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Synthesis of a folate functionalized PEGylated poly(propylene imine) dendrimer as prospective targeted drug delivery system

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ABSTRACT

Based on fourth generation diaminobutane poly(propylene imine) dendrimer, a novel targeted drug nanocarrier was prepared, bearing protective PEG chains and a folate targeting ligand. As a control a PEGylated derivative without folate was also synthesized. The encapsulation and release properties of these PEGylated derivatives were investigated employing etoposide, an anticancer hydrophobic drug. Enhanced solubility of etoposide was achieved inside the dendrimeric scaffold which was subsequently released in a controlled manner. These properties coupled with specificity towards the folate receptor and the low toxicity render folate functionalized PEGylated poly(propylene imine) dendrimer promising candidate for targeted drug delivery.

Dendrimers are highly branched and monodispersed macromolecules with well-defined morphology, nano-sized architecture which are prepared by multi-step synthetic procedures.[1,2] They consist of a central core, branching units and surface functional groups. Appropriate functionalization of the surface groups has proved as a fruitful strategy for preparing diversified materials including systems for drug delivery.[3–6] Encapsulation or conjugation of bioactive molecules in these functional dendrimers may reduce their toxicity, enhance their solubility and prolong blood clearance.[7,8] Additionally, appropriate dendrimer-based drug delivery systems can enable controlled or triggered release[9] and specific delivery of bioactive molecules to the diseased or damaged tissue.[10]

The role of poly(ethylene glycol) chains (PEG chains) is crucial among other groups employed for the functionalization of surface dendritic groups. Thus, PEGylation in addition to enhancing water solubility and reducing immunogenicity of the carrier, it also protects the so-obtained PEGylated dendritic derivatives in the biological milieu as it has long been established for liposomes,[11,12] prolonging their circulation time, which is a property of critical importance for drug delivery systems. Additionally, in certain cases PEG coating enhances solubilization efficiency for hydrophobic compounds.[12–14] Furthermore, the introduction of a folate group at the end of PEG chains induces a targeting property to the dendritic carrier due to its endocytosis into folate receptor-bearing cells.[15,16] The folate receptor is known to be significantly over-expressed in a wide variety of human cancers and on activated macrophages.[17] Folate-mediated targeting has already been used with liposomes,[18,19] dendrimers[20–22] and hyperbranched dendritic polymers[23,24] and nanoparticles.[25,26]

In the present study, a PEGylated poly(propylene imine) dendrimer is synthesized which in addition to the protection exercised by the PEG chains it is also functionalized by a folate targeting ligand at the end of the one PEG chain. In this manner the folate group, due to its location at the distal end of PEG chain, is accessible to interact with complementary receptor. A targeted dendrimeric drug delivery system is therefore produced which, in this preliminary study, is physicochemically characterized with the prospect of being applied as drug delivery for the lipophilic drug etoposide, which is encapsulated in its nanocavities.

In addition, a PEGylated poly(propylene imine) dendrimer without folate has also been prepared to be used as a control. Specifically, functional polymers derived from poly(propylene imine) dendrimers of fourth generation (DAB) were prepared bearing at their external surface poly(ethylene glycol) chains with or without folate group. For investigating this potential application, experiments have been performed, employing etoposide, an anticancer hydrophobic drug.[27] Etoposide is applied for a variety of malignancies but it is strongly lipophilic[28] and exhibits haematological toxicity[29] which are major obstacles for its administration. The encapsulation and release properties of this prospective dendrimeric drug nanocarrier as well as preliminary toxicity and cell internalization studies were conducted.

