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Synthesis and ligation ability of mono-aminooxy-functionalized dendrigraft poly-L-lysine (DGL)

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ABSTRACT

A bifunctional tetraethylene glycol (TEG) linker was prepared and used as an initiator for the synthesis of the first two generations of dendrigraft poly-L-lysines (DGL). The key steps involved the desymmetrization of TEG by introduction of an amine group after activation of a terminal hydroxyl group and of a conveniently protected aminooxy functionality at the other end. Initiation of L-lysine *N*-carboxyanhydride polymerization by the terminal amine yielded generations 1 and 2 of DGL in which a subsequent functionalization of the aminooxy group by ligation with entities bearing an aldehyde group turned out to be feasible.

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Arborescent polymers form a special class of macromolecules with a hyperbranched architecture that is derived from a multistage synthetic protocol.[1] Among this particular class of polymers, dendrimers have a symmetrically organized and, in principle, perfectly controlled structure, monodispersed size, and chain end functionality control.[2] However, the purity of the reagents and the difficulty in achieving high yields in their stepwise synthesis usually generate numerous structural defects. Although not ideally perfect, other families, such as hyperbranched polymers (non-symmetrical and polydisperse structures) and dendrigrafts (structure intermediate between dendrimers and hyperbranched polymers), usually present similar properties while being much easily accessible to synthesis.[3] Dendrigrafts, the most recently introduced family, are prepared by employing reactive oligomers or polymers through repeated protection/deprotection steps.[3] The possibility of obtaining a high molecular weight and functionalized dendrigraft at very low generation numbers combined with the use of relatively inexpensive monomers offers the potential to synthesize and develop dendrigraft polymers possessing dendrimer-like properties at a much lower cost and at a multigram scale.[4] Recently, our group reported a new synthetic route to dendrigraft lysine polymers (DGLs).[5] These few nanometer-sized objects[6] were synthesized by an original process based on the polycondensation of N^{ε}-trifluoroacetyl-L-lysine-*N*-carboxyanhydride (Lys(Tfa)-NCA) in buffered water at pH values close to neutrality. According to this process, the spontaneous precipitation of the growing protected polymer allows the control of the molecular weight. As a matter of fact, oligo-L-lysine prepared as a first generation of these materials (**G1**) served as a backbone for subsequent polymerization of Lys(Tfa)-NCA, permitting the preparation of higher generations (**G2**–**G5**) through an easy work-up (Fig. 1).[5]

These molecular materials display a higher branching ratio compared to hyperbranched lysine polymers prepared through other procedure,[7] which gives them a behavior very similar to ideal lysine dendrimers as, for example, the properties associated with their polycationic structure electrostatically interacting with cell membranes. Indeed, partial guanidinylation of the surface amino groups enhanced the ability of the second generation DGL **G2** to cross model liposomal and cellular membranes.[8] These properties combined with their biodegradability and biocompatibility validate that DGLs are promising tools for many biological applications[9] either in their naked form or after overall or partial derivation (possibly by functionalization of the core or the surface). From this point of view, taking advantage of the synthetic protocol to introduce a fixed number of functional groups on these objects is strongly appealing. With this aim, we conceived structures based on a DGL architecture involving a linking arm bearing a free amine group introduced as a potential initiator of DGL **G1** synthesis. Here we report the synthesis of a bifunctional tetraethylene glycol (TEG) linker to check the validity of this strategy using a masked aminooxy group, subsequently available to undergo a selective ligation giving an oxime bond at low concentration[10] without competing interactions of the numerous free amines present in DGLs. This group is in principle compatible with the conditions of DGL synthesis and its further ability to undergo a subsequent derivation by selective oxime formation may be used in ligation with a wide range of biochemical targets including antibodies.

