Gadolinium-Conjugated FA-PEG-PAMAM-COOH Nanoparticles as Potential Tumor-Targeted Circulation-Prolonged Macromolecular MRI Contrast Agents

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ABSTRACT: The aim of research is to develop potential tumor-targeted circulation-prolonged macromolecular magnetic resonance imaging (MRI) contrast agents without the use of low molecular gadolinium (Gd) ligands. The contrast agents were based on polymer–metal complex nanoparticles with controllable particle size to achieve the active and passive tumor-targeted potential. In particular, poly (amidoamine) (PAMAM) dendrimer with 32 carboxylic groups was modified with folate-conjugated poly (ethyleneglycol) amine (FA-PEG-NH_2, M_w: 2 k and 4 kDa). FA-PEG-PAMAM-Gd macromolecular MRI contrast agents were prepared by the complex reaction between the carboxylic groups in PAMAM and $GdCl_3$. The structure of FA-PEG-PAMAM-COOH was confirmed by nuclear magnetic resonance (^{1}H-NMR), Fourier transform infrared (FTIR) spectra, and electrospray ionization mass spectra (ESI-MS). The mass percentage content of Gd (III) in FA-PEG-PAMAM-Gd was measured by inductively coupled plasma-atomic emission spectrometer (ICP-AES). The sizes of these nanoparticles were about 70 nm measured by transmission electron microscopy, suggestion of their passive targeting potential to tumor tissue. In comparison with clinically available small molecular Gadopentetate dimeglumine, FA-PEG-PAMAM-Gd showed comparable cytotoxicity and higher relaxation rate, suggestion of their great potential as tumor-targeted nanosized macromolecular MRI contrast agents due to the overexpressed FA receptor in human tumor cell surfaces. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 1805–1814, 2010

Key words: FA-PEG-PAMAM-Gd nanoparticles; MRI contrast agents; cytotoxicity; relaxivity

INTRODUCTION

Magnetic resonance imaging (MRI) is one of the most useful noninvasive methodologies today in clinical medicine for assessing anatomy and function of tissues and organs, and its novel applications such as imaging of gene expression and real-time *in vivo* evaluation for drug delivery are actively studied.[1,2] This methodology is characterized by its excellent temporal and spatial resolution, its nonexposure to radiation and long effective imaging windows.[3,4] However, MRI is less sensitive than nuclear medicine when used to monitor small tissue lesions, molecular activity, or cellular activities.[5,6] With the aid of paramagnetic contrast agents such as chelated Gd-diethylenetriaminepentaacetate (Gd-DTPA) or Gd-tetraazacyclododecanetetraacetic acid (Gd-DOTA), the imaging contrast and sensitivity can be enhanced by shortening the T_1-relaxation time of water protons, which appear brighter in the T_1-weighted tumor image.[7] Several kinds of low molecular MRI contrast agents based on Gd-DTPA and Gd-DOTA have been approved by American Food and Drug Administration and widely used in clinical diagnosis of tumors.[8] However, these low molecular weight contrast agents have serious problems such as short half-life in blood, low relaxivity rate, and lack of

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specificity to target organs and tissues for early diagnosis of tumor, which limit their application for MRI contrast agents. To achieve the early diagnosis of tumors, research efforts have been made in developing macromolecular MRI contrast agents by the conjugation of DTPA or other chelating units and polymer carriers. Polymers such as poly (ethylene glycol) (PEG),[9] dextran,[10] poly (L-lysine),[11] poly (glutamic acid),[2] poly [*N*-(2-hydroxypropyl) methacrylamide],[12] α, β-poly [*N*-(2-aminoethyl) aspartamide],[13] disulfide-based biodegradable synthetic polymers,[14] and dendrimer[15,16] have been investigated as the carriers of gadolinium complexes. The result macromolecular MRI contrast agents showed great promise for enhancing contrast, sensitivity, diagnostic imaging time, and specificity to tumor tissues due to the targeting moieties such as antibody and folate conjugated to the polymers.[17,18] However, a major drawback of existing macromolecular MRI contrast agents is that they suffer from tedious and expensive synthesis approach of their chelating units, which could drastically affect their practical applications in the diagnosis of tumors on large scale.

Previous reports also showed that the modification of macromolecular MRI contrast agents with PEG could enhance their stability in bloodstream, and thus prolong their blood circulation, along with high concentration of macromolecular MRI contrast agents in the vascular space.[14] While a high concentration of macromolecular MRI contrast agents in the vascular space often creates a disadvantageous background in imaging and systemic toxic action. As an ideal macromolecular MRI contrast agent, besides its low systemic toxicity, it is necessary not only to increase the signal intensity at the tumor site but also to lower the signal intensity in the vascular space, which can be achieved by the conjugation of targeting ligands to the macromolecular MRI contrast agent. Folic acid (FA) is one of the well-studied targeting ligands used for this strategy. It was anticipated that the macromolecular MRI contrast agents modified with FA could reach the tumor sites via receptor-guided active targeting due to the overexpressed FA receptors in tumor cell surfaces.[19,20]

As well-known, the pore size of blood capillary in tumor tissue is about 100–800 nm, rather than that in normal tissue (8 nm).[21] Herein, to achieve the passive targeting approach to tumor tissue, novel nanosized contrast agents of FA-PEG-PAMAM-Gd with controllable particle size were developed. In this way, a simple mixing of $GdCl_3$ with FA-PEG-PAMAM-COOH in aqueous media led to a spontaneous formation of narrowly distributed nanoparticles, where the cores of PAMAM were coated by hydrophilic poly(ethylene glycol) (PEG) chains. The half-life of these nanoparticles in blood can be increased by PEGylation because PEG significantly decreases the uptake of the agent by macrophages in the liver and spleen.[22–24]

From the aforementioned considerations for an ideal macromolecular MRI contrast agent, this article describes a facile and inexpensive approach to develop a potential tumor-targeted and circulation-prolonged macromolecular MRI contrast agent without the use of low molecular gadolinium ligands. Thus, FA-PEG-PAMAM-Gd nanosized macromolecular MRI contrast agents were prepared and characterized by nuclear magnetic resonance (^{1}H-NMR), Fourier transform infrared (FTIR) spectra, inductively coupled plasma-atomic emission spectrometer (ICP-AES), and electrospray ionization (ESI) mass spectra. The cytotoxicity, relaxation rate, and *in vitro* imaging of FA-PEG-PAMAM-Gd were also tested. Their tumor-targeted profile in mice will be observed by further experiments and published in next article.