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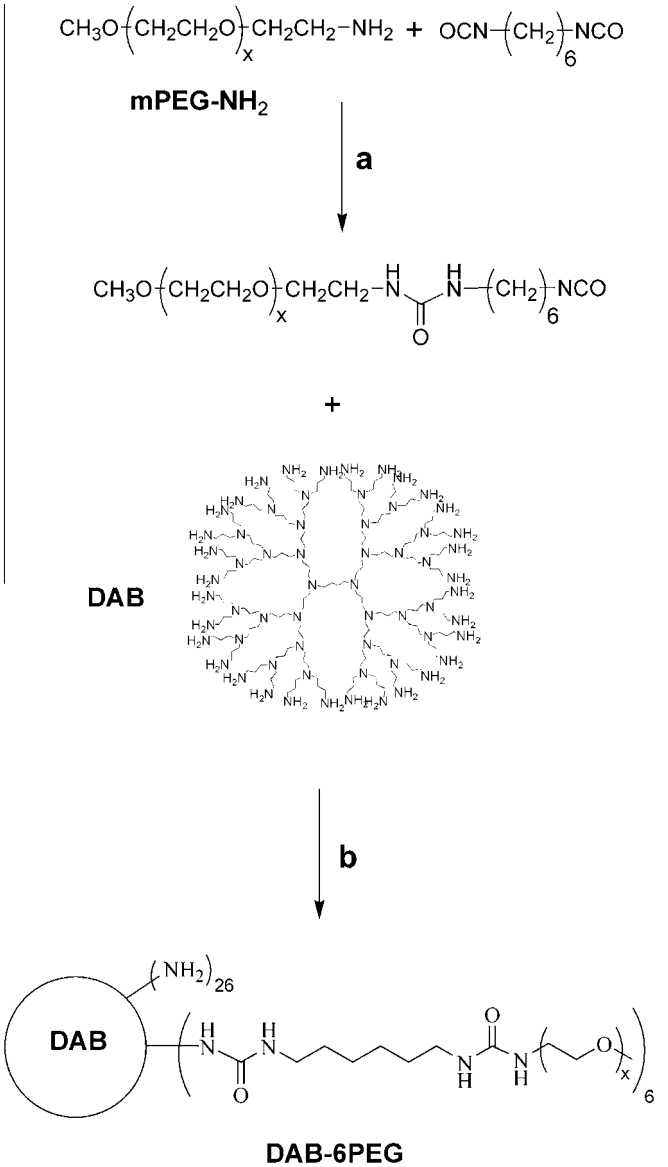
PEGylation of diaminobutane poly(propylene imine) dendrimers of the fourth generation, DAB, was achieved by a method analogous to one previously reported.[30] Thus, functionalization of DAB with poly(ethylene glycol) chains, that is, DAB-6PEG was achieved in two steps as shown in Scheme 1. In the first step, one of the isocyanate groups of 1,6-diisocyanatohexane was interacted with the amino group of methoxy poly(ethylene glycol)-amine (mPEG-NH_2) in dry dichloromethane at room temperature for 4 h under argon atmosphere, while in the second step the activated PEG without purification, reacted immediately with amino groups at the external surface of the DAB in the same solvent at room temperature for 24 h, under argon atmosphere. The final product, DAB-6PEG was received following precipitation with isopropanol in 75% yield. The structure of PEGylated dendrimer was established by FT-IR as well as by proton and carbon NMR spectroscopy (see Supplementary data). The average number of attached PEG chains per polymer was found to be 6, as established by ^{1}H NMR spectrum. Specifically, the degree of substitution was determined from the integration of the peaks at ~3.10 and 3.25 ppm attributed to methylenes next to the newly formed urea moieties (PEGCH_2NHCONHCH_2– and DAB-CH_2NHCONHCH_2–) and the peaks at ~2.45 ppm attributed to α methylene relative to tertiary amines.



