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Dextran Gadolinium Complex Containing Folate Groups as a Potential Magnetic Resonance Imaging Contrast Agent*

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Abstract Folate-containing dextran ligand (FA-Dextran-DTPA) was synthesized by the incorporation of diethylenetriamine-pentaacetic acid (DTPA) and folate (FA) as a tumor-targeting group into dextran as a polymer carrier. This ligand was further reacted with gadolinium chloride to make a dextran gadolinium complex FA-Dextran-DTPA-Gd. The ligand and its gadolinium complex were characterized by ^{1}H-NMR, FTIR, UV-Vis, average particle sizes and zeta potential, as well. *In vitro* properties including relaxivity, cytotoxicity assay, cellular uptake assay, and magnetic resonance imaging (MRI) were also evaluated. Compared with Gd-DTPA, FA-Dextran-DTPA-Gd possessed obviously higher relaxation effectiveness and lower cytotoxicity to HeLa cells. FA-Dextran-DTPA-Gd had a high affinity to the H460 and MDA-MB-231 tumor cells and can be taken up selectively by these tumor cells. Moreover, FA-Dextran-DTPA-Gd showed enhanced signal intensities (SI) of MRI and enhanced the contrast of MR images of tumor cells. These results indicated that FA-Dextran-DTPA-Gd showed the potential as a tumor-targeting contrast agent in MRI.

Keywords: Magnetic resonance imaging (MRI); Contrast agent; Tumor-targeting; Dextran; Diethylenetriamine-pentaacetic acid (DTPA); Folic acid (FA).

INTRODUCTION

Magnetic resonance imaging (MRI) is one of the most powerful convenient and noninvasive clinical imaging modalities for visualizing the anatomic structure and function of the body[1–3]. In recent years, macromolecular gadolinium complexes have received increased attention to be the important use as potential MRI contrast agents in cardiovascular and lymphatic systems[4–7]. Although some low molecular weight gadolinium complexes, for example gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA), are the clinical used MRI contrast agents, some disadvantages including rapid excretion by the kidneys, weak signal and lack specificity or low affinity, restrict their applications in the pathological tissues such as cancers, lymphatics and lymph nodes *in vivo*[8–10]. An ideal contrast agent should be designed as the tissue- or organ-targeting materials with high relaxivity, low toxicity and side effects, suitable long duration and excretion time, and high imaging contrast enhancement with low dose, *in vivo*, all coupled to low overall cost[11–16].

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Macromolecular gadolinium complexes are commonly synthesized by binding of Gd-DTPA and gadolinium 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acids (Gd-DOTA) to water-soluble polymer carriers[17–19]. Some biological molecules including monoclonal antibodies, albumin, β-cyclodextrin, and polysaccharides, and some synthetic polymers such as poly(amino acid), fullerenes and dendrimers, have also been investigated as polymer carriers for MRI contrast agents. Macromolecular MRI contrast agents usually exhibit more effective relaxation rates than that of low molecular weight metal complexes alone and improve the relaxivity per gadolinium atom due to a slowly tumbling system and an increase in rotational correlation time. On the other hand, macromolecular contrast agents may show prolonged intravascular retention due to bulky molecular volume, and can be potential for magnetic resonance angiography (MRA). In addition, when a tumor-targeting group is attached to macromolecular gadolinium chelate, it can be endowed with tumor-specific property[20–24].

Dextran, as a polysaccharide of D-glucose monomers linked by glycosidic bonds, has attracted great interest for the use as a biodegradable material in drug delivery and tissue engineering because of its good medicine permeability, low immunogenity, good biocompatibility and biodegradability[25]. In previous work, dextran gadolinium complexes Dextran-DTPA-Gd greatly enhanced the contrast of MR images of normal politeal lymph nodes and reactive hyperplasia of politeal lymph nodes in rabbits and provided prolonged duration in lymphatic system with lower injection doses[26].

