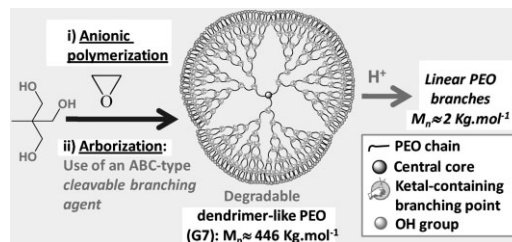


# Dendritic Carrier Based on PEG: Design and Degradation of Acid-sensitive Dendrimer-like Poly(ethylene oxide)s<sup>a</sup>

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Degradable dendrimer-like PEO's were designed using an original ABC-type branching agent featuring a cleavable ketal group, following an iterative divergent approach based on the anionic ring opening polymerization (AROP) of ethylene oxide and arborization of PEO chain ends. A seventh generation dendrimer-like PEO carrying 192 peripheral hydroxyls and exhibiting a molar mass of  $446 \text{ kg} \cdot \text{mol}^{-1}$  was obtained in this way. The chemical degradation of these dendritic scaffolds was next successfully accomplished under acidic conditions, forming linear PEO chains of low molar mass ( $\approx 2 \text{ kg} \cdot \text{mol}^{-1}$ ), as monitored by <sup>1</sup>H NMR, SEC, and MALDI-TOF mass spectrometry as well as by AFM.



## Introduction

Since the introduction of the “drug delivery” concept by Ringsdorf,<sup>[1]</sup> the delivery of bioactive molecules using

polymeric materials has emerged as an interdisciplinary research area.<sup>[2]</sup> Many of the clinical drugs indeed exhibit drawbacks such as low aqueous solubility, instability under physiological conditions, high systemic toxicity, etc. that could be overcome through their conjugation to a polymeric component that helps enhancing their therapeutic efficacy. One of the prerequisites for an enhanced permeability and retention (EPR)<sup>[3]</sup> effect of a drug within the targeted tumor is indeed its association with polymeric carriers,<sup>[4]</sup> which allows for the extravasation and accumulation of such polymeric drugs into the tumor tissue over time. When the polymer exhibits stealth properties, such as poly(ethylene oxide) (PEO) — also referred to as poly(ethylene glycol) (PEG) — the bioconjugates formed can travel during prolonged circulation times in the body without being neutralized by macrophages of the immune system. After uptake of such polymer therapeutics by endocytosis and drug release by either low pH or the presence of lysosomal enzymes at the cellular level, then comes the time of the polymeric carrier exocytosis. For a non-degradable polymer such as PEO, it is essential that its molar mass be limited below  $40 \text{ kg} \cdot \text{mol}^{-1}$  to ensure its renal elimination. The accumulation of a non-biodegradable polymer of higher molar mass may indeed cause serious kidney damage.<sup>[5]</sup>

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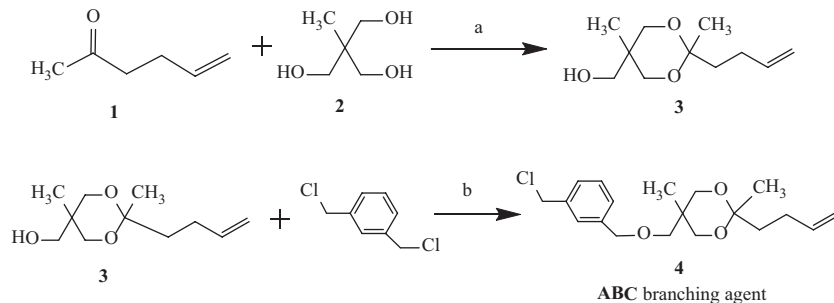
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Many polymers have been investigated as carriers for the delivery of drugs or proteins.<sup>[6]</sup> For instance, poly(L-glutamic acid)<sup>[6i]</sup> conjugates are biodegradable but prone to an uptake by macrophages before reaching the target sites.<sup>[7]</sup> As for poly[N-(2-hydroxypropyl)methacrylamide]<sup>[6h,8]</sup> and PEO,<sup>[6j,6k,9]</sup> they are non-biodegradable and have been mainly used in a range of molar mass below the renal elimination threshold of 40 kg · mol<sup>-1</sup>. To impart biodegradability to PEO, several teams have derived linear PEO chains that include both pendant functional groups<sup>[10]</sup> for drug attachment and intracatenary fragile bonds — esters,<sup>[10a,10b,11]</sup> ortho esters,<sup>[12]</sup> amide,<sup>[10c]</sup> acetal,<sup>[10d,10e,13]</sup> and disulfide —<sup>[10f,14]</sup> sensitive to pH or reductive conditions. Generally obtained by polycondensation of PEG telechelics, these PEG derivatives exhibit, however, a broad molar mass distribution and ill-defined structures.

