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Gadolinium(III) chelated conjugated polymer as a potential MRI contrast agent

Qingling Xu [a,1], Litao Zhu [b], Minghui Yu [a,1], Fude Feng [a,1], Lingling An [a,1], Chengfen Xing [a,1], Shu Wang [a,*]

[a] Beijing National Laboratory for Molecular Science, Key Laboratory of Organic Solids, Institute of Chemistry, Chinese Academy of Sciences, No. 2 Zhongguancun North First street, Beijing 100190, PR China
[b] State Key Laboratory of Cognitive Neuroscience and Learning, Beijing Normal University, Beijing 100875, PR China

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ABSTRACT

Nuclear magnetic resonance imaging (MRI) has become a powerful technique in clinical diagnostics. In this work, a new MRI contrast agent by covalently linking Gd(III) chelates to the side chain of conjugated polymer (PF–Gd) is synthesized by Suzuki cross-coupling reaction. The PF–Gd exhibits a higher relaxivity and a pronounced enhancement in contrast than that of $(NMG)_2$–Gd–DTPA widely used for clinical diagnosis. This work should be feasible to potentially lead to a new class of imaging contrast agents.

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1. Introduction

Nuclear magnetic resonance imaging (MRI) has become a powerful technique in clinical diagnostics [1]. The image is based on the NMR signal from the protons of water, where the signal intensity depends on the water concentration and relaxation times (T_1 and T_2). The ability of contrast agents to reduce the longitudinal relaxation time (T_1) of water protons is evaluated by relaxivity (R_1) [1]. Paramagnetic Gd(III) complexes [2], such as Gd(III)–DTPA, Gd(III)–DOTA and their derivatives, are widely used as contrast agents for MRI to enhance image contrast due to their abilities to shorten the relaxation times. However, their relaxivities are still relatively low to effectively image diseased tissues. To improve the relaxivity, a strategy has been demonstrated to reduce the molecule tumbling by attaching Gd(III) chelate to macromolecules [3]. However, due to the internal rotation of flexible chain, the linear polymer-based contrast agents improve relaxivity slightly. Although poly(amidoamide) (PAMAM) dendrimers can increase relaxivity greatly, relaxivity per gadolinium levels off for a limited water exchange rate at higher generation [4]. It's necessary to find new macromolecular structures to get more insight into increasing the relaxivity.

Conjugated polymers (CPs) have been used as optical platforms in highly sensitive bioassays for proteins, nucleic acids and small molecules [5,6]. They are characterized by a delocalized electronic structure, where the multiple and single bonds appear in turn along the backbone to make CPs more rigid than flexible polymers. To the best of our knowledge, there is no report that takes advantage of the CPs to improve the relaxivity of Gd(III) complex. In this paper, we introduce the first CP–Gd(III) conjugate (PF–Gd, see Scheme 1 for the chemical structure) as contrast agent that is designed to improve the relaxivity.

2. Results and discussion

Scheme 2 shows the synthetic entry into the monomer **7** and the polymer PF–Gd. Reaction of 2,7-dibromo-9,9-bis(6′-bromohexyl)-fluorene (**1**) with potassium acetate in DMSO provided 2,7-dibromo-9-(6′-bromohexyl)-9-(6′-hydroxylhexyl)fluorene (**2**) in 37% yield. Compound **3** was obtained by reaction of **2** with 2-(benzylideneamino)phenol in the presence of 18-crown-6 and K_2CO_3 in acetone in 74% yield. After reaction of **3** with ethyl bromoacetate in the presence of N,N-diisopropyl-ethylamine (DIEA) and NaI in anhydrous DMF, the ester **4** was obtained in 65% yield. The hydroxy group of **4** was bromized by CBr_4/PPh_3 to get **5** in 83% yield, which was treated with cyclen in $CHCl_3$ to afford compound **6** in 58% yield. The monomer **7** was obtained by reaction of *tert*-butyl bromoacetate with **6** in acetonitrile in 83% yield. The ester-protected copolymer PF-ester was prepared by Suzuki cross-coupling reaction [7] between **7**, **8** and **9** (with a molar feed ratio of 0.2:0.8:1.0) in the presence of 2.0 M K_2CO_3 aqueous solution and $Pd(PPh_3)_4$ in THF. The PF-ester was treated with KOH solution in CH_3OH followed by Boc-deprotecting with trifluoroacetic acid (TFA)

* Corresponding author. Tel./fax: +86 10 6263 6680.
E-mail address: wangshu@iccas.ac.cn (S. Wang).
[1] Tel./fax: +86 10 6263 6680.