EXPERIMENTAL

Materials

Methanol (anhydrous) and dimethyl sulfoxide (DMSO) were distilled before use. FA and *N*-hydroxysuccinimide (NHS) were purchased from Sinopharm Chemical Reagent Co., Shanghai, China. PAMAM-COOH dendrimer (G3.0, containing 32 terminal carboxylic groups, ethylenediamine as core) and Gadolinium (III) Chloride hexahydrate ($GdCl_3{\cdot}6H_2O$) were obtained from Sigma–Aldrich (Shanghai, China). Poly (ethylene glycol)-bis-amine (H_2N-PEG-NH_2) from Beijing Kaizheng Biotechnology Development Co. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) was bought from GL Biochem (Shanghai, China) Co. Gadopentetate meglumine injection was purchased from Xudong Haipu Pharmaceutical Co., Shanghai, China.

Thiazolyl blue formazan (MTT, powder), propidium iodide (PI, >95%), acridine orange (AO, Dye content 90%), and RPMI-1640 and F-12K cell culture medium were purchased from Gibco, NY, USA. Trypsin (1 : 250) powder was bought from Invitrogen, Shanghai, China. Streptomycin, ampicillin solution and fetal bovine serum (FBS) were purchased from Every Green Organism Engineering Materials Co., Hangzhou, China. The aqueous solutions of K_2CO_3 and NaOH were filtered through a filter membrane of 0.2 μm pore size (PN4612, PALL Corporation, USA) before use.

Measurements

^{1}H-NMR spectra were collected on a BRUKER 500MHz NMR spectrometer in DMSO-d_6 or D_2O. Fourier transform infrared (FTIR) spectra were

recorded on a Nicolet Nexus 670 by using pressed KBr pellets. Tests of longitudinal relaxation time (T_1) were performed with a SIEMENS MAGNETOM Trio I-class 3T-MRI. Inductively coupled plasma (ICP) measurements were performed with a Thermo Electron IRIS Intrepid II XSP atomic emission spectrometer (AES) instrument under an argon atmosphere. Optical density (OD) was determined by a BIO-TEK PowerWave XS Microplate Spectrophotometer. Electrospray ionization mass spectra (ESI-MS) were recorded on a Micromass Quattro II Triple Quadrupole mass spectrometer. The samples dissolved in ultrapure water were introduced into ESI source through a syringe pump at the rate of 5 μL/min. The ESI capillary was set at 3.5 KV, and the cone voltage was 40 V. The hydrodynamic diameter and size distributions of the contrast agents were measured with dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments, UK). Transmission electron microscopy (TEM) was performed on JEM-100C II microscope (LIBRA 120, Carl Zeiss, Germany). Flow Cytometry (FCM) measurement was on FACS Calibur (BD Bioscience, USA) using the CELL Quest software (BD Bioscience, USA) for data analysis.

Cell lines and culture

KB (human nasopharyngeal cell line) and A549 (human lung adenocarcinoma cells) purchased from cell bank of Chinese Academy of Sciences, Shanghai, China, were cultured in RPMI-1640 and F-12K culture medium separately, supplemented with 10% FBS, streptomycin at 100 μg/mL, and penicillin at 100 U/mL. All cells were incubated at 37°C in humidified 5% CO_2 atmosphere. Cells were split by using trypsin/EDTA solution when almost confluent.

Preparation of folate-polyethyleneglycol (FA-PEG$_{2K}$-NH$_2$)

FA-PEG$_{2k}$-NH$_2$ conjugate was synthesized according to the previous method.[12] Briefly, FA (2 mmol, 0.88 g) was activated with equal molar of EDC and NHS in DMSO, and then H_2N-PEG$_{2K}$-NH$_2$ (Mw: 2 kDa, 2 mmol, 4.0 g) in DMSO was slowly added to the solution under nitrogen atmosphere in dark at room temperature with stirring for 12 h. The reaction mixture was quenched with distilled water, dialyzed by using Spectra/Pro membrane (MWCO = 1000) against sodium bicarbonate buffer (pH 9.0, 5 m*M*) for 4 days, and then against deionized water for 3 days, respectively. The dialyzate was further purified by cation-exchange chromatography on a SP-Sepharose column (Pharmacia) followed by anion-exchange chromatography on a DEAE-Sepharose column (Pharmacia). The final product was eluted with ammonium acetate (pH 8.0, 10 m*M*). After lyophilization, the result FA-PEG$_{2K}$-NH$_2$ was a fluffy light yellow solid, yield: 46.8%, 2.28 g.

Preparation of FA-PEG$_{2K}$-PAMAM-COOH

The DMSO solution of FA-PEG$_{2K}$-NH$_2$ (0.8 mmol, 1.8 g) was added dropwise to the DMSO solution of PAMAM-COOH (0.1 mmol, 0.56 g), EDC (0.15 mmol), and NHS (0.15 mmol). The mixture was stirred under nitrogen atmosphere in dark at room temperature for 4 days, then thoroughly dialyzed using Spectra/Pro membrane (molecular weight cut-off size: 3500 Da) against deionized water at room temperature for 3 days. The lyophilized product was stored at −4°C before use and labeled as FA-PEG$_{2K}$-PAMAM-COOH.

FA-PEG$_{4K}$-PAMAM-COOH was also synthesized according to the similar procedure by using corresponding H_2N-PEG-NH$_2$ with molecular weight of 4 kDa.

Preparation of FA-PEG$_{2k}$-PAMAM-Gd complexes

In a typical procedure, Gadolinium chloride hexahydrate (0.48 mmol, 0.18 g) was dissolved in 2 mL of double distilled water, and the pH of solution was adjusted to 5–7 with 0.1*M* of hydrochloric acid. FA-PEG$_{2K}$-PAMAM-COOH (1.0 g, containing carboxylic groups 0.96 mmol) dissolved in 5 mL of double distilled water was added dropwise to the solution of $GdCl_3 \cdot 6H_2O$. The mixture was gently stirred for 48 h at 37°C under dark. FA-PEG$_{2K}$-PAMAM-Gd complexes were purified by using gel chromatography on a Sephadex-G25 column to remove free Gd^{3+}. The lyophilized product was stored at −4°C. The mass percentage content of Gd (III) in FA-PEG$_{2k}$-PAMAM-Gd was measured by ICP-AES.

