Liver-targeting macromolecular MRI contrast agents

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Abstract Macromolecular ligands with liver-targeting group (pyridoxamine, PM) PHEA-DTPA-PM and PAEA-DTPA-PM were prepared by the incorporation of different amount of diethylenetriaminepentaacetic acid monopyridoxamine group (DTPA-PM) into poly-α, β-[N-(2-hydroxyethyl)-*L*-aspartamide] (PHEA) and poly-α, β-[N-(2-aminoethyl)-*L*-aspartamide] (PAEA). The macromolecular ligands thus obtained were further complexed with gadolinium chloride to give macromolecular MRI contrast agents with different Gd(III) contents. These macromolecular ligands and their gadolinium complexes were characterized by ^{1}H NMR, IR, UV and elementary analysis. Relaxivity studies showed that these polyaspartamide gadolinium complexes possess higher relaxation effectiveness than that of the clinically used Gd-DTPA. Magnetic resonance imaging of the liver in rats and experimental data of biodistribution in mice indicate that these macromolecular MRI contrast agents containing pyridoxamine exhibit liver-targeting property.

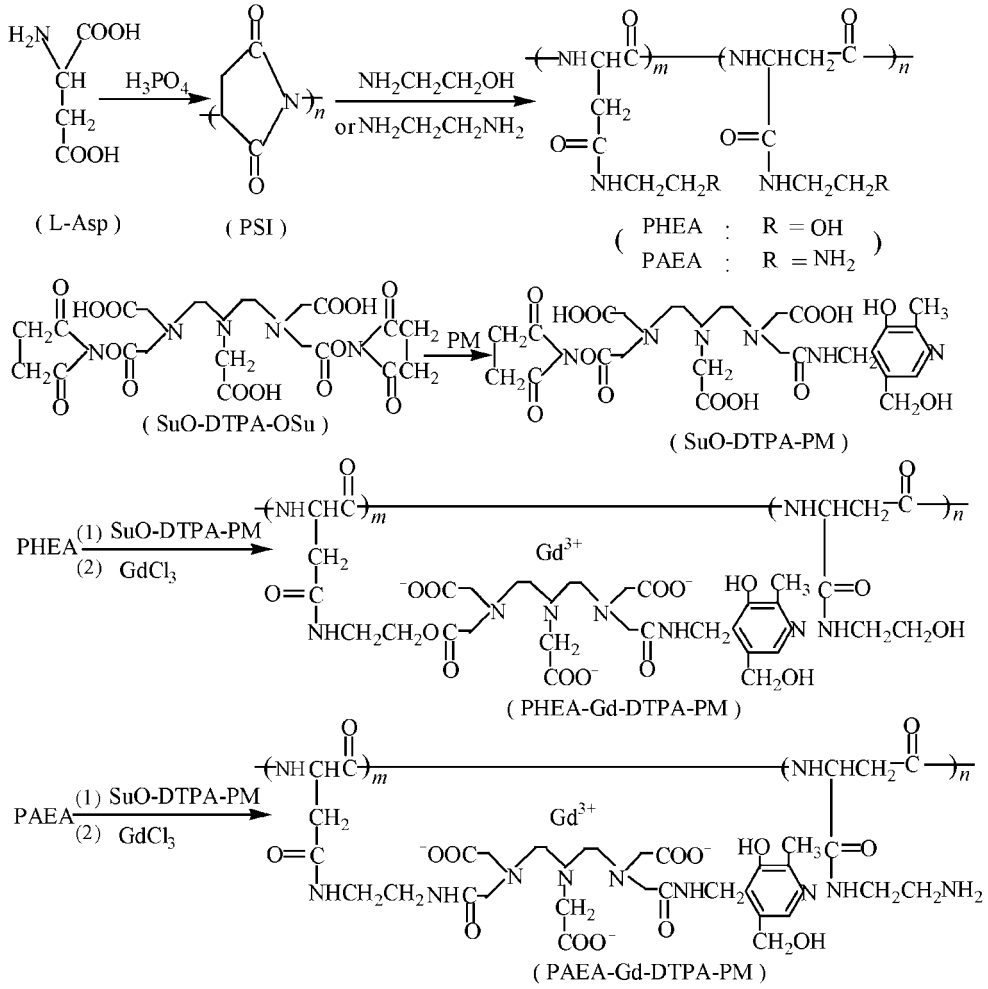
Keywords: polyaspartamide, gadolinium complexes, magnetic resonance imaging (MRI), relaxivity, liver-targeting.