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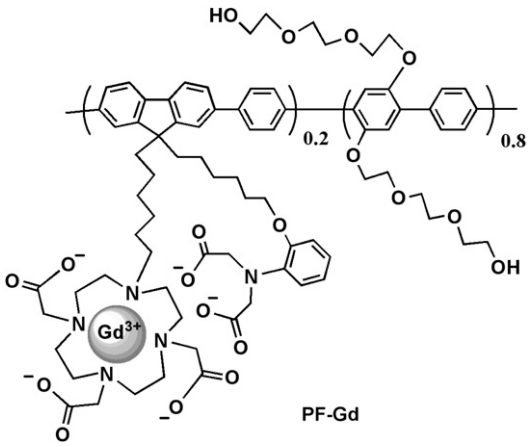


Scheme 1. The chemical structure of PF–Gd.

to give water-soluble PF. The ^{1}H NMR spectroscopy of PF is given in Fig. 1b. On comparing with that of ester-protected PF-ester (Fig. 1a), we could find that the proton peaks of $-COOCH_2CH_3$ (δ_{CH_2} = 3.99 ppm, δ_{CH_3} = 1.08 ppm) and Boc (δ_{CH_3} = 1.35 ppm) groups in PF-ester disappeared. These results indicated that the protected ethyl and Boc groups were gotten rid of entirely upon treatments with KOH and TFA, respectively. The PF was mixed with $GdCl_3$ in DMSO/H_2O solution to afford target PF–Gd complex, where the Gd(III) content was analyzed using ICP spectrometer.

The ability of PF–Gd to enhance the contrast in MRI images was studied by measuring T_1 of water protons as a function of Gd(III) concentration in aqueous solution. In these experiments the gadopentetate dimeglumine ((NMG)$_2$–Gd–DTPA) [8] which is widely used in clinical diagnosis, was used for comparison. As shown in Fig. 2a, the T_1-weighted images showed that PF–Gd could more efficiently enhance the contrast in comparison with (NMG)$_2$–Gd–DTPA at the same Gd(III) concentration. The relaxivity (R_1) was obtained by taking the slope of a plot of T_1^{-1} versus Gd(III) concentration (Fig. 2b). The R_1 value of PF–Gd (12.57 $mM^{-1}\,s^{-1}$) was 3.5 times higher than that of (NMG)$_2$–Gd–DTPA (3.64 $mM^{-1}\,s^{-1}$). The rigidity of the conjugated polymer backbone may result in an increase in the rotational correlation time and subsequently an increase in relaxivity. It was noted that the formation of aggregation [9] due to the amphiphilic characteristic of PF–Gd could also play roles in the increase of relaxivity.

Because of the importance for in vivo use, the biocompatibility of PF–Gd was studied by typical MTT assay method, in which the pulmonary adenocarcinoma cell (A549) was incubated with PF–Gd. The conversion of soluble MTT into formazan is directly related to mitochondrial activity and subsequently to cell viability [14]. As shown in Fig. 3, the cell viability decreases down to 37% as the Gd^{3+} concentration increases from 0.1 to 3.2 μM with IC_{50} value of 2.6 μM. The results show that the PF–Gd is cytotoxic to the A498 cell.

3. Conclusions

In summary, we have developed a new MRI contrast agent by covalently linking Gd(III) chelates to the side chain of conjugated polymer. The new agent exhibits a higher relaxivity and a more pronounced enhancement in contrast than that of (NMG)$_2$–Gd–DTPA widely used for clinical diagnosis. This work should be feasible to potentially lead to a new class of imaging contrast agents. The drawback of this new agent is its cytotoxicity to the cell and the lack of biodegradability, which restricts its in vivo application. The design of other new MRI contrast agent based on conjugated polymers [15] with good biocompatibility is underway in our group.