The linker was synthesized starting from TEG **1** as shown in Scheme 1. The desymmetrization of diol **1** (introduced in large

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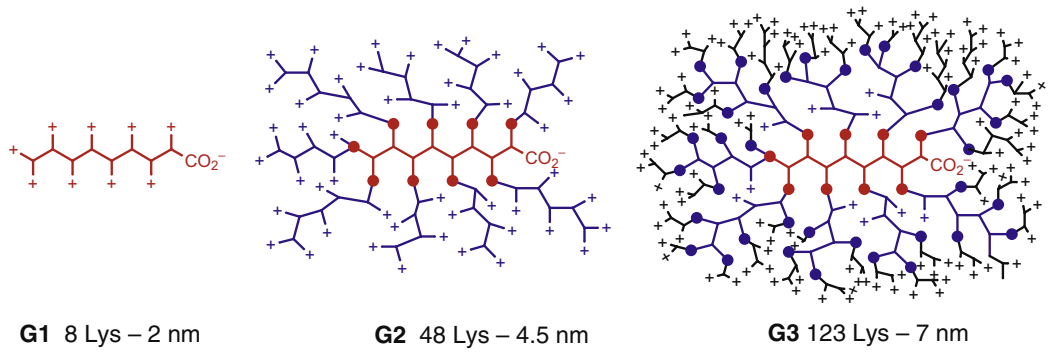
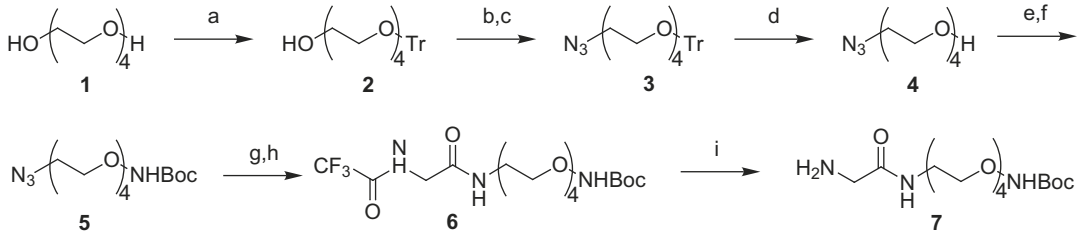


Figure 1. Schematic structure of DGL generations **G1**, **G2**, and **G3**.

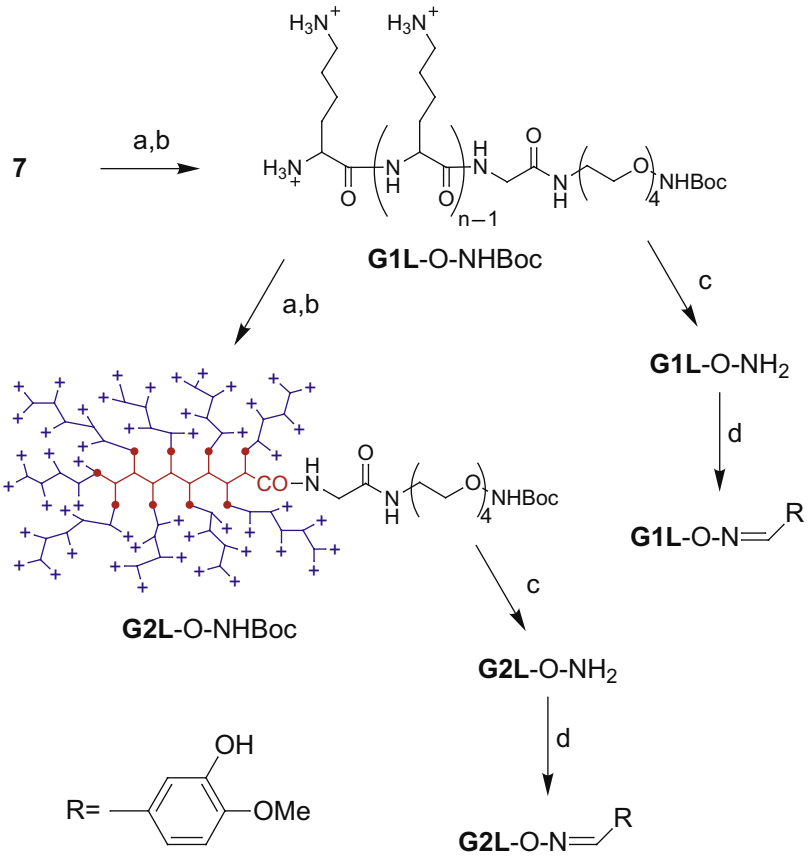
excess) was performed with TrCl (Tr = trityl) and pyridine.[11] The reaction afforded selectively the monoprotected compound **2**, which, upon the introduction of this bulky group, becomes more hydrophobic facilitating the recovery of the product by a simple extraction procedure. The protected TEG **2** was converted into azide **4** using a procedure adapted from Tahtaoui et al.[12] by mesylation of the free hydroxyl group with MsCl and Et_3N and then reaction with NaN_3 in acetonitrile. The azide **3** used as a masked amine precursor was functionalized at the other extremity of TEG after removal of trityl group protecting group. The alcohol **4** was activated by MsCl and the intermediate was reacted with Boc-NHOH and DBU[13] to afford compound **5**,[14] which bears the desired protected aminooxy functionality. The Boc protective group was selected because of its resistance to the conditions of polymerization of Lys(Tfa)-NCA and deprotection of the Tfa group. Last, the amine termination was formed by reduction of the corresponding azide into amine in good yields, according to Staudinger's procedure.

