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Synthesis and characterization of multifunctional hyperbranched polyesters as prospective contrast agents for targeted MRI

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ABSTRACT

Based on a commercially available hyperbranched aliphatic polyester, novel multifunctional gadolinium complexes were prepared bearing protective PEG chains, a folate targeting ligand and EDTA or DTPA chelate moieties. Their relatively high water relaxivity values coupled with biodegradability of the hyperbranched scaffold, folate receptor specificity render these non-toxic dendritic polymers promising candidates for MRI applications.

Low molecular weight contrast agents based on gadolinium chelates are widely used for enhancing the contrast in magnetic resonance imaging (MRI).[1–3] Gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA, Magnevist®) and Gadolinium-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (Gd-DOTA, Dotarem®) complexes as well as their derivatives are used in MRI for the diagnosis of a wide range of pathologies.[4] They are characterized by predominant positive signal enhancement in T_1-weighted MRI, tissue distribution and particularly high safety profile.[5,6] These complexes, however, exhibit rapid clearance rates from vascular circulation and fast renal excretion requiring, therefore, high dosage administration. Another shortcoming of these molecules is their non-specific tissue distribution. Due to these shortcomings, intense efforts are being made for developing target-specific agents coupled with high contrast efficiency.[7]

The conjugation of low molecular weight gadolinium chelates to macromolecules has been used to increase the rotational correlation time resulting in a relaxivity improvement per gadolinium atom.[6] Gd^{3+} complexes based on linear polymers,[8,9] dendrimers,[10–12] and hyperbranched polymers[12] have been developed in order to enhance sensitivity and decrease clearance rates. Specifically, dendrimers and hyperbranched polymers (collectively characterized as dendritic polymers) which are highly branched nano-sized macromolecules, consisting of a central core, branching units, and terminal groups can conveniently be functionalized with appropriate moieties affording a diversity of functional materials including MRI contrast agents,[11–13] drug,[14–16] and gene[17–19] delivery systems.

In this study, multifunctional dendritic polymers were synthesized and characterized bearing gadolinium chelates, a protective coating and a tissue-specific targeting moiety. Specifically, ethylenediaminetetracetic acid (EDTA) or diethylenetriaminepentaacetic acid (DTDA) groups, which can form gadolinium chelates,[20] were introduced on the surface of the hyperbranched polyester Boltorn™ H40 (BH40) which is biodegradable[21] and of low polydispersity after appropriate fractionation.[22] The introduction of poly(ethylene glycol) chains (PEG) at the external surface protects the synthesized contrast agents in the biological milieu, prolonging their circulation time, which is a property of critical importance for contrast agents.[8,23] Additionally, the introduction of a folate group at the end of PEG chain provides tissue specificity through receptor-mediated endocytosis.[24,25] PEGylated BH40 is known to be easily hydrolyzed in the presence of lipases (ca. 50% hydrolysis of ester bonds in 24 h),[21] while folate receptors are over-expressed in a wide variety of human cancers and on activated macrophages.[26] Water relaxation studies as well as cell toxicities were investigated in vitro using folate receptor positive (HeLa) and folate receptor negative (A549) tumor cell lines.

The functionalization of hyperbranched aliphatic polyester BH40 with EDTA or DTPA groups and poly(ethylene glycol) chains, with one of the latter chains bearing the folate targeting ligand at its end, that is, BH40-EDTA-PEG-Folate and BH40-DTPA-PEG-Folate was achieved employing the steps shown in Schemes 1–3. BH40 was initially interacted with EDTA or DTPA dianhydride in dry