It is reported that folate-based compounds can be internalized into folate receptor (FR)-expressing cells and have preferential selective uptake and retention by several cancers including breast, lung, cervical, ovarian, colorectal, and renal cancers. These tumor cells over-express the high affinity folate receptor, a glycosylphosphatidylinositol-anchored protein. Currently, the folate conjugates have been successfully delivered to FR-expressing cancer cells and used as the radiopharmaceutical agents, MRI contrast agents, near-infrared fluorescence imaging probes, anticancer drugs and targeted gene delivery systems. Because of low FR expression in normal tissues, folic acid (folate, FA) can be chosen as a tumor-targeting group and then attached to macromolecular gadolinium chelate to produce tumor-targeting macromolecular MRI contrast agents，which can be exploited to target folate-linked contrast agents specifically to FR-expressing tumors, thereby avoiding uptake by most healthy tissues that express few FR[27–34].

In this work, folic acid and Gd-DTPA were incorporated to dextran to synthesize a macromolecular complex (FA-Dextran-DTPA) (Scheme 1). Dextran was chosen as a polymer carrier due to its good water solubility, biocompatibility, biodegradability, medicine permeability and low immunogenity. This complex was characterized and *in vitro* properties were also evaluated. The aim was to generate a water-soluble tumor-targeting macromolecular gadolinium complex containing folic acid as a tumor-specific selective group and Gd-DTPA as a MRI functional group. This macromolecular gadolinium complex will be potential to be used as a contrast agent for tumor-targeting MR imaging and diagnosis to tumors in lymphatic systems.

