Synthesis and characterization of a novel paramagnetic macromolecular complex [Gd(TTDASQ–protamine)]†

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Received 6th April 2006, Accepted 8th September 2006
First published as an Advance Article on the web 18th September 2006
DOI: 10.1039/b604783a

Adenocarcinomas in rats and humans frequently contain perivascular, degranulating mast cells that release heparin. Protamine is a low-molecular weight, cationic polypeptide that binds to heparin and neutralizes its anticoagulant properties. A novel magnetic resonance imaging (MRI) contrast agent containing protamine was synthesized. TTDASQ, the derivative of TTDA (3,6,10-tri(carboxymethyl)-3,6,10-triazadodecanedioic acid), was also synthesized and the kinetic stability of $[Gd(TTDASQ)]^-$ chelate containing phosphate buffer and $ZnCl_2$ to measure the relaxation rate (R_1) at 20 MHz was studied by transmetallation with Zn(II). The water-exchange rate (k_{ex}^{298}) of $[Gd(TTDASQ)]^-$ is 6.4×10^6 s^{-1} at 25.0 ± 0.1 °C which was obtained from the reduced ^{17}O relaxation rates ($1/T_{1r}$ and $1/T_{2r}$) and chemical shift (ω_r) of $H_2{}^{17}O$, and it is compared with that previously reported for the other gadolinium(III) complex, [Gd(DO3ASQ)]. The binding affinity assay showed that the $(TTDASQ)_3$–pro_{19} has higher activity toward heparin. On the other hand, the effect of heparin on the relaxivity of the $[Gd(TTDASQ)_3–pro_{19}]$ conjugate shows the binding strength (K_A) is 7669 dm^3 mol^{-1} at pH 7.4 and the relaxivity ($r^b{}_1$) of the $[Gd(TTDASQ)_3–pro_{19}]$–heparin adduct is 30.9 dm^3 $mmol^{-1}$ s^{-1}.

Introduction

The study of macromolecular Gd(III) complexes for magnetic resonance imaging (MRI) has intensified in recent decades.[1–3] These macromolecular systems provide both an enhanced ability to catalyze solvent proton relaxation rates through the occurrence of a long molecular reorientational time τ_R and an increased lifetime of the contrast agents in the circulating blood by avoiding the extravasation of the contrast of the small molecular Gd(III) complexes commonly employed in MRI investigations.[4]

To synthesize tissue specific macromolecule contrast agents, protamine has been used as a conjugated substance.[5] Because of the extensive degranulation of mast cells with deposition of heparin in adenocarcinomas, the mast cell heparin might function as an anticoagulant.[6,7] Protamines are low-molecular weight proteins that are rich in arginine and strongly cationic. In the presence of heparin, which is strongly acidic, protamines and heparin rapidly form a stable salt in which the anticoagulant activity of heparin is neutralized.[8] *In vivo*, protamines are rapidly cleared from circulation, with a plasma half-life estimated to be about 5 min.[8] Heparin–protamine complexes are also believed to be metabolized by fibrinolysine.[5]

The water-residence lifetime has a slight influence on relaxivity for small molecular contrast agents but a significant influence on relaxivity for macromolecular contrast agents. Hence, the ligand TTDA (3,6,10-tri(carboxymethyl)-3,6,10-triazadodecanedioic acid) was chosen because the water-residence lifetime of TTDA and its derivatives are significantly lower than that of DTPA (3,6,9-tri(carboxymethyl)-3,6,9-triazaundecanedioic acid) and reached the optimal value.[9–11] On the other hand, squaric esters were chosen as a linker between a Gd(III) complex and NH_2 moieties of protamine, since it has been recently reported that squaric esters readily react with amino groups under mild conditions and in good yield.[2,12]

This report describes the synthesis of a TTDASQ–protamine conjugate (Scheme 1). The molecular weight of the TTDASQ–protamine conjugate was measured by electrophoresis. The kinetic stability of $[Gd(TTDASQ)]^-$ chelate containing phosphate buffer and $ZnCl_2$ to measure the relaxation rate (R_1) at 20 MHz was studied by transmetallation with Zn(II). The water-exchange rate (k_{ex}^{298}) and rotational correlation time (τ_R) of $[Gd(TTDASQ)]^-$