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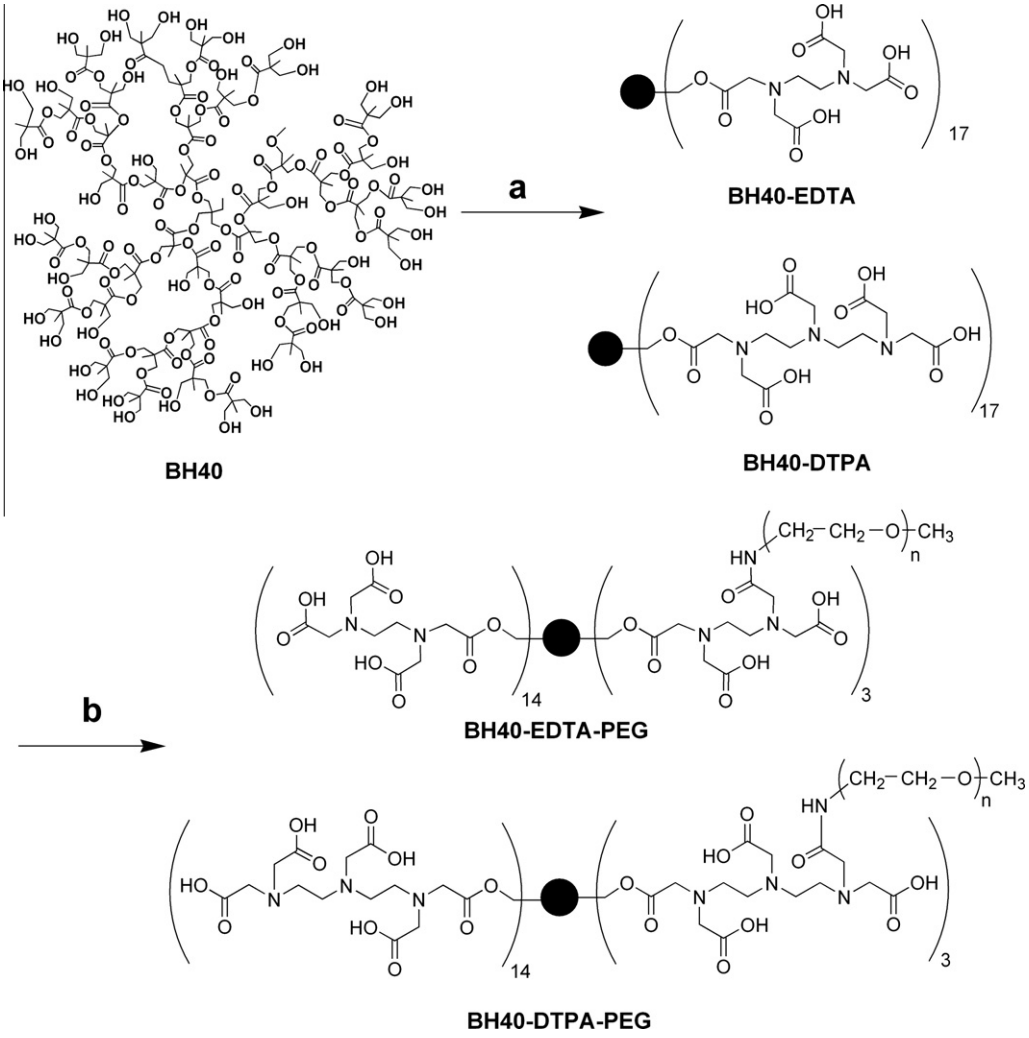
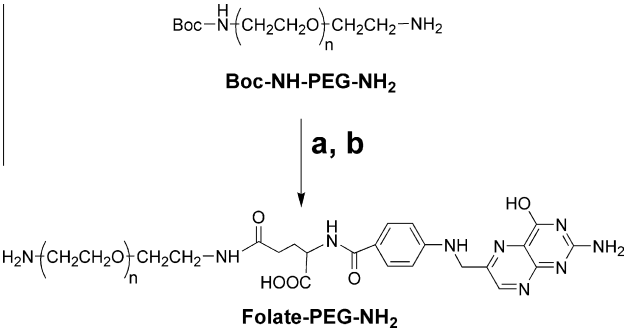