EXPERIMENTAL

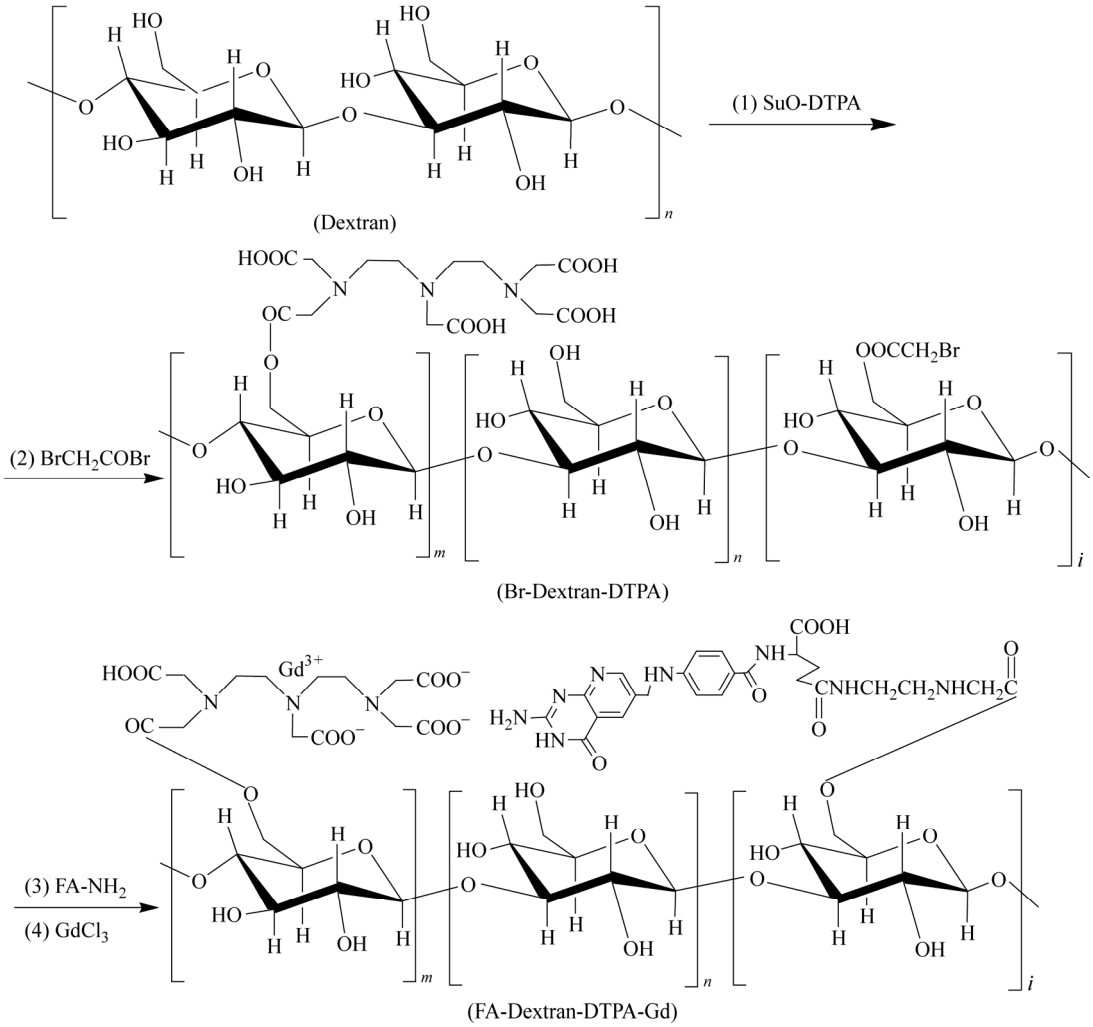
Materials

All chemicals and solvents were of analytical grade. DTPA mono(hydroxysuccinimidyl) ester (SuO-DTPA)[35] and amino-derivatized folic acid (FA-NH_2)[36] were synthesized according to the methods established in the literatures. Folic acid was first reacted with ethylenebisamine using diisopropylcarbodiimide as the coupling agent in DMSO.

Measurements

The compounds were characterized using a Varian Mercury-VX300 NMR spectrometer (Varian, Inc. Corporate, Palo Alto, CA, United States of America), a Nicolet IS10 Fourier transform infrared (FTIR) spectrophotometer (Thermo Fisher Scientific Inc., Madison, WI, United States of America), a UV-Vis spectrophotometer (UV-2800 series, Unico, Shanghai, China) and a zeta potential and laser diffraction particle size analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd, United Kingdom). The concentration of the paramagnetic species [Gd^{3+}] was

measured by an Intrepid XSP Radial inductively-coupled plasma emission spectrometer (ICP-AES, IRIS Intrepid II, Thermo Fisher Scientific Inc., Madison, WI, United States of America). The solvent longitudinal relaxation time (T_1) for gadolinium complex in distilled water was determined by a Varian Mercury-VX300 NMR spectrometer. MR image was performed on a 3.0 Tesla Magnetom Tiro Tim MR Scanner (Siemens, Germany). The H460 lung carcinoma cells, MDA-MB-231 breast carcinoma cells, and T40D breast carcinoma cells were provided by the China Center for Type Culture Collection of Wuhan University, China and were cultured according to the method described in the literature[37].



Scheme 1 Synthetic route of FA-Dextran-DTPA-Gd

Synthesis of FA-Dextran-DTPA

The solution of DTPA mono(hydroxysuccinimidyl) ester (SuO-DTPA, 6.1 g, 12.7 mmol) in DMSO (100 mL) was added dropwise to a solution of dextran (2.0 g, 12.7 mmol, 1 equiv.) in 150 mL of DMSO and 8 mL of triethylamine with rapid stirring at room temperature. The reaction continued stirring for 2 h at room temperature and a further 7 days at 60 °C. The resultant mixture was filtered and precipitated with ethanol and ethyl ether. The precipitate was reprecipitated from DMF using ethanol and anhydrous ether (*V*/*V*: 1/1), filtered and dried under vacuum. After dialysis against distilled water, the dialyzed solution was evaporated and the solid residue was dried under vacuum to yield a white dextran ligand containing DTPA groups (Dextran-DTPA, 2.7 g,

56.1%)[25]. ^{1}H-NMR (D_2O, δ): 4.9 (d, OCH_2O), 3.8, 3.7 (d, OCH), 3.6 (m, CCH), 3.5–3.3 (s, NCH_2CO), 3.3–3.2 (m, CHC), 3.1 (t, NCH_2CH_2N); IR (KBr, cm^{-1}): 3421 (OH), 2924 (C—H), 1750, 1645 (COO), 1322 (C—N), 1010 (C—O).