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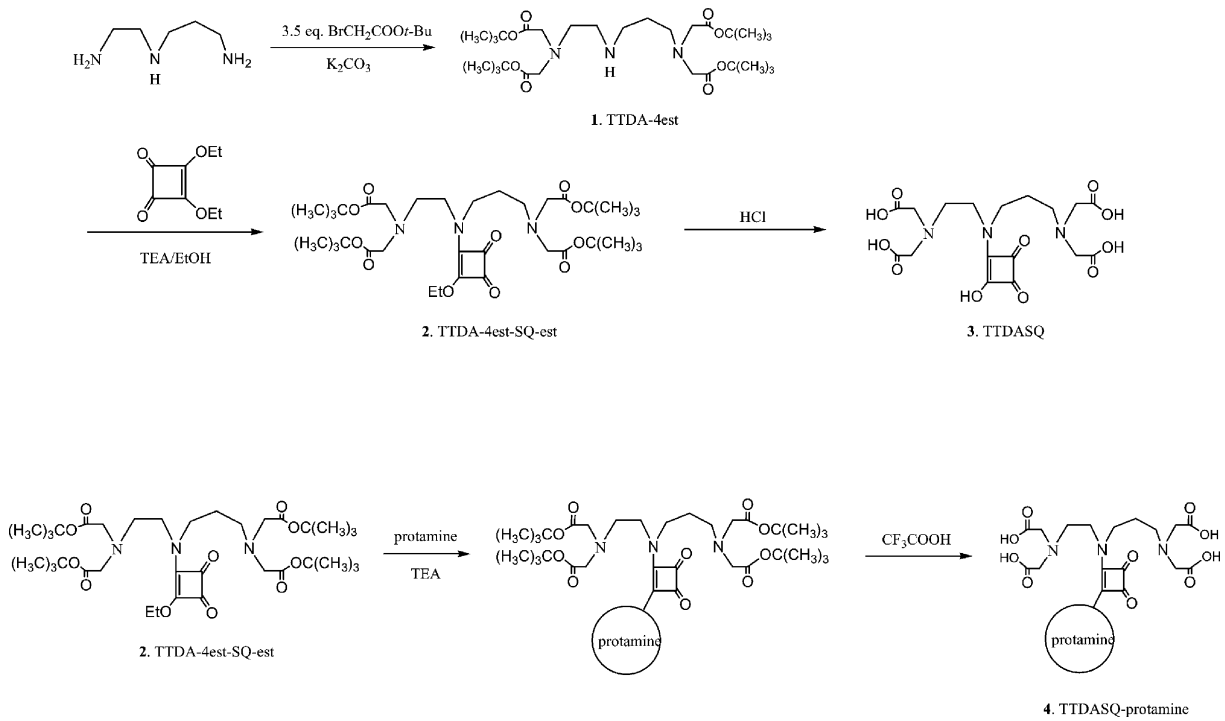
† Electronic supplementary information (ESI) available: Table 1S: Crystal data and structure refinement for TTDA-4est·HBr. X-Ray structure determination details. Table 2S: Bond lengths (Å) and angles (°) for TTDA-4est·HBr. Table 3S: Hydrogen bonds for TTDA-4est·HBr (Å and °). Table 4S: Temperature dependence of reduced transverse and longitudinal ^{17}O relaxation rates and reduced angular frequencies of solutions containing $[Gd(TTDASQ)]^-$. Fig. 1S: Molecular view of TTDA-4est·HBr. Fig. 2S: The relative water proton paramagnetic longitudinal relaxation rate $R_{1p}(t)/R_{1p}(0)$ *vs.* time for $[Gd(TTDASQ)]^-$ and $[Gd(DTPA)]^{2-}$. Fig. 3S: The elution curve of $[(TTDASQ)_n–pro19]$ and TTDASQ on HPLC. Fig. 4S: The elution curves of protamine and $(TTDASQ)_3$–pro19 using gradient 0–2 mol dm^{-3} NaCl as an eluent and heparin affinity column. S1: Variable-temperature ^{17}O NMR measurements. See DOI: 10.1039/b604783a



Scheme 1

were obtained from the reduced ^{17}O relaxation rate ($1/T_{1r}$ and $1/T_{2r}$) and chemical shifts (ω_r) of $H_2{}^{17}O$. Finally, an affinity experiment was used to assess binding ability in order to determine whether [Gd(TTDASQ)$_3$–pro$_{19}$] has an affinity for heparin.