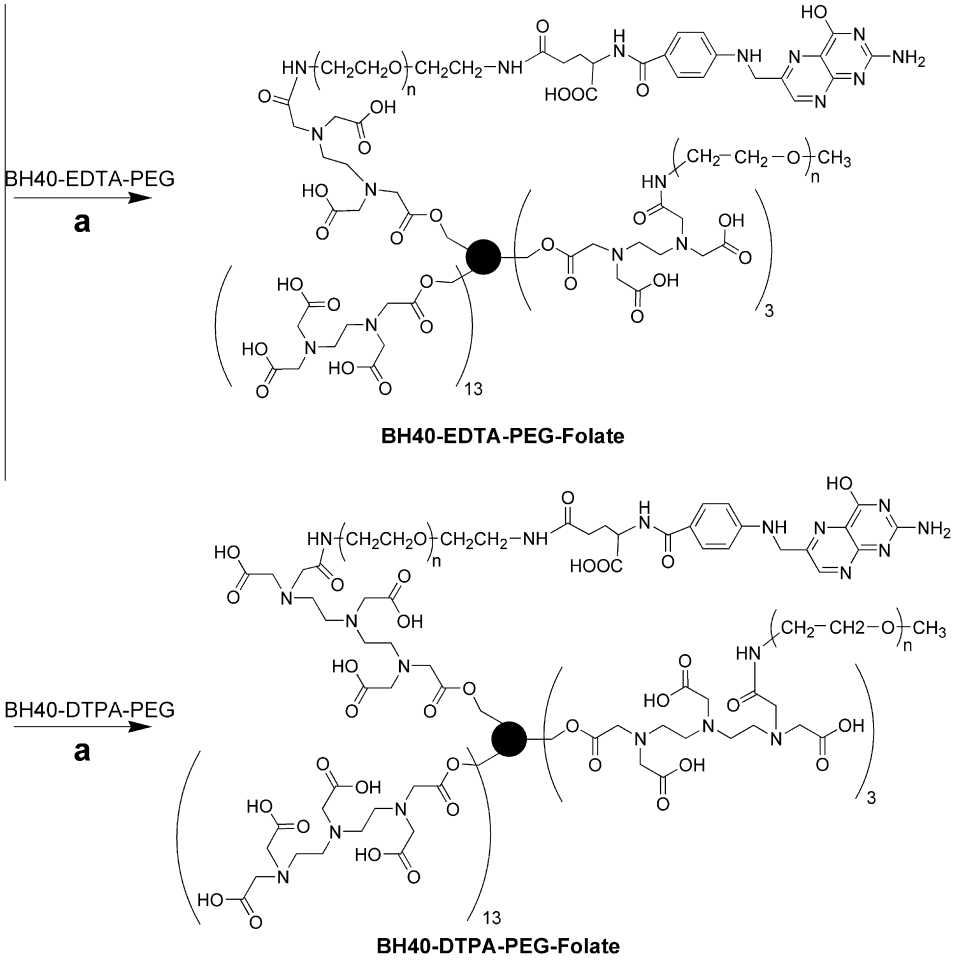
Scheme 1. Reagents and conditions: Synthesis of BH40-EDTA and BH40-EDTA: (a) EDTA or EDTA dianhydride (33 equiv), dry pyridine, 24 h, room temperature, 82% and 74% yield, respectively. Synthesis of BH40-EDTA-PEG and BH40-EDTA-PEG: (b) mPEG-NH_2 (3.3 equiv), EDC (3.3 equiv), NHS (3.3 equiv), 50 mM MES buffer, overnight, room temperature, 63% and 66% yield, respectively.