Dextran-DTPA (2.0 g, 12.35 mmol) was dissolved in 20 mL of DMSO and then bromoacetyl bromide (0.43 mL, 4.94 mmol, 40% molar ratio of repeat units of dextran) was added to the reaction solution. Pyridine (0.977 g, 12.35 mmol) was slowly added dropwise to the mixture solution at 0 °C and continued stirring for 72 h at room temperature. The mixture was filtered and then precipitated using ethanol. The precipitated solid was dialyzed against distilled water and then the solution was evaporated. The solid residue was dried under vacuum to yield Br-Dextran-DTPA (2.126 g, 85.42%). 2.0 g Br-Dextran-DTPA was dissolved in 20 mL of DMSO and then was added a solution of the amino-derivatized folic acid (FA-NH_2, 2.38 g, 4.94 mmol) dissolved in 10mL of distilled water. Subsequently, 25% tetrabutylammonium hydroxide solution (Dosage 0.8%–1.2%) was added and the mixture was stirred for 48 h at room temperature. The reaction solution was filtered and precipitated with ethanol. The precipitate was dialyzed against distilled water. The dialyzed solution was evaporated and the solid residue was dried under vacuum to yield a purple dextran ligand containing DTPA and FA groups (FA-Dextran-DTPA, 1.53 g, 76.5%).

FA-Dextran-DTPA: ^{1}H-NMR (DMSO-d_6, δ): 9.00, 8.98, 8.63, 8.12, 7.81 (m, H of pteridine), 7.53, 6.65, 6.54 (m, —C_6H_4—), 5.59 (s, —NH_2), 4.68, 4.35, 4.21, 4.20 (s, —CH_2—), 3.52–3.24 (s, NCH_2CO), 2.7–2.21 (m, CHC, NCH_2CH_2N). IR (KBr, ν_{max}, cm^{-1}): 3423 (OH), 1605 (C=C), 2980 (aryl CH), 2786, 2720 (aldehyde CH), 1682, 1637 (CO), 1612, 1575 (aryl C—C), 1356, 1348 (NO). UV-Vis (H_2O, λ_{max}, nm): 283, 368.

Preparation of Dextran Gadolinium Chelate

FA-Dextran-DTPA (2.0 g, 2.26 mmol) was dissolved in 30 mL of distilled water and gadolinium chloride ($GdCl_3$, 0.65 g, 2.459 mmol) was added. The mixture was stirred for 1 h, adjusted with NaOH solution (2 mol/mL) to pH 5, and continued to stir for 12 h at room temperature. After dialysis against distilled water, the dialyzed solution was evaporated and the solid residue was dried under vacuum to yield a dextran gadolinium complex FA-Dextran-DTPA-Gd (1.76 g, 88%). FA-Dextran-DTPA-Gd: IR (KBr, cm^{-1}): 3423 (OH), 2924 (C—H), 1630, 1450 (COO), 1322 (C—N), 1010 (C—O). UV-Vis (H_2O, λ_{max}, nm): 279, 366. The average mole ratio of attached Gd-DTPA and FA groups to dextran repeat units (mol%): Gd-DTPA, 6.63 (as determined by an Intrepid XSP Radial inductively-coupled plasma emission spectrometer (ICP-AES, IRIS Intrepid II)) and FA, 4.5 (as determined from ^{1}H-NMR).

Relaxivity

In the absence of solute-solute interactions, the solvent relaxation rates are linearly dependent on the concentration of the paramagnetic species ([M]); Relaxivity, r_1, is defined as the slope of this dependence (1):

$$(1/T_1)_{obsd} = (1/T_1)_d + r_1[M] \quad (1)$$

where $(1/T_1)_{obsd}$ is the observed solvent relaxation rate in the presence of a paramagnetic species, $(1/T_1)_d$ is the solvent relaxation rate in the absence of a paramagnatic species. In this experiment, the concentrations of paramagnetic species [Gd^{3+}] were measured by an Intrepid XSP Radial inductively-coupled plasma emission spectrometer (ICP-AES, IRIS Intrepid II). The solvent longitudinal relaxation time (T_1) measurements for gadolinium complex was carried out on a 10^{-3} mol/L solution of gadolinium complexes in distilled water. Thus r_1 for gadolinium complex in distilled water could be calculated.