Experimental

Materials

3,4-Diethoxy-3-cyclobutene-1,2-dione was purchased from Fluka Co. Protamine sulfate was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Heparin affinity column (HiTrap heparin 1 × 5 mL) was purchased from Amersham Pharmacia Biotech. Co. All other reagents used for the synthesis of the ligands were purchased from commercial sources unless otherwise noted. ^{17}O-enriched water (20.3%) was purchased from Isotec Inc. The ^{13}C NMR spectra were referenced internally relative to 3-(trimethylsilyl)-1-propanesulfonic acid for D_2O.

Preparations

Synthesis of 3,6,10-triazadodecane-3,10-tetraacetic acid, (tetra)-*tert*-butyl ester hydrobromide (TTDA-4est, 1). To a stirred solution of the *N*-(2-aminoethyl)-1,3-propanediamine (5.15 g, 43.96 mmol) in CH_3CN (250 mL) was added potassium carbonate (5 g, 36.18 mmol), and the mixture was stirred for 30 min at room temperature. *tert*-Butyl bromoacetate (22.7 mL, 153.86 mmol) was slowly added and refluxed for 48 h. The potassium carbonate was removed by filtering the reaction mixture with a Büchner funnel and then washed with CH_3CN. The filtrate was evaporated by rotary evaporation. To the residue was added H_2O and extracted with chloroform (3 × 50 mL). The chloroform phase was evaporated under reduced pressure. The product was purified by column chromatography (SiO_2, ethyl acetate–acetone 50 : 50). After evaporation, the solvent ethyl acetate was added to the brown oil, which was allowed to stand at room temperature overnight. The crystalline product **1** was isolated by filtration, washed with ethyl acetate, and dried *in vacuo*. The product was recrystallized from ethyl acetate, yielding 12.58 g (49.9%): mp 105.1–106.4 °C. Anal. (%). Calc. (found) for $C_{29}H_{55}N_3O_8$ (fw = 573.77): C, 60.71 (60.59); H, 9.66 (9.75); N, 7.32 (7.21). ^1H NMR ($CDCl_3$, 400 MHz), δ 3.56 (s, 4H, CH_2COOH), 3.51 (s, 4H, CH_2COOH), 3.27 (t, J = 5.6 Hz, 2H, ethylene backbone), 3.22 (t, J = 5.6 Hz, 2H, propylene backbone), 3.06 (t, J = 5.6 Hz, 2H, ethylene backbone), 2.90 (t, J = 5.6 Hz, 2H, propylene backbone), 2.07 (m, 2H, $NCH_2CH_2CH_2N$), 1.45 (s, 36H, $C(CH_3)_3$). ^{13}C NMR ($CDCl_3$, 400 MHz), δ 171.1, 170.8, 81.6, 81.3, 55.2, 54.9, 52.1, 50.3, 49.2, 45.9, 28.1, 22.9.

Synthesis of 6-(2-ethoxy-3,4-dioxo-1-cyclobutenyl)-3,6,10-triazadodecane-3,10-tetraacetic acid, (tetra)-*tert*-butyl ester (TTDA-4est-SQ-est, 2). To a stirred solution of the tetraester **1** (4.81 g, 8.38 mmol) in ethanol (50 mL) was added triethylamine (1.2 mL), and the mixture was stirred for 30 min at room temperature. Diethyl squarate (1.71 g, 10.06 mmol) was added in one portion. The resulting mixture was stirred overnight at room temperature and then evaporated *in vacuo*. To the residue was added H_2O and extracted with chloroform (3 × 50 mL). The chloroform phase was evaporated under reduced pressure. The product was purified by column chromatography (SiO_2, $CHCl_3$–acetone 9 : 1), yielding 4.51 g (80%) of **2** as a colorless viscous oil. Anal. (%). Calc. (found) for $C_{35}H_{59}N_3O_{11}$ (fw = 697.86): C, 60.24 (60.11); H, 8.52 (8.57); N, 6.02 (6.21). ^1H NMR ($CDCl_3$, 400 MHz), δ 4.76 (q, 2H, OCH_2CH_3), 3.79 (t, J = 6.8 Hz, 2H, ethylene backbone), 3.55 (t, J = 6.8 Hz, 2H, propylene backbone), 3.46 (s, 4H, CH_2COOH), 3.41 (s, 4H, CH_2COOH),