Scheme 2. Synthesis of Folate-PEG-NH_2. Reagents and conditions: (a) folic acid (1 equiv), DCC (1 equiv), NHS (1 equiv), triethylamine (1 equiv), dry DMSO, overnight, room temperature, addition of Boc-NH-PEG-NH_2 (0.25 equiv) in pyridine, overnight, room temperature, 82% yield; (b) deprotection/neutralization; i—TFA/$CHCl_3$, 3 h, room temperature; ii—$CHCl_3$, triethylamine, 2 h, room temperature, 96% yield.

pyridine at room temperature for 24 h (Scheme 1), affording the EDTA or DTPA polyester derivatives, BH40-EDTA or BH40-DTPA, respectively (82% and 74% yield). The average number of EDTA and DTPA moieties per polymer was 17, as revealed by ^{1}H NMR spectra (see Supplementary data). In the second step, PEG chains were introduced to these polyester derivatives by the reaction of methoxy poly(ethylene glycol)-amine with BH40-EDTA and BH40-DTPA (Scheme 1) affording the PEGylated derivatives, BH40-EDTA-PEG and BH40-DTPA-PEG, respectively (63% and 66% yield, respectively). This coupling reactions were performed using a 20% excess of both 1-ethyl-3(3-dimethyl aminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) in aqueous 50 mM 2-morpholinoethane sulfonic acid (MES) buffer (pH 5.5) at room temperature for 24 h.[27] The completion of the reaction was confirmed by the ninhydrin test and the final products were received after dialysis against water followed by lyophilization. The average number of attached PEG chains per polymer was found to be 3, as established by ^{1}H NMR spectra (see Supplementary data).

In the final step, the Folate-PEG-NH_2 intermediate was prepared by a method analogous to one previously reported (Scheme 2).[28] Briefly, folic acid was activated to its hydroxysuccinimidylester, using *N*-hydroxysuccinimide (NHS) and *N*-dicyclohexylcarbodiimide (DCC), and subsequently reacted with α-*tert*-butyloxycarbonylamino-ω-amino poly(ethylene glycol) (H_2N-PEG-NH-Boc), affording the amino protected Folate-PEG derivative in 82% yield. The cleavage of Boc group was achieved using TFA/$CHCl_3$ (1:1 v/v) to afford Folate-PEG-NH_2 in 96% yield. This compound was subsequently interacted with BH40-EDTA-PEG and BH40-DTPA-PEG



Scheme 3. Synthesis of BH40-EDTA-PEG-Folate and BH40-EDTA-PEG-Folate. Reagents and conditions: (a) Folate-PEG-NH_2 (2 equiv), HBTU (2 equiv), HOBt (2 equiv), triethylamine (4 equiv), dry DMF, overnight, room temperature, 55% and 54% yield, respectively.

using HBTU/HOBt/DIEA as coupling reagents (Scheme 3) to provide the final products, BH40-EDTA-PEG-Folate and BH40-DTPA-PEG-Folate, respectively (55% and 54% yield, respectively). The folate/polymer ratio was also determined by UV spectroscopy and found to be 0.92–0.95, which was in line with results from proton NMR experiments. The structure of the final products was further established by ^{13}C NMR spectroscopy (see Supplementary data).

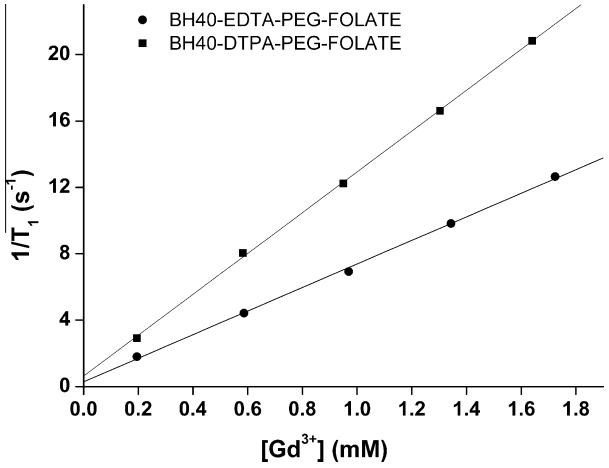


Figure 1. Longitudinal relaxation rates ($1/T_1$) versus the concentration of Gd^{3+} for BH40-EDTA-PEG-Folate (circles) and BH40-DTPA-PEG-Folate (squares) complexes.

