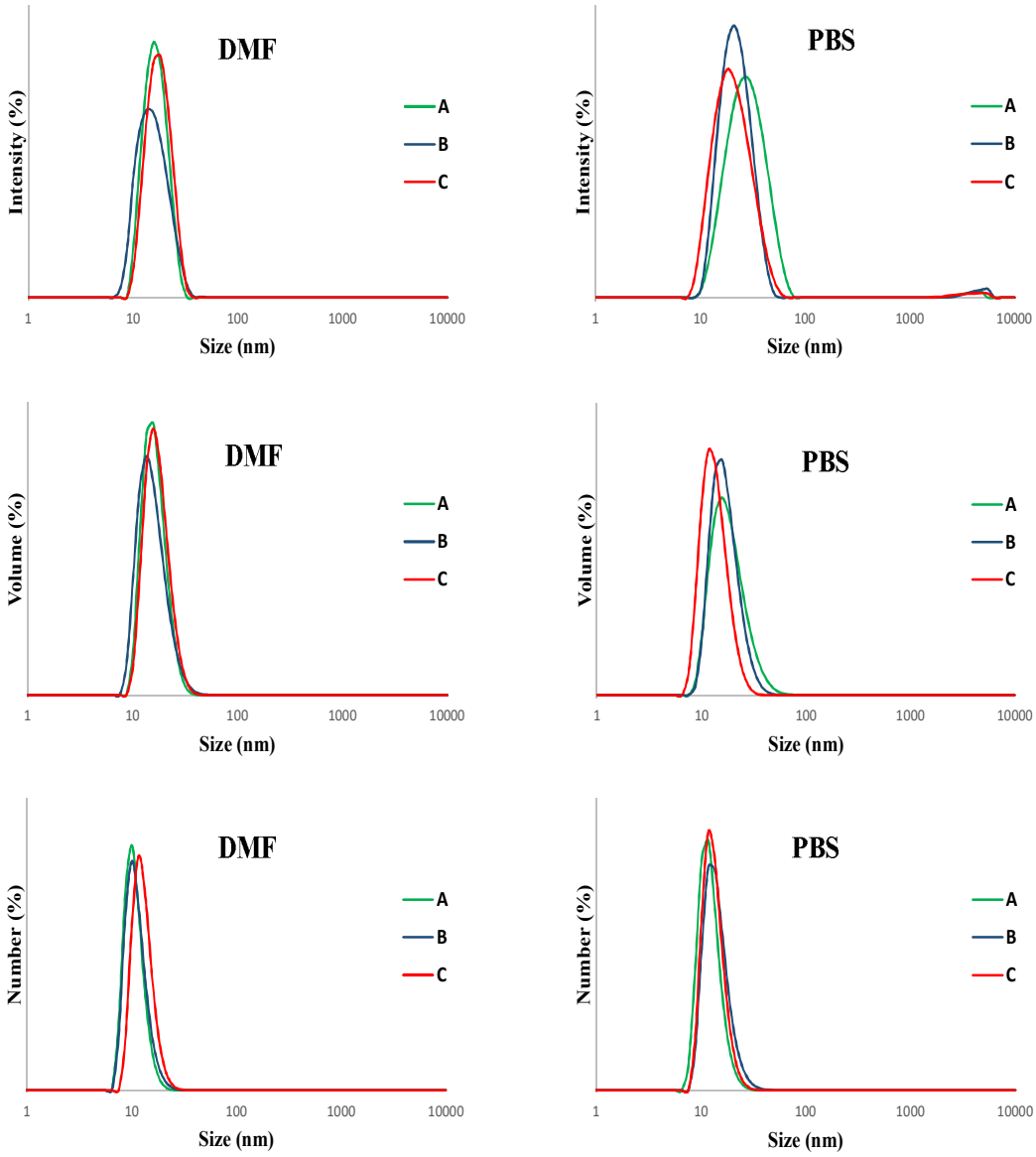


Figure S3. Hydrodynamic diameter distributions of the arborescent copolymers determined by DLS in DMF (left) and in PBS solution (right): (A) G1PBG$_{15}$-*eg*-PEO, (B) G1PBG$_{29}$-*eg*-PEO, and (C) G1PBG$_{65}$-*eg*-PEO.