2.97 (t, $J = 6.4$ Hz, 2H, ethylene backbone), 2.75 (t, $J = 6.4$ Hz, 2H, propylene backbone), 1.80 (qnt, 2H, $NCH_2CH_2CH_2N$), 1.46 (s, 36H, $C(CH_3)_3$ and 3H, OCH_2CH_3). ^{13}C NMR ($CDCl_3$, 200 MHz), δ 188.7, 182.1, 175.8, 172.2, 170.1, 170.0, 80.7, 80.6, 69.2, 55.7, 55.6, 52.7, 50.9, 47.8, 47.7, 27.9, 27.4, 15.6.

Synthesis of 6-(2-hydroxy-3,4-dioxo-1-cyclobutenyl)-3,6,10-triazadodecane-3,10-tetraacetic acid (TTDASQ, 3). To a stirred solution of the **2** (2.5 g, 3.58 mmol) was added 3 mol dm^{-3} HCl (20 mL), and the mixture was stirred for 12 h at room temperature. After rotary evaporation, the oil was dissolved in 20 mL of distilled water and made alkaline with aqueous ammonia to pH 11.0, and the solution applied to an AG1 × 8 anion exchange resin column (200–400 mesh, HCO_2-form, 100 mL of resin, 3.0 cm column diameter). The product was eluted with 500 mL of water and a formic acid gradient. The product **3** came off in 5.5 mol dm^{-3} formic acid, yielding 1.9 g (74.2%). Anal. (%). Calc. (found) for $C_{17}H_{23}N_3O_{11}$ (fw = 445.38): C, 45.85 (45.73); H, 5.21 (5.30); N, 9.44 (9.58). ^1H NMR (D_2O, 400 MHz), δ 4.17 (s, 4H, CH_2COOH), 4.12 (s, 4H, CH_2COOH), 3.98 (t, $J =$ 6.4 Hz, 2H, backbone), 3.68 (t, $J = 6.4$ Hz, 2H, backbone), 3.66 (t, $J = 8$ Hz, 2H, backbone), 3.36 (t, $J = 8$ Hz, 2H, backbone), 2.06 (qnt, 2H, $NCH_2CH_2CH_2N$). ^{13}C NMR (D_2O, 400 MHz), δ 191.0, 186.0, 177.7, 167.8, 167.7, 54.8, 54.6, 53.7, 53.5, 46.2, 43.4, 22.8.

Synthesis of TTDASQ–protamine conjugate (4). Protamine sulfate (0.56 g, 0.32 mmol) was dissolved in 3 mL of H_2O; triethylamine (5.7 mL) was added to the solution and, after 10 min, a solution of **2** (1.81 g, 2.6 mmol) in 8 mL of ethanol was added in one portion. The mixture was stirred at room temperature for 72 h, with the disappearance of **2** (SiO_2, $CHCl_3$–acetone 9 : 1) monitored by TLC. The mixture was evaporated in vacuum and the residue dissolved in neat trifluoroacetic acid (25 mL) then stirred for 12 h at room temperature. After the solution was evaporated, the residue was dissolved in 3 mL of methanol. By slow addition of diethyl ether (80 mL), the white precipitated material was collected by centrifugation, repeatedly washed with diethyl ether, and dried *in vacuo*. The white precipitated material was dissolved in distilled water (5 mL), and the solution applied to a Sephadex G-50 column (100 mL of resin, 2.0 cm column diameter) equilibrated with 0.01 mol dm^{-3} phosphate buffer, pH 7.0. After collecting all the solution of the first peak, the conjugate was collected by lyophilize as a white amorphous powder.

The presence of unconjugated TTDASQ monomer has been ruled out on the basis of HPLC analysis. The experimental setup of HPLC analysis is as follows: Hamilton PRP-1 column (5 μm, 250 × 4 mm), UV detector at 280 nm, isocratic elution with 0.01 mol dm^{-3} phosphate buffer, pH 6.3; flow rate = 0.75 mL min^{-1}.