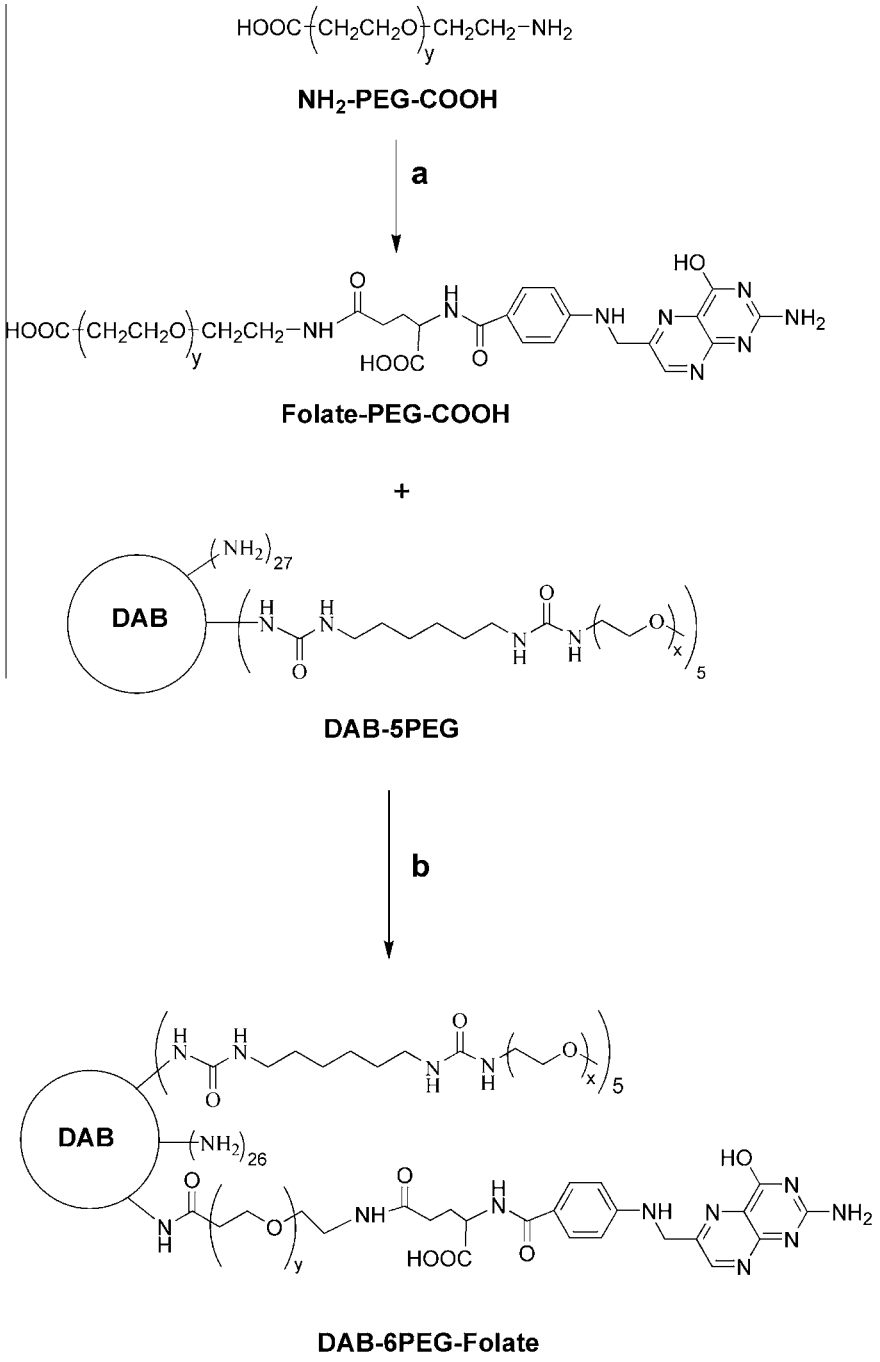
Scheme 1. Synthesis of DAB-6PEG. Reagents and conditions: (a) mPEG-NH_2 (1.5 equiv), 1,6-diisocyanatohexane (1.5 equiv), dry CH_2Cl_2, room temperature, 4 h, (b) addition of DAB (0.2 equiv) in dry CH_2Cl_2, room temperature, 24 h, 75% yield.

For the preparation of the folate functionalized PEGylated DAB derivative, that is, DAB-6PEG-Folate, a multi-step process was used (Scheme 2). Initially, the Folate-PEG-COOH intermediate was prepared by a method analogous to one previously described (Scheme 2).[31] Briefly, folic acid was activated to its hydroxysuccinimidylester, using *N*-hydroxysuccinimide (NHS) and *N*-dicyclohexylcarbodiimide (DCC), and subsequently reacted with α-amino-ω-carboxy poly(ethylene glycol) (H_2N-PEG-COOH), affording the carboxylate Folate-PEG derivative (Folate-PEG-COOH) in 85% yield. The structure of Folate-PEG-COOH was confirmed by proton and carbon NMR spectroscopy (see Supplementary data). Subsequently, a PEGylated DAB with five PEG chain (DAB-5PEG) was prepared by a method analogous of the previously prepared PEGylated derivative. The final multifunctional derivative was obtained by reacting DAB-5PEG with Folate-PEG-COOH (Scheme 2) using HBTU/HOBt/DIEA as coupling reagents (89% yield). The structure of the compound was confirmed by FT-IR as well as by ^{1}H NMR spectroscopy (see Supplementary data). On the average, one PEG chain bearing folate at its distal end was attached at the dendritic scaffold as determined from the integral ratio of the signal at 8.60 ppm corresponding to the methine group at position 7 of the pterin ring of folate, relative to the signal at ~2.45 ppm attributed to α methylene relative to tertiary amines of DAB. Additionally, the folate/polymer ratio was also determined by UV spectroscopy and found to be 0.94–0.96, which was in line with results from proton NMR experiments.