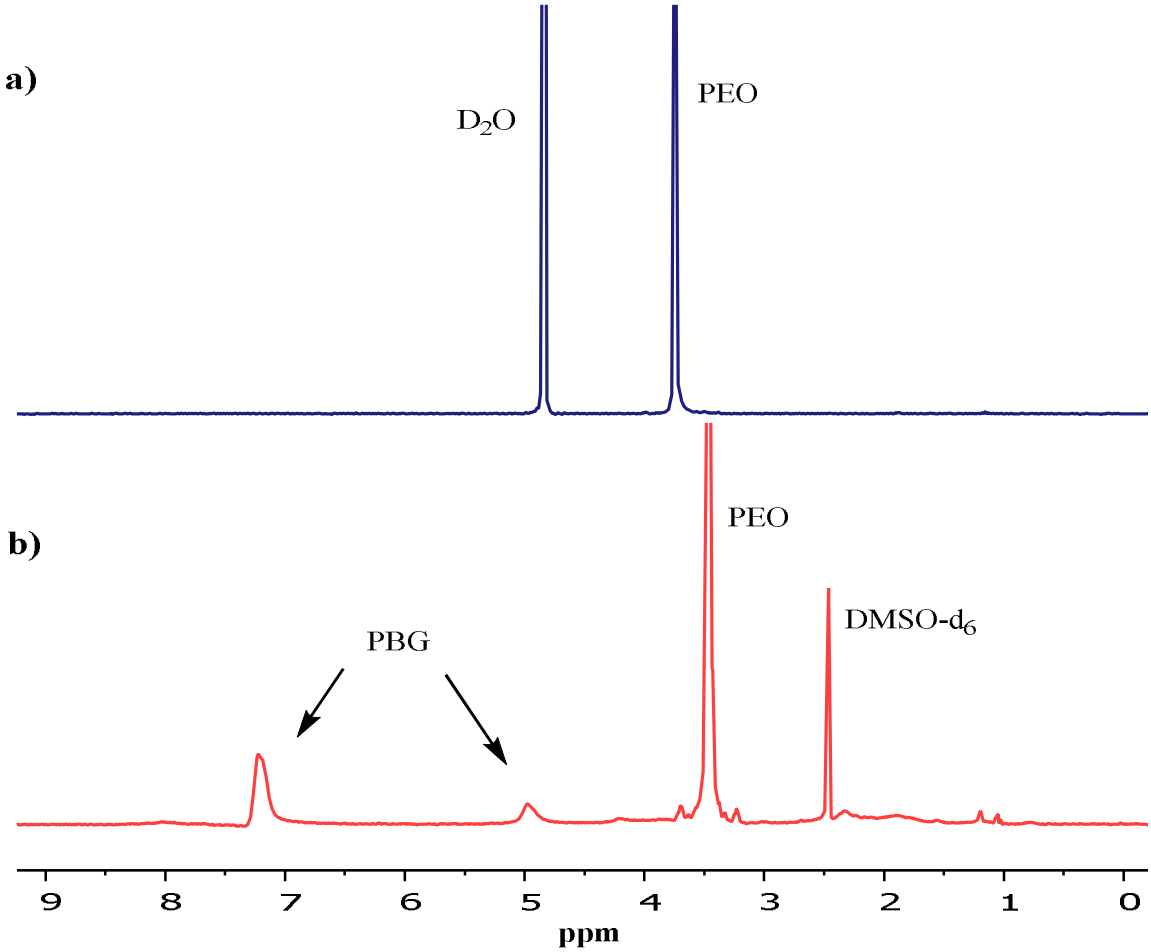


Figure S4. ^{1}H NMR spectra for G2PBG$_{15}$-*eg*-PEO in (a) D_2O and (b) deuterated DMSO.