Sample preparation for ^{17}O longitudinal and transverse relaxation rates measurement. $Gd(ClO_4)_3$ stock solution were prepared by dissolving a slight excess of Gd_2O_3 in perchloric acid. The pH of the solutions was adjusted to 4 after filtration. The lanthanide concentration was determined by chelatometric titration with Na_2H_2EDTA solution using xylenol orange indictor. All solutions were prepared by weight. For the preparation of the complex $[Gd(TTDASQ)]^-$, weighed quantities of solid ligand was dissolved in deionised water, and then a weighed amount of $Gd(ClO_4)_3$ stock solution was added dropwise to form the chelate complex with a ligand excess 2–3%. The pH of all solution was adjusted using 1 mol dm^{-3} NaOH or $HClO_4$. The solution was then evaporated under reduced pressured and an equivalent weight of 5.5% ^{17}O-enriched water was added. All complexes are proved the absence of free metal ions by xylenol orange test.

^{17}O NMR measurements

The measurement of the ^{17}O transverse relaxation rates was carried out with Varian Gemini-400 (9.4 T, 54.2 MHz) spectrometers equipped with a 10-mm probe by using an external D_2O lock. The Varian 600 temperature control unit was used to stabilize the temperature in the range 278–338 K. Solutions containing 5.5% of the ^{17}O isotope were used. The inversion recovery method was applied to measure longitudinal relaxation rates, $1/T_1$, and the Carr–Purcell–Meiboom–Gill spin–echo technique was used to obtain transverse relaxation rates, $1/T_2$. In order to eliminate magnetic susceptibility corrections to chemical shift, the solution was introduced into spherical glass containers fitting into ordinary 10-mm NMR tubes.

Transmetallation experiment

The technique is based on measurement of the evolution of the water proton paramagnetic longitudinal relaxation rate (R_1^p) of a buffered solution (phosphate buffer, pH 7) containing 2.5 mmol dm^{-3} $[Gd(TTDASQ)]^-$ complex and 2.5 mmol dm^{-3} $ZnCl_2$. The longitudinal relaxation time (T_1) was measured at 37.0 ± 0.1 °C and 20 MHz (Bruker Minispec 120), by means of the standard inversion–recovery pulse sequence.

Electrophoresis

The molecular weight of the TTDASQ–protamine conjugate was assessed by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a 4% stacking gel and a 15% protein separating gel on a BioRad Protean II electrophoresis apparatus. A low-molecular weight marker was used for standards to determine the sizes of the conjugate and unreacted protamine. Gels were stained with Coomassie blue R-250 dye after being run at 200 V for 50 min.

TTDASQ measurement in $(TTDASQ)_n$–pro_{19} ligand

A series of concentrations of $GdCl_3$ ((1–9) × 10^{-3} mmol dm^{-3}) were added into $(TTDASQ)_n$–pro_{19} (1 × 10^{-3} mmol dm^{-3}), separately, and the excess $GdCl_3$ was excluded by ultrafiltration equipment with YM1 membrane. The conjugated number (n) of $(TTDASQ)_n$–pro_{19} was assessed by ICP-OES(Inductively Coupled Plasma-Optical Emission Spectrometry) (Perkin Elmer, OPTIMA 2000DV, USA).

Data analysis

The simultaneous least-squares fitting of ^{17}O NMR data and the binding parameters to heparin were determined by fitting the experimental data using the program SCIENTIST for WINDOWS by MICROMATH, version 2.0.

Crystallography

Crystallographic details for TTDA-4est·HBr are given in the ESI† CCDC reference number 603492.

For crystallographic data in CIF or other electronic format see DOI: 10.1039/b604783a