Before initiating the polymerization of Lys-NCA at pH 6.5, we introduced an N-terminal glycine residue by conventional peptide coupling of Tfa-Gly with *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC). This modification was intended to improve the initiation rate in the pH 6.5 buffered polymerization mixture since the N-terminal glycine **6**[15] (p*K* 7.5–8) is likely to act as a better initiator than the original amino group (p*K* 9–10). The last step of this procedure was the mild removal of Tfa-protecting group by reaction with a mixture of NH_3 in MeOH/H_2O that led to compound **7**.[16] This molecule bears at one extremity the amine function which can initiate the polymerization of Lys(Tfa)-NCA to synthesize DGLs (Scheme 2), whereas the other extremity bears a protected aminooxy substituent, able to react with aldehyde groups for a selective coupling to carrier proteins or antibodies.

Lys(Tfa)-NCA was prepared as previously described,[5] from N^{α}-carbamoyl-N^{ε}-trifluoroacetyl lysine C-Lys(Tfa)[17] and its polycondensation was performed without isolating the NCA intermediate in aqueous media at pH 6.5 and 0 °C leading to linear oligo-Lys(Tfa) **8** as a solid precipitate, which was characterized by MALDI-TOF mass spectrometry and ^1H NMR.[18] Tfa-protecting groups were removed by treatment with a solution of NH_3 in MeOH/H_2O affording the crude product in 40% overall yield from C-Lys(Tfa) with a structure consistent with the functionalized linear peptide **G1L**-O-NHBoc by capillary electrophoresis (CE) analysis,[19] which was compared to that of the peptide **G1** previously prepared in the absence of initiator.[5] The second generation of functionalized dendrigraft poly-L-lysines (DGL) was synthesized using **G1L**-O-NHBoc as a macro-initiator of Lys(Tfa)-NCA polymerization according to the work-up described earlier.[5] After removal of Tfa protecting groups, the new dendritic material **G2L**-O-NHBoc was shown by CE[19] to exhibit a pattern different from that of the non-functionalized material **G2**.[5] A proof of the principle of the ability of the synthesized materials **G1L**-O-NH_2 and **G2L**-O-NH_2 to undergo a ligation with compounds bearing an aldehyde group was obtained by studying their reaction with vanillin which presents a characteristic UV spectrum with a maximum of absorption at 300 nm (Scheme 2). To this aim, the Boc-protecting groups of **G1L**-O-NHBoc and **G2L**-O-NHBoc were removed by treatment with trifluoroacetic acid (TFA) and 100 μM aqueous solutions of the resulting materials **G1L**-O-NH_2 and **G2L**-O-NH_2 acidified to pH ~2 with TFA (0.1% v/v) were incubated at room temperature with 100 μM vanillin using a procedure similar to that generally used for biomolecules.[20] The RP HPLC analysis[21] of the reaction medium at 48 h indicated a decrease in free vanillin (48% and 26% for **G1L**-O-NH_2 and **G2L**-O-NH_2, respectively) coupled with the formation of products with an absorption at 300 nm, characteristic of a vanillin derivative, and at a retention time consistent with those observed for the non-functionalized materials **G1** and **G2** (Fig. 2). The additional LC–MS(ESI) analysis of **G1L**-O-NH_2 reaction solutions using conditions similar to those of the HPLC analysis of Fig. 2 confirmed the attribution of HPLC peaks to the oxyamine-derivatized dendrigraft (n = 2–6; e.g., m/z 453.84, z = 2, n = 5) and the presence of oxime adducts (n = 2–6; e.g., m/z 456.81, z = 2, n = 4; 520.84, z = 2, n = 5) in the absence or presence of vanillin, respectively.