Relaxivity measurements

Solutions of FA-PEG$_{2k}$-PAMAM-Gd and Gadopentetate dimeglumine were prepared in double distilled water at Gd concentrations of 0.001, 0.01, 0.02, 0.04, 0.1, 0.2, 0.3, and 0.4 m*M* and put into centrifuge tube with volume of 15 mL separately. The longitudinal relaxation time (T_1) for the eight solutions and water were measured at 3 T, 10 MHz, 20°C using an inversion recovery pulse sequence (Siemens Tim 3 T MRI scanner). Relaxation rate (r_1) ($r_1 = 1/T_1$) was calculated from a linear least squares fit of the slope of the line obtained from following formula by plotting $1/T_1$ vs. Gd concentration in corresponding FA-PEG-PAMAM-Gd solution.

$$1/T_{wat} = 1/T_{obs} + r_1 \times [C] \quad (1)$$

where T_{wat} was the relaxation time of water; T_{obs} was the relaxation time of solution of FA-PEG$_{2k}$-

PAMAM-Gd; *C* was the corresponding Gd concentration of each FA-PEG_{2k}-PAMAM-Gd solution.

Cytotoxicity of FA-PEG_{2k}-PAMAM-Gd contrast agents by MTT assay

Cytotoxicity of FA-PEG_{2k}-PAMAM-Gd macromolecular MRI contrast agent was determined by evaluation of the viability of KB and A549 by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Commercial available low molecular MRI contrast agent Gadopentetate meglumine was used as control. Briefly, cells were seeded in 96 well plates at an initial density of 1×10^4 cells/well in 100 μL of RPMI-1640 growth medium and incubated for 18–20 h to reach 80% confluency at the time of treatment. Growth medium was replaced with 100 μL fresh serum-free media containing various amounts of FA-PEG_{2k}-PAMAM-Gd complexes or Gadopentetate meglumine (25, 50, 75, 100, 125, 150, 175, and 200 μg/mL). Cells were incubated for 24 h or 48 h, respectively. And then, the culture medium was replaced by 100 μL of MTT solution (0.5 mg/mL). After further incubation for 4 h in incubator, 100 μL of dimethyl sulfoxide (DMSO) was added to each well to replace the culture medium and dissolve the insoluble formazan-containing crystals. The OD was measured at 570 nm using an automatic BIO-TEK microplate reader (Powerwave XS, USA), and the cell viability was calculated from following equation:

$$\text{Cell viability}(\%) = (\text{OD}_{\text{sample}}/\text{OD}_{\text{control}}) \times 100 \quad (2)$$

where $\text{OD}_{\text{sample}}$ represents an OD value from a well treated with samples and $\text{OD}_{\text{control}}$ from a well treated with phosphate buffered saline (PBS) only. Each experiment was carried out in triplicate. Means and corresponding standard deviations (mean ± SD) were shown as results.

Cytotoxicity of FA-PEG_{2k}-PAMAM-Gd contrast agents by FCM assay

The typical flow cytometry (FCM) experiment was described as following. Aliquots of 1×10^6 A549 human lung epithelial cell lines were cultured with RPMI-1640 culture medium in petri dish at 37°C and 5% CO_2-containing incubator. Growth medium was replaced with 10 mL fresh culture medium containing 1 mg sample of FA-PEG_{4K}-PAMAM-Gd. After incubation for 24 h, all of the cells were collected and digested with 0.25% trypsin, followed by fixing with 75% ethanol and incubating for 24 h at −4°C. Before FCM, the cells were washed with PBS and stained with propidium iodide (PI, 5 mL/L propidine iodide and 0.5% Triton X-100) for 30 min after removal of ethanol by centrifuge. All samples were analyzed in a FACS Calibur (BD Bioscience) using the CELL Quest 7.0 software (BD Bioscience). The control group was A549 cells only.

Dynamic light scattering measurements

The samples were loaded in disposable sizing cuvette cell (DTS 0012) and dispersed in ultrapure water at 25°C. The polystyrene latex was chose as control. The concentration of FA-PEG-PAMAM-Gd was kept constant at 1.0 mg/mL. The measurements were averaged in triplicate.

In vitro contrast effect

Briefly, the prepared contrast agent samples (~ 1.5 mL) with different concentrations were placed in Eppendorf tubes. The tubes were placed on a tube rack, which was placed in the center of the 12 channels head surface coil for the measurement of the *in vitro* T1-weighted MR images at 3 T MRI scanner. Gadopentetate dimeglumine (G) and ultrapure water (W) were used as controls.

RESULTS AND DISCUSSION

Synthesis and characterization of FA-PEG_{2k}-NH_2

FA-PEG_{2k}-NH_2 was synthesized through carbodiimide-mediated coupling reaction of carboxylic groups in FA and amino groups in NH_2-PEG_{2k}-NH_2 (M_W 2000) (Fig. 1). To obtain the mono-substituted target product of FA-PEG_{2k}-NH_2, thoroughly diluted DMSO solution of NH_2-PEG_{2k}-NH_2 was added dropwise to the solution of FA at an equimolar quantity. The result FA-PEG_{2k}-NH_2 was purified by cation-exchange chromatography on a SP-Sepharose column followed by anion-exchange chromatography on a DEAE-Sepharose column. The chemical structure of purified FA-PEG_{2k}-NH_2 was confirmed by ^{1}H NMR spectrum, and detail chemical shifts in DMSO-d_6 were shown in Figure 2. The peaks at 6.80, 7.80, and 8.71 ppm and the multiple peaks at 3.3–3.7 ppm were assigned to aromatic protons in FA and methylene (—CH_2CH_2O—) in PEG, respectively. The integral area ratio of methylene in PEG and the single proton peak at 8.71 ppm assigned to pterin ring in FA was 185.6, very similar to its theoretic value of 181 when the molar ratio of FA to H_2N-PEG-NH_2 was 1 : 1, which further confirmed the chemical composition of FA-PEG-NH_2. The FTIR spectra of FA-PEG_{2k}-NH_2, H_2N-PEG_{2k}-NH_2, and FA were shown in Figure 3. The bands at 1113.64 cm^{-1} in H_2N-PEG_{2k}-NH_2 and 1105.84 cm^{-1} in FA-PEG_{2k}-NH_2 were attributed to the stretching vibration of C—O in PEG. The characteristic adsorption bands at 1644.60 in Figure 3(a) was assigned to the stretching vibration of CO in —CONH—, further confirmed the chemical structure of FA-PEG_{2k}-NH_2.