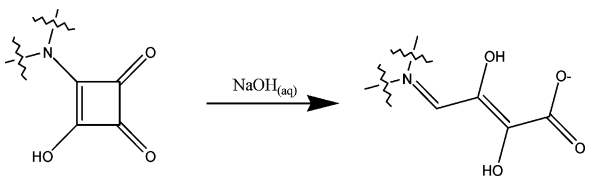
Results and discussion

Synthesis of TTDASQ

The TTDASQ is made in an efficient manner in three steps giving an overall yield of 46.3%. The asymmetric triamine was mixed with 3.5 eq. *tert*-butyl bromoacetate and anhydrous potassium carbonate in dry acetonitrile. After the mixture was refluxed for 18 h, the ligand (as its monohydrobromide salt) was isolated by flash silica column chromatography. The major product (TTDA-4est) had all-terminal nitrogen substituents and was identified by the X-ray crystallography with data deposited as ESI† (Fig. 1S and Tables 1S–3S). Followed by addition of triethylamine and diethyl squarate to the above ligand in absolute ethanol, the solution was stirred at room temperature for 48 h. Hydrolysis with 3 mol dm^{-3} hydrochloric acid and purification by ion-exchange resin afforded the free ligand as a hygroscopic powder. This methodology allows for the preparation of gram quantities of the TTDASQ ligand.

Transmetallation

The protonation constants for TTDASQ and its stability constants with metal ions were determined by potentiometric titration at 25.0 ± 0.1 °C and ionic strength 0.10 mol dm^{-3} in Me_4NNO_3. However, these values obtained are unacceptable and undesirable due to the squarate ring opening under basic conditions (eqn (1)).[13,14] Hence, the stability of the gadolinium(III) complex with TTDASQ must be evaluated by the transmetallation method.[15]



(1)

The kinetic stability of $[Gd(TTDASQ)]^-$ chelate was evaluated in solution containing phosphate buffer and $ZnCl_2$ by measuring the ^1H relaxation rate (R_1) upon transmetallation with Zn(II) at 20 MHz and 37.0 ± 0.1 °C. The results of transmetallation experiments have been deposited as ESI† (Fig. 2S). Table 1 shows the percentage of $[Gd(TTDASQ)]^-$ existing in the Zn(II) solution after 3 days. The sequence of the kinetic stability decreased in the following order: $[Gd(DTPA)]^{2-}$ > $[Gd(TTDA\text{-}BOM)]^{2-}$ (TTDA-BOM = (*R*,*S*)-4-carboxy-5,9,12-tris(carboxymethyl)-1-phenyl-2-oxa-5,9,12-triazatetradecan-14-oic acid)[11] > $[Gd(TTDASQ)]^-$ ≫ [Gd(DTPA-BMA)]. Therefore, the kinetic stability toward Zn^{2+} transmetallation of $[Gd(TTDASQ)]^-$ is lower than those of $[Gd(DTPA)]^{2-}$ and $[Gd(TTDA\text{-}BOM)]^{2-}$ but is strikingly more kinetically stable than [Gd(DTPA-BMA)].

Table 1 Values of $R^p_{(t=3d)}/R^p_{(t=0)}$ for the gadolinium(III) complexes after three days of transmetallation with Zn^{2+} at 20 MHz and 37.0 ± 0.1 °C

Complex	$100R^p_{(t=3d)}/R^p_{(t=0)}$ (%)
$[Gd(TTDASQ)]^-$	31.1
$[Gd(DTPA)]^{2-}$	49.79
$[Gd(TTDA\text{-}BOM)]^{2-}$ [a]	43.9
[Gd(DTPA-BMA)] [b]	9

[a] Data from ref. 11. [b] Data from ref. 15.

Synthesis of the conjugate with protamine

The tetraester **2** (TTDA-4est-SQ-est) was employed for the synthesis of the TTDASQ–protamine conjugate. Owing to the low solubility of protamine in solvent other than water, the coupling reaction was run in a water–ethanol mixture in the presence of triethylamine. The crude conjugate was dissolved in a small amount of methanol followed by addition of diethyl ether leading to a white precipitate. This material was purified by a Sephadex G-50 column and then lyophilized. TTDASQ–protamine conjugate was identified by HPLC (ESI,† Fig. 3S). The lyophilized product, which was purified by a Sephadex column, has only one absorption peak at wavelength 280 nm (UV detector), and the retention time is 8.904 min as shown in Fig. 3S(A). The retention time of the small molecule TTDASQ **3** is 11.835 min under the same HPLC condition shown in Fig. 3S(B). However, two of the possible compounds in Fig. 3S(A) are free protamine and TTDASQ–protamine conjugate. Free protamine can be ruled out because it does not have any absorption at wavelength 280 nm. Therefore, the absorption peak in Fig. 3S(A) must be due to the TTDASQ–protamine conjugate. Because the number of monomeric units in the protamine chain is 19,[16] the TTDASQ–protamine conjugate can be denoted $(TTDASQ)_n$–pro_{19}.