In the last 5 years, we have developed synthetic strategies and prepared by divergent approach well-defined multihydroxy-ended,<sup>[15]</sup> but also pH controlled size-varying dendrimer-like PEO's,<sup>[16]</sup> and besides heterodifunctional bouquet-type<sup>[17]</sup> and asymmetrical Janus-type dendrimer-like PEO's.<sup>[18]</sup> Such dendritic PEGs were obtained by repetition of anionic ring opening polymerization (AROP) of EO combined with suitable branching reactions (= arborization reactions). One outcome of our synthetic efforts was the discovery that dendritic PEO carriers with multiple reactive sites exhibit better in vivo anti-inflammatory activity than their linear and even star counterparts when fitted with glycosidic end units.<sup>[19]</sup> However, these glycoside-ended dendrimer-like PEO's were not biodegradable and were of molar masses higher than the renal clearance threshold, hence they could accumulate in the kidneys and provoke the so-called storage disease syndrome.<sup>[4]</sup>

In this contribution, we report the synthesis of dendrimer-like PEO's that are degradable under acidic conditions, due to the presence of ketal moieties at their branching points. An acidic environment — typically pH = 5 — is found within the tumors at the cellular level, which means that the hydrolytic degradation of ketals would only occur after uptake by the cells.<sup>[20]</sup> A degradable dendrimer-like PEO up to generation seven carrying 192 peripheral hydroxyl groups, which represent as many functional sites to be fitted with the drug to deliver and receptor-targeting ligands, and exhibiting a molar mass of 446 kg · mol<sup>-1</sup> was synthesized divergently by reiterative AROP of EO and an arborization reaction using a newly designed ABC-type branching agent (Scheme 1). The degradation of these dendrimer-like PEO's under acidic