In vitro Cytotoxicity Assay

HeLa cells (2×10^5/mL) were plated in 96 wells plates in the growth medium (the RPMI-1640 media: 10% fetal bovine serum (Gibco.Co., USA), 100 units/mL penicillium, 100 μg/mL streptomycin) and the number of cells in each well was 2×10^4. The cells were incubated for 24 h in an incubator (37 °C, 5% CO_2) and the growth medium was then removed and replaced with 100 μL of the growth medium containing gadolinium complex Gd-DTPA or FA-Dextran-DTPA-Gd. After a 48 h incubation, 20 μL of a MTT (thiazolyl blue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 5.0 mg/mL) solution in the phosphate buffer saline

solution (PBS) was added to each well. The cells were incubated for 3 h again and 100 μL of DMSO was then added and shaken for 30 min at room temperature, after which the growth medium was removed. The optical density (OD_{570}) was measured at 570 nm with a DG-3022A ELISA-Reader and expressed as a percentage relative to control cells (no gadolinium complex).

Cellular Uptake Assay

H460 lung carcinoma cells, MDA-MB-231 breast carcinoma cells and T40D breast carcinoma cells (2×10^5/mL) were plated in 12 wells plates in the growth medium (the RPMI-1640 media: 10% fetal bovine serum (Gibco.Co., USA), 100 units/mL penicillium, 100 μg/mL streptomycin), respectively and the number of cells in each well was 5×10^4. These cells were incubated for 48h in an incubator (37 °C, 5% CO_2) and the growth medium was then removed and replaced with 1 mL of the growth medium containing gadolinium complex FA-Dextran-DTPA-Gd (0.002 mmolGd/mL) or pure growth medium. After 2 h incubation, the growth medium was then removed again and washed using PBS for three times. The cell morphology and density were observed in an inverted microscope. These cells were digested using trypsin and transferred into centrifuge tubes. Subsequently, these cells were centrifuged and washed again using PBS twice. The cell suspensions were transferred into centrifuge tubes (1.5 mL) to prepare the samples for MRI test.

MR Imaging

MR imaging of the solution of FA-Dextran-DTPA-Gd in RPMI-1640 media (20 mmolGd/L) and the suspensions of tumor cells including H460 lung carcinoma cells, MDA-MB-231 breast carcinoma cells and T40D breast carcinoma cells incubated with FA-Dextran-DTPA-Gd, respectively were carried out on a 3.0 Tesla Magnetom Tiro Tim MR Scanner. The T_1-weighted images were obtained with a T_1-weighted spin-echo sequence [Repetition time (TR) 400 msec, echo time (TE) 17.9 msec, rotating angle: 90°, the field of view is 12.7 mm, with an image matrix of 332 × 332 and FOV of 14 cm × 14 cm. A region of interest (ROI) of signal intensity (ROI) was 20 mm^2, number of excitation (NEX) was 2–4, and slice thickness was 5 mm.].

RESULTS AND DISCUSSION

Synthesis and Characterization

A water-soluble tumor-targeting dextran containing FA and Gd-DTPA groups was successfully synthesized and studied herein. Low molecular weight Gd-DTPA and FA as a tumor-targeting group were incorporated into dextran to make macromolecular contrast agent FA-Dextran-DTPA-Gd. ^{1}H-NMR spectra of FA-Dextran-DTPA showed the characteristic peaks of NCH_2COO structure of DTPA groups and pteridine structure of folate groups, indicating that DTPA and FA were covalently bound to dextran. IR spectra of free ligand FA-Dextran-DTPA showed characteristic absorption peaks of carboxyl at 1682–1637 cm^{-1}, while these peaks disappeared and strong absorption peaks at 1630–1600 cm^{-1} were present in IR spectra of FA-Dextran-DTPA-Gd. Moreover, UV-Vis spectra of FA-Dextran-DTPA-Gd showed characteristic absorption peaks (279, 366 nm) of folate groups. All results evidenced the formation of gadolinium complex. The substituted molar ratios of FA and DTPA structures to the glucose repeat units of FA-Dextran-DTPA-Gd were 4.5% and 6.63%, respectively, which were calculated based on ^{1}H-NMR.

