Sequential Functionalization of Janus-Type Dendrimer-Like Poly(ethylene oxide)s with Camptothecin and Folic Acid

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ABSTRACT: Janus-type dendrimer-like poly(ethylene oxide)s (PEOs) of 1st, 2nd, and 3rd generation carrying terminal hydroxyl functions on one side and cleavable ketal groups on the other were used as substrates to conjugate folic acid as a folate receptor and camptothecin (CPT) as a therapeutic drug in a sequential fashion. The conjugation of both FA and CPT was accomplished by "click chemistry" based on the 1,3 dipolar cycloaddition coupling reaction. First, the hydroxyl functions present at one face of Janus-type dendrimer-like PEOs were transformed into alkyne groups through a simple Williamson-type etherification reaction. Next, the ketals carried by the other face of the dendrimer-like PEOs were hydrolyzed, yielding twice as many hydroxyls which were subsequently subjected to an esterification reaction using 2-bromopropionic bromide. Before substituting azides for the bromide of 2-bromopropionate esters just generated in the presence of NaN_3, an azido-containing amidified FA derivative was reacted through click chemistry with alkyne functions introduced on the other face of the dendrimer-like PEOs. A purposely designed alkyne-functionalized biomolecule derived from CPT was conjugated to the azido functions carried by the dendritic PEOs by a second "click reaction." In this case, twice as many CPT as FA moieties were finally conjugated to the two faces of the Janus-type dendrimer-like PEOs, the numbers of folate and CPT introduced being 2 and 4, 4 and 8, and 8 and 16 for samples of 1st, 2nd, and 3rd generation, respectively (route A). An alternate route for functionalizing the dendrimer-like PEO of 1st generation consisted, first, in conjugating the azido-containing CPT onto the alkyne groups present on one face of the dendritic PEO scaffold. The alkyne-functionalized FA was further introduced by click chemistry after the bromides of 2-bromopropionate esters were chemically transformed into azido groups. The corresponding prodrug thus contains 2 CPT and 4 FA external moieties (route B). Every reaction step product was thoroughly characterized by ^1H NMR spectroscopy. A preliminary investigation into the water solution properties of these functionalized dendritic PEOs is also presented. © 2011 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 49: 2839–2849, 2011

KEYWORDS: bioconjugates; camptothecin; conjugation; dendrimer; dendrimer-like; folic acid; poly(ethylene oxide)

INTRODUCTION Many challenges have to be overcome before a potent antitumor natural drug such as camptothecin (CPT) can be used in anticancer therapy.[1] One challenge stems in part from the instability of its E-lactone ring, resulting in the formation of toxic ring-opened carboxylate form.[2] Another problem relates to its poor water solubility, making necessary the conjugation of CPT for its delivery in the body. As it was established that the conjugation of the 20—OH of CPT could stabilize the E-lactone ring,[2] a variety of strategies have been contemplated to derivatize CPT at this position with the help of prodrugs and polymer conjugates.[3–7] Among the approaches currently under investigation, polymer conjugates offer undisputed advantages that arise from their ability to stabilize the lactone ring, to improve both the water solubility of the drug and its biological distribution, to increase circulation times within the body, to reduce its systemic toxicity, and to enhance the therapeutic efficacy. One of the most versatile biocompatible polymer used to this end is poly(ethylene oxide) (PEO), also referred to as PEG for polyethylene glycol, owing to its unique properties such as its chemical stability, its solubility in aqueous media, its nontoxicity, and its low immunogenicity and antigenicity.[8,9] Attempts at "PEGylating" CPT through a readily hydrolysable ester function have been extensively investigated.[3,10–21] Dextran,[22,23] poly(2-hydroxypropyl)methacrylamide,[24] poly(L-glutamic acid),[4,25,26] amongst others have been employed as other water soluble and biocompatible polymers for conjugation of CPT. In some cases, folic acid was conjugated at the

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other end of PEO providing bioconjugates targeting folate receptors in malicious cells.[12,13,16,17,19,20] Owing to its high affinity to the folate receptor and its nonimmunogenicity, and because it over-expresses on many tumors,[27] FA has been the most examined for its targeting role whereas CPT is often employed as a model drug.[16,17,26] However, the presence of only two conjugating sites at its chain ends limits the biological applications of linear PEG. As reported by Khandare et al.,[28] the anticancer activity of paclitaxel when conjugated to poly(amidoamine) (PAMAM) dendrimers is much higher than when the drug is conjugated to linear PEG, although the bioavailability of the drug could be improved in both cases.

On the other hand, conjugation of more than one category of biomolecules, that is, a drug, a targeting entity and a probe for imaging, on symmetrical dendrimers is not straightforward. Yet, Majoros et al. have functionalized the periphery of a PAMAM dendrimer of fifth generation with FA as the targeting moiety, fluorescein isothioisocyanate as the fluorescence probe and taxol as the drug.[29,30] In this example, however, the multifunctionalization of the PAMAM dendrimer occurred randomly. Among the different polymeric nanoparticles that are being investigated as multifunctionalized prodrugs carrying out multiple tasks at a time,[31,32] multifaced dendrimers—also called Janus-type or asymmetrically arranged dendrimers—with different types of peripheral functions for multifunctionalization have been designed following highly precise synthetic pathways.[33–38] However, *in vivo* studies performed from such multifaced dendrimers conjugated with biomolecules have shown that they seldom reach their target because of their cytotoxicity and of their uptake by the reticular endothelial system.[30,31] This can be overcome, however, by grafting linear PEO chains (PEGylation) on the outer surface of the dendritic scaffold. PEGylating the periphery of dendritic nanodevices indeed provides a protection from opsonization, enhancing their circulation time.[39–48]

Dendrimer-like PEOs provides a more direct solution combining the multivalency of the dendritic architecture with the specific properties of PEO mentioned earlier.[49] They essentially differ from regular dendrimers by the size of their polymeric generations between the branching points and by the fact that these generations are grown by a living chain polymerization. Dendrimer-like PEOs thus possess multiple peripheral functions that can be of different type and are not expected to be recognized by the body immune system due to the stealth effect of PEO. In the past five years, we designed a variety of such dendrimer-like PEOs. We first developed a new methodology of synthesis of high generation symmetrical dendrimer-like PEOs that involves the reiteration of (i) polymerization of ethylene oxide and (ii) arborization of PEO chain ends.[50,51] More recently, Janus-type dendrimer-like PEOs bearing orthogonal functions on their surface were also designed up to the generation 6.[50–52] We also prepared bifunctional dendrimer-like PEOs including an inner functionalization with poly(acrylic acid) chains.[53] Besides, bouquet-type dendrimer-like PEOs that could accommodate bovin serum albumin at the core and a therapeutic agent at their periphery were designed.[54]