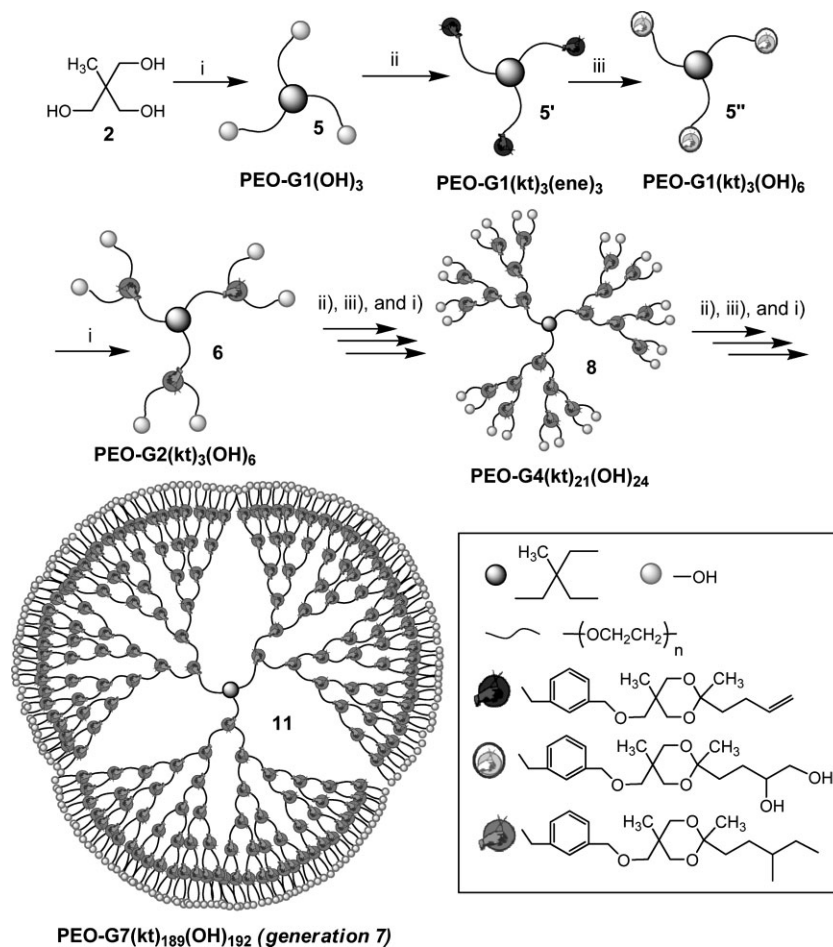
**Scheme 1.** Synthesis of the ABC-type cleavable branching agent. (a) Camphorsulfonic acid (10 mol-%), benzene (reflux, dean-stark trap); (b) NaH (1.5 equiv.), dried THF, 48 h.

conditions was monitored by <sup>1</sup>H NMR spectroscopy and could be visualized by atomic force microscopy (AFM). The products of degradation were characterized by GPC and matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry, providing for the first time a direct evidence that PEO branches grown generation after generation by the divergent approach are all well-controlled and uniform in size, as those grown by a convergent approach.<sup>[21]</sup>

## Results and Discussion

A three-arm PEO star, **5**, was first prepared by AROP of EO from 1,1,1-tris(hydroxymethyl)ethane (**2**, Scheme 2).<sup>[15–18]</sup> **5** was then end-modified with **4** under phase transfer conditions at 50 °C for 48 h. The resulting functionalized star, **5'**, carrying three ketal and three vinyl groups, respectively, was then subjected to osmylation, which consisted in the chemical modification of the three vinyl groups into six hydroxyls, while the three ketal rings remained intact as potentially cleavable linkages under acidic conditions. These three-arm PEO star derivatives, **5**, **5'**, **5''**, were characterized by <sup>1</sup>H NMR spectroscopy (Figure S1 of Supporting Information) and MALDI-TOF mass spectrometry (Figure S2 of Supporting Information). Both methods confirmed the quantitative derivatization at each step.

The star precursor **5''** was then used to grow the PEO branches of second generation. Only 30% of the six OH groups of **5''** were deprotonated using DPMK and DMSO as the polymerization solvent.<sup>[15–18]</sup> Next, the six PEO branches of 1 000 g · mol<sup>-1</sup> each were grown upon EO addition, affording the second generation dendrimer-like PEO **6**. A symmetrical and unimodal size exclusion chromatography (SEC) trace with a marked shift to the higher molar mass region with regard to that of **5** could be observed (Figure 1), indicative of a complete initiation and living polymerization of EO. The <sup>1</sup>H NMR spectrum of **6** retained the same characteristic peaks as those of