Scheme 1. Reagents and conditions: (a) TrCl, pyridine, 45 °C, overnight, quantitative; (b) MsCl, Et_3N, CH_2Cl_2, overnight, 73%; (c) NaN_3, CH_3CN, 95 °C, 48 h, 76%; (d) PTSA, MeOH, 87%; (e) MsCl, Et_3N, CH_2Cl_2, overnight, 88%; (f) BocNHOH, DBU, 63%; (g) PPh_3, CH_2Cl_2/H_2O, 60 °C, quantitative; (h) Tfa-Gly, EDC, 66%; (i) NH_3 in MeOH/H_2O, 40 °C, quantitative.



Scheme 2. Formation of aminooxy-functionalyzed poly-lysine **G1L**-O-NH_2 and **G2L**-O-NH_2 and subsequent ligation with a model aldehyde RCHO (vanillin). Reagents and conditions: (a) Lys(Tfa)-NCA, pH6.5; (b) NH_3 in H_2O/MeOH (4:1); (c) TFA; (d) vanillin 0.1 mM, pH 2 (TFA 0.1% v/v in water).

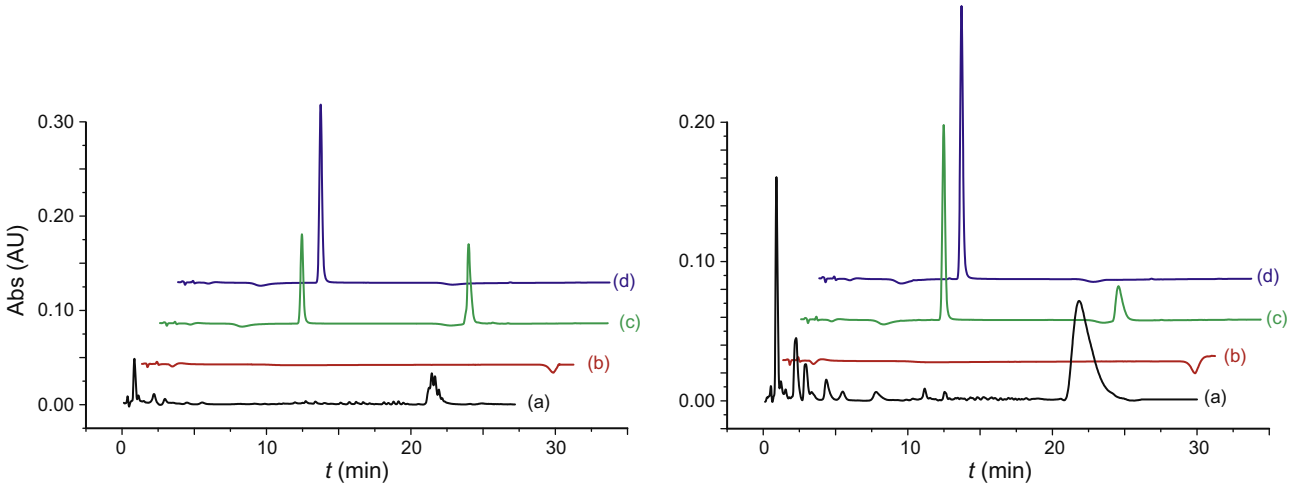


Figure 2. RP HPLC monitoring[21] of the ligation of vanillin onto aminooxy-functionalized poly-lysines **G1L**-O-NH_2 (left panel) and **G2L**-O-NH_2 (right panel). (a) 0.1 mM **G*x*L**-O-NH_2 (*x* = 1 or 2) in water at pH 2 (UV detection 210 nm); (b) 0.1 mM of **G*x*L**-O-NH_2 in water at pH 2 (UV detection 300 nm); (c) 48 h reaction of 0.1 mM of **G*x*L**-O-NH_2 and 0.1 mM of vanillin in water at pH 2 and rt (UV detection 300 nm); (d) 48 h reaction of 0.1 mM of the corresponding native **G*x*** (*x* = 1, left panel or 2, right panel) and 0.1 mM of vanillin in water at pH 2 and rt (UV detection 300 nm).