The average particle size and zeta potential of FA-Dextran-DTPA and FA-Dextran-DTPA-Gd solutions in distilled water are listed in Table 1. The average particle sizes of FA-Dextran-DTPA and FA-Dextran-DTPA-Gd decreased whilst the solution concentrations increased. The reason for the different sizes at different concentrations maybe was that the dextran chains were readily to extend free in the low concentrations (0.0001 g/mL), however, the dextran chains became to curl up in the high concentrations (0.01 g/mL). After complexation, the average particle size of FA-Dextran-DTPA-Gd solutions were less about 2-fold than that of the corresponding ligand FA-Dextran-DTPA solutions in distilled water. Some negative carboxyl groups COO^- of FA-Dextran-DTPA made the FA-Dextran-DTPA chains extend more. Meanwhile, the negative zeta potential of FA-Dextran-DTPA solutions (–10 ± 0.4 mV) also proved this results. However, gadolinium ions just neutralized the negative carboxyl groups COO^- of FA-Dextran-DTPA in the complexation process. Therefore

the zeta potential of FA-Dextran-DTPA-Gd solutions was 0 mv and the average particle size of FA-Dextran-DTPA-Gd solutions reduced greatly.

Table 1. Average particle size and zeta potential of dextran ligand and complex solutions

Sample solution	Particle size (nm)			Zeta potential (mV)
	0.0001 g/mL	0.001 g/mL	0.01 g/mL	
FA-Dextran-DTPA	789.5 ± 39.5	623.6 ± 31.2	514.6 ± 25.7	−10 ± 0.4
FA-Dextran-DTPA-Gd	405.3 ± 20.3	334.2 ± 16.7	327.5 ± 16.4	0

Relaxivity

According to the Eq. (1), the curve was made by the value of solvent relaxation rate $(1/T_1)_{obsd}-(1/T_1)_d$ as the *y*-axis versus the concentration of gadolinium ions [Gd^{3+}] as the *x*-axis (shown in Fig. 1). The relaxivity of gadolinium chelate accordingly enhanced when the concentration of gadolinium ion [M] increased. Then the slope value of simulation linearity of 6.066 $mmol^{-1}\cdot L\cdot s^{-1}$ represented the relaxivity r_1. Gadolinium complex FA-Dextran-DTPA-Gd possessed higher relaxation effectiveness than that of Gd-DTPA (3.63 $mmol^{-1}\cdot L\cdot s^{-1}$)[25] at the same condition. Therefore, FA-Dextran-DTPA-Gd exhibited the effective relaxation rates and improved the relaxivity per gadolinium atom.