4. Experimental section

4.1. Material and instrumentation

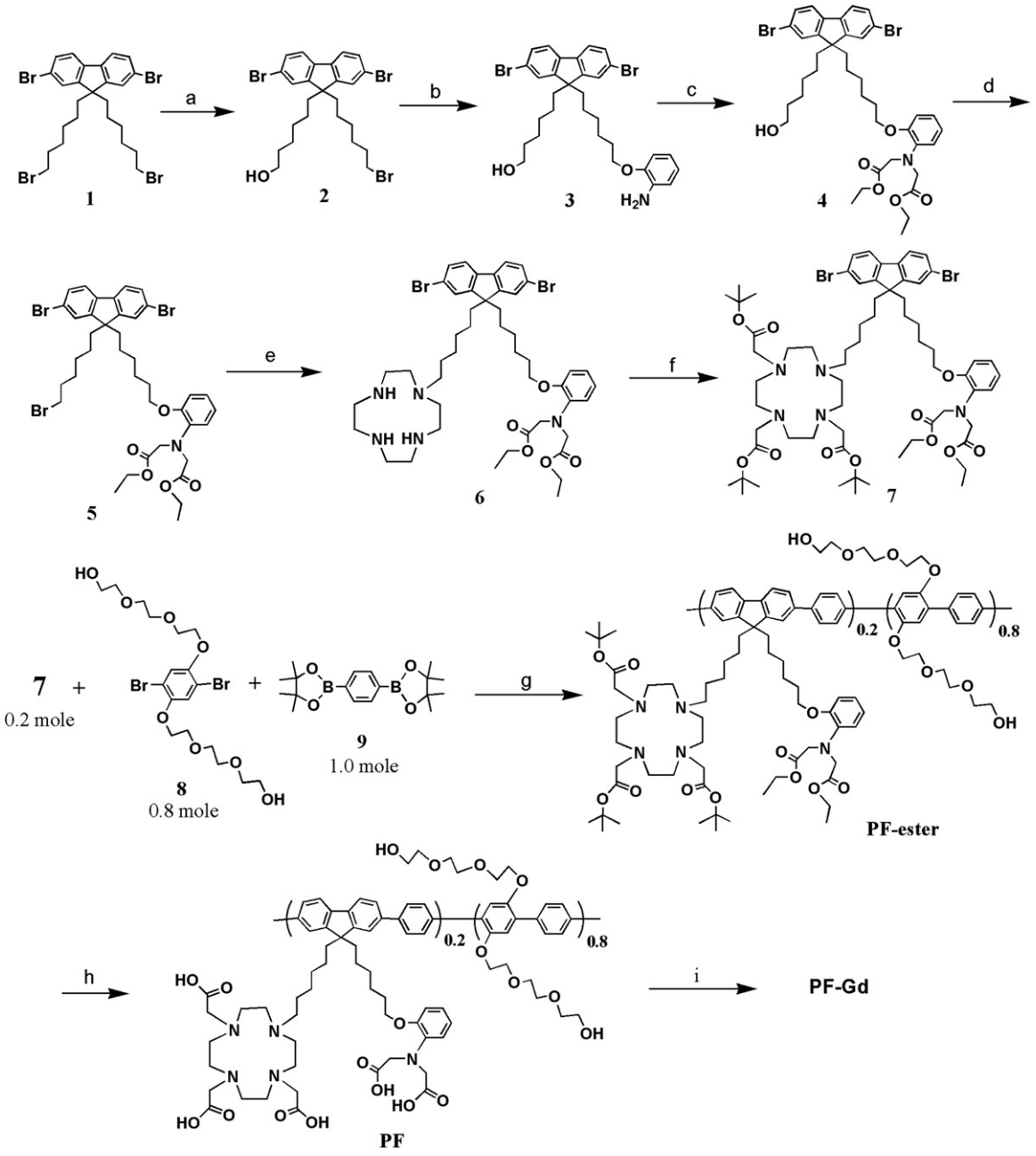
All chemicals were purchased from Arcos or Alfa Aesar, and used as received. 2,7-Dibromo-9,9-bis(6′-bromohexyl)fluorene [10], 2-(benzylideneamino)phenol [11], cyclen [12] and compound **9** [13] were prepared according to the procedures in literatures. Pulmonary adenocarcinoma cell (A549) was purchased from cell culture center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China) and grown in a humidified atmosphere containing 5% CO_2 at 37 °C. The concentration of Gd(III) was determined by ICP spectrometer. Magnetic resonance imaging (MRI) was performed on a Siemens 3T scanner (MAGNETOM Trio, A Tim System) with a TxRx Head coil at Imaging Center for Brain Research of Beijing Normal University. T_1 values were measured using an Inversion Recovery TSE (IR-TSE) imaging sequence with varying IR time. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer. Mass spectra were recorded on Bruker Biflex MALDI-TOF spectrometer. Elemental analyses were carried out with a Flash EA1112 instrument. The gel permeation chromatography (GPC) measurements were performed on Water-410 system against polystyrene standard with THF as the eluent.