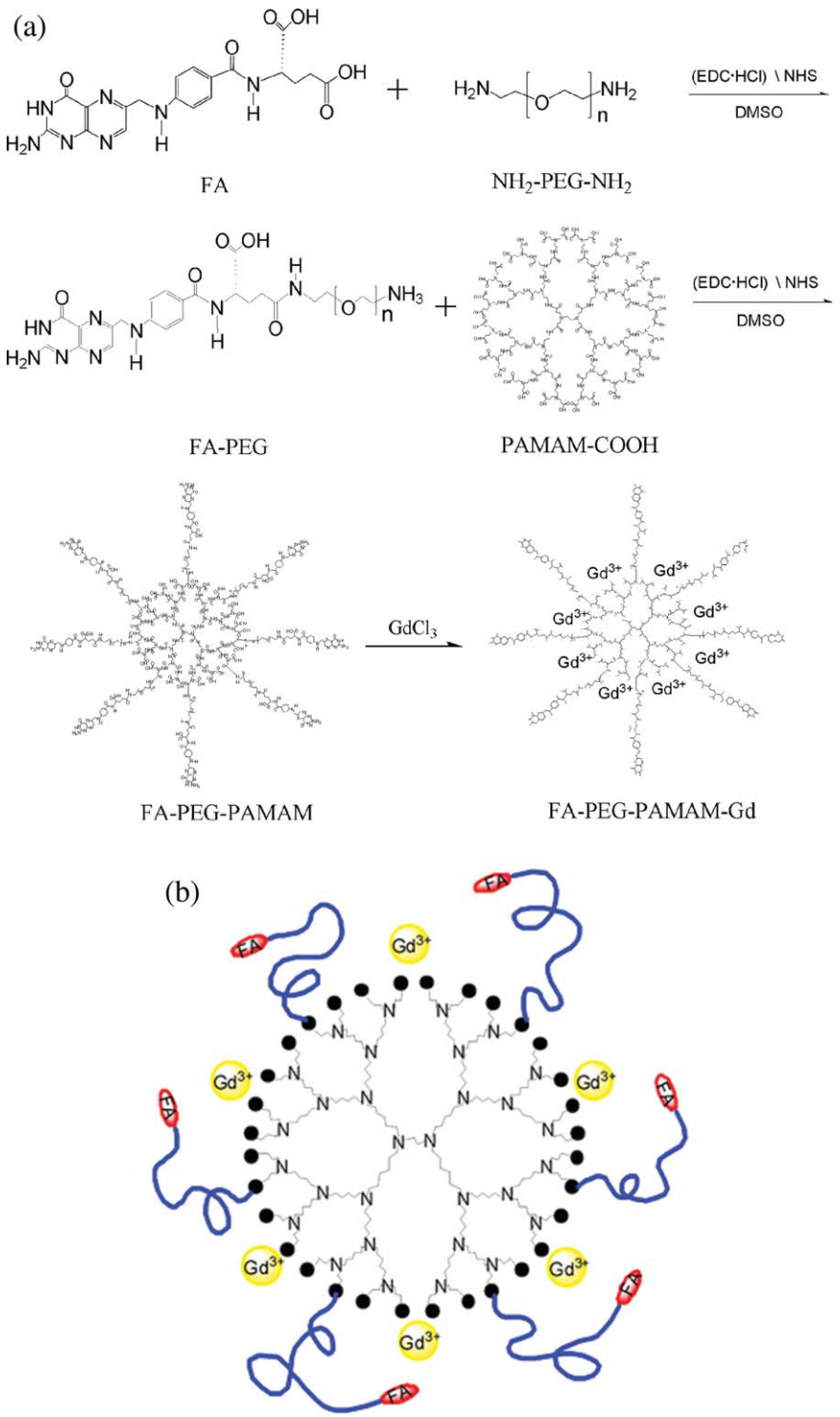

Figure 1 Synthesis scheme (a) and structure model (b) of FA-PEG-PAMAM-Gd. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Synthesis and characterization of FA-PEG-PAMAM-COOH

FA-PEG_{2k}-PAMAM-COOH was also synthesized through EDC-mediated coupling reaction of carboxylic groups in PAMAM-COOH and amino groups in FA-PEG_{2k}-NH_2. To improve the chelating ability and content of gadolinium in the result macromolecular MRI contrast agent, it was a key issue to synthesize the macromolecular MRI ligand with enough chelating groups. So the feed molar ratio of FA-PEG_{2k}-NH_2 to PAMAM-COOH was controlled to be about 8 : 1. After thorough dialysis at deionized water, the lyophilized FA-PEG_{2k}-PAMAM-COOH was ointment-like solid with light yellow color and easy to