BH40-EDTA-PEG-Folate and BH40-DTPA-PEG-Folate formed complexes with Gd^{3+} by a method analogous to one previously reported.[29] The resulting stoichiometry of the Gd^{3+}:DTPA groups in the BH40-DTPA-PEG-Folate polymer was 0.95, while the respective stoichiometry for the EDTA polymeric derivative was 0.32. Given that each EDTA group attached on the dendritic scaffold has three free carboxylic groups available for complexation (while in the case that a PEG chain is also covalently attached to EDTA, only two free carboxylic groups are present) it can be envisaged that two EDTA groups attached at spatially neighboring branches are taking part in the complexation of one Gd^{3+} ion. The stability of the complexes, is demonstrated by the fact that at the above stoichiometries no free gadolinium is detected in their aqueous solutions employing the xylenol orange method, and also that they are non-toxic even at high (1000 μM) gadolinium concentrations (vide infra).

MRI contrast agents enhance the $1/T_1$ nuclear magnetic resonance (NMR) relaxation rate of water protons in tissues or organs and subsequently alter the signal intensity in the magnetic resonance image relatively to parts where the contrast agent is not present. The relaxation mechanism responsible for the $1/T_1$ enhancement is the dipolar relaxation due to the direct interaction between the water protons and the magnetic moment of the unpaired electrons of the contrast agent.[6] The enhancement of the relaxation rate depends on the concentration of the paramagnetic ions and therefore the efficiency of the contrast agent is measured by the relaxivity parameter commonly defined by the equation

$$1/T_1(C) = 1/T_1(0) + r_1 C, \quad (1)$$

where $1/T_1(C)$ is the proton relaxation rate in the presence of the contrast agent, $1/T_1(0)$ is the relaxation rate of the pure solvent, C is the concentration of the contrast agent and r_1 is the relaxivity measured in $mM^{-1}\,s^{-1}$.

As shown in Figure 1, the proton inverse relaxation times were linearly proportional to the Gd^{3+} concentration according to Eq. (1). The relaxivities determined for Gd^{3+} complexes of BH40-EDTA-PEG-Folate and BH40-DTPA-PEG-Folate were $7.10_{\pm 0.15}$ and $12.30_{\pm 0.15}\,mM^{-1}\,s^{-1}$, respectively. These values are considerably higher than that of the low molecular weight analog commonly used in clinical practice; the r_1 value determined for the Gd-DTPA complex (Magnevist®) was found to be $3.93_{\pm 0.12}\,mM^{-1}\,s^{-1}$ under the same experimental conditions. These data suggest that, due to the macromolecular nature of the compounds their rotation is slowed down and the Gd^{3+} complexes introduced into hyperbranched polymers have an increased rotational correlation lifetime which is known to result in higher relaxivity values.[11] It should be also noted that the Gd^{3+}-DTPA hyperbranched complex exhibits a higher relaxivity value compared to the EDTA analog. Taking into consideration that the same hyperbranched scaffold and the same number of PEG chains are present in both cases, and therefore the rotational lifetimes must be similar, the observed variation in relaxivity values can be attributed to a difference in the water exchange rates. It is known[1,6,10a] that when rotational correlation times are short, as in this case, the relaxivity of complexes can strongly depend on their water exchange rate and that there is an optimum value of this parameter that maximizes relaxivity. Therefore, the observed difference in relaxivity values between the EDTA and DTPA hyperbranched complexes can be attributed to a water exchange rate for the DTPA derivative that is closer to the optimal value for the given rotational correlation lifetime of the complexes.