In this contribution, we describe the precise and sequential conjugation using "click chemistry"[55–61] of Janus-type dendrimer-like PEOs from the first to the third generation, with folic acid (FA) as targeting group on one side and camptothecin (CPT) as drug on the other side. Recent examples of sequential orthogonal functionalization of biomolecules or polymers by variant approaches in click chemistry have been reported. For instance, Wolfbeis and coworkers have applied both copper-free and copper-mediated click reaction for the sequential labeling of biological targets.[62] Haddleton and coworkers have synthesized alkyne-functional macromonomers via catalytic chain transfer polymerization, followed by functionalization with sugar azides and thiols, using both Cu(I)-catalyzed azide–alkyne cycloaddition and thiol–ene Michael addition reactions.[63] As for Lutz and coworkers, they have reported the orthogonal modification of polystyrene chain-ends via sequential nitrile oxide–alkyne and azide–alkyne Huisgen cycloadditions, yielding heterotelechelic polystyrenes.[64]

The dendritic substrates based on PEO described here can accommodate more than one drug and FA molecules, the multivalent interactions of folates with the receptors being essential for the efficient delivery of the medicine to the target.[5] For this purpose, the peripheral groups of the parent Janus-type dendrimer-like PEOs were suitably derivatized both into alkynes and potent azido groups (bromides), and further reacted consecutively with a modified FA in the form of a folate alkyne and an azido-functionalized CPT, one after another. Additionally, we propose a variant for the conjugation of the dendrimer-like PEO of 1st generation used as substrate, by changing the order of conjugation of CPT and FA as compared to the former case. A dendrimer-like PEO prodrug with a different ratio of FA to CPT could thus be derived.

EXPERIMENTAL

Materials

Dichloromethane and dimethyl sulfoxide (DMSO) were distilled over CaH_2 prior to use. (*S*)-(+)-CPT (95%) was purchased from TCI Europe. All other chemicals and solvents (Aldrich) were used as received without further purification. The Janus-type dendrimer-like PEOs of 1st, 2nd, and 3rd generation, noted PEO-Gk1(ket)$_2$-Ga1(OH)$_2$ ($M_{n\ NMR}$ = 7,500 g mol^{-1}, PDI = 1.05), PEO-Gk2(ket)$_4$-Ga2(OH)$_4$ ($M_{n\ NMR}$ = 14,500 g mol^{-1}, PDI = 1.07), and PEO-Gk3(ket)$_8$-Ga3(OH)$_8$ ($M_{n\ NMR}$ = 36,500 g mol^{-1}, PDI = 1.07), respectively, were synthesized by anionic ring-opening polymerization of ethylene oxide carried out using Schlenk-type equipments, followed by arborization of PEO chains ends with appropriate branching agents. All details of these syntheses are described in our previously published report.[52] 2-aminoethyl azide was prepared following the procedure reported by Inverarity et al.[65]

^{1}H NMR (δ_{ppm}, $CDCl_3$): 3.34 (t, 2H, CH_2N_3), 2.86 (t, 2H, CH_2NH_2), 1.34 (s, 2H, CH_2NH_2).

Synthesis of Azido-Containing (2) and of Alkyne-Functionalized CPT (3)

The azido-CPT derivative (**2**) was synthesized following a similar procedure to that reported by Parrish and Emrick[66] Similarly, 5-hexynoic acid was used instead of 6-azidohexanoic acid to prepare the alkyne-containing CPT derivative (**3**); yield = 85%.

^{1}H NMR (δ_{ppm}, $CDCl_3$): 8.40 (s, 1H, C-7*H*), 8.24 (d, 1H, C-12*H*), 7.94 (d, 1H, C-9*H*), 7.84 (t, 1H, C-11*H*), 7.68 (t, 1H, C-10*H*), 7.21 (s, 1H, C-14*H*), 5.68 (d, 1H, C-17H_2), 5.40 (d, 1H, C-17H_2), 5.29 (s, 2H, C-5*H*), 2.66 (m, 2H, OOCCH_2), 2.29 (m, 3H, OOCCH$_2$CH_2CH_2(1)), 2.16 (m, 1H, OOCCH$_2$CH$_2$CH_2(1)), 2.03 (s, 1H, —C≡C*H*), 1.86 (m, 2H, C-19H_2), 1.01 (t, 3H, C-18H_3). ^{13}C NMR ($CDCl_3$, ppm): 172.2, 167.6, 157.5, 152.4, 148.8, 146.2, 146.0, 131.4, 130.9, 129.6, 128.6, 128.4, 128.3, 128.2, 120.4, 96.2, 83.1, 76.0, 69.6, 67.2, 50.1, 32.5, 32.0, 23.3, 17.8, 7.7.

Synthesis of Folate Azide (5) and of Alkyne-Functionalized Folic Acid (6)

These two derivatives were prepared following already published procedures.[67] Under a nitrogen atmosphere, 2-aminoethyl azide (0.34 g, 4.00 mmol) and dicyclohexylcarbodiimide (DCC) (1.88 g, 9.15 mmol) were added to a solution of folic acid (1.61 g, 3.66 mmol) in anhydrous DMSO (64 mL) and pyridine (32 mL), and the mixture was stirred overnight at room temperature. The precipitated products were filtered off and the filtrate was gradually poured into a vigorously stirred solution of cooled diethyl ether (1 L). The yellow precipitate was collected by filtration, and dried under vacuum to yield 1.67 g (yield = 95%) of the amidated product (**5**) as a yellow solid.

FTIR (cm^{-1}): 2105; ^{1}H NMR (DMSO-d6): d 11.45 (br s, 1H), 8.64 (s, 1H), 8.06 (br m, 2H, CONH), 7.65 (d, 2H), 6.93 (br t, 2H, NH), 6.63 (d, 2H), 4.48 (d, 2H), 4.35 (m, 1H), 3.33 (m, 2H, CH_2N$_3$), 3.22 (m, 2H, CH_2NH), 2.29–2.15 (m, 2H), 1.98–1.82 (m, 2H); ^{13}C NMR (DMSO-d6): 174.0, 172.1, 166.3, 160.8, 156.5, 153.7, 150.7, 148.6, 129.0, 127.9, 121.3, 111.1, 52.7, 49.9, 45.9, 38.1, 30.4, 26.9.

Similarly, the alkyne-functionalized folic acid derivative (**6**) was prepared in 95% yield using propargyl amine instead of 2-aminoethyl azide. ^{1}H NMR (DMSO-d6): d 11.45 (br s, 1H), 8.64 (s, 1H), 8.26 (br m, 1H, CONH), 8.00 (br m, 1H, CONH), 7.65 (d, 2H), 6.93 (br t, 2H, NH), 6.63 (d, 2H), 4.48 (d, 2H), 4.35 (m, 1H), 3.83 (m, 2H, CH_2NH), 3.06 (t, 1H, —C≡C*H*), 2.29–2.15 (m, 2H), 1.98–1.82 (m, 2H); ^{13}C NMR (DMSO-d6): 174.0, 171.6, 166.2, 161.0, 156.5, 153.8, 150.7, 148.6, 129.0, 127.9, 121.3, 111.1, 81.2, 72.8, 52.7, 45.9, 31.7, 30.4, 26.9.