4.2. Synthesis of 2,7-dibromo-9-(6′-bromohexyl)-9-(6′-hydroxylhexyl)fluorene (**2**)

A mixture of 2,7-dibromo-9,9-bis(6′-bromohexyl) fluorine (**1**) (4.46 g, 6.86 mmol) and potassium acetate (0.0672 g, 0.685 mmol) in 50 mL DMSO was stirred at 80 °C for 4 h. After cooling to room temperature, 50 mL of H_2O was added and the mixture was extracted with CH_2Cl_2 (40 mL × 3). The organic layer was washed with H_2O (50 mL × 3) and then was dried over anhydrous $MgSO_4$. The solvent was removed at reduced pressure and the resulting residue was purified by silica gel column chromatography with petroleum ether/ethyl acetate 4:1) as eluent to yield **2** as a white solid (1.47 g, 37%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.52 (d, 2H), 7.47–7.43 (m, 4H), 3.53 (t, 2H), 3.29 (t, 2H), 1.92 (m, 4H), 1.67 (m, 2H), 1.38 (m, 2H), 1.20 (m, 2H), 1.11 (m, 6H), 0.59 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 152.22, 139.02, 130.25, 126.08, 121.51, 121.18, 62.75, 55.54, 40.03, 33.82, 32.57, 29.52, 28.91, 27.71, 25.26, 23.55, 23.44; MS (MALDI-TOF): 588.0 ($M + H^+$); $C_{25}H_{31}OBr_3$: Calcd. C 51.13, H 5.32; Found C 51.15, H 5.33.

4.3. Synthesis of compound **3**

A mixture of **2** (1.47 g, 2.5 mmol), 2-(benzylideneamino)phenol (0.59 g, 3 mmol), 18-crown-6, and K_2CO_3 (0.41 g, 3 mmol) in 10 mL acetone was refluxed for 1.5 days. After cooling to room temperature, the solvent was removed under reduced pressure, and then 15 mL of water was added. The mixture was extracted with CH_2Cl_2 (10 mL × 3). The organic layer was washed with 20% NaOH aqueous solution (10 mL × 6), then dried over anhydrous Na_2SO_4. The solvent was removed at reduced pressure and the resulting residue was purified by silica gel column chromatography with petroleum ether/ethyl acetate 4:1) as eluent to afford **3** as sticky oil (1.14 g, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.52 (d, 2H), 7.46 (d, 4H), 6.78–6.67 (m, 4H), 3.87 (t, 2H), 3.53 (t, 2H), 2.05–1.91 (m, 4H), 1.63 (t, 2H), 1.38 (t, 2H), 1.26–1.21 (m, 2H), 1.13 (d, 6H), 0.62 (t, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 152.32, 146.65, 139.05, 136.20, 130.25, 126.11, 121.51, 121.20, 120.90, 118.42, 115.03, 111.44, 68.00, 62.76, 55.59, 40.05, 32.56, 29.55, 29.19, 25.74, 25.29, 23.62, 23.58; MS (MALDI-TOF): 616.2