Macromolecular magnetic resonance imaging (MRI) contrast agent can be prepared by the incorporation of a paramagnetic metal chelated complex to a macromolecule. The macromolecular paramagnetic metal complex thus obtained usually exhibits more effective relaxation than that of the metal complex alone since an increase in rotational correlation time can improve relaxivity. In addition, when an organ-targeting group is attached to this macromolecular metal complex, it could be endowed with organ-targeting property. On the other hand, macromolecular MRI contrast agents may show prolonged intravascular retention due to its bulky molecular volume, and it can be used clinically as a blood pool contrast agent[1, 2].

Polyaspartamide is a water-soluble, biologically well tolerated synthetic polymer that has been proposed as a plasma extender and a drug carrier. It is nontoxic, nonantigenic and biodegradable in living systems, and can be further modified via the reactive pendant group[3].

In this work, pyridoxamine (PM) was chosen as a liver-targeting group[4]. PM-containing DTPA mono(N-hydroxysuccinimide) ester (SuO-DTPA-PM) was prepared by reacting PM with DTPA bis(N-hydroxysuccinimide) ester. The PM-containing DTPA active ester thus obtained was further incorporated into poly-α, β-[N-(2-hydroxyethy1)-*L*-aspartamide] (PHEA) and poly-α, β-

[N-(2-aminoethy1)-*L*-aspartamide] (PAEA) to give liver-targeting macromolecular ligands PHEA-DTPA-PM and PAEA-DTPA-PM. Finally, by the metalation of the ligands with Gd(III), two kinds of macromolecular gadolinium complexes were synthesized. The synthetic process is shown as scheme 1.



Scheme 1. Synthetic route and molecular structures for MRI contrast agents.

1 Experimental

1.1 Instruments and reagents

IR spectra were recorded on a Nicolet-170SX FT-IR spectrophotometer. ^{1}H NMR data were obtained on a JEOL FX-90Q NMR (90 MHz) spectrometer. The elementary analysis was performed on a Carlo Erba 1106 analyzer. The concentrations of the paramagnetic species [Gd^{3+}] were measured by ICP Atomscan-2000 spectrometer. UV spectra were recorded on a Shimadzu UV-240 spectrophotometer. The molecular weight and polydispersity of poly(amino acid) were measured by GPC (waters 2960D separation module, ultrahydrogel 2000 column, poly(ethylene

glycol) standard, 0.1 mol • L^{-1} KH_2PO_4 solvent, 0.500 mL • min^{-1} flow, 35℃ column temperature and 30℃ detector temperature). Biodistribution in mice was reported by an SN-695 gamma counter RIA program Ver 5.33 and Kunming mice weighing (20±2) g were used. MR images were performed using a BIOSPEC 47/30 system (Bruker Instruments, 4.7 T) and SD rats weighing 150 g were used. Longitudinal relaxation time (T_1) for paramagnetic metal complexes was determined by a Bruker WP-80SY NMR (80 MHz，1.88 T) spectrometer. The optical densities (OD_{570}) were measured with a DG-3022A ELISA-Reader. HeLa cells and L-02 cells were provided by China Center for Type Culture Collection of Wuhan University.

L-aspartic acid, N-hydroxysuccinimide and poly-*L*-lysine (PLys, M_w: 3×10^4—7×10^4) were biochemical reagents. Diethylenetriaminepentaacetic acid, ethylenediamine, aminoethanol and gadolinium chloride were analytical reagents. The growth medium was the RPMI-1640 media (10% fetal bovine serum (Gibco. Co. USA), 100 units/mL penicillium, 100 μg/mL streptomycin). DTPA bis(N-hydroxysuccinimide) ester (SuO-DTPA-OSu) was prepared according to ref. [5]. Pyridoxamine (PM) was prepared according to ref. [6]. All the other chemicals and solvents were analytical reagents.