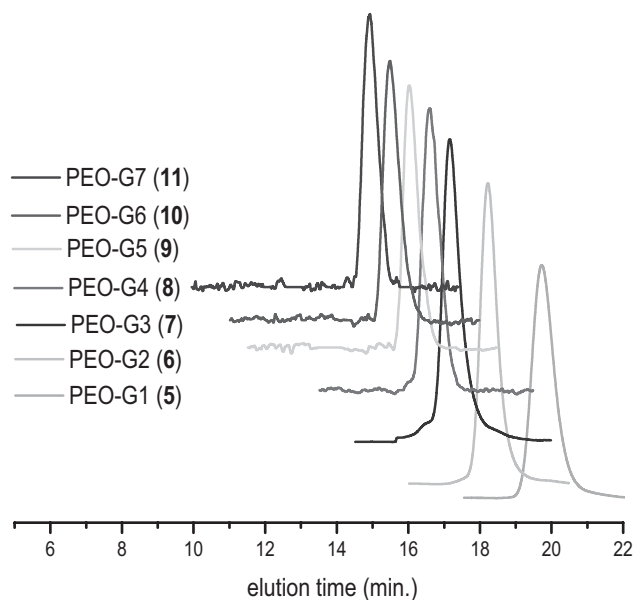
**Scheme 2.** Synthesis of acid-sensitive degradable dendrimer-like PEO's. (i) a: DPMK (0.3 equiv. of hydroxyls), DMSO; b: ethylene oxide, 3 d, r. t.; (ii) **4** (1.5 equiv.), TBAB, NaOH, THF/H<sub>2</sub>O, 50 °C, 48 h; (iii) *N*-methylmorpholine-*N*-oxide (1.2 equiv.), 4 wt.-% OsO<sub>4</sub> (0.2 equiv.%) (CH<sub>3</sub>COCH<sub>3</sub>/H<sub>2</sub>O/*t*-BuOH (5:5:1), r. t., 24 h.

its precursor **5''**, except that only one type of terminal primary hydroxyl groups appear at 2.7 ppm, and that the peak (c) assigned to EO repeating units increases substantially after polymerization (Figure S3 of Supporting Information). Using signals *b* and *k* at around 0.9 ppm of methyl protons belonging to the core and branching point, respectively, the molar mass of the second generation dendrimer-like PEO could be determined (Table 1). Furthermore, the average length of the PEO branches grown from the six hydroxyl groups of **5''** could be calculated using the three following assumptions: (i) the molar mass of precursor **5** remained unchanged after the successive chemical reactions; (ii) the arborization that transformed the 3 terminal hydroxyls of **5** into **6** was quantitative; (iii) all the six hydroxyl groups initiated the AROP of EO upon deprotonation. Experimental data agree well with targeted values on the basis of the feed ratio between EO and **5''** (Table 1).

Following the same iterative procedure based on PEO chain ends arborization and AROP of EO, dendrimer-like

PEO's with ketal-containing linkages could be synthesized, generation after generation, up to generation seven. Samples of all generations and their intermediates were characterized by <sup>1</sup>H NMR and SEC. Narrow and unimodal molar mass distributions were obtained for all samples (Figure 1). Using the same assumptions as those adopted for sample **6**, the total molar mass of the dendrimer-like PEO's synthesized, *M*<sub>Gn</sub> NMR, and the molar mass of each PEO chain grown at every generation, *M*<sub>Gn/PEO</sub>, could be calculated (Table 1).<sup>[22]</sup> Note that each branch length between two branching points actually fluctuates around 1 000 g · mol<sup>-1</sup> at any generation considered.

Sample **11** (generation 7), exhibiting a high enough molar mass (446 kg · mol<sup>-1</sup>) to be easily detectable by AFM, appeared as evenly distributed thin disk-like objects with an average diameter of 100 nm when deposited as individual molecules on mica.<sup>[15a]</sup> When characterized in aqueous solution by DLS, a monomodal particle size distribution with an average diameter of 58.8 nm of unimolecules is observed (Figure S6A of Supporting



**Figure 1.** SEC traces (DMF) of dendrimer-like PEO's from generations 1–7 (from right to left), using RI detection (see also Table 1).

Information). This is consistent with the molecular structure and AFM results (Figure S6B of Supporting Information).