Identification of Gd(III) complex with $(TTDASQ)_n$–pro_{19}

The dropwise addition of a $GdCl_3$ solution to 1×10^{-3} mmol dm^{-3} aqueous solution of $(TTDASQ)_n$–pro_{19} at room temperature led to the prompt chelation of gadolinium(III) ions by the chelate moieties on the protamine substrate. The formation of the $(TTDASQ)_n$–pro_{19} gadolinium(III) complex was monitored by measuring the concentration of gadolinium(III) through ICP-OES. The results of gadolinium(III) concentration *vs*. the ratio of $[Gd^{3+}]/[protamine]$ is shown in Fig. 1. The curve reaches the maximum at a gadolinium(III) concentration of 3×10^{-3} mmol dm^{-3}, hence, the value of *n* in $Gd[(TTDASQ)_n\text{–}pro_{19}]$ is 3. The molecular weight of $[Gd(TTDASQ)_3\text{–}pro_{19}]$ determined by electrophoresis is about 6000.

Relaxometric studies of the Gd(III) complex

The longitudinal water proton relaxivity (r_{1p}) is contributed from water molecules in the inner and outer coordination spheres:

$$r_{1p} = R^{is}_{1p} + R^{os}_{1p} \quad (2)$$

The outer-sphere term, R^{os}_{1p}, describes the contribution arising from the water molecules diffusing near the paramagnetic chelate.[17] The inner sphere term, R^{is}_{1p}, can be ascribed to the difference of relaxivity. The paramagnetic contribution of the solvent longitudinal relaxivity is obtained[18] by eqn (3)

$$R^{is}_{1p} = Cq/[55.6(T_{1M} + \tau_M)] \quad (3)$$

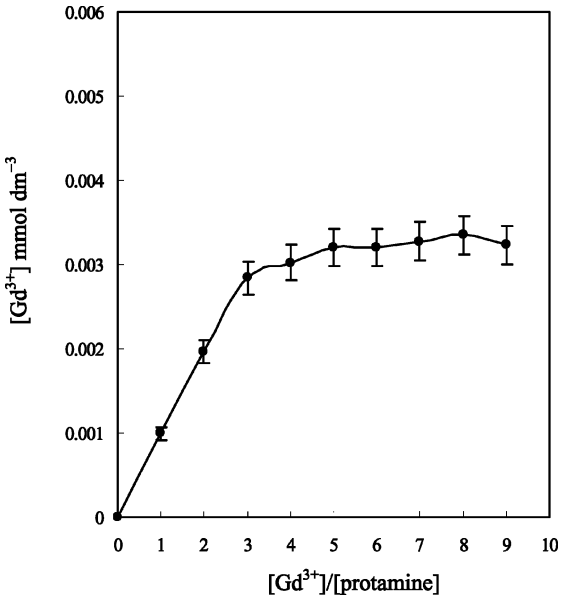


Fig. 1 Plot of 0.001 mmol dm^{-3} $(TTDASQ)_n$–pro_{19} ligand solutions as a function of the Gd(III) ion concentration through ICP-OES.

where C is the molar concentration of the gadolinium(III) complex, q is the number of water molecules bound to metal ion, T_{1M} is the longitudinal relaxation time of the bound water protons and τ_M is the residence lifetime of the bound water.

Because of the opposite temperature dependence of T_{1M} and τ_M, two cases can be considered: (1) fast water-exchange ($T_{1M} \gg \tau_M$), R_{1p}^{is} increases by decreasing the temperature; (2) slow water-exchange ($T_{1M} \ll \tau_M$), R_{1p}^{is} decreases by decreasing the temperature. Fig. 2 shows the temperature dependence of the relaxivity (r_1) for the $[Gd(TTDASQ)]^-$ complex at 20 MHz in the temperature range 278–333 K. A monoexponential decrease of the observed relaxivity upon increasing the temperature in the range 278–333 K was not found for the $[Gd(TTDASQ)]^-$ complex. This is characteristic of the slow chemical exchange behavior, which occurs when the residence lifetime of the coordinated water molecule is much longer than the longitudinal relaxation time of the bound water proton.