Conjugation of CPT and Folic Acid Derivatives Onto Janus-Type Dendrimer-Like PEOs by Click Chemistry

These chemical modifications were carried out under an inert atmosphere of nitrogen using Schlenk equipments.

Synthesis of PEO-Gk1(Br)$_4$-Ga1(FA)$_2$

Into a 3-neck flask, PEO-Gk1(Br)$_4$-Ga1(yne)$_2$ (0.65 g, 0.16 meq.) folate azide **5** (0.12 g, 0.24 mmol) and 10 mL of DMSO were added. After the materials were dissolved, sodium ascorbate (16 mg, 0.08 mmol) and copper sulfate (8 mg, 0.03mmol) were added. The reaction mixture was stirred for 48 h at room temperature. After dilution with water (50 mL), the solution was dialyzed against deionized water for 24 h to remove the salts and DMSO. The precipitates were removed by centrifugation and the obtained solution was lyophilized, affording the conjugated PEO (0.6 g, 82%); the ^{1}H NMR characterization of this PEO derivative is shown in Figure 3.

Synthesis of PEO-Gk1(N$_3$)$_4$-Ga1(FA)$_2$

PEO-Gk1(Br)$_4$-Ga1(FA)$_2$ (0.6 g, 0.27 meq.), NaN_3 (53 mg, 0.81 mmol) and DMSO (10 mL) were introduced into a 3-neck flask. The suspension was stirred at room temperature for overnight. After the precipitates were removed by filtration, the filtrate was precipitated into an excess of diethyl ether. The product was isolated after filtration and dried under vacuum (yield = 95%). FTIR (cm^{-1}): 2110; the ^{1}H NMR spectrum of this compound is displayed in Figure 3.

Characterization

^{1}H NMR spectra were recorded on a Bruker AC 400 spectrometer. Bruker Tensor 27 spectrometer was used for FTIR analysis. Dynamic light scattering (DLS) experiments were performed using an ALV Laser Goniometer, which consists of a 22 mW HeNe linear polarized laser with 632.8 nm wavelength and an ALV-5000/EPP Multiple Tau Digital Correlator with 125 ns initial sampling time. The samples were kept at constant temperature (25 °C) during all experiments. The accessible scattering angular range varied from 40° up to 150°. The solutions were introduced into 10 mm diameter glass cells. The minimum sample volume required for the experiment was 1 mL. The data acquisition was realized using the ALV-Correlator Control Software, and the counting time varied for each sample from 300 s up to 600 s. The hydrodynamic radius (R_H) of these populations of dendrimer-like PEOs was then calculated from the diffusion coefficient using the Stokes-Einstein relation $D = kT/6\pi\eta R_H$, where η is the viscosity of the medium (water). Micelles were prepared by dissolving 10 mg of conjugates in 2 mL of DMSO and adding to vigorously stirred deionized water (8 mL). DMSO was then eliminated by dialysis against deionized water with a dialysis tube (10 mL) (cut-off of 3500 Da) for two days. The micelles solution was passed through 0.45 μm filter before characterization.

RESULTS AND DISCUSSION

The three Janus-type dendrimer-like PEOs consisting of one, two and three generations of PEO arms on each side are denoted as PEO-Gk1(ket)$_2$-Ga1(OH)$_2$ (M_n = 7,500 g mol^{-1}), PEO-Gk2(ket)$_4$-Ga2(OH)$_4$ (M_n = 14,500 g mol^{-1}) and PEO-Gk3(ket)$_8$-Ga3(OH)$_8$ (M_n = 36,500 g mol^{-1}), respectively (see Fig. 1). In these notations, Gkn refers to the *n*th generation of the PEO dendron synthesized from a ketal-containing (k) branching agent, whereas Gan refers to the *n*th generation of the other PEO dendron constructed with allyl chloride (a) as branching agent. Finally, (ket)$_m$ and (OH)$_m$ represent the total number of hydroxyls and ketal rings, respectively, present on each face of these dendritic PEOs. As