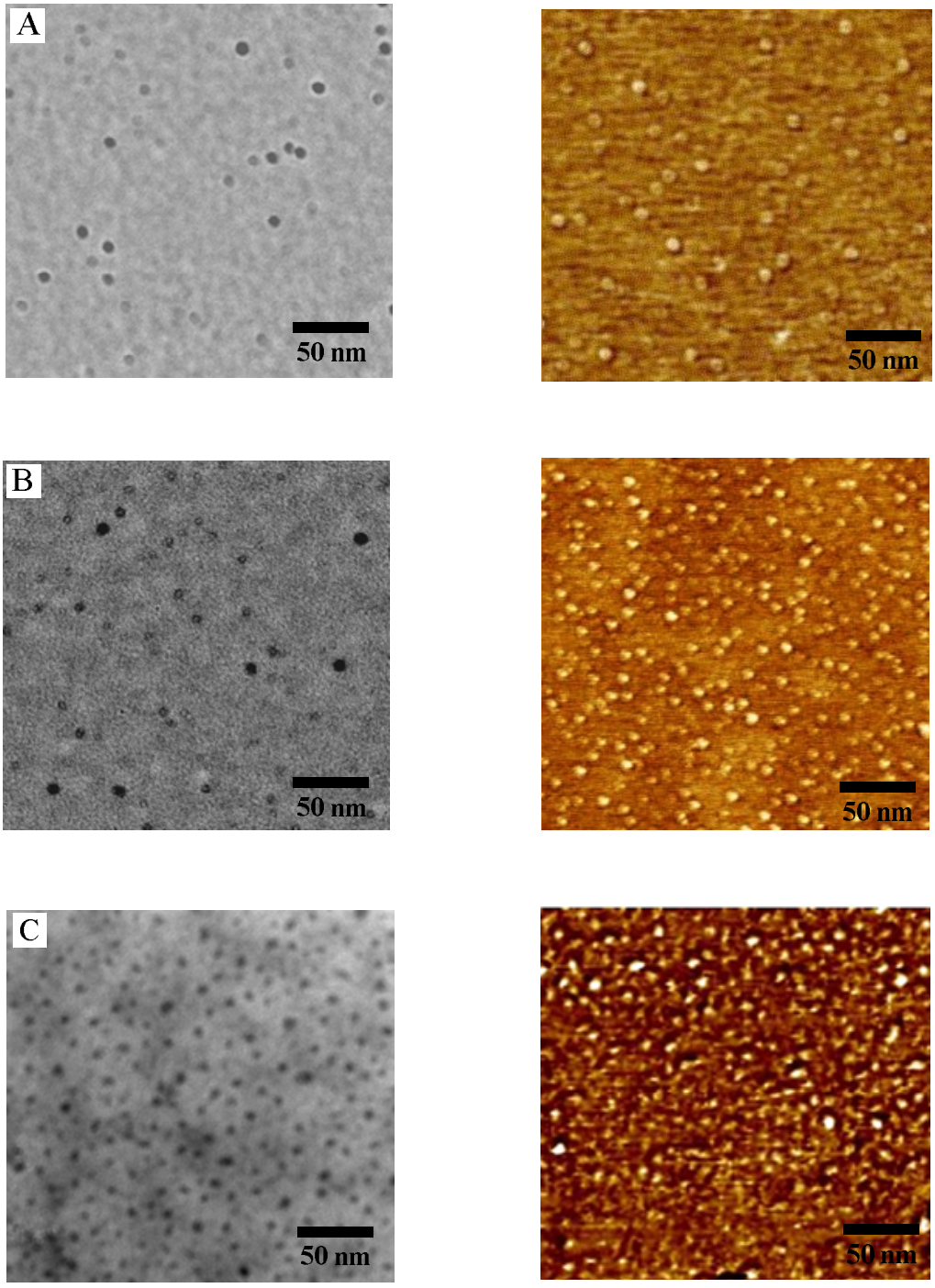


Figure S5. TEM (left) and AFM phase (right) images for chain end-grafted arborescent copolymers: (A) G1PBG$_{15}$-*eg*-PEO, (B) G1PBG$_{29}$-*eg*-PEO, and (C) G1PBG$_{65}$-*eg*-PEO.