The specificity of the oxyamine function of **G1L**-O-NH_2 and **G2L**-O-NH_2 (with respect to the numerous free amino groups) in reacting with the aldehyde group of vanillin was demonstrated by the absence of ligated products starting from **G1** and **G2** species (Fig. 2). The results of these experiments indicate (i) that native DGLs **G1** and **G2** do not lead to the formation of a ligated product by reaction of amines on an aldehyde and (ii) that the ligation between DGLs bearing an aminooxy-functionalized PEG arm is effective at the sub-millimolar concentrations required for the reactions of functionalized biomacromolecules.

In summary, we have reported the synthesis of a Boc-aminooxy-TEG-amine in nine steps. We also demonstrated that this compound is suitable to initiate the oligomerization of Lys(Tfa)-NCA and allows the formation of structures bearing a single aminooxy

group subsequently useful for selective ligations with aldehydes. In spite of the number of lysine amino groups on the surface of DGL, no interference compromising the ligation selectivity was observed at low concentrations, as needed for the synthesis of bioconjugates.

Acknowledgments

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14. *Experimental procedure for the synthesis of compound* **5**: To a cold solution (0 °C) of **4** (1 g, 4.56 mmol) and triethylamine (0.76 mL, 5.47 mmol, 1.2 equiv) in CH_2Cl_2 (6.2 mL) was added MsCl (0.39 mL, 5.02 mmol, 1.1 equiv). The reaction mixture was stirred at 0 °C for 2 h and rt overnight, then filtered and the filtrate solution was concentrated. The residue was purified by silica gel chromatography (EtOAc/hexane, 90:10) to give a colorless liquid (1.19 g, 88%). A fraction of this compound (340 mg, 1.14 mmol) was reacted in THF (0.6 mL) with BocNHOH (213 mg, 1.60 mmol, 1.4 equiv) and DBU (0.26 mL, 1.72 mmol, 1.5 equiv) according to the procedure of Ref. 13. CH_2Cl_2 (10 mL) was added and the organic layer was washed with 2 M NH_4Cl (4 × 5 mL) and brine (2 × 5 mL), dried (Na_2SO_4), and concentrated. The product was purified by silica gel chromatography (EtOAc/petroleum ether, 1:3) to give compound **5** as a brown oil (241 mg, 63%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.41 (s, 9H, CH_3); 3.32 (t, J = 5.03 Hz, 2H, CH_2); 3.60–3.73 (m, 12H, CH_2O); 3.98 (t, 2H, CH_2); 7.8 (1H, s, N*H*). ^{13}C ($CDCl_3$, 75 MHz) δ = 28.15 (CH_3); 50.59 (CH_2N_3); 69.15; 69.96; 70.48; 70.54; 70.6; 81.27 (C); 156.7 (*C*=O). HRMS (ESI+): calcd: $C_{13}H_{27}N_4O_6$: 335.1931; found: 335.1925.
15. *Experimental procedure for the synthesis of compound* **6**: PPh_3 (345 mg, 1.32 mmol, 2 equiv) was added to azide **5** (220 mg, 0.658 mmol) in THF/H_2O (8:2, 6.30 mL) and stirred at 60 °C for 24 h. Then, the mixture was concentrated under vacuum and EtOAc (10 mL) was added to the residue. The organic layer was extracted with 10% $KHSO_4$ (3 × 2 mL). The mixed aqueous layers were brought to pH 9 with Na_2CO_3 and the resulting solution was freeze-dried. The solid residue was treated with EtOAc; salts were removed by filtration and the filtrate was concentrated under vacuum. The oily residue containing a free amino group (230 mg, 0.746 mmol) was acylated using Tfa-Gly (152 mg, 0.895 mmol, 