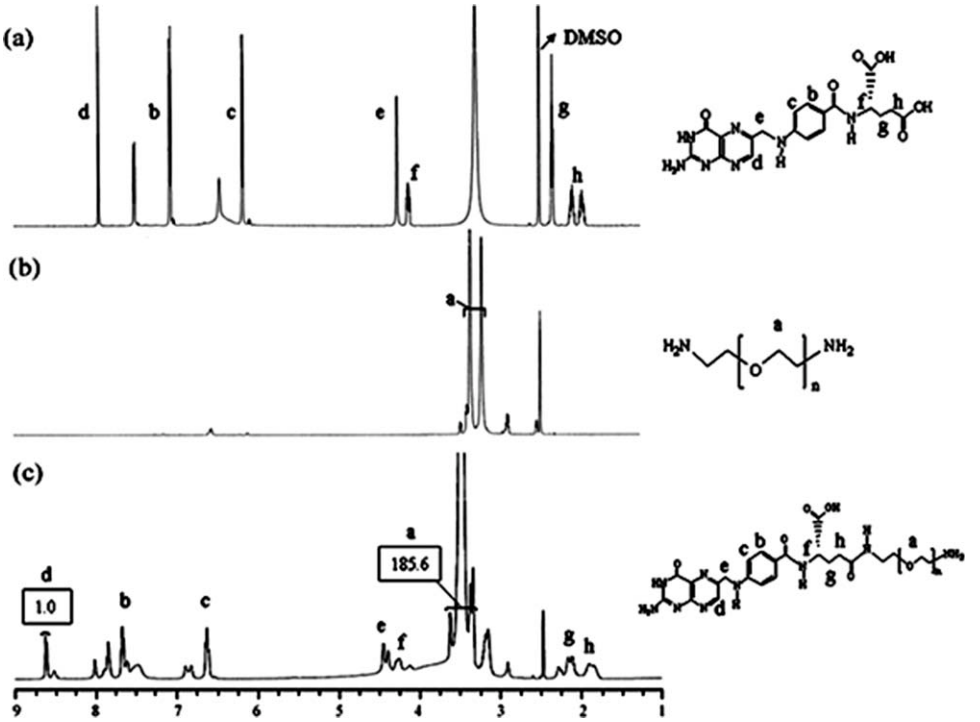

Figure 2 ^{1}H-NMR spectrum of (a) Folic acid, (b) NH_2-PEG-NH_2, and (c) FA-PEG_{2K}-NH_2 in DMSO-d_6. The multiple peaks of a (3.3–3.7) was assigned to methylene (—CH_2CH_2O—) in PEG, the single proton peak of *d* (8.71) assigned to pterin ring in folic acid.

be dissolved in water. Representative ^{1}H-NMR of purified FA-PEG_{2k}-PAMAM-COOH was shown in Figure 4. The proton peaks at 6.83, 7.68, and 8.63 ppm were assigned to the aromatic cycles in FA, and the peaks at 3.3–3.8 ppm and 2.8–3.0 ppm were attributed to protons in PEG and PAMAM-COOH, respectively. Molar ratio of FA-PEG_{2k} to PAMAM-COOH calculated from the integral area ratio of proton at 8.63 ppm in FA to the methylene peak at 2.8–3.0 ppm in PAMAM-COOH was about 6.7 (Table I), which indicated that about seven carboxylic groups in each PAMAM-COOH molecule have been conjugated with FA-PEG_{2k}-NH_2. So the free carboxylic groups in each FA-PEG_{2k}-PAMAM-COOH molecule were about 25, which can be complexed with gadolinium ions.

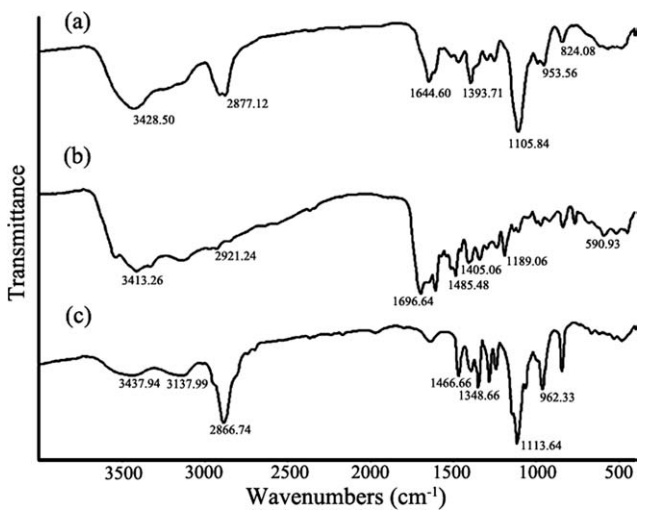

Figure 3 FTIR spectra of (a) FA-PEG_{2K}-NH_2, (b) FA, and (c) NH_2-PEG-NH_2.

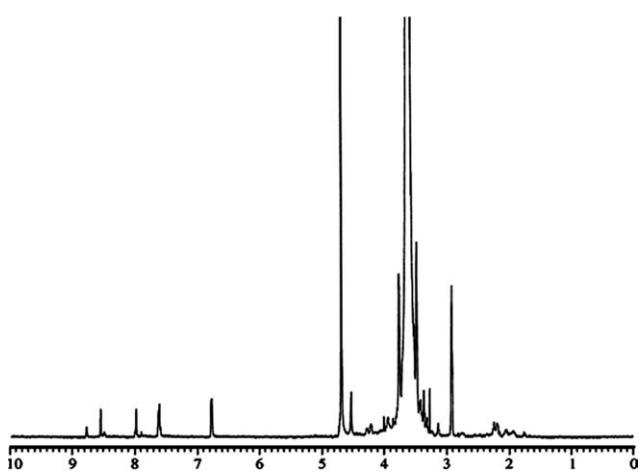

Figure 4 ^{1}H NMR spectrum of FA-PEG_{2K}-PAMAM-COOH in D_2O. The proton peaks at 6.83, 7.68, and 8.63 ppm were assigned to the aromatic cycles in FA, and the peaks at 3.3–3.8 ppm and 2.8–3.0 ppm were attributed to protons in PEG and PAMAM-COOH, respectively. The integral area ratio (6.7) of proton at 8.63 ppm to the methylene peak at 2.8–3.0 ppm indicated that about seven carboxylic groups in each PAMAM-COOH molecule have been conjugated with FA-PEG_{2k}-NH_2.

TABLE I
Properties of FA-PEG-PAMAM-COOH Complexes and FA-PEG-PAMAM-Gd Contrast Agents

Polymers	Size (nm)[a]	PDI[a]	FA-PEG: PAMAM (mol mol^{-1}) Theoretical values	FA-PEG: PAMAM (mol mol^{-1}) Measured values[b]	Gd (%) Theoretical values	Gd (%) Measured values[c]	Molecular weight Calculated values	Molecular weight Measured values[d]
FA-PEG$_{2k}$-PAMAM-COOH	63.3	0.22	8	6.7	–	–	22 687	22 680
FA-PEG$_{4k}$-PAMAM-COOH	76.8	0.24	8	6.0	–	–	32 240	32 236
FA-PEG$_{2k}$-PAMAM-Gd	61.2	0.20	8	6.7	15.3	16	–	–
FA-PEG$_{4k}$-PAMAM-Gd	75.5	0.25	8	6.0	19.2	18	–	–

[a] Determined by DLS.
[b] Determined by ^{1}H NMR.
[c] Determined by ICP-AES.
[d] Determined by ESI-MS.

The molecular weights of FA-PEG$_{2k}$-PAMAM and FA-PEG$_{4k}$-PAMAM measured by ESI were 22,680 and 32,236, respectively, similar to that of the calculated values (Table I).