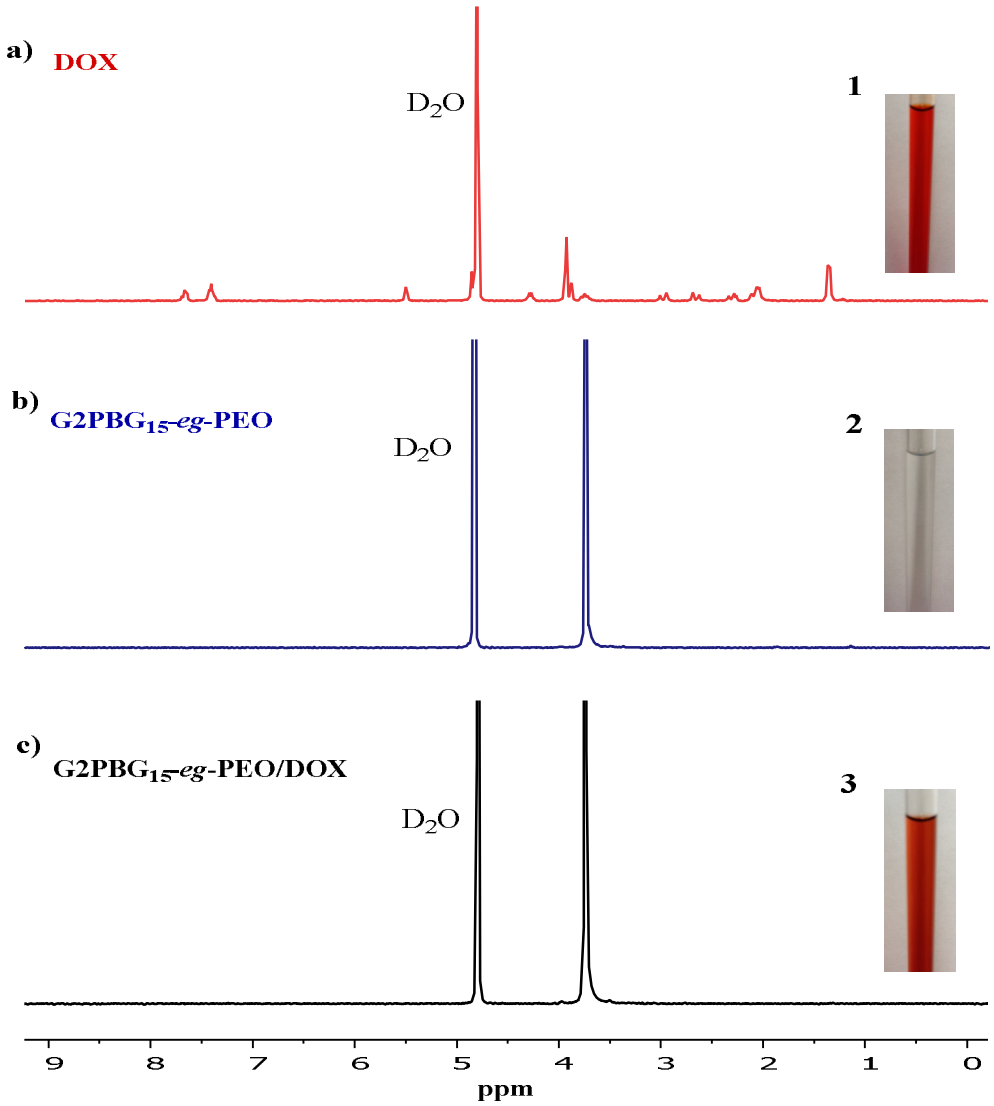


Figure S6. ^{1}H NMR spectra for (a) DOX in D_2O, (b) G2PBG$_{15}$-*eg*-PEO in D_2O and (c) G2PBG$_{15}$-*eg*-PEO/DOX in D_2O. The spectra show the appearance of the DOX signals upon encapsulation in G2PBG$_{15}$-*eg*-PEO.