1.2 Synthesis of macromolecular gadolinium complexes

1.2.1 Preparation of PHEA and PAEA. Polysuccinimide (PSI), poly-α, β-[N-(2-hydroxyethy1)-*L*-aspartamide] (PHEA) and poly-α, β-[N-(2-aminoethy1)-*L*-aspartamide] (PAEA) were synthesized according to ref. [7]. The molecular weight and polydispersity of poly(amino acid) are shown in table 1.

Table 1 M_n, M_w and polydispersity of poly(amino acid)

Poly(amino acid)	$M_n\times10^4$	$M_w\times10^4$	Polydispersity
PHEA	2.61	3.77	1.44
PAEA	1.16	1.91	1.65

1.2.2 Synthesis of SuO-DTPA-PM. 0.57 g (3.4 mmol) of pyridoxamine (PM) in 30 mL of DMF-H_2O (*v*/*v*=3∶5) was added slowly to a solution of SuO-DTPA-OSu (2.0 g, 3.4 mmol) in DMF (20 mL) with rapidly stirring at −15—−20℃. The reaction mixture was stirred for 4 h at the same temperature and for 6 h at 20℃, then precipitated by pouring into 600 mL ethanol-ether (*v*/*v*=1/6) and filtered. The precipitate was dried for 2 h at room temperature under vacuum to yield 1.74 g (80%) product, m. p. 103—104℃.

IR(KBr,cm^{-1}): 3400(OH), 3062(C=C), 2962(CH_2CH_2), 1725(COOH), 1656(CONH), 1390 (). ^{1}H NMR (DMSO-d_6, δ): 8.5 (s, 1H), 4.5(s, 2H), 3.9—4.2 (s, 2H), 3.5 (m, 8H), 3.0 (s, 3H), 2.85 (s, 6H),2.7 (s, 4H), 2.3—2.5 (t, 4H). Anal: calcd. for ($C_{26}H_{36}N_6O_{13}$)(%): C 48.75, H 5.63, N 13.13; found(%): C 48.41, H 5.66, N 13.03.

1.2.3 Synthesis of macromolecular ligands PHEA-DTPA-PM(L_{1-4}). 0.20 g (0.316 mmol) of

SuO-DTPA-PM (mole ratio of SuO-DTPA-PM to PHEA repeat units:10%) was dissolved in DMF (20 mL), and then added into a PHEA (0.5g, 3.16 mmol) in DMF (20 mL) solution with rapidly stirring at 20℃. The reaction mixture was stirred for 48 h at the same temperature and precipitated by pouring into ethanol (300 mL). After filtration, the precipitate was recrystallized from DMF-ethanol (*v*/*v*=1/6) and dried for 6 h at 60℃ under vacuum to give product PHEA-DTPA-PM (L_1)(0.36 g, 54.5%).

IR(KBr, cm^{-1}): 3316(OH), 3069(C=C), 1662(COOH), 1645, 1547(CONH). ^1H NMR (D_2O, δ): 7.9 (), 3.9 (CCH_2), 3.6—3.3 (CH_2CH_2), 3.0 (CH_3), 2.82 (NCH_2CO). UV(H_2O, nm): 282. PHEA-DTPA-PM(L_{2-4})(mole ratio of SuO-DTPA-PM to PHEA repeat units: 20%, 60%, 100%) were similarly synthesized.

1.2.4 Synthesis of macromolecular ligands PAEA-DTPA-PM(L_{5-7}). 0.33 g (0.51 mmol) of SuO-DTPA-PM (mole ratio of SuO-DTPA-PM to PAEA repeat units: 20%) was dissolved in DMF (20 mL), and then added into a PAEA (0.40 g, 2.55 mmol) in DMF-H_2O (*v*/*v*=1/5, 30 mL) solution with rapidly stirring at 0℃. The reaction mixture was stirred for 8 h at the same temperature and for 24 h at 20℃. The reaction mixture was evaporated and the residue solution was precipitated by pouring into 500 mL ethanol-ether (*v*/*v*=1/4). The precipitate was collected and dialysed against distilled water. After lyophilization, the product PAEA-DTPA- PM(L_5) was obtained (0.58 g, 86.4%).