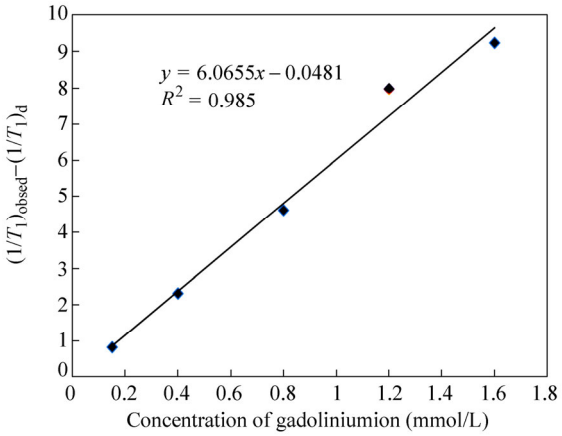


Fig. 1 Relaxivity of gadolinium complex in water solution

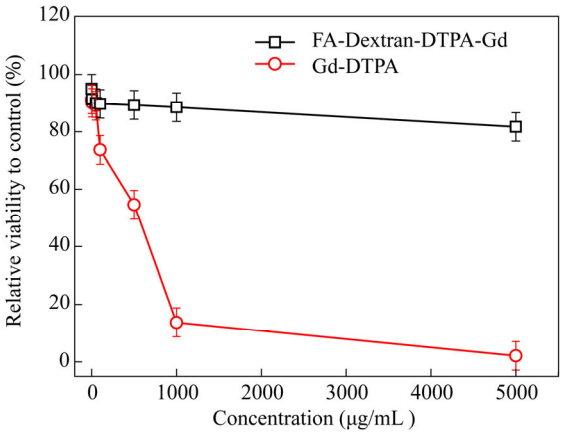


Fig. 2 *In vitro* cytotoxicity assay of gadolinium complex to HeLa cells

In vitro Cytotoxicity Assay

The effects of gadolinium complex to HeLa cell growth and metabolism are shown in Fig. 2. At the concentration (500 μg/mL) of FA-Dextran-DTPA-Gd or Gd-DTPA in the growth medium, the viabilities of HeLa cells incubated with FA-Dextran-DTPA-Gd or Gd-DTPA were 89.36% and 54.57%, respectively, relative to control. Meanwhile, the viabilities of HeLa cells incubated with FA-Dextran-DTPA-Gd or Gd-DTPA retained 81.69% and 2.07%, respectively, relative to control, at the concentration (5000 μg/mL) of FA-Dextran-DTPA-Gd or Gd-DTPA in the growth medium. It illustrated that FA-Dextran-DTPA-Gd possessed lower cytotoxicity to HeLa cells than that of Gd-DTPA.

Cellular Uptake Assay and MR Imaging

The T_1-weight MR images of the solution of FA-Dextran-DTPA-Gd in RPMI-1640 media (20 mmolGd/L) at 30 min are shown in Fig. 3. Compared to pure RPMI-1640 solution, the T_1 signal intensity of FA-Dextran-DTPA-Gd solution in RPMI-1640 media was obviously enhanced and MRI images displayed brighter during the detection time.

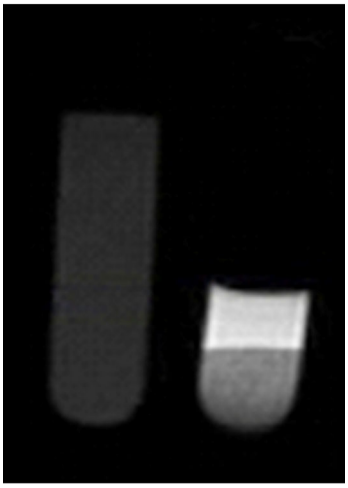


Fig. 3 MR imaging of FA-Dextran-DTPA-Gd in the RPMI-1640 solution
(Left: RPMI-1640 solution; Right: FA-Dextran-DTPA-Gd in RPMI-1640 solution)

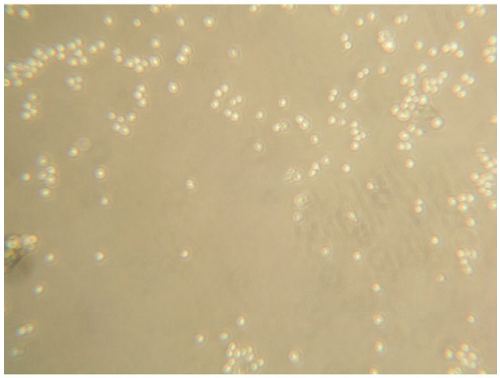


Fig. 4 Picture of H460 lung carcinoma cells incubated with FA-Dextran-DTPA-Gd under the inverted microscope

The picture of H460 lung carcinoma cells incubated with FA-Dextran-DTPA-Gd under the inverted microscope is shown in Fig. 4. T_1-weight MR images of the suspensions of tumor cells including H460 lung carcinoma cells, MDA-MB-231 breast carcinoma cells and T40D breast carcinoma cells incubated with FA-Dextran-DTPA-Gd, respectively at 30 min are shown in Fig. 5. The positional distribution of tumor cells suspensions in MRI are listed in Table 2. Compared to MDA-MB-231 cell suspensions M-1, T_1 signal intensity of MDA-MB-231 cell suspensions M-2 and M-4 were obviously enhanced and MRI images displayed brighter during the detection time. However, T_1 signal intensity of H460 cell suspensions (H-2, H-3 and H-4) and T40D cell suspensions (T-2, T-3 and T-4) were enhanced less and MRI images displayed little brighter during the detection time than those of H460 cell suspensions H-1 and T40D cell suspensions T-1, respectively.