Scheme 2. Synthesis of PF–Gd: (a) KOAc, DMSO, 80 °C, 37%; (b) 2-(benzylideneamino)phenol, 18-crown-6, K_2CO_3, acetone, reflux, 74%; (c) ethyl bromoacetate, DIEA, NaI, DMF, 100 °C, 65%; (d) PPh_3, CBr_4, CH_2Cl_2, 0 °C, 83%; (e) cyclen, $CHCl_3$, room temperature, 58%; (f) *tert*-butyl bromoacetate, K_2CO_3, acetonitrile, room temperature, 83%; (g) 2.0 M K_2CO_3, $Pd(PPh_3)_4$, THF/water, 80 °C; (h) first step: KOH, CH_3OH, DMSO, 80 °C, second step: TFA, room temperature; (i) $GdCl_3$, DMSO/water, room temperature.

$(M + H^+)$, 638.1 $(M + Na^+)$; $C_{31}H_{37}O_2NBr_2$: Calcd. C 60.50, H 6.06; N 2.28; Found C 60.61, H 6.26, N 1.81.

4.4. Synthesis of compound **4**

A mixture of **3** (0.97 g, 1.57 mmol), ethyl bromoacetate (0.53 mL, 4.7 mmol), DIEA (0.68 mL, 3.9 mmol) and NaI (0.47 g, 3.14 mmol) in 8.4 mL anhydrous DMF was stirred at 100 °C under N_2 atmosphere for 49 h. After cooling to room temperature, 30 mL of CH_2Cl_2 was added, and the organic layer was washed with H_2O (15 mL × 6). The organic layer was dried over anhydrous $MgSO_4$ and was removed under reduced pressure. The residue was purified by silica gel column chromatography with petroleum ether/ethyl acetate 4:1) as eluent to afford **4** as sticky oil (0.81 g, 65%). ^1H NMR (400 MHz, $CDCl_3$) δ: 7.52 (d, 2H), 7.45 (d, 4H), 6.88–6.75 (m, 4H), 4.17–4.10 (m, 8H), 3.83 (t, 2H), 3.51 (t, 2H), 1.93 (m, 4H), 1.60 (m, 2H), 1.37 (m, 2H), 1.23 (m, 8H), 1.11 (b, 6H), 0.61 (b, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.51, 152.29, 150.65, 139.03, 130.23, 126.08, 122.05, 121.49, 121.18, 120.78, 118.90, 112.95, 68.40, 62.72, 60.52, 55.57, 53.60, 53.42, 40.06, 32.54, 29.62, 29.53, 29.07, 25.66, 25.27, 23.65, 23.57, 14.20; MS (MALDI-TOF): 788.2 $(M + H^+)$, 810.2 $(M + Na^+)$; $C_{39}H_{49}O_6NBr_2$: Calcd. C 59.47, H 6.27; N 1.78; Found C 58.84, H 6.25, N 1.80.

4.5. Synthesis of compound **5**

To the solution of **4** (0.76 g, 0.96 mmol) in 24 mL anhydrous CH_2Cl_2 under N_2 atmosphere was added PPh_3 (0.50 g, 1.92 mmol). After cooling to 0 °C, CBr_4 (0.64 g, 1.92 mmol) was added under exclusion of