The degradation behavior of three representative dendrimer-like PEO's, **6**, **7**, **11** was next evaluated under extreme acidic conditions (pH=1). Complete cleavage of ketal groups is expected to give rise to a mixture of different kinds of PEO branches (see Scheme S1 of Supporting Information). For instance, in the case of **7** and **11**, along with the three-arm PEO, **12**, two different two-armed PEOs, **13** and **14**, respectively, should be formed after complete degradation of the parent dendrimer. The three types of PEO branches correspond, respectively, to the PEO star precursor located at the core, to inner PEO arms and to PEO arms

located in the peripheral layers. For **6**, however, only **12** and **14** are generated upon degradation. As the generation number increases, the content of **12** in the final mixture decreases.

Products recovered after degradation were characterized by  $^1\text{H}$  NMR spectroscopy (Figure S4 of Supporting Information), in which the peak due to the methyl groups connected to the ketal ring disappears completely, confirming the complete degradation. This is also illustrated by GPC showing a monomodal, symmetric, and narrow distribution of degradation products (Figure S5 of Supporting Information). The apparent molar mass of the products of degradation is around  $2\,000\text{ g}\cdot\text{mol}^{-1}$ , corresponding to two-armed PEO's. Because the  $\bar{M}_n$  value of **12** and **14** revolves around  $3\,400$  and  $2\,300\text{ g}\cdot\text{mol}^{-1}$ , their higher proportion in **6** comparatively to **7** and **11** results in a broader dispersity in the case of the degradation products of **6** compared to those of **7** and **11**.

MALDI-TOF mass spectrometry provided a even more accurate insight into the internal structure of these dendrimer-like PEO's (Figure S7 of Supporting Information). For the parent sample **6**, two populations of degraded chains assignable to **12** and **14** are detected, whereas all the three populations corresponding to **12**, **13**, and **14** are observed from the degraded dendrimer-like PEO **7**. As for sample **11**, the proportion of **12** is too low to be detected and only the two populations of **13** and **14** can be identified (Figure S7C of Supporting Information). By taking into account the terminal groups of the three kinds of PEO's produced after degradation, the experimental peak values appear in excellent agreement with those calculated from the expected structures (Scheme S1 of Supporting Information). It should be stressed that the two populations corresponding to **13** and **14** exhibit almost the same degrees of polymerization of EO ( $\overline{DP}=44$ ), which represents two times the value targeted for each branch (molar mass  $1\,000\text{ g}\cdot\text{mol}^{-1}$ ,

**Table 1.** Molecular characteristics of ketal-containing dendrimer-like PEO's, before and after degradation.

Dendrimer-like PEO samples	$\bar{M}_n(\text{NMR})^{\text{a}}$ ( $\times 10^3$ )	Per PEO chain <sup>b)</sup>		$\bar{M}_n(\text{GPC})$ ( $\times 10^3$ )	PDI	$\text{N}_{\text{OH}}^{\text{c)}$	$\bar{M}_n(\text{GPC})^{\text{d)}$
		$\bar{M}_n(\text{theo})$	$\bar{M}_n(\text{NMR})^{\text{a)}$				
G1(OH) <sub>3</sub> ( <b>5</b> )	2.70	1 000	860	2.02	1.06	3	
G2(kt) <sub>3</sub> (OH) <sub>6</sub> ( <b>6</b> )	10.6	1 000	1 140	7.80	1.06	6	1 840/1.12
G3(kt) <sub>9</sub> (OH) <sub>12</sub> ( <b>7</b> )	24.7	1 000	1 020	16.1	1.05	12	1 750/1.11
G4(kt) <sub>21</sub> (OH) <sub>24</sub> ( <b>8</b> )	53.9	1 000	1 070	30.8	1.05	24	
G5(kt) <sub>45</sub> (OH) <sub>48</sub> ( <b>9</b> )	111	1 000	1 040	48.8	1.06	48	
G6(kt) <sub>93</sub> (OH) <sub>96</sub> ( <b>10</b> )	229	1 000	1 060	82.2	1.08	96	
G7(kt) <sub>189</sub> (OH) <sub>192</sub> ( <b>11</b> )	446	1 000	950	124	1.09	192	1 780/1.05