Cells used were the human lung carcinoma cell line A549 with no folate receptor (FR) expression and the HeLa cell line originating from human cervical cancer used as a FR-positive model. Cytotoxicity of BH40-EDTA-PEG-Folate and BH40-DTPA-PEG-Folate Gd^{3+} complexes was evaluated employing the above cell lines. Cells were incubated at various concentrations of the two complexes (20, 50, 200, 500, and 1000 μM with respect to Gd^{3+}) for 3 h in the presence or absence of 10% fetal bovine serum (FBS), while control cells were allowed in media not containing any complexes. XTT assays were performed both immediately following incubation and 24 h later to determine the compound toxicity by assessing mitochondrial redox function.

The cytotoxicities for the two cell lines studied are shown in Figures 2 and 3. The EDTA dendritic complex toxicity remained negligible in all cases, within experimental errors ($p > 0.5$ as shown by paired Student's t-tests) regardless of experimental parameters, that is, incubation time, cell line, presence or absence of fetal bovine serum (+/− FBS). The DTPA dendritic complex, showed also negligible cytotoxicity within experimental errors when the XTT assay performed immediately following a 3 h incubation. A residual cytotoxicity (~20%) was observed in the absence of FBS and only for the FR-positive HeLa cells. When the XTT assay was

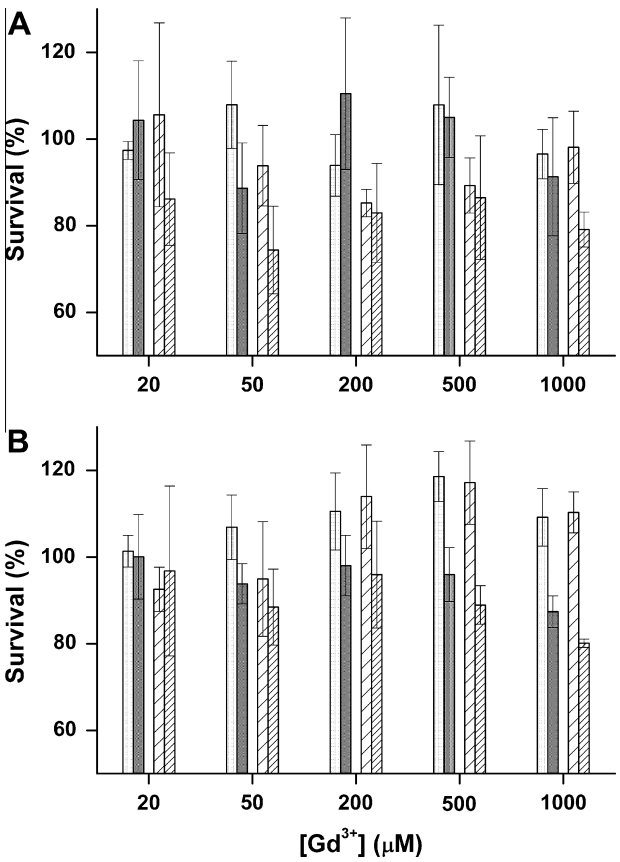


Figure 2. Cell viability of A549 and HeLa cells incubated for 3 h with Gd^{3+} complexes of BH40-EDTA-PEG-Folate and BH40-DTPA-PEG-Folate as demonstrated by standard XTT assays immediately following incubation in the absence (A) and presence (B) of FBS. EDTA complex with A549: light gray columns; DTPA complex with A549: gray columns; EDTA complex with HeLa: hatched columns; DTPA complex with HeLa: densely hatched columns.