The size exclusion chromatograms of DAB-6PEG and DAB-6PEG-Folate showed one sharp peak with a polydispersity index of 1.04 and 1.07, respectively (see Supplementary data).

The cellular membrane transport of the PEGylated dendrimers was monitored by employing labeled dendrimeric derivatives. Their preparation was achieved by reacting the PEGylated derivatives (1 equiv) with fluorescein isothiocyanate (FITC, 1.1 equiv), in freshly distilled DMF, for 24 h at room temperature in the dark to avoid photobleaching of the probe. The resulting products were purified by dialysis against water (mol. weight cut-off: 1200) and finally obtained following lyophilization. As established from ^{1}H NMR spectrum, one fluorescein moiety was attached to one molecule of PEGylated dendrimer by integrating the peak at ~3.15 ppm, attributed to the CH_2 of the dendrimeric derivatives relative to the urea group and the peak at 6.55 ppm attributed to the ortho-aromatic protons relative to the hydroxyl groups of FITC.

For assessing the loading capacity and release properties of the above PEGylated dendrimeric derivatives, etoposide was employed (see Supplementary data). The encapsulated etoposide in aqueous dendrimeric solutions (0.14 mM) was determined by UV absorption spectroscopy. Comparison of solubilization ability in the parent and PEGylated dendrimers is shown in Table 1. It is seen that the concentration of encapsulated etoposide in dendrimers is higher than in phosphate buffer saline (PBS), that is, 0.2 mM. Specifically, solubility of etoposide is increased by a factor of ~14 in DAB aqueous solution, or otherwise ~21 molecules of drug are solubilized per dendrimeric molecule. This increase is attributed to the acidic character of etoposide (pK_a = 9.8), although the formation of a charge-transfer complex between the aromatic rings of etoposide and the tertiary amino groups of dendrimer, as previously established for DAB encapsulated pyrene,[14,32,33] cannot be ruled out. In case of PEGylated derivatives, the amount of the encapsulated etoposide is almost the same with the parent dendrimer. This contradicts other resent reports,[12–14,23,32] where PEG coating enhances solubilization efficiency for hydrophobic compounds. It is therefore concluded that etoposide is only incorporated inside the dendrimeric core.



Scheme 2. (i) Synthesis of Folate-PEG-COOH. Reagents and conditions: (a) folic acid (1 equiv), DCC (1 equiv), NHS (1 equiv), triethylamine (1 equiv), dry DMSO, overnight, addition of NH_2-PEG-COOH in dry pyridine, overnight, room temperature, 85% yield. (ii) Synthesis of DAB-6PEG-Folate. Reagents and conditions: (b) Folate-PEG-COOH (1.2 equiv), HBTU (2.4 equiv), HOBt (2.4 equiv), DIPEA (4.8 equiv), dry DMF, overnight, room temperature, 89% yield.

Table 1
Comparative solubility of etoposide in DAB and PEGylated dendrimeric derivatives

Dendritic derivative	[Dendrimer]/ mM	[Etoposide]/ mM	Etoposide/dendrimer molar ratio
DAB	0.14	2.92 ± 0.12	20.86 ± 0.86
DAB-6PEG	0.14	2.86 ± 0.09	20.43 ± 0.64
DAB-6PEG-Folate	0.14	2.84 ± 0.11	20.28 ± 0.78

The in vitro release of etoposide from dendrimers was studied in PBS, pH 7.4 at 37 °C, using the dialysis method (see Supplementary data). The etoposide released in the outer phase was measured by UV spectroscopy. The release profiles of etoposide from both parent and PEGylated DAB dendrimers as well as the pure drug release profile are shown in Figure 1. Pure etoposide completely diffused (~100%) through the dialysis membrane with a linear release rate within 5 h, while ~60% etoposide was released from DAB dendrimer in the first 4 h, followed by the relatively slow release of about 15% of the loaded drug in the next 3 h. (Fig. 1). In addition, even slower release rate is observed when PEGylated derivatives were employed. Specifically, ~40% of drug was released in a steady rate from DAB-6PEG and DAB-6PEG-Folate within the first 4 h, while a relatively slow release of about 25% of etoposide is ob-