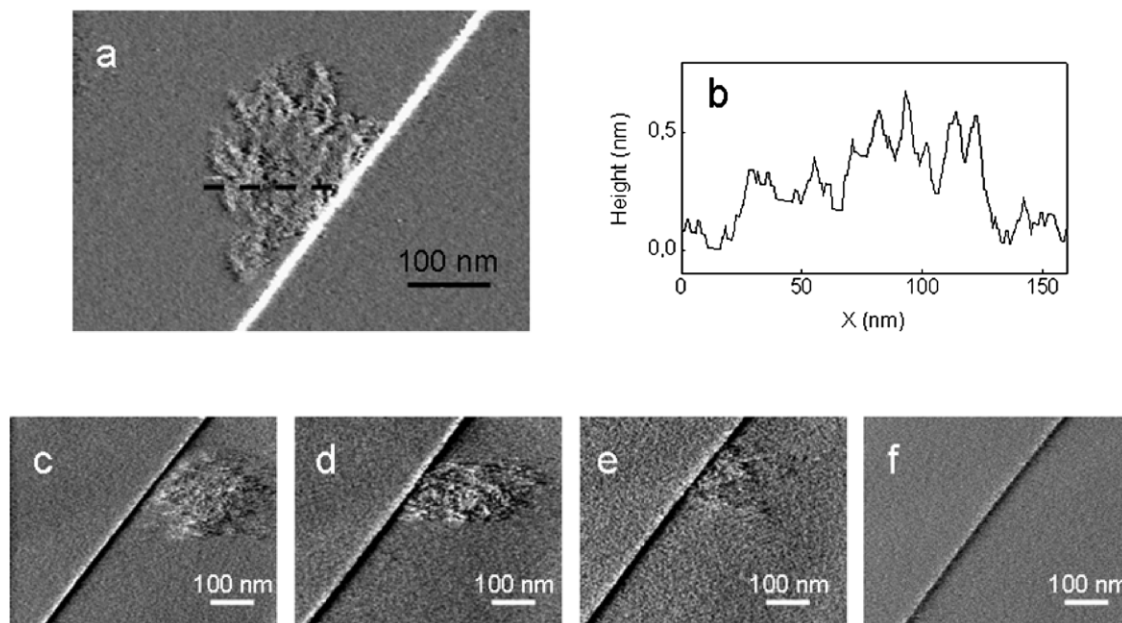
<sup>a)</sup>The total molar masses were calculated by formula 1 and 2<sup>[22]</sup>; <sup>b)</sup>The average chain length grown from the precursors of their respective generations; <sup>c)</sup>Theoretical values of peripheral hydroxyl groups; <sup>d)</sup>GPC analysis results of degraded products of dendrimer-like PEO's.

$\overline{DP}$   $22 \pm 1$ ) during the polymerization of EO. This further corroborates the results determined by  $^1\text{H}$  NMR (Table 1). Due to the presence of cleavable ketal linkages, a direct evidence of the uniformity of high generation dendrimer-like polymers prepared by a divergent strategy can thus be provided for the first time.

Next, we investigated the cleavage reaction of the ketal groups by  $^1\text{H}$ NMR spectroscopy at pH = 5.5. The dendrimer-like PEO of the highest generation, **11** ( $446 \text{ kg} \cdot \text{mol}^{-1}$ ), was dissolved in a buffer solution of pH 5.5 and transferred into an NMR tube. For comparison purpose, the same sample was placed under neutral conditions. Because of the formation of a ketone upon acidic cleavage, the signal of the methyl group connected to the ketone function was detected at 2.22 ppm without any interference (Figure S4 of Supporting Information). The dendrimer-like PEO underwent a fast degradation up to 65%, during the first 80 h (Figure S8 of Supporting Information). As the terminal groups of the dendritic PEG are OH functions, no hydrophobic effect is brought at the periphery of our dendrimer. Since no induction period was noted on the degradation profile (Figure S8 of Supporting Information), which could have reflected the fact that the aqueous acidic reagent could not easily access to the periphery, it is likely that both the peripheral ketal groups and those of the interior of the dendritic scaffolds are similarly accessible. Most of the ketal moieties (90%) could be hydrolyzed after 1 week. The sample used in this investigation with its high molar mass and degree of branching was actually found to exhibit a