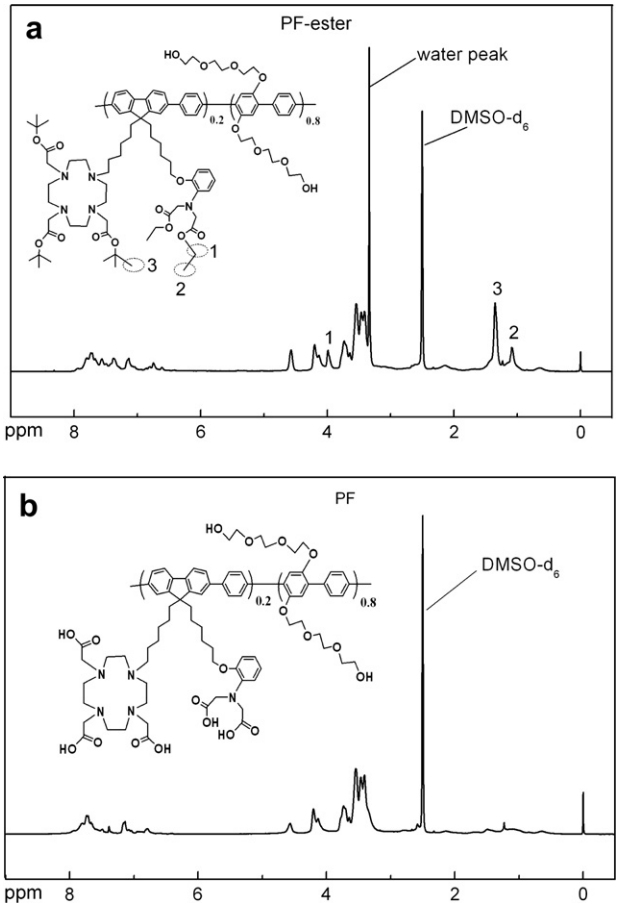


Fig. 1. ^{1}H NMR spectra of PF-ester (a) and PF in DMSO-d_6 (b) at room temperature.

light and the resulting solution was stirred for 3 h. The organic layer was removed under reduced pressure and the residue was purified by silica gel column chromatography with petroleum ether/ethyl acetate 4:1) as eluent to afford **5** as brown oil (0.68 g, 83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.52 (d, 2H), 7.47–7.44 (m, 4H), 6.81–6.76 (m, 4H), 4.14 (q, 4H), 4.10 (s, 4H), 3.83 (t, 2H), 3.29 (t, 2H), 1.93 (m, 4H), 1.66 (m, 2H), 1.58 (m, 2H), 1.25–1.19 (m, 10H), 1.10(m, 4H), 0.60 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.49, 152.19, 150.65, 139.03, 130.27, 126.06, 122.06, 121.51, 121.19, 120.78, 118.91, 112.93, 68.39, 60.52, 55.54, 53.59, 40.10, 39.98, 33.79, 32.56, 29.62, 29.06, 28.91, 27.71, 25.65, 23.63, 23.42, 14.20; MS (MALDI-TOF): 850.1 ($M + H^+$); $C_{39}H_{48}O_5NBr_3$: Calcd. C 55.07, H 5.69; N 1.65; Found C 55.00, H 6.04, N 1.99.

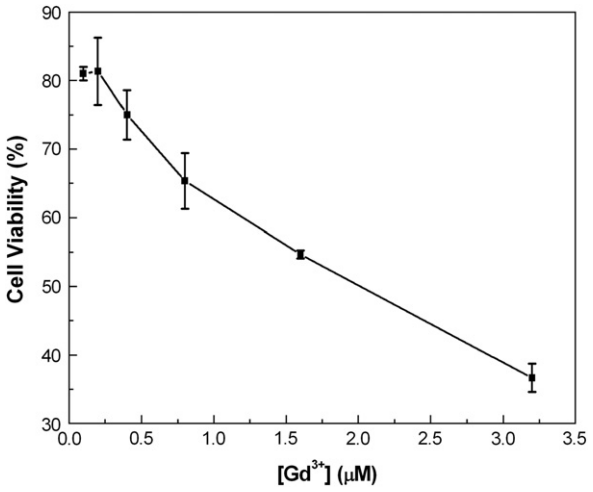


Fig. 3. Cell viability as a function of Gd^{3+} concentrations by typical MTT assay. Cells were subcultured in 96-well plates the day before the experiment at a density of 6×10^4 cells/well, and cultured for 24 h. Then cells were treated with PF–Gd with different concentrations for 24 h respectively. [Gd^{3+}] = 0.1–3.2 μM, [MTT] = 1.0 mg/mL (100 μL/well).