IR(KBr, cm^{-1}): 3281(OH), 3062(C=C), 1665(COOH), 1652, 1540(CONH). ^1H NMR (D_2O, δ): 7.9 (), 3.9 (CCH_2), 3.6—3.2 (CH_2CH_2), 3.0 (CH_3), 2.82 (NCH_2CO). UV(H_2O, nm): 282. PAEA-DTPA-PM(L_6, L_7) (mole ratio of SuO-DTPA-PM to PAEA repeat units: 60%, 100%) were similarly synthesized.

1.2.5 Sythesis of macromolecular gadolinium complexes. 5 mmol of PHEA-DTPA-PM(L_{1-4}) or PAEA-DTPA-PM(L_{5-7}) in distilled water was added into a solution of $GdCl_3$ (7.5 mmol) in distilled water with stirring at room temperature. The reaction mixture was stirred for 1 h, adjusted the reaction solution to pH = 5 with dilute NaOH solution, and continually stirred for 12 h at room temperature. After dialysis and evaporation of water under vacuum, macromolecular gadolinium complexes were obtained (table 2).

PHEA-Gd-DTPA-PM: IR(KBr, cm^{-1}): 3468—3436 (OH), 2938 (C═C), 1644 (COO), 1638, 1536 (CONH).

PAEA-Gd-DTPA-PM: IR(KBr, cm^{-1}): 3436 (NH_2), 2935 (C═C), 1661 (COO), 1645, 1596 (CONH).

1.3 Relaxivity of metal complexes

The proton longitudinal relaxation times (T_1) for PHEA-Gd-DTPA-PM, PAEA-Gd-DTPA-

PM and Gd-DTPA in distilled water at 25℃ were measured by a Bruker WP-80SY NMR(80 MHz, 1.88 T) spectrometer. The experimental data are listed in table 3.

Table 2 Elementary analysis of macromolecular gadolinium complexes

Polymer metal complexes	Mole ratio of SuO-DTPA-PM/Polymeric repeat units (%)	Elem. anal.(%)			
		Gd [a)]	C	N	H
PHEA-Gd-DTPA-PM					
M_1	10	1.70	49.80	18.10	6.19
M_2	40	2.24	45.62	16.53	5.66
M_3	60	2.65	45.46	16.40	5.63
M_4	100	2.90	45.37	16.33	5.62
PAEA-Gd-DTPA-PM					
M_5	20	10.76	42.77	16.32	4.92
M_6	60	11.04	42.65	16.17	4.80
M_7	100	13.91	41.51	14.70	4.77

a) Measured by ICP Atomscan-2000 spectrometer.

Table 3 Experimental data of relaxivity

Metal complexes	$[Gd^{3+}]$/mmol • L^{-1}	$T_{1\,obsd}$/s	R_1/(mmol • L^{-1} • s)$^{-1}$
Gd-DTPA	3.3066	0.05323	5.58
PHEA-Gd-DTPA-PM			
M_1	1.8708	0.03801	13.88
M_2	2.3630	0.02816	14.88
M_3	1.4206	0.04266	16.26
M_4	1.8708	0.02723	19.45
PAEA-Gd-DTPA-PM			
M_5	1.2410	0.07890	9.94
M_6	2.7710	0.03365	10.61
M_7	1.8390	0.03740	14.35

Temp: 25℃, NMR frequency: 80 MHz, T_{1d} =2.95 s.

1.4 *In vitro* cytotoxicity assay of polyaminoacid

HeLa cells were plated in 24 well plates in the growth medium. The cells grew for 24 h and then the growth medium was removed and replaced with growth medium containing polyaminoacid. After 15 h incubation, the cells were washed with the Hank's solution without Ca^{2+} or Mg^{2+} and digested by the trypsin solution, then collected by centrifugalization and dyed for 5 min using Trypan blue assay. So the dead cells were dyed by Trypan blue and the active cells were not dyed by Trypan blue. The optical density was measured at microscope and expressed as a percent relative to control.