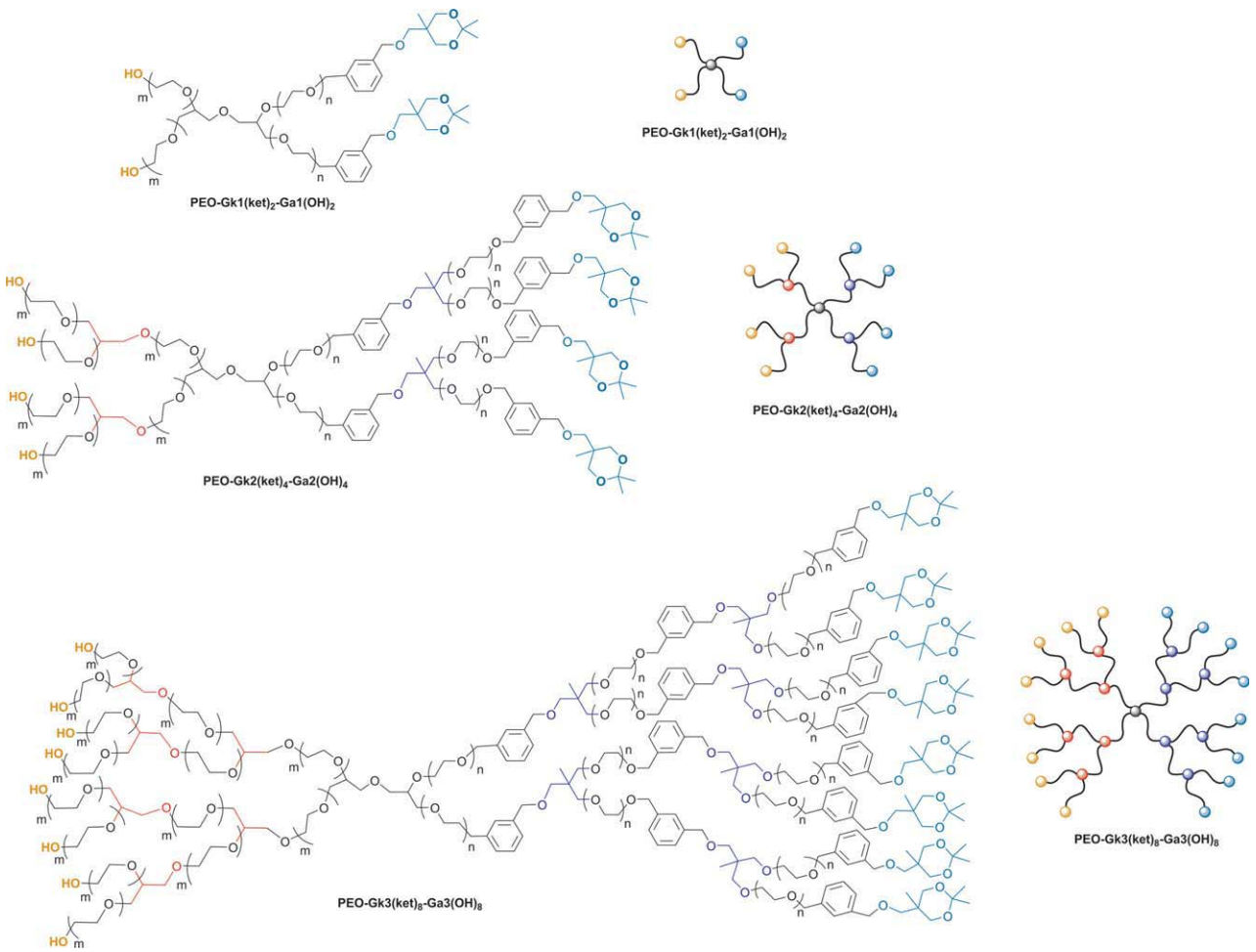


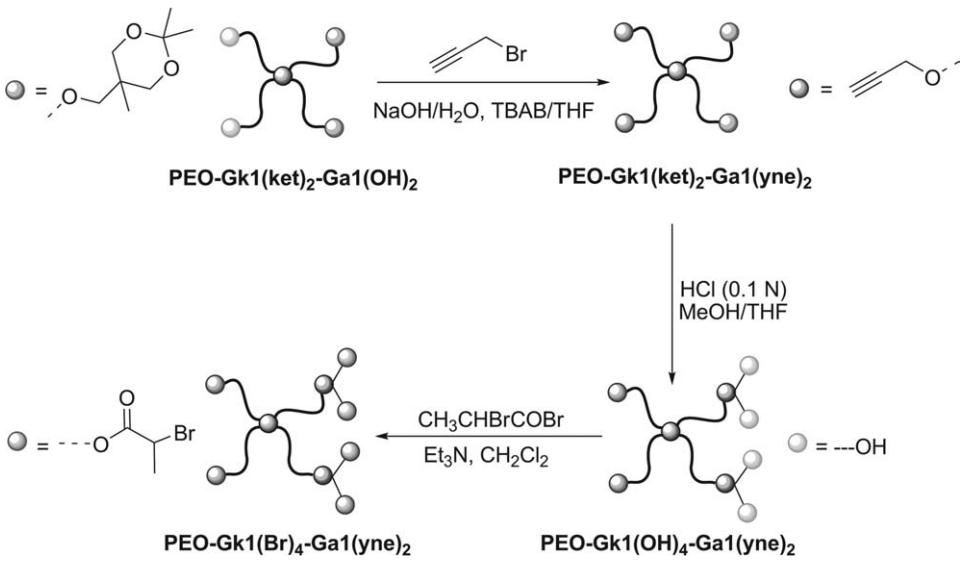
FIGURE 1 Structure of the three Janus-type dendrimer-like PEOs used for conjugation.

reported elsewhere,[52] the synthesis of these dendrimer-like PEOs carrying orthogonal functional groups on their surface is based on the reiteration of an alternate and divergent procedure, combining the anionic ring-opening polymerization of ethylene oxide and the selective arborization of PEO branches.

To achieve a high reaction efficiency, conjugation of biomolecules onto PEG is generally based on an esterification or amidation through an activation step of a carboxylic acid group.[3,10–21] To conjugate our Janus-type dendrimer-like PEOs, we turned here to a more reliable coupling reaction of biologically active molecules, that is, by the copper-catalyzed Huisgen's 1,3-dipolar cycloaddition between an alkyne-containing reagent and an azido-containing antagonist reagent, which refers to as a click chemistry. [55–61] The chemical modification of PEO-Gk1(ket)$_2$-Ga1(OH)$_2$, PEO-Gk2(ket)$_4$-Ga2(OH)$_4$ and PEO-Gk3(ket)$_8$-Ga3(OH)$_8$ through such a sequential functionalization is illustrated in Scheme 1.

The hydroxyls carried by one side of the dendritic PEOs were thus derivatized into alkynes by a Williamson etherification reaction using propargyl bromide as functionalizing agent. Then, the terminal ketal groups of the other face of the dendritic scaffold were deprotected under acidic conditions, thus releasing the hydroxyl groups. The latter functions were then esterified with 2-bromopropionic bromide and the corresponding bromides adjacent to the ester groups were subsequently transformed into azido groups.[68] Figure 2 shows the representative ^{1}H NMR spectra after consecutive derivatizations of PEO-Gk1(ket)$_2$-Ga1(OH)$_2$ into PEO-Gk1(ket)$_2$-Ga1(yne)$_2$, PEO-Gk1(OH)$_4$-Ga1(yne)$_2$ and PEO-Gk1(Br)$_4$-Ga1(yne)$_2$. The notation (yne)$_m$ refers here to the presence of m alkyne functions at the periphery of one PEO dendron. After alkynation of the two terminal hydroxyls present at one side of the dendrimer, two new peaks, c and e that can be ascribed to the methylene and methine protons appear at 4.21 and 2.45 ppm, respectively. The ratio of the c:e:g peaks is equal to 2:1:3, as expected, indicative of a quantitative transformation of the two hydroxyls into two alkynes. The peak denoted as f at 1.5 and 1.6 ppm due to the resonance of the methyl of the ketal rings vanishes [Fig. 2(B)], confirming the complete deprotection of the hydroxyls at the other side of the Janus-type dendrimer-like PEO.

The ^{1}H NMR spectrum of the esterification product generated by treatment with 2-bromopropionic bromide is shown in Figure 2(C). The signals denoted as h at 4.60 ppm and at



SCHEME 1 Chemical modification of Janus-type dendrimer-like PEOs of 1st generation (part I): introduction of peripheral alkyne and bromoesters moieties. For a sake of simplicity, the chemical modification is shown here only for PEO derivative of first generation (see also Scheme 4).

4.20-4.10 ppm assignable to the methine proton of the secondary bromide and to the ester methylene protons overlap with other peaks (c and i). The signal denoted as j due to the resonance of the methyl protons of bromopropionate groups appears as a doublet separately at 1.78 ppm. In addition, the peak g attributable to the methyl groups of the branching point shifts to 1.1 ppm, owing to the influence of the terminal ester group, as already observed in our previous studies.[52] Using the peak j as a reference for integration, the ratio of e:j:g peaks is equal to 1:6:3, again attesting to the efficient esterification of the four hydroxyl groups on one PEO dendron. Importantly, the alkyne groups previously introduced is not affected at all by this esterification step.