similar degradation pattern at pH 5.5 to that of a linear cleavable homolog, as reported by Duncan and coworkers<sup>[13c]</sup> In the latter case, however, the degradation was observed under neutral conditions. In our case, no degradation product was observed from the dendrimer-like PEO placed during one week under the same neutral conditions, whereas degradation occurred smoothly at pH 7.4 from the linear homologs, with a noticeable degradation over a 3 week period. The globular structure of the dendrimer-like PEO may well have protected the inner ketal rings from hydrolysis under neutral conditions. Since such high molar mass dendrimer-like PEO's could be cleaved under weak acidic conditions, this type of PEO thus appears suitable as drug delivery scaffold with molar mass greater than the renal threshold, without concern that a storage disease syndrome might emerge on repeated administrations.<sup>[4]</sup>

The course of the degradation of **11** was finally monitored by AFM, providing a direct means of visualization of the induced change of morphology. Upon exposition of freshly cleaved graphite to a solution of **11**, objects of 120–140 nm diameter and few Ångströms height were observed on the mica surface upon analysis by AFM. A typical example is presented in Figure 2A, B. No modification of the adsorbed objects was detected 16 h after rinsing with a 0.1 M LiCl solution at neutral pH. In stark contrast, they progressively decomposed after the pH of the solution was reduced to 3.5; under these conditions, the degradation process could be easily monitored in a reasonable time scale by AFM.



**Figure 2.** Adsorption on graphite and acid-induced degradation of PEO-G7(ket)<sub>189</sub>(OH)<sub>192</sub> **11**. (A) Deflection AFM micrograph showing an individual molecule adsorbed nearby a step in the graphite substrate. (B) Height profile along the dashed line. (C–F) Sequence of deflection AFM micrographs measured at 15 min interval. The first image was measured 40 min after exposure of the dendrimer-like PEO coated graphite substrate to an acid aqueous environment (pH 3.5).

Panels C–F in Figure 2 show a sequence of AFM deflection micrographs taken at 15 min intervals at the same place on the graphite substrate, starting 40 min after exposing the preadsorbed dendrimer-like PEO molecules to acidic environment. The size of the molecule adsorbed nearby a step in the graphite surface gradually decreased; it eventually disappeared after 1 h of exposure to acid. These results establish the fact that our ketal-containing dendrimer-like PEO's can be degraded under acidic conditions. A more systematic investigation into the kinetics of the degradation under physiological conditions is in progress.

## Conclusion

Acid-sensitive dendrimer-like PEO's were synthesized by AROP of EO and quantitative functionalization/arborization reaction, using a purposely designed ABC-type branching agent featuring an acid sensitive ketal group. The well-defined structure of these dendrimer-like polymers could be attested through their characterization by NMR, SEC, and MALDI-TOF MS, and be further supported by analysis of the products of degradation. This is the first time that a direct evidence is provided about the uniformity of the branches constituting dendrimer-like polymers synthesized through an iterative divergent methodology. The degradation of the seventh generation dendrimer-like PEO, whose initial molar mass reaches a value as high as  $446 \text{ kg} \cdot \text{mol}^{-1}$ , occurs readily under acidic conditions, with a behavior similar to its linear homolog placed under similar conditions when end-fitted with the proper drug and appropriate receptor-targeting ligands. Although a more systematic investigation into the degradation profile as a function of pH and the generation number is needed, these high molar mass, multifunctional biodegradable dendritic PEGs thus fulfill the requirements of polymer carriers, and are expected to serve as a stable polymer-conjugate unimolecular nanoparticles in drug delivery applications. Work is in progress to conjugate targeting molecules at their periphery by chemical modification of the terminal OH groups, while the presence of the phenyl groups at the branching points could be beneficial to encapsulate a drug at the interior of these dendrimers.

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