1.5 *In vitro* cytotoxicity assay of macromolecular gadolinium complexes

L-02 cells (2×10^5/mL) were plated in 96-well plates in the growth medium and the number of cells in each well is 2×10^4. The cells were incubated for 24 h in incubator (37℃, 5% CO_2) and 100 μL of the growth medium containing gadolinium complex was added. After 72 h incubation, the cells were washed with the growth medium, 20 μL of the MTT solution (1.0 mg/mL) was added and incubated for 4 h, then washed again with 3% fetal calf serum and phosphate buffer saline solution (3% FCS-PBS), 100 μL of DMSO added and shaken for 30 min at room temperature. The optical density (OD_{570}) was measured at 570 nm with a DG-3022A ELISA-Reader and expressed as a percent relative to control.

1.6 MR imaging

MR imaging experiments were performed on a BIOSPEC 47/30 system (Bruker instruments, 4.7 T). MR imaging of the liver was carried out on the rat under anesthesia. SD rats (150 g) were anesthetized with urethane (20%, 1 g/kg), positioned pronely and fixed to a polystyrene cradle with adhesive tape to minimize respiratory motion. Coronal images of the liver were performed before SD rats had received the injection of gadolinium complexes. Gd-DTPA (0.1 mmol/kg, Magnevist), PHEA-Gd-DTPA-PM (M_3, 0.06 mmol/kg) or PAEA-Gd-DTPA-PM (M_6, 0.08 mmol/kg) were injected via the tail. Coronal images of the liver were obtained with a T_1-weighted spin-echo sequence (TR=500 ms, TE=15 ms, the field of view is 40 mm, with an image matrix of 256×256. Slice thickness is 2 mm, 8 multislices, average 8, with a l mm interslice gap).

1.7 *In vivo* biodistribution

In vivo biodistribution of mice was measured on an SN-695 gamma counter RIA program Ver 5.33. PAEA-DTPA-PM (L_7) was labeled with $^{99m}TcO_4^-$ and reduced with fresh tin (II) tartrate and vitamin C at room temperature (radiochemical purity >95%). Forty-five mice (Kunming, (20 ±2) g) were divided into nine groups, and each mouse was injected with ^{99m}Tc-labeled PAEA-DTPA-PM solution via the tail and sacrificed at different time intervals postadministration. Samples of blood, lung, liver, spleen, kidney, muscle, heart, bone, and small intestine were taken. Tissues were blotted to remove excess blood and weighed, analyzed by an SN-695 gamma counter RIA program Ver 5.33. Percentage of injected dose per organ (%ID/g) was calculated.

2 Results and discussion

2.1 Synthesis and characterization

The elementary analysis of macromolecular gadolinium complexes is shown in table 2. In this work, PHEA-Gd-DTPA-PM and PAEA-Gd-DTPA-PM in aqueous solution after dialysis show absorption at 282 nm, however, PHEA and PAEA show no UV absorption at the above-mentioned wavelengths. Thus the UV data in aqueous solution give the evidence of the incorporation of DTPA-PM into PHEA and PAEA.

2.2 Relaxivity of metal complexes

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[2]:

$$(1/T_1)_{obsd}=(1/T_1)_d+R_1(M),$$

where $(1/T_1)_{obsd}$ is the observed solvent relaxation rate in the presence of a paramagnetic species, and $(1/T_1)_d$ is the solvent relaxation rate in the absence of a paramagnatic species. In this experiment, the concentrations of the paramagnetic species [Gd^{3+}] were measured by ICP Atomscan-2000 spectrometer. Thus R_1 for paramagnetic metal complexes in distilled water could be worked out (table 3).

Table 3 illustrates that all polyaspartamide gadolinium complexes possess higher relaxation effectiveness than that of the corresponding small molecular complex: Gd-DTPA.

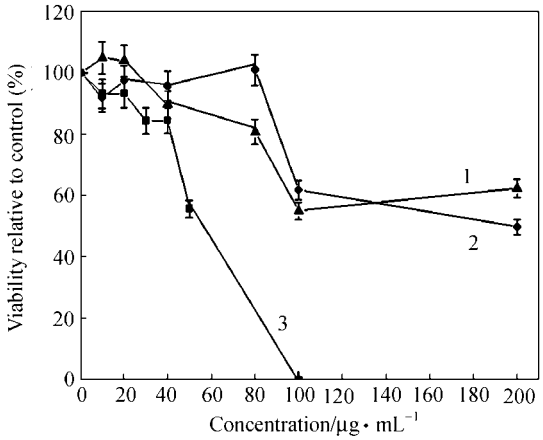


Fig. 1. Cytotoxicity assay of PLys, PHEA and PAEA in HeLa cells. 1, PHEA; 2, PAEA; 3, PLys.