Similarly, samples of higher generation noted PEO-Gk2(ket)$_4$-Ga2(OH)$_4$ and PEO-Gk3(ket)$_8$-Ga3(OH)$_8$ were successfully obtained (Schemes 1 and 3).

Therefore, the two types of peripheral groups introduced on the Janus-type dendrimer-like PEO of 2nd and 3rd generation, namely PEO-Gk2(Br)$_8$-Ga2(yne)$_4$, and PEO-Gk3(Br)$_{16}$-Ga3(yne)$_8$, could serve for further click reactions with biologically active molecules. Folic acid and CPT were selected

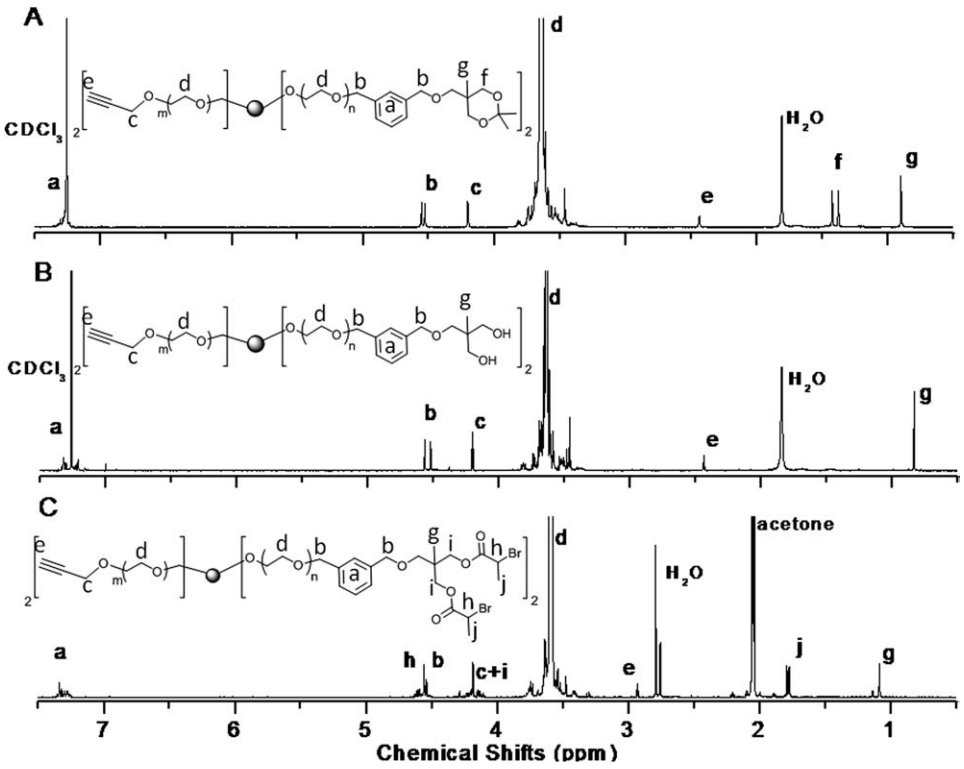
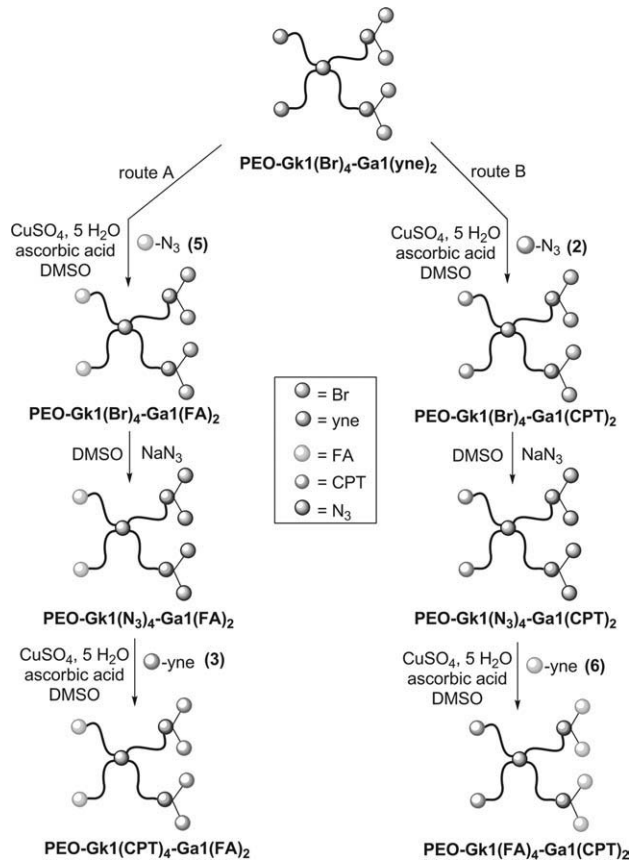


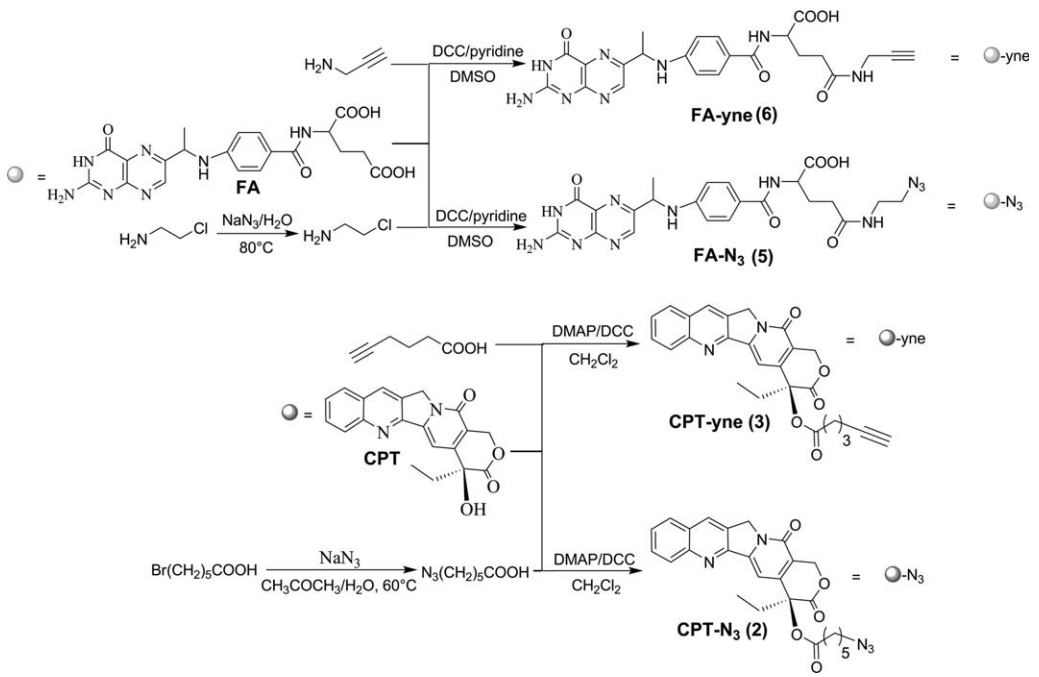
FIGURE 2 ^{1}H NMR spectra of PEO-Gk1(ket)$_2$-Ga1(yne)$_2$ (A, in $CDCl_3$), PEO-Gk1(OH)$_4$-Ga1(yne)$_2$ (B, in $CDCl_3$), and PEO-Gk1(Br)$_4$-Ga1(yne)$_2$ (C, in acetone-d$_6$).