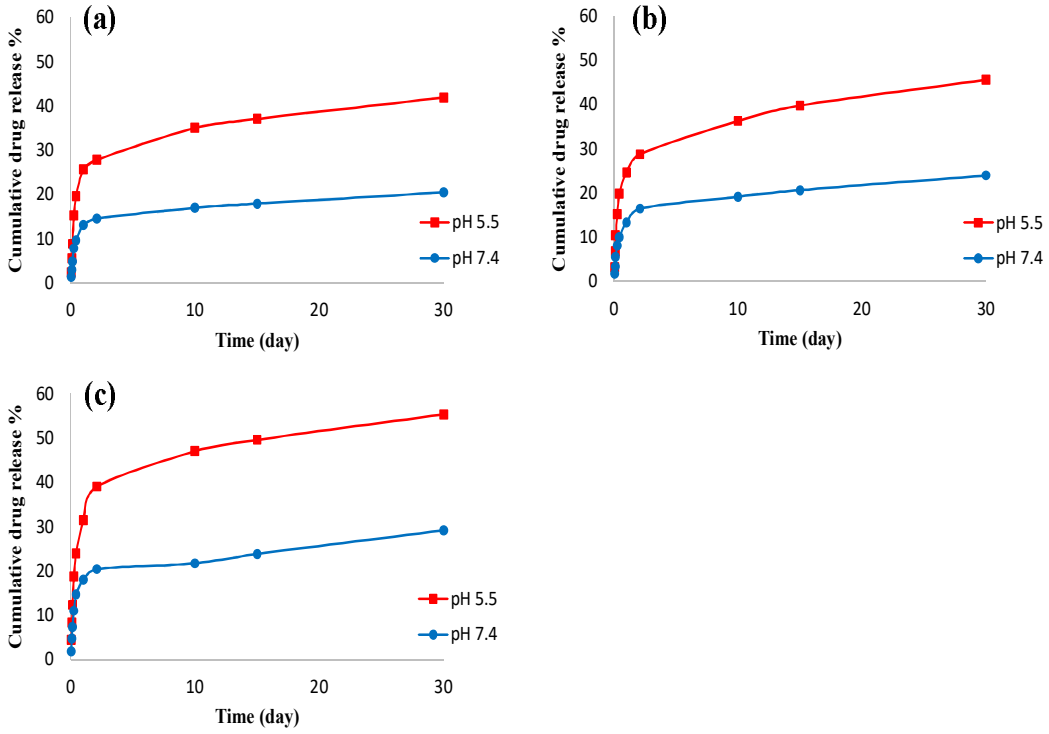


Figure S7. *In vitro* DOX release profiles over 30 days from (a) G2PBG$_{15}$-*eg*-PEO/DOX, (b) G2PBG$_{29}$-*eg*-PEO/DOX and (c) G2PBG$_{65}$-*eg*-PEO/DOX micelles in PBS (pH 7.4 and 5.5) at 37 °C.