2.3 Cytotoxicity assay of polyaminoacid

The effect of PHEA and PAEA on cell growth and metabolism of HeLa cells *in vitro* was determined as a function of polymer concentration and compared to polylysine (fig. 1). The preliminary results show that over the concentration range tested, the cells incubated with PHEA and PAEA retain 55.1% and 61.9% viability at 100 μg/mL, while in the presence of poly-*L*-lysine (PLys), HeLa cells show no viability under the same concentration. At a higher concentration (200 μg/mL) of PHEA and PAEA, the cells still retain 62.4% and 49.8% viability respectively, relative to control.

2.4 Cytotoxicity assay of metal complexes

The effect of metal complexes on cell growth and metabolism of L-02 cells *in vitro* is shown in fig. 2. At the concentration (25600 μg/mL) of gadolinium complexes in the growth medium, the L-02 cells incubated with Gd-DTPA and PHEA-Gd-DTPA-PM (M_3) retain respectively above 4.0% and 88.7% viability respectively relative to control, illustrating that PHEA-Gd-DTPA-PM (M_3) possesses obviously lower cytotoxicity to L-02 cells than that of Gd-DTPA. However, L-02 cells incubated with PAEA-Gd-DTPA- PM (M_6) over the same concentration range had lower viability because the cells were covered by PAEA-Gd-DTPA-PM (M_6) and could not grow up.

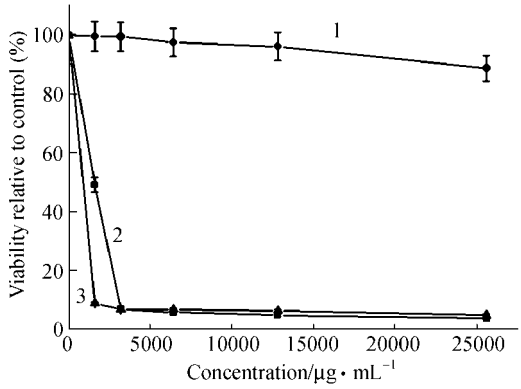


Fig. 2. Cytotoxicity assay of metal complexes in L-02 cells. 1, PHEA-Gd-DTPA-PM (M_3); 2, Gd-DTPA; 3, PAEA-Gd-DTPA-PM (M_6).

2.5 MR imaging

Compared to the signal intensity (SI) of the liver in the rat injected with Gd-DTPA (0.1 mmol/kg), it demonstrates that SI of the liver in the rat injected with PHEA-Gd- DTPA-PM (M_3, 0.06 mmol/kg) or PAEA-Gd- DTPA-PM (M_6, 0.08 mmol/kg) was obviously enhanced, the irradiated portion of the liver was brighter and the demarcation became clearer (fig.3).