1.2 equiv) in the presence of EDC (243 mg, 1.26 mmol, 1.7 equiv) in THF (5.7 mL). The reaction mixture was stirred at rt for 3 days. Then, THF was removed under vacuum, CH_2Cl_2 (20 mL) was added, and the organic layer was washed with 10% $KHSO_4$ (2 × 20 mL), brine (10 mL), and dried (Na_2SO_4). The residue was purified by silica gel chromatography (CH_2Cl_2/MeOH, 98:2–90:10, v/v) to give the product **6** as a yellow oil (227 mg, 66%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.40 (s, 9H, CH_3), 3.41 (t, 2H, CH_2), 3.50–3.72 (m, 12H, CH_2O), 3.90 (m, 4H, CH_2ONH + $NHCH_2CO$), 7.21 (s, 1H, N*H*), 7.62 (s, 1H, N*H*), 8.01 (s, 1H, N*H*). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 28.19 (CH_3); 39.56 (CH_2CH_2NH); 42.45 (CH_2NH); 68.98 (CH_2ONH); 69.50; 69.80; 70.10; 70.17; 70.32; 70.36; 75.20; 81.75 (*C*); 115.74 (q, CF_3); 157.11 (O–NH–*C*=O); 157.45 (q, CF_3C=O); 167.07 (CH_2C=O). HRMS (ESI+): calcd: $C_{17}H_{31}F_3N_3O_8$: 462.2063; found: 462.2069.
16. *Experimental procedure for the synthesis of compound* **7**: the Tfa-protected linker **6** (200 mg, 0.43 mmol) was stirred at 40 °C overnight in the presence of 5 mL of solution of 28% NH_3/H_2O/MeOH (7:14:4). NH_3 and MeOH were removed under vacuum and the resulting aqueous solution was freeze-dried. ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.38 (s, 9H, CH_3); 3.38 (t, 2H, CH_2); 3.49–3.69 (m, 14H, CH_2O + $NHCH_2CO$); 3.92 (m, 2H, CH_2); 6.07 (s, 2H, NH_2); 8.16 (s, 1H, N*H*–CO); 8.45 (s, 1H, N*H*). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 28.04 (CH_3); 39.09 (CH_2NH); 41.25 (CH_2NH_2); 68.27 (CH_2ONH); 69.69; 69.81; 69.92; 70.21; 70.34; 75; 81.56 (*C*); 157.96 (O*C*=O); 167.75 (CH_2C=O). HRMS (ESI+): calcd: $C_{15}H_{32}N_3O_7$: 366.2240; found: 366.2238.
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18. MALDI-TOF MS (CHCA matrix): m/z = 1060.4, 1284.5, 1508.6, 1732.6, 1956.7, 2180.6, 2409.6; H-[Lys(Tfa)]$_n$-Gly-(CH_2–CH_2–O)$_4$-NHBoc Na^+ (n = 3–9). **G1L**-ONH-Boc**:** NMR ^{1}H (D_2O, 300 MHz) δ = 1.2–1.8 (30H, m, $CH_2\alpha,\beta,\gamma$); 1.36 (9H, s, CH_3 Boc); 2.89 (10H, m, $CH_2\delta$); 3.32 (2H, m, CH_2–NH); 3.60 (12H, m, CH_2); 3.81 (2H, m, CH_2 Gly); 3.91 (2H, m, CH_2–O–NH–); 4.19 (5H, m, CH α).
19. *CE analyses*: Fused silica capillaries of dimensions 50 μm id × 58.5 cm length (50 cm to detector window) were used. Prior to use, new capillaries were conditioned by washing with 1 M NaOH (930 mbar for 30 min), 0.1 M NaOH (930 mbar for 20 min), then with background electrolyte (930 mbar for 10 min). The background electrolyte was aqueous phosphate buffer (H_3PO_4 125 mM + NaH_2PO_4 125 mM, pH 2.5). DGL samples were made of 5 g L^{-1} solutions of polymer in background electrolyte, then hydrodynamically injected by application of a positive pressure 18 mbar for 9 s. Separations were carried out at 25 °C by applying a constant voltage of +20 kV with UV detection at λ = 200 and 214 nm. RT **G1**: 4.3–4.8 min, RT **G1L**-O-NH-Boc: 5.3–5.8 min. RT **G2**: 8–10 min and RT **G2L**-O-NH-Boc: 15–18 min.
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21. Hypersil BDS C18 (50 × 2.1 mm, 3 μm); flow 0.2 mL min^{-1}; eluent A: water TFA 0.1% v/v; B: CH_3CN TFA 0.1% v/v; 100% to 0% A in 40 min.