SCHEME 3 Chemical modification of Janus-type dendrimer-like PEOs of 1st generation (part II): introduction of peripheral folate and camptothecin functions.

for conjugation with these PEO-based dendritic scaffolds. Accordingly, both FA and CPT were first chemically modified with antagonist functions, as illustrated in Scheme 2. An azido group was readily introduced onto FA through an amidation reaction using 2-aminoethyl azide, yielding the folate azide (**5**). As for CPT, its tertiary hydroxyl group was esterified with 5-hexynoic acid, according to an already published procedure,[66] affording the alkyne-modified CPT (**3**). As verified by ^{1}H and ^{13}C NMR spectroscopy (see Experimental), the analyzed structures exactly corresponded to the expected FA and CPT derivatives.

Next was the conjugation by click chemistry of the dendrimer-like PEOs with these biomolecules, first following the route A shown in Scheme 3. To this end, the catalytic system based on $CuSO_4$ and ascorbic acid was preferred over CuBr/PMDETA to avoid a possible loss of bromide by elimination from the bromoester functions at the other side of the Janus-type dendrimer-like PEO. A slight excess of **5** was thus added to ensure a complete conjugation. The ^{1}H NMR spectrum of the dendritic PEO thus derivatized is shown in Figure 3(A). Compared with the spectrum of its precursor PEO-Gk1$(Br)_4$-Ga1$(yne)_2$ [Fig. 2(C)], one can note that the signals of propargyl group at 4.21 and 2.94 ppm vanished, and a new series of characteristic peaks (k, m, o) appearing at 8.66, 7.65 and 6.64 ppm due to the resonance of protons of FA moieties confirmed that conjugation occurred efficiently. From the signal of methyl group at 0.99 ppm taken as a reference, the ratio of peaks k:m:o:h:j:g was equal to 1:2:2:2:6:3, indicating that the reaction of the PEO-Gk1$(Br)_4$-Ga1$(yne)_2$ with **5** was complete. Importantly, the bromide groups carried by the other side of the dendrimer-like PEO remained intact under the conditions of the click reaction.



SCHEME 2 Synthesis of alkyne- and azido-containing folate and camptothecin derivatives.