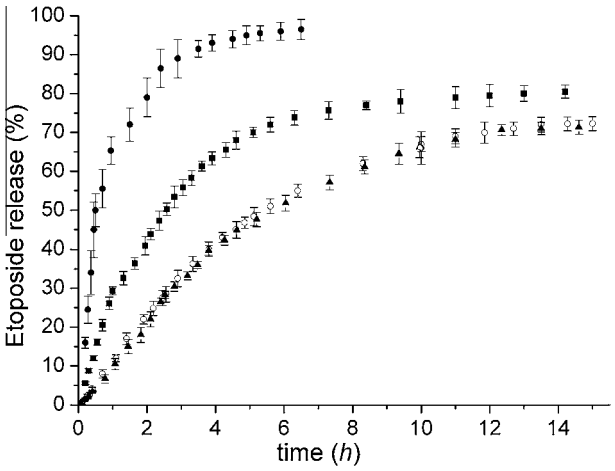


Figure 1. Release in vitro profiles of etoposide from aqueous solution of DAB and PEGylated dendrimeric derivatives (0.14 mM) in phosphate buffer saline (pH 7.4) at 37 °C as well as pure drug release profile. Free etoposide: solid circles; DAB: solid squares; DAB-6PEG: open circles; DAB-6PEG-Folate: triangles.

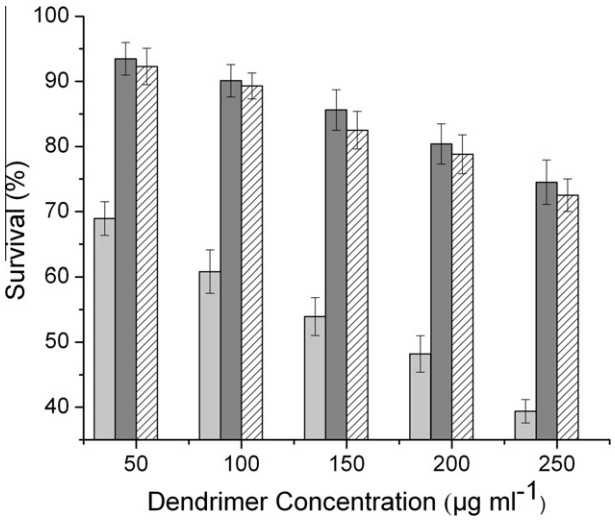


Figure 2. Cell viability of MDA-MB-468 cells incubated for 24 h with dendrimeric derivatives as demonstrated by flow cytometry using propidium iodide staining of fixed cells. DAB: light grey columns; DAB-6PEG: grey columns; DAB-6PEG-Folate: hatched columns.

served during the next 7 h. It is obvious that etoposide is released at a slower rate when PEG chains are present on the surface of the dendrimer than when the non-PEGylated dendrimer is used. In addition, the introduction of folate moiety at the surface of DAB-6PEG did not influence the in vitro release profile of etoposide, as observed in Figure 1.

The cytotoxicity of the parent DAB and dendrimeric functional derivatives was assessed by flow cytometry using propidium iodide staining of fixed cells. Following the protocol described in Supplementary data, human breast adenocarcinoma cells (MDA-MB-468) were seeded in 6-well plates and incubated for 48 h with various concentrations of the dendrimeric derivatives ranging from 50 to 250 μg mL^{-1}. The % percentage of cell death was estimated by the percentage of sub-G1 events. As shown in Figure 2, the viability (%) of cells gradually decreased as the concentration of dendrimeric derivatives increased. In addition, it is clear that the introduction of PEG chains at the external surface of dendrimer leads to a concom-