4.6. Synthesis of compound **6**

A solution of cyclen (0.16 g, 0.94 mmol) in 3 mL $CHCl_3$ was passed through neutral alumina, and then **5** (0.32 g, 0.38 mmol) in 1 mL $CHCl_3$ was added dropwise under N_2 atmosphere. The resulting solution was stirred at room temperature for **2** days. The organic layer was removed under reduced pressure and the residue was purified by silica gel column chromatography with chloroform/methanol/aqueous NH_4OH (8:2:0.2) as eluent to yield **6** as brown oil (0.206 g, 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.52 (d, 2H), 7.45 (d, 4H), 6.84–6.76 (m, 4H), 4.14 (q, 4H), 4.10 (s, 4H), 3.82 (t, 2H), 2.75 (t, 4H), 2.60 (t, 4H), 2.55 (t, 4H), 2.48 (t, 4H), 2.30 (t, 2H), 1.91 (m, 4H), 1.59 (m, 2H), 1.30 (m, 2H), 1.26–1.21 (m, 8H), 1.08 (m, 6H), 0.60 (br, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.36, 152.32, 150.55, 138.93, 130.12, 126.00, 121.95, 121.39, 121.15, 120.69, 118.76, 112.87, 68.32, 60.41, 55.53, 54.18, 53.51, 51.36, 47.04, 46.00, 45.22, 40.03, 39.87, 29.54, 28.99, 26.86, 25.58, 23.67, 23.58, 14.16; MS (MALDI-TOF): 942.5 ($M + H^+$), 964.4 ($M + Na^+$); $C_{47}H_{67}O_5N_5Br_2$: Calcd. C 59.93, H 7.17; N 7.44; Found C 58.99, H 7.21, N 6.94.

4.7. Synthesis of compound **7**

To a mixture of **6** (0.1 g, 0.11 mmol) and K_2CO_3 (0.18 g, 1.3 mmol) in 5 mL acetonitrile was added a solution of *tert*-butyl bromoacetate

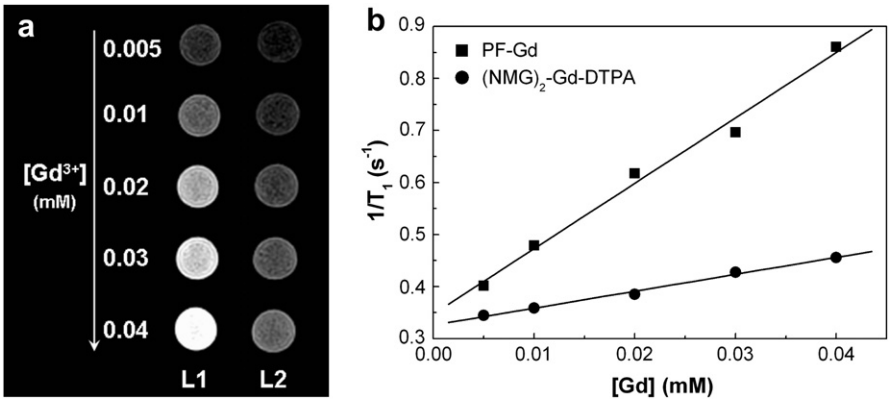


Fig. 2. (a) T_1-weighted magnetic resonance images of PF–Gd (L1) and $(NMG)_2$–Gd–DTPA (L2) at various Gd^{3+} concentrations. (b) Water proton longitudinal relaxation rate ($1/T_1$) of PF–Gd and $(NMG)_2$–Gd–DTPA as a function of Gd^{3+} concentration.

(0.12 g, 0.62 mmol) in 5 mL acetonitrile slowly at room temperature. After stirring for 4 h, the mixture was filtered, and the liquid was concentrated under reduced pressure. The residue was purified by silica gel column chromatography with CH_2Cl_2/methanol (47:3) as the eluent to afford **7** (0.113 g, 83%) as a lightly yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.51–7.48 (m, 2H), 7.44–7.39 (m, 4H), 6.83–6.71 (m, 4H), 4.10 (q, 4H), 4.06 (s, 4H), 3.79 (t, 2H), 3.70–2.00 (m, 22H), 1.90 (m, 4H), 1.55 (m, 2H), 1.46 (m, 2H), 1.40 (m, 27H), 1.22–1.10 (m, 16H), 0.55 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.46, 169.86, 152.10, 150.60, 138.98, 130.28, 125.98, 122.02, 121.47, 121.24, 120.74, 118.84, 112.92, 81.67, 68.36, 60.49, 56.84, 55.49, 55.35, 53.56, 52.89, 52.67, 50.16, 47.66, 40.04, 39.88, 29.58, 29.00, 28.05, 26.21, 25.62, 23.60, 23.45, 14.16; MS (MALDI-TOF): 1284.4 (M^+).