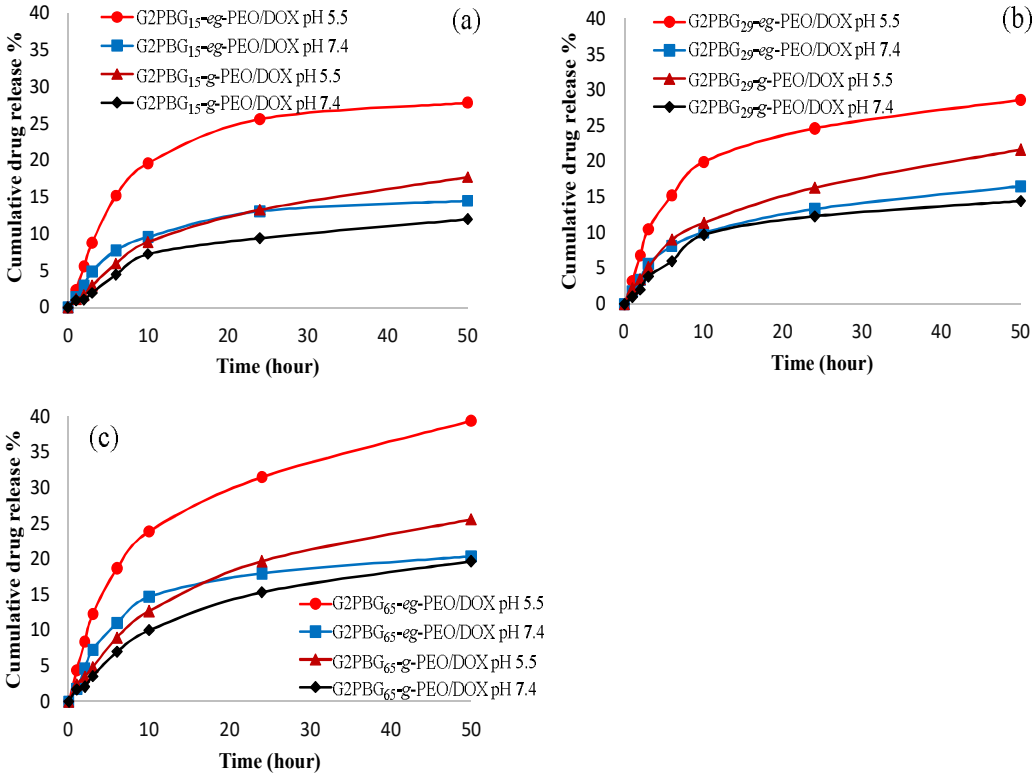


Figure S8. *In vitro* DOX release profiles from (a) G2PBG$_{15}$-*eg*-PEO/DOX and G2PBG$_{15}$-*g*-PEO/DOX, (b) G2PBG$_{29}$-*eg*-PEO/DOX and G2PBG$_{29}$-*g*-PEO/DOX, (c) G2PBG$_{65}$-*eg*-PEO/DOX and G2PBG$_{65}$-*g*-PEO/DOX unimolecular micelles in PBS (pH 7.4 and 5.5) at 37 °C.

References

1. Whitton, G.; Gauthier, M. Arborescent micelles: Dendritic poly(γ-benzyl l-glutamate) cores grafted with hydrophilic chain segments. *Journal of Polymer Science Part A: Polymer Chemistry* **2016**, *54*, 1197-1209, doi:10.1002/pola.27943.
2. Knobler, Y.; Bittner, S.; Frankel, M. 749. Reaction of N-carboxy-α-amino-acid anhydrides with hydrochlorides of hydroxylamine, O-alkylhydroxylamines, and amines; syntheses of amino-hydroxamic acids, amido-oxy-peptides, and α-amino-acid amides. *Journal of the Chemical Society* **1964**, 3941-3951, doi:10.1039/JR9640003941.
3. Alsehli, M.; Gauthier, M. Unimolecular micelles from randomly grafted arborescent copolymers with different core branching densities: Encapsulation of doxorubicin and in vitro release study. Materials 2023, 16, 2461, doi:10.3390/ma16062461.
4. Alsehli, M.; Gauthier, M. Arborescent Polypeptides for Sustained Drug Delivery. *MRS Online Proceedings Library (OPL)* **2016**, *1819*, imrc2015s2014d-o2015, doi:10.1557/opl.2016.70.