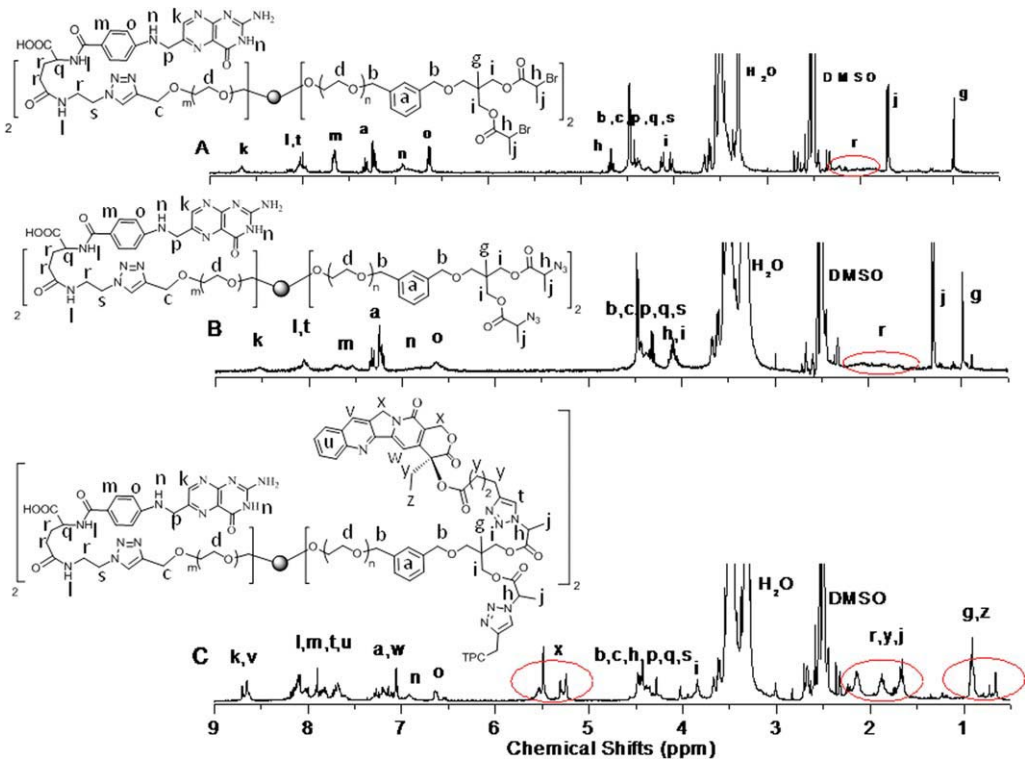


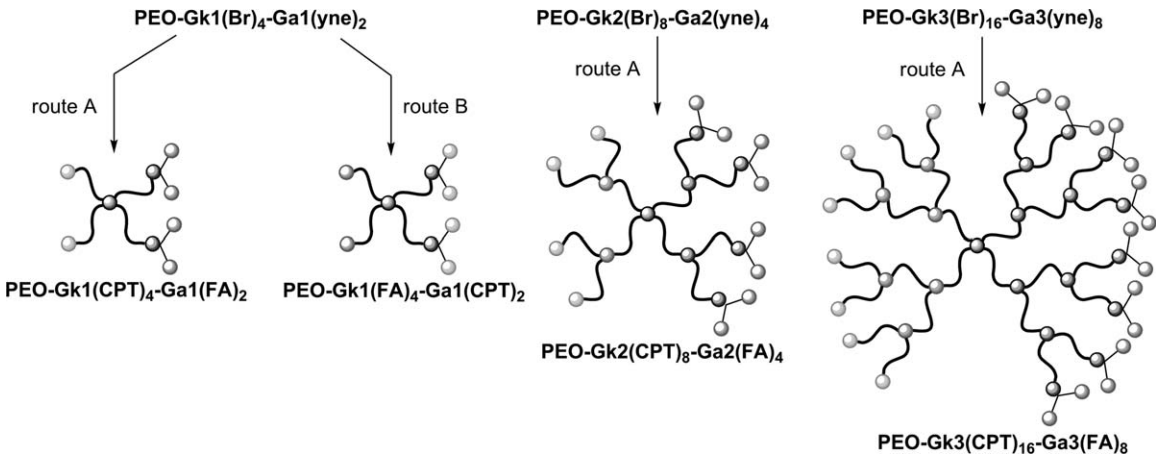
FIGURE 3 Representative ^{1}H NMR spectra of conjugate products (in DMSO-d_6): (A) PEO-Gk1Br$_4$-Ga1(FA)$_2$, (B) PEO-Gk1(N3)$_4$-Ga1(FA)$_2$, and (C) PEO-Gk1(CPT)$_4$-Ga1(FA)$_2$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The other face of this dendrimer-like PEO was then submitted to an azidification reaction before conjugation with the alkyne-modified CPT (**3**). The displacement of the bromide of the bromoester groups by azides using NaN_3 occurred readily. As seen in Figure 3(B), indeed, the signal of the methine group completely vanished and another peak appeared at 4.11 ppm and overlapped with peak i, after displacement by the azide. This chemical modification could also be ascertained by the fact that the signal of the methyl protons of the bromopropionate group which previously appeared at 1.70 ppm [Fig. 3(A)] was shifted upfield at 1.30 ppm after treatment with NaN_3 [Fig. 3(B)]. Under the same "click reaction" conditions as those previously used, twice as many CPT moieties as FA ones were thus conjugated on the other side of the Janus-type dendrimer-like PEO of 1st generation. The peaks due to aromatic protons of CPT were found to overlap with those due to FA, inducing an increase of the signal intensity in that region. Another characteristic peak denoted as x and due to the protons in position 5 and 17 of the CPT rings appears distinctly around 5.5 ppm [Fig. 3(C)], supporting the occurrence of conjugation.

Following the same route A of Scheme 3, the two other Janus-type dendrimer-like PEOs of 2nd and 3rd generation, PEO-Gk2(Br)$_8$-Ga2(yne)$_4$ and PEO-Gk3(Br)$_{16}$-Ga3(yne)$_8$, were also conjugated, with twice as many CPT on one side as FA on the other. The structure of all dendrimer-like PEOs synthesized in this work is provided in Scheme 4.

As shown in route B of Scheme 3, changing the order of conjugation allowed us to achieve dendritic PEO conjugates carrying, in this case, twice as many FA as CPT moieties. For this purpose, CPT and FA were initially modified with the appropriate functionalizing agents, as depicted in Scheme 2. Similar reaction conditions as those described earlier for the synthesis of **3** and **5** were implemented. Thus, 6-azidohexanoic acid was prepared by azidification from 6-bromohexanoic acid and was further esterified with the tertiary hydroxyls of CPT, affording the azido-containing CPT (**2**).[66] Similarly, FA was reacted with propagyl amine yielding the alkyne-functionalized FA (**6**). The latter FA derivative was further conjugated by click chemistry onto the azido-containing dendrimer-like PEO-Gk1(N$_3$)$_4$-Ga1(CPT)$_2$ arising from the same PEO-Gk1(Br)$_4$-Ga1(yne)$_2$ substrate as that employed for route A (Scheme 3). Thus, a dendrimer-like PEO of 1st generation, denoted as PEO-Gk1(FA)$_4$-Ga1(CPT)$_2$, carrying two peripheral CPT moieties at one side and four FA groups at the other could be obtained through repeated click chemistry. The ^{1}H NMR spectra of all conjugates are consistent with the expected structure in each case and the peaks can be assigned unambiguously, as shown in Figures 3 and 4.

It should be mentioned that the water solution properties of the different Janus-type dendrimer-like PEOs changed dramatically after conjugation. The hydrophobic character of both CPT and FA moieties obviously made the dendritic PEO conjugates much less soluble in water, as compared to the



SCHEME 4 Synthetic pathway to Janus-type dendrimer-like PEOs with peripheral camptothecin dans folate moieties.

parent substrates before conjugation. First inspection by ^{1}H NMR suggested the formation of micellar aggregates with CPT moieties—and partly the folates—being wrapped by the PEO chains. For instance, the ^{1}H NMR spectrum of PEO-Gk1(CPT)$_4$-Ga1(FA)$_2$ run in a mixture of DMSO-d$_6$ and D$_2$O did not show the resonance of the peaks due to the CPT moieties (Fig. 5), in particular that appearing at 5.5 ppm due to the protons at C5 and C17 positions [see Fig. 3(C)]. In contrast, the characteristic protons of the folate moieties could be partly detected, for instance, peaks k, m, o (Fig. 5), suggesting that these latter groups were not entirely shielded by PEO.

Water solution properties of all the four PEO conjugates shown in Scheme 4 were further investigated by dynamic light scattering (DLS). Because of the aforementioned hydrophobicity of folates and CPT moieties, the dendritic PEO conjugates could not be directly dissolved in water. Hence, they were first dissolved in DMSO then dialyzed against water, and the micellar aggregates formed in this way were analyzed by DLS (Fig. 6). The prodrug based on the Janus-type