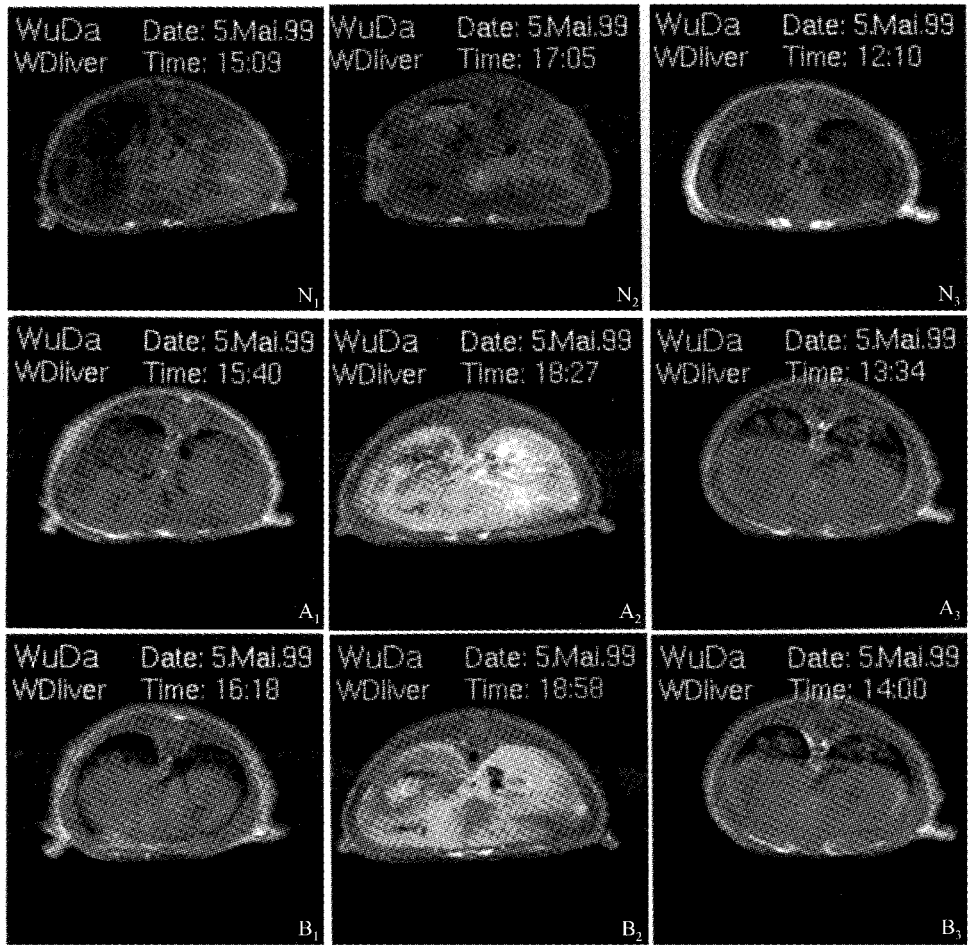


Fig. 3. N_1, N_2 and N_3 are the T_1-weighted images of the rat received no injection of MRI contrast agent; A_1 and B_1 are the T_1-weighted images of the rat received injection of Gd-DTPA (0.1 mmol/kg, Magnevist) after 6 and 44 min; A_2 and B_2 are the T_1-weighted images of the rat received injection of PHEA-Gd-DTPA-PM (M_3, 0.06 mmol/kg) after 15 min and 46 min; A_3 and B_3 are the T_1-weighted images of the rat received injection of PAEA-Gd-DTPA-PM (M_6, 0.08 mmol/kg) after 20 and 46 min.

2.6 *In vivo* biodistribution

Fig.4 shows that the rapid decrease of the macromolecular MRI contrast agent in blood, heart and spleen correlates with its increasing capture by the liver, indicating that these macromolecular MRI contrast agents containing pyridoxamine were taken up specifically by the liver.

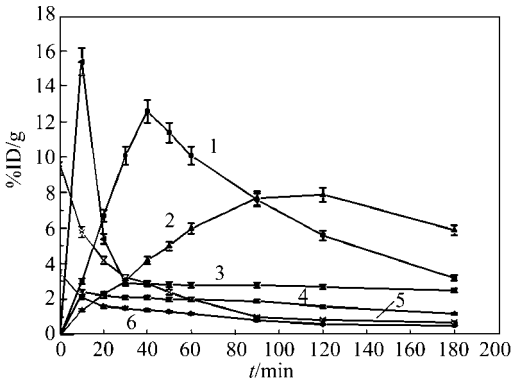


Fig. 4. Organ distribution at the different times after injection. 1, Liver; 2, kidney; 3, spleen; 4, lung; 5, blood; 6, heart.

3 Conclusion

Polyaspartamide gadolinium chelated complexes containing pyridoxamine moiety

can be targeted to specific organs such as liver and spleen. They possess obviously higher relaxation effectiveness than that of Gd-DTPA. MR images show that the signal intensity (SI) of the liver in rat injected PHEA-Gd-DTPA-PM or PAEA-Gd-DTPA-PM is obviously enhanced.

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