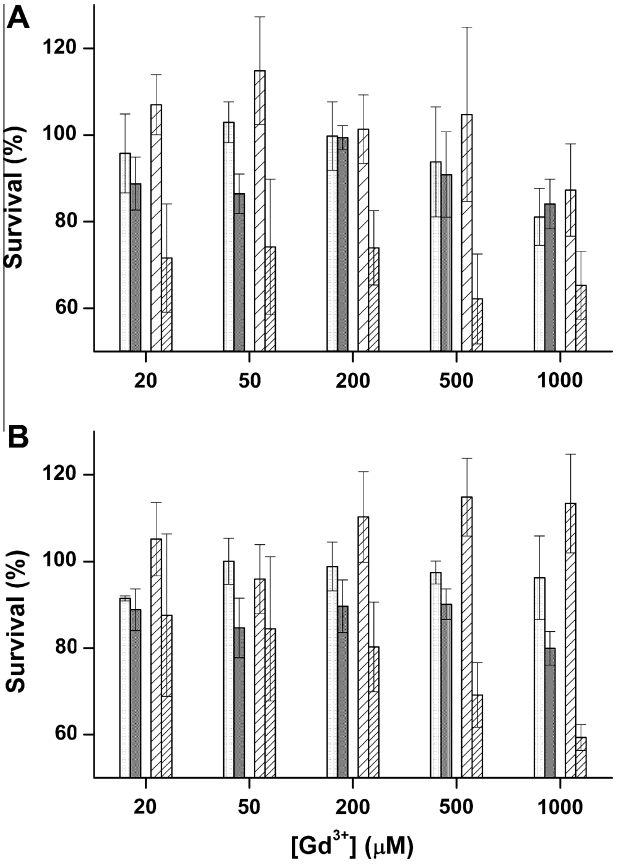


Figure 3. Cell viability of A549 and HeLa cells incubated for 3 h with Gd^{3+} complexes of BH40-EDTA-PEG-Folate and BH40-DTPA-PEG-Folate as demonstrated by standard XTT assays 24 h later in the absence (A) and presence (B) of FBS. EDTA complex with A549: light gray columns; DTPA complex with A549: gray columns; EDTA complex with HeLa: hatched columns; DTPA complex with HeLa: densely hatched columns.

repeated 24 h following a 3 h incubation of HeLa cells with DTPA complex, the cytotoxicity was enhanced both in the presence and absence of FBS (Fig. 3). This cytotoxicity reached ~40% at the highest Gd^{3+} concentration (1000 μM) in HeLa cells, and was statistically significant ($p < 0.001$ as revealed by paired Student's *t*-tests). As noted above, unlike A549 which are FR-negative, HeLa cells express FR and the observed cytotoxicity indicates enhanced interaction of these cells with folate functionalized complexes, leading to a more efficient internalization.

Given that minor, but statistically significant, cytotoxicity is only observed in FR-positive HeLa cells but not in FR-negative A549 cells, this toxicity cannot be attributed to free non-complexed Gd^{3+} but rather to the DTPA-folate functionalized derivative being efficiently transferred intracellularly. Indeed, free Gd^{3+} toxicity would have been FR independent and expected to have the same impact on HeLa as well as A549 cells. It is also well known that the toxicity of gadolinium containing MRI contrast agents, is based upon the amount of Gd^{3+} that dissociates from its chelate.[30] Free Gd^{3+} is extremely toxic even at concentrations as low as 20 μM for various cell lines,[31,32] that is, at concentrations 50 times lower than those employed in our experiments. Both complexes are therefore stable in water as well as in cell culture media and non-toxic.

In conclusion, employing a biodegradable hyperbranched polyester, novel contrast agents bearing gadolinium chelate moieties were prepared, having in addition a protective PEG-coating for enhanced circulation in biological milieu and a folate targeting ligand. The molar relaxivities of BH40-EDTA-PEG-Folate and BH40-DTPA-PEG-Folate gadolinium complexes were found to be 2- to 3-fold higher than that of the clinically used [Gd(DTPA)] complex. The cytotoxicity of the gadolinium hyperbranched complexes was comparatively assessed in a human lung carcinoma cell line (A549), not expressing folate receptors, and in a folate receptor-positive human cervical carcinoma cell line (HeLa). Preliminary studies reveal that these dendritic Gd complexes are non-toxic and exhibit folate receptor specificity. Further studies of cellular uptake, in vivo biodistribution, clearance rates from vascular circulation and MRI in mice are underway.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.05.055.

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