Synthesis and characterization of FA-PEG-PAMAM-Gd complexes

To improve the mass percentage of Gd (III) in FA-PEG-PAMAM-Gd, the excess $GdCl_3$ (about twofolds of FA-PEG-PAMAM-COOH) was added to complex with FA-PEG-PAMAM-COOH. As $GdCl_3$ was easy to be hydrolyzed when the pH value was above 7, the pH value of reaction medium was maintained between 5 and 7. After purification by gel chromatography on a Sephadex-G25 column, the lyophilized FA-PEG-PAMAM-Gd was easy to be dissolved in water. The mass percentage content of Gd (III) in FA-PEG$_{2K}$-PAMAM-Gd measured by ICP-AES was about 16%, very similar to its theoretic value of 15.3%. The mass percentage content of Gd (III) in FA-PEG$_{4K}$-PAMAM-Gd was about 18% (Table I).

The size of FA-PEG$_{2K}$-PAMAM-Gd was about 60 nm, and for FA-PEG$_{2K}$-PAMAM-Gd, it was 80 nm. The electron microscopic analysis of FA-PEG$_{2K}$-PAMAM-Gd proves them to be nanometric, which was evident from TEM photographs (Fig. 7). The different sizes between these FA-PEG$_{2K}$-PAMAM-Gd contrast agents revealed a change in particle size through PEG.[25] This result suggested that the particle size can be changed by conjugation of different chains of PEG.

Relaxation rate of FA-PEG-PAMAM-Gds

To know better the potentials of FA-PEG-PAMAM-Gd as macromolecular MRI contrast agent, their relaxation rates were measured on a Siemens Tim 3 T MRI scanner. Commercial available low molecular MRI contrast agent (Gadopentetate dimeglumine) was used as positive controls. As shown in Figure 5, the r_1 of FA-PEG$_{2K}$-PAMAM-Gd and FA-PEG$_{4K}$-PAMAM-Gd were 20.27 and 64.88 mM^{-1} s^{-1}, respectively, sharply higher than that of Gadopentetate dimeglumine (3.28 mM^{-1} s^{-1}), suggestion of their potentials as macromolecular MRI contrast agents. With an increase in the molecular weight of PEG, the r_1 of FA-PEG-PAMAM-Gd showed a tendency to increase. It was thought that the higher molecular weight of FA-PEG-PAMAM-Gd resulted in the longer rotational correlation lifetime of Gd, along with increased r_1.

Cytotoxicity of FA-PEG-PAMAM-Gd complexes

The *in vitro* cytotoxicity of FA-PEG-PAMAM-Gd complexes was evaluated with KB and A549 cell lines by MTT assay. Representative concentration-growth inhibition curves showing the effects of treatments with FA-PEG$_{2K}$-PAMAM-Gd, FA-PEG$_{4K}$-PAMAM-Gd, and Gadopentetate dimeglumine on the growth of cells after 24 h were shown in Figure 6(a,b). FA-PEG$_{2K}$-PAMAM-Gd, FA-PEG$_{4K}$-PAMAM-Gd, and Gadopentetate dimeglumine showed about 80–100% of cell viability in two different cell lines,

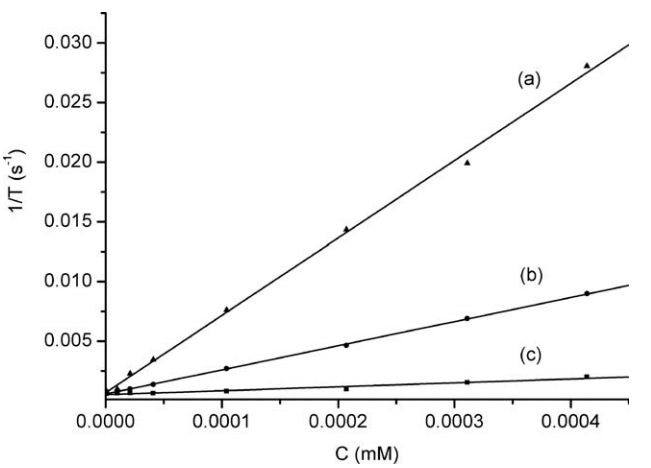

Figure 5 Relaxivity of (a) FA-PEG$_{4K}$-PAMAM-Gd, (b) FA-PEG$_{2K}$-PAMAM-Gd, and (c) Gadopentetate dimeglumine.