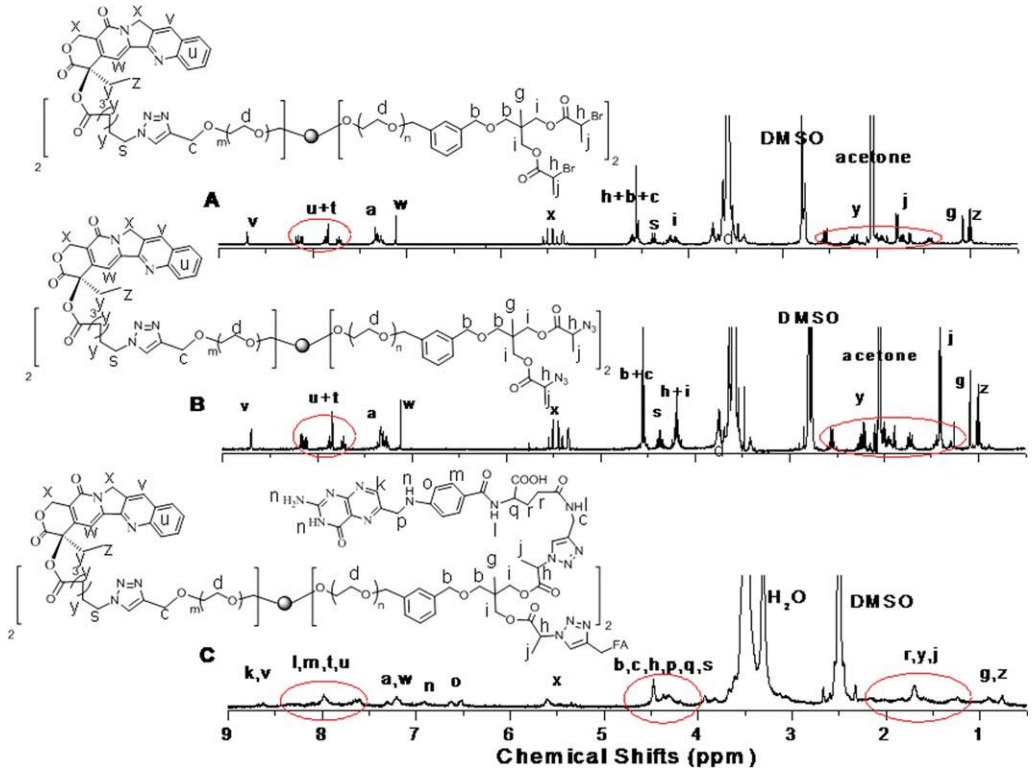


FIGURE 4 ^{1}H NMR spectra of conjugate products following the route B of Scheme 2: (A) PEO-Gk1Br$_4$-Ga1(CPT)$_2$ (acetone-d$_6$), (B) PEO-Gk1(N3)$_4$-Ga1(CPT)$_2$ (acetone-d$_6$), and (C) PEO-Gk1(FA)$_4$-Ga1(CPT)$_2$ (DMSO-d$_6$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

dendrimer-like PEO of 1st generation, PEO-Gk1$(CPT)_4$-Ga1$(FA)_2$, showed one major population of large size (D_H = 220 nm) along with two other less intense populations of lower degree of aggregation. Upon increasing the number of generations, two populations for PEO-Gk2$(CPT)_8$-Ga2$(FA)_4$ and only one single population for PEO-Gk3$(CPT)_{16}$-Ga3$(FA)_8$ were observed, respectively. The size of these micellar aggregates decreased from 220 to 192 to 146 nm for PEO-Gk1$(CPT)_4$-Ga1$(FA)_2$, PEO-Gk2$(CPT)_8$-Ga2$(FA)_4$, and PEO-Gk3$(CPT)_{16}$-Ga3$(FA)_8$, respectively. This decrease in size might be explained by an increase of compactness as the generation number increased.

Although the weight ratios of PEO chains to CPT and FA moieties do not vary significantly between the three dendritic PEO bioconjugates, their respective degree of branching drastically varies. As reported by Wang et al.,[69] a low degree of branching as in the 1st generation cannot prevent intermolecular associations of the hydrophobic components, the PEO arms forming a relatively loose outer shell. In contrast, for the 2nd and 3rd dendrimer-like PEOs carrying CPT and FA functions exhibit a higher degree of branching, and the formation of a relatively compact shield may be favored, preventing intermolecular hydrophobic aggregations from developing to a large extent. As for the conjugate of 1st generation obtained by the alternate route of functionalization and possessing the lowest content of hydrophobic CPT, PEO-Gk1$(FA)_4$-Ga1$(CPT)_2$, only one population of micellar aggregate is observed by DLS with a value of D_H = 124 nm. The folate part of this compound can well be detected by ^1H NMR, which indicates that FA moieties are not embedded in the core of the aggregate, but rather reside in the outer shell ready to target its receptor site. A more thorough investigation into the solution properties of these conjugated dendrimer-like PEOs is in progress. A biological evaluation of these materials *in vitro* and *in vivo* is also currently ongoing.

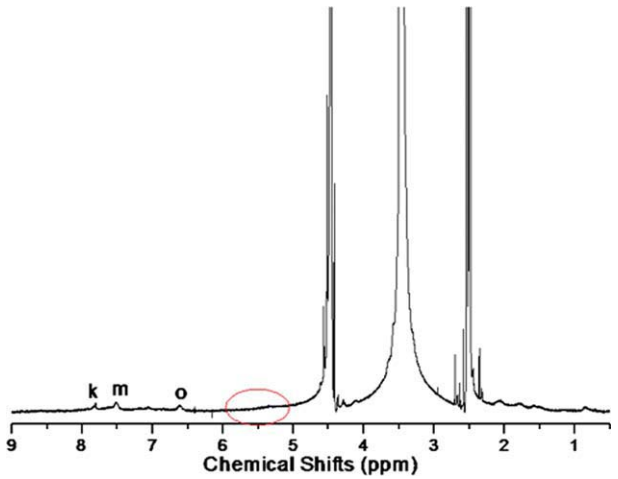


FIGURE 5 ^1H NMR spectrum of conjugate products PEO-Gk1$(CPT)_4$-Ga1$(FA)_2$ in the mixture of DMSO-d_6 and D_2O (1: 4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

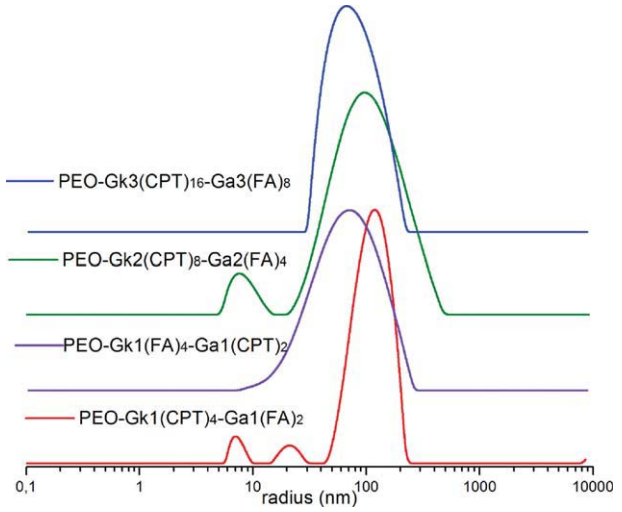


FIGURE 6 Dynamic light scattering results of different Janus-type PEO dendrimer conjugates with camptothecin and folic acid in water. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

Asymmetric dendritic scaffolds consisting of true PEO arms between the branching points, referred to as Janus-type dendrimer-like PEOs, can serve to derive bioconjugates carrying multiple folic acid and CPT moieties. The multivalency provided by the dendritic architecture can thus be combined with the specific properties of PEO, including biocompatibility, water solubility, nontoxicity, and nonrecognition by the immune system. The multiple folate and CPT moieties of these bioconjugates are aimed to target receptor sites and to treat the infected targets, respectively. Such polymeric prodrugs are expected to exhibit better biological activities than linear PEO counterparts. Click chemistry proves a particularly efficient coupling method to conjugate biologically molecules sequentially onto these Janus-type dendrimer-like PEOs. This can be achieved thanks to consecutive and alternate chemical modifications of the peripheral groups, one dendritic face after the other. The number of conjugated biomolecules carried by these prodrugs can be adjusted by varying the generation number of the dendritic PEO substrates and/or the order of functionalization. The biological activity of these novel dendritic bioconjugates is in progress, in particular in order to scrutinize the optimal number of CPT and folic acid molecules that are required for an efficient application.

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