4.8. Synthesis of PF-ester

A mixture of **7** (50 mg, 0.0389 mmol), **8** (82.8 mg, 0.1556 mmol), 4,4,5,5-tetramethyl-2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-1,3,2-dioxaboronane (64.2 mg, 0.1945 mmol) in 5 mL THF and 1 mL 2 M K_2CO_3 was degassed, and catalytic amount of $Pd(PPh_3)_4$ was added under nitrogen atmosphere. The resulting mixture was stirred at 80 °C for 38 h. After cooling to room temperature, 100 mL of water was added and mixture was extracted with chloroform. The organic phase was dried over anhydrous $MgSO_4$. After the solvent was concentrated to a small volume, the residue was precipitated in ethyl ether. The crude polymers were purified by precipitation from chloroform into ethyl ether again and the precipitation was collected and dried under vacuum to get PF-ester (73.7 mg). ^{1}H NMR (400 MHz, DMSO) δ: 7.94–7.67 (br), 7.56 (br), 7.49 (br), 7.37 (br), 7.14–7.05 (br), 6.86–6.62 (br), 4.57 (br), 4.20 (br), 4.13 (br), 3.99 (br), 3.74 (br), 3.65 (br), 3.54 (br), 3.46 (br), 3.41 (br), 3.22–3.10 (br), 2.67–2.61 (br), 2.33 (br), 2.15 (br), 1.67 (br), 1.35 (br), 1.23 (br), 1.08 (br), 0.65 (br); $M_w = 4600$, PDI = 1.51.

4.9. Synthesis of PF

To a solution of PF-ester (21.4 mg) in 1 mL DMSO was added a solution of KOH (19.4) in 1 mL CH_3OH, and the resulting solution was stirred at 80 °C for 24 h. After cooling to room temperature, the CH_3OH was removed under reduced pressure, and then 1 mL of TFA was added. The resulting mixture was stirred for 12 h at room temperature, and then the solvent was removed under reduced pressure. The residue was dialyzed to deionised water for 2 days. The precipitation was collected and dried under vacuum to give PF (14.6 mg). ^{1}H NMR (400 MHz, DMSO) δ: 7.94–7.65 (br), 7.49 (br), 7.39 (br), 7.16–7.04 (br), 6.95–6.79 (br), 4.57 (br), 4.20 (br), 4.13 (br), 3.86 (br), 3.73 (br), 3.64 (br), 3.54 (br), 3.46 (br), 3.40 (br), 2.80 (br), 2.67 (br), 2.58 (br), 2.33 (br), 2.13 (br), 1.68 (br), 1.49 (br), 1.23 (br), 0.85 (br), 0.65 (br).

4.10. The preparation of PF–Gd

To a solution of PF (11 mg) in 2 mL DMSO was added 3.84 mL of 1 mM $GdCl_3$ aqueous solution, and the mixture was stirred for 24 h at room temperature. The precipitation was collected and washed by water, then dried under vacuum to afford PF–Gd (5.9 mg). The concentration of Gd(III) was determined by ICP spectrometer.

4.11. Cell viability assay by MTT

Cell viability was assayed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Cells were subcultured in 96-well plates the day before the experiment at a density of 6×10^4 cells/well, and cultured for 24 h. Then cells were treated with polymer PF–Gd with different concentrations for 24 h respectively. Then the culture media were discarded and MTT (1 mg/mL, 100 μL/well) was added to the wells, which was followed by incubation at 37 °C for 4 h. The supernatant was abandoned, and 150 μL DMSO per well to solubilize the formazan was added and the sample was shaken for an additional 10 min. This procedure was repeated three times. The absorbance values of the wells were then read with a microplate reader at a wavelength of 520 nm. The cell viability rate (VR) was calculated according to the following equation [14]:

$$\mathrm{VR}\% = \frac{A_{\text{experimental group}}}{A_{\text{control group}}} \times 100\%$$

where the control group was not treated and the experimental group was treated with polymer PF–Gd at different concentrations for 48 h. IC_{50} was analysed by the statistic software SPSS (Ver. 13.0).

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