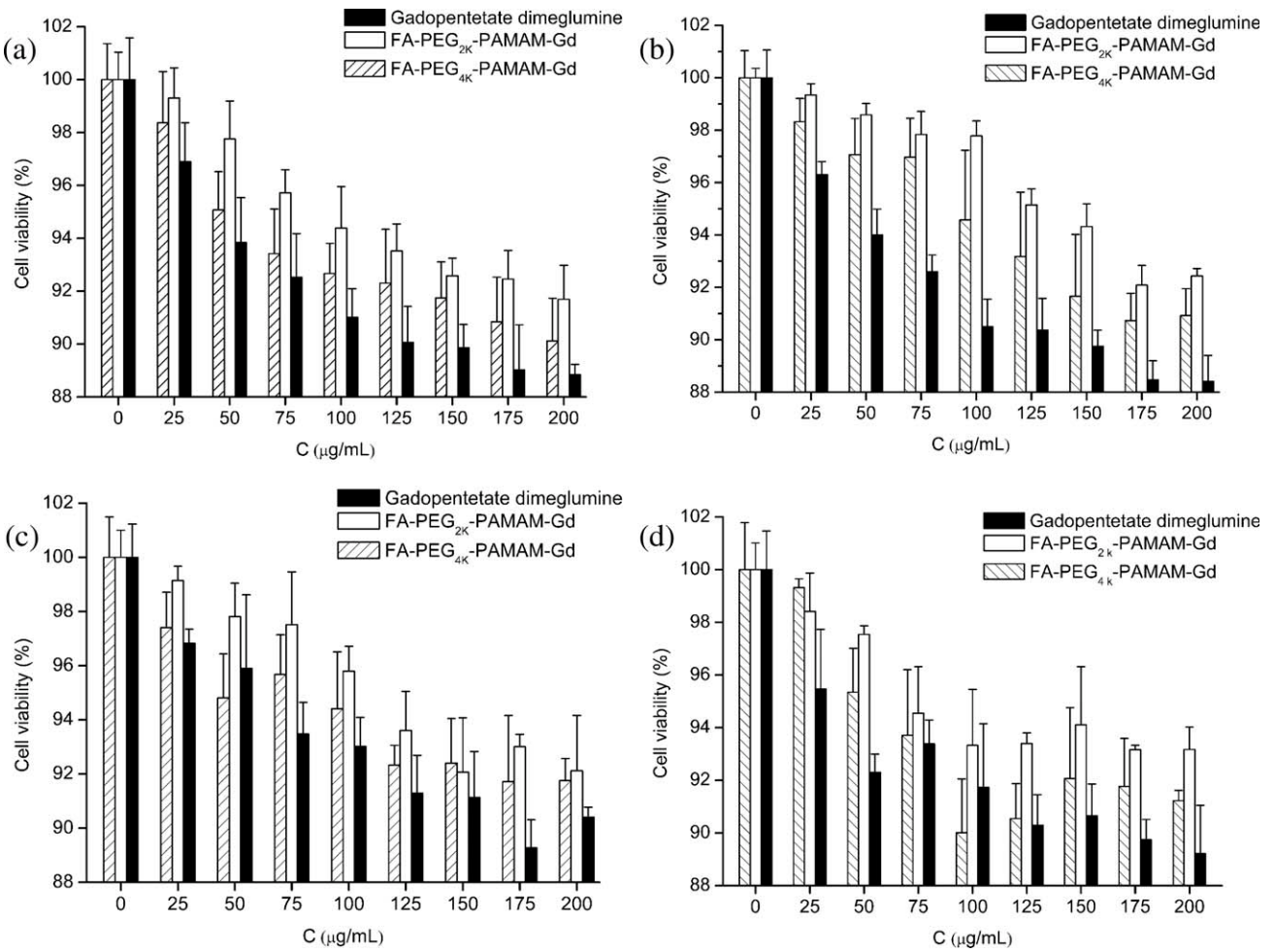

Figure 6 Cell viability of FA-PEG_{2K}-PAMAM-Gd, FA-PEG_{4K}-PAMAM-Gd, and Gadopentetate dimeglumine at various concentrations in different cell lines (mean ± SD $n = 3$) (a) A549 and (b) KB for 24 h, (c) A549 and (d) KB for 48 h.

even at 200 μg/mL of high Gd (III) dose, suggestion of their rather good biocompatibilities.

A549 and KB cancer cells were incubated with FA-PEG_{2K}-PAMAM-Gd, FA-PEG_{4K}-PAMAM-Gd, and Gadopentetate dimeglumine for 48 h, respectively. The results of MTT assay for 48 h incubation were similar to that of 24 h. As shown in Figure 6(c) (A549) and d (KB), the viability of FA-PEG_{2k}-PAMAM-Gd was generally higher than that of FA-PEG_{4k}-PAMAM-Gd. This result was mainly contributed to the graft degrees of FA-PEG conjugation to PAMAM because the conjugation of PEG can decrease the cytotoxicity of PAMAM.[26]

FCM is one of the routine techniques of analyzing cytotoxic activity as a complementary data to MTT assays in this article. A549 cells were incubated with FA-PEG_{2k}-PAMAM-Gd or FA-PEG_{4k}-PAMAM-Gd for 24 h, respectively. Figure 7 was the results of the analysis on the basis of their FL2 fluorescence (emission 585 nm, excitation at 488 nm) using Cell Quest 7.0 software. As shown from Figure 7, the diploid of the treated group (FA-PEG_{2k}-PAMAM-Gd) [Fig. 7(a)] in the G1, G2, and S phase of the cell cycle were 61.82%, 16.28%, and 21.89%, respectively. The diploid of the treated group (FA-PEG_{4k}-PAMAM-Gd) [Fig. 7(b)] in the G1, G2, and S phase of the cell cycle were 58.85%, 15.93%, and 25.22%. Similarly, the diploid of the control group [Fig. 7(c)] were 59.69%, 9.05%, and 31.26%. The G2/G1 values of the two treated groups and the control group were 1.62, 1.64, and 1.65, respectively, which indicated that the cells both in the treated groups and in the control group grew normally. At the same time, no cell apoptosis peak was observed before the G1 phase both in the treated and the control groups.

All of these results obtained from MTT and FCM assays indicated that the samples had no effect on the diploid (or DNA) in the cell growth and had a high biocompatibility to the cells.

Particle sizes and size distributions

The hydrodynamic diameter of particle sizes and size distributions of the contrast agents were