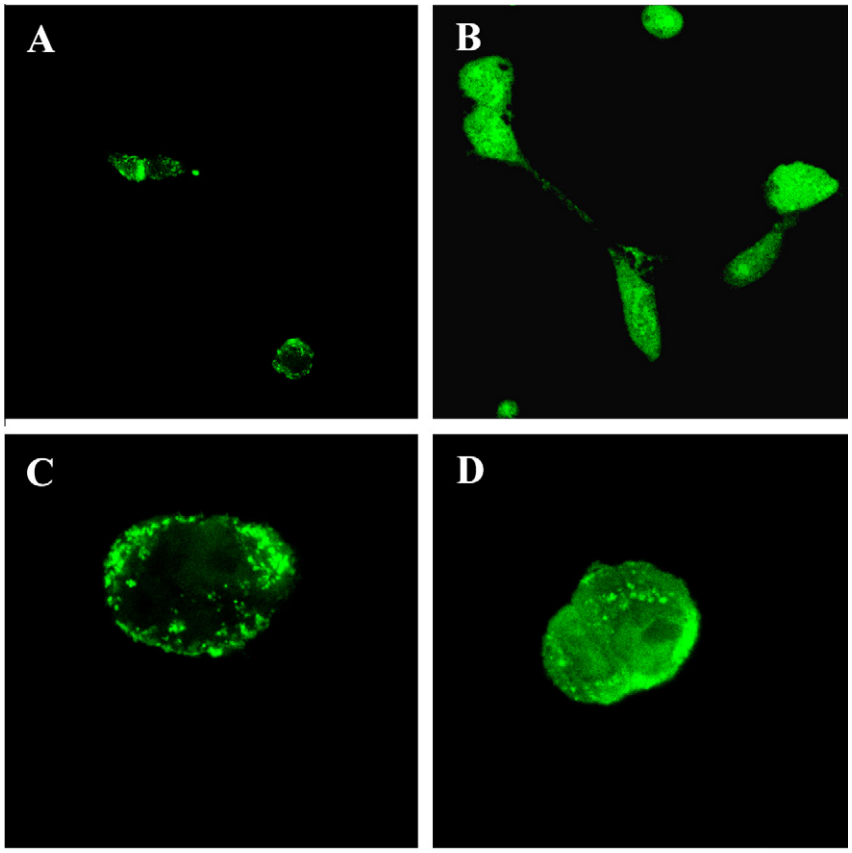


Figure 3. Confocal microscopy on MDA-MB-468 cells incubated with 200 μg mL^{-1} DAB-6PEG-FITC (A and C) and DAB-6PEG-Folate-FITC (B and D) for 24 h.

itant decrease of the dendrimer's cytotoxicity. This reduction was attributed to the decrease of the external primary amino groups coupled with the PEG coating located at the dendrimeric surface.[34] On the other hand the introduction of folate moiety at the surface of DAB-6PEG did not affect its cytotoxicity.

Cellular uptake of DAB-6PEG and DAB-6PEG-Folate was assessed by using confocal microscopy in order to investigate the ability of folate moieties to enhance the internalization of DAB-6PEG into MDA.MB.468 cells, which overexpress the folate receptors (see Supplementary data). For this purpose, cells seeded on round glass cover-slips in 35 mm Petri dishes, were incubated with 200 μg mL^{-1} DAB-6PEG-FITC and DAB-6PEG-Folate-FITC for 24 h and subsequently imaged for FITC fluorescence using confocal microscopy. Images A and C in Figure 3 depict cells incubated with DAB-6PEG-FITC, while images B and D depict cells incubated with DAB-6PEG-Folate-FITC. DAB-PEG and DAB-PEG-Folate images were acquired using the same laser power for excitation. Control confocal images of cells without any added polymer have shown complete lack of fluorescence (data not shown). Taking into account that the fluorescence intensity of the images B and D in Figure 3 is much higher compared to that of images A and C, it is evident that internalization was more efficient with DAB-6PEG-Folate-FITC. It is therefore obvious from these that folate moieties enhance internalization of DAB-6PEG by MDA.MB.468 cells.

In conclusion, employing fourth generation DAB dendrimer, a novel multifunctional drug delivery system was prepared, bearing protective PEG coating for enhanced circulation in biological milieu and a folate targeting ligand for enhancing cell specificity. Encapsulation of the anticancer drug etoposide was achieved due to non-covalent interactions inside the dendrimeric scaffold and it was found that 20-21 molecules of drug are solubilized per dendritic polymer. The in vitro release of encapsulated etoposide from PEGylated dendrimers was found to be slower and sustained, comparable however to the non-functionalized dendrimer. The release profiles therefore revealed the controlled release nature of the PEGylated dendrimers. Cytotoxicity and cellular uptake studies reveal that folate PEGylated derivative has low toxicity, and also exhibits folate receptor specificity. Further studies of in vitro biological activity employing a variety of folate receptor-positive cell lines as well as in vivo animal studies are currently in progress in order to evaluate this system as a potential targeted drug nanocarrier.

Supplementary data

Supplementary data (detailed synthetic procedures, spectroscopic data for intermediates and final products, size exclusion chromatograms of mPEG-NH_2, DAB-6PEG and DAB-6PEG-Folate as well as biochemical assays for the final products) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.058.

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