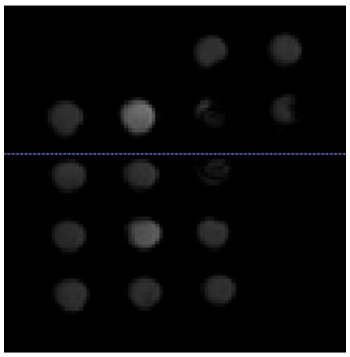


Fig. 5 MR imaging of tumor cells
(Positional distribution of cells is given in Table 2. Tumors cells H: H460 lung carcinoma cells, H-1: H460 carcinoma cells without incubation with FA-Dextran-DTPA-Gd solution, H-2, H-3, H-4: H460 carcinoma cells with incubation with FA-Dextran-DTPA-Gd solution, respectively. Tumors cells M: MDA-MB-231 breast carcinoma cells, M-1: MDA-MB-231 carcinoma cells without incubation with FA-Dextran-DTPA-Gd solution; M-2, M-3, M-4: MDA-MB-231 carcinoma cells with incubation with FA-Dextran-DTPA-Gd solution, respectively. Tumors cells T: T40D breast carcinoma cells, T-1: T40D carcinoma cells without incubation with FA-Dextran-DTPA-Gd solution; T-2, T-3, T-4: T40D carcinoma cells with incubation with FA-Dextran-DTPA-Gd solution, respectively.)

Table 2. Positional distribution of cells in cellular uptaking assay for MRI

		Trypsin + PBS	Trypsin + PBS
H-4	M-4	T-4	Trypsin + PBS
H-3	M-3	T-3	
H-2	M-2	T-2	
H-1	M-1	T-1	

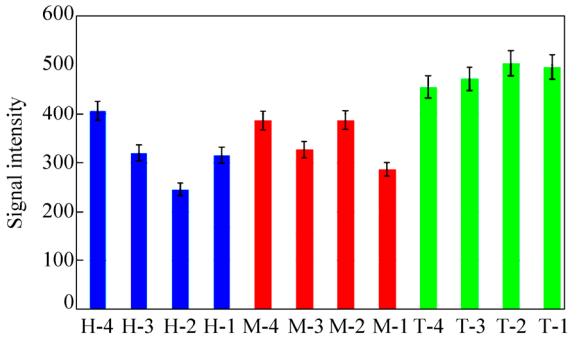


Fig. 6 MRI signal intensities of tumor cells
(Positional distribution of cells is given in Table 2.)

Based on the results shown in Fig. 6, MRI signal enhancements of MDA-MB-231 tumor cell suspensions (M-2, M-3 and M-4) and H460 cell suspensions (H-3 and H-4) were higher in comparison with those of MDA-MB-231 cell suspensions M-1 and H460 cell suspensions H-1, respectively. However, Compared to T40D cell suspensions T-1, MRI signal intensities of T40D cell suspensions (T-2, T-3 and T-4) showed no enhancements. It revealed that FA-Dextran-DTPA-Gd accumulated specially and remained in MDA-MB-231 breast carcinoma cells and H460 lung carcinoma cells significantly, and possessed high MRI signal enhancements. These results demonstrated that FA-Dextran-DTPA-Gd had a high affinity and can be taken up selectively by MDA-MB-231 breast carcinoma cells and H460 lung carcinoma cells and then enhanced the contrast of MR images.

CONCLUSIONS

A water-soluble dextran gadolinium complex FA-Dextran-DTPA-Gd was synthesized by attachment of folate (FA) as a tumor-targeting group and Gd-DTPA to dextran. Compared with Gd-DTPA, FA-Dextran-DTPA-Gd possessed obviously higher relaxation effectiveness and lower cytotoxicity to HeLa cells. Moreover, FA-Dextran-DTPA-Gd enhanced the contrast of MR images of MDA-MB-231 breast carcinoma cells and H460 lung carcinoma cells. Therefore FA-Dextran-DTPA-Gd can be taken up selectively by tumors and showed the potential as a contrast agent in targeted MRI for tumors.

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