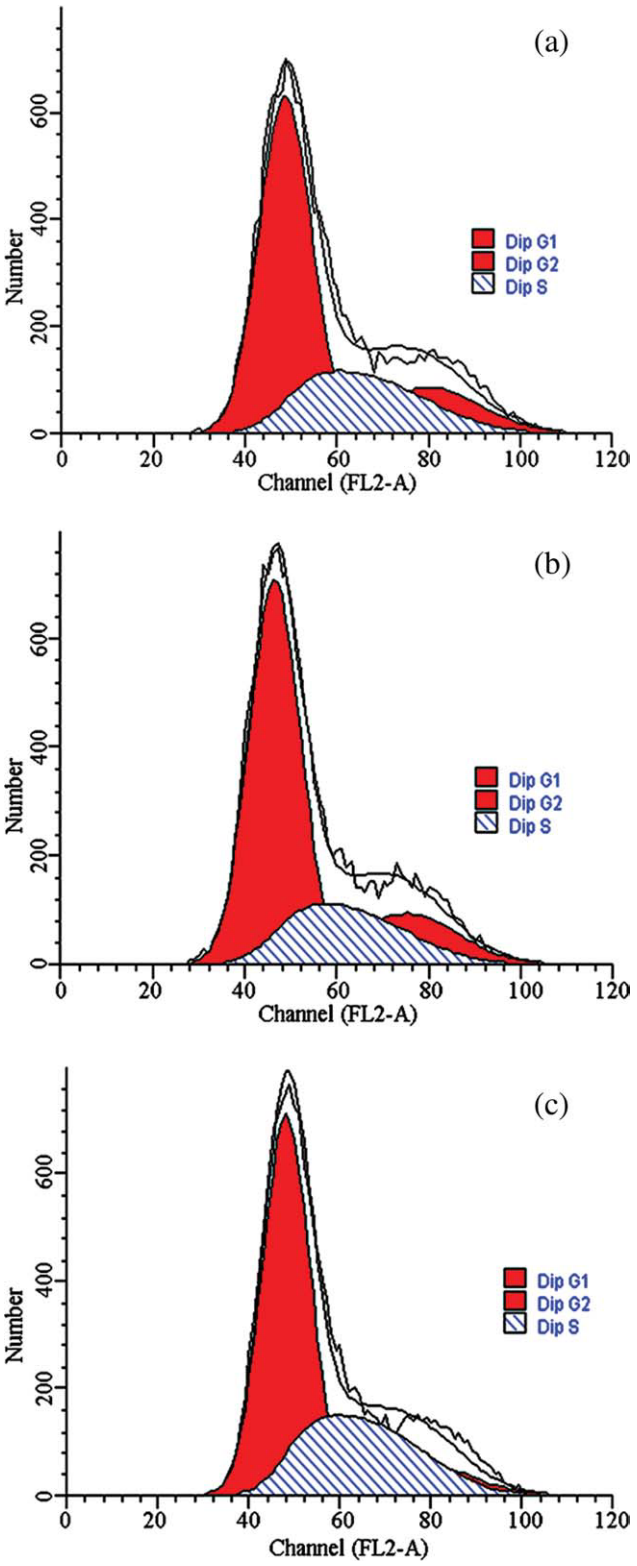

Figure 7 The analysis of flow cytometric detection based on the FL2 fluorescence (emission 585 nm, excitation at 488 nm) using Cell Quest 7.0 software. Propidium iodide (PI) stained samples. (a) A549 cells were incubated with FA-PEG_{2k}-PAMAM-Gd for 24 h; (b) A549 cells were incubated with FA-PEG_{4k}-PAMAM-Gd for 24 h; (c) A549 cells only (as a control). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

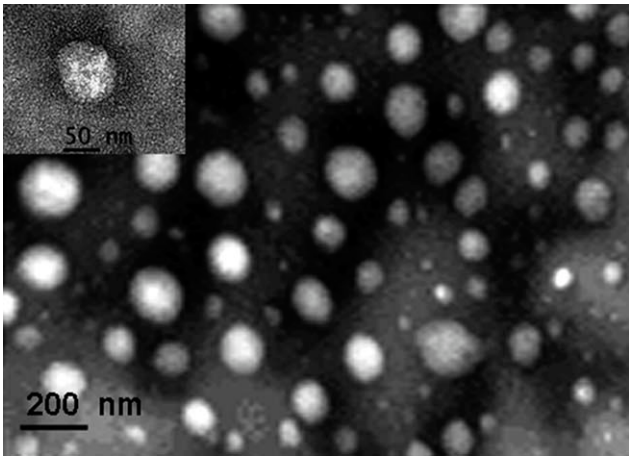

Figure 8 TEM image of FA-PEG_{4K}-PAMAM-Gd.

measured by dynamic light scattering. As shown in Table I, the mean diameters of these contrast agents were all small than 100 nm, with PDI of 0.2. These nanoparticles with the range of 50–100 nm suggested their good passive targeting to the tumor.[27]

To know the morphologies of the complex, representative TEM images of FA-PEG_{4k}-PAMAM-Gd were observed and shown in Figure 8. The size of FA-PEG_{4k}-PAMAM-Gd was about 70 nm with uniform dispersivity, similar as measured by DLS. Nanoparticles with this size scope (<100 nm) can concentrate in the tumor for the passive targeting, and apparently improve the local drug concentration, prolong retention time, and decrease the poison and side effects caused by the systemic distribution.[28,29]

In vitro MRI

T1-weighted MR images of the FA-PEG-PAMAM-Gds were shown as Figure 9. In this image, when the concentration of Gd was above 2.484×10^{-2} m*M*, the image contrast showed similar patterns, but

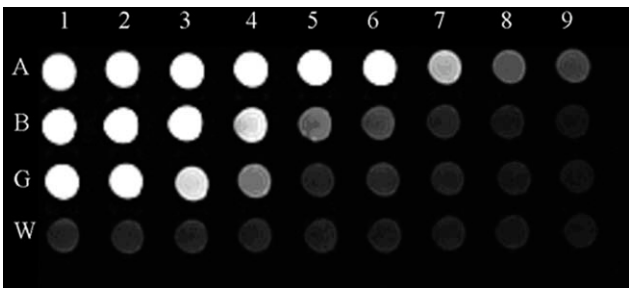

Figure 9 *In vitro* contrast effect of FA-PEG-PAMAM-Gd and Gadopentetate dimeglumine measured by MRI. (A) FA-PEG_{4k}-PAMAM-Gd, (B) FA-PEG_{2k}-PAMAM-Gd, (G) Gadopentetate dimeglumine, and (W) Ultrapure water. Gd concentrations of A-1, A-2, A-3, A-4, A-5, A-6, A-7, A-8, and A-9 were 4.14×10^{-2}, 3.31×10^{-2}, 2.48×10^{-2}, 2.07×10^{-2}, 1.03×10^{-2}, 4.14×10^{-3}, 2.07×10^{-3}, 1.03×10^{-3}, and 1.01×10^{-4} m*M*, respectively.

when the concentration of Gd was below this value, the image contrast of FA-PEG-PAMAM-Gd seemed better than that of Gadopentetate dimeglumine. From Figure 9, the signal intensity value of FA-PEG_{4k}-PAMAM-Gd in 4.14×10^{-3} m*M* was similar with the signal intensity value of Gadopentetate dimeglumine in 3.31×10^{-2} m*M*. These results showed that the prepared samples had better contrast imaging at a lower concentration than that of Gadopentetate dimeglumine, which meant much lower dosage of FA-PEG_{4k}-PAMAM-Gd should be used for obtaining the same MR image in clinic application. The finding that the highest molecular weight MRI contrast agent of FA-PEG_{4k}-PAMAM-Gd gave the lowest detection limit would make it potentially the best candidate for further molecular imaging purposes.

CONCLUSIONS

FA-PEG-PAMAM-Gd macromolecular MRI contrast agents were prepared without the use of low molecular gadolinium (Gd) ligands. With an increase of PEG molecular weight in FA-PEG-PAMAM-Gd, the corresponding r_1 increased. The size of these contrast agents were about 70 nm with passive targeting property. The low cytotoxicity, good biocompatible, and high relaxation rate of FA-PEG-PAMAM-Gds suggested their great potential as tumor-targeted macromolecular MRI contrast agent.

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