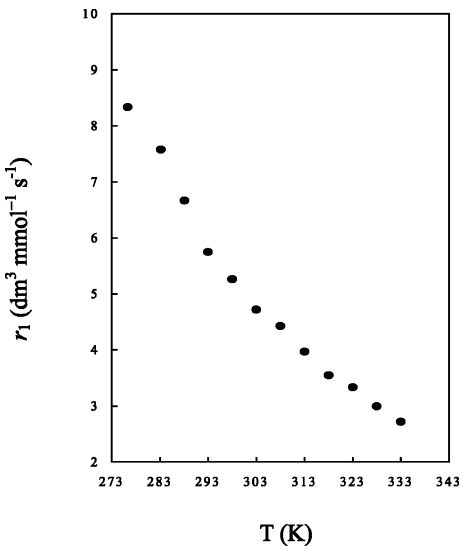


Fig. 2 Temperature dependence of the relaxivity for $[Gd(TTDASQ)]^-$ at 20 MHz and pH 7.0.

The relaxivity r_1 values for gadolinium(III) complex at various pH values are shown in Fig. 3. The relaxivity r_1 curve of $[Gd(TTDASQ)]^-$ complex exhibits no pH dependence over the pH range 1.5–8.0. In other words, no ligand dissociation for $[Gd(TTDASQ)]^-$ complexes occurred at this pH range of the solution. It is also concluded that the hydration number of $[Gd(TTDASQ)]^-$ complex remains constant in this pH range 1.5–8.0. When the pH is higher than 8.0, the relaxivity r_1 of $[Gd(TTDASQ)]^-$ complex decreased. This may be attributed to hydrolysis of the complexes leading to partial precipitation of the hydroxide since the behavior of r_1 with pH is irreversible.

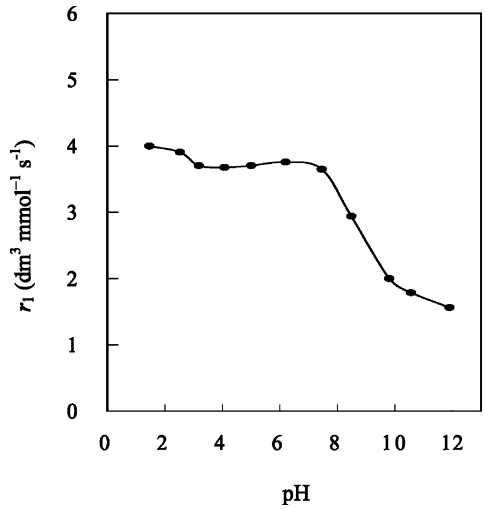


Fig. 3 pH dependence of the relaxivity for the $[Gd(TTDASQ)]^-$ at 20 MHz and 37 ± 0.1 °C.

Water-exchange lifetime studies of Gd(III) complex

The ^{17}O NMR relaxation rates and angular frequencies of the Gd(III) complex solutions, $1/T_1$, $1/T_2$ and ω, and of the acidified water reference, $1/T_{1A}$, $1/T_{2A}$ and ω_A, were measured at 9.4 T. Moreover, the reduced relaxation rates and chemical shift, $1/T_{1r}$, $1/T_{2r}$ and $\Delta\omega_r$, can also be calculated. The data of $1/T_{1r}$, $1/T_{2r}$ and $\Delta\omega_r$ which shown in Table 4S (ESI†) are fitted simultaneously according to eqn (1S)–(10S) (ESI, S1†).[19–24] The results for $[Gd(TTDASQ)]^-$ complex are plotted in Fig. 4 with the corresponding curve representing the result of the best fitting of the data according to equations.

A good fit of the data with the parameters in Table 2 is obtained for $[Gd(TTDASQ)]^-$. The accurate estimation of k_{ex}^{298} value is 6.4×10^6 s^{-1} for $[Gd(TTDASQ)]^-$. This value is significantly lower than those reported for $[Gd(TTDA)]^{2-}$, $[Gd(TTDA\text{-}BOM)]^{2-}$, $[Gd(TTDA\text{-}MA)]^-$ (TTDA-MA = 6-[(methylcarbamoyl)methyl]-3,6,10-tri(carboxymethyl)-3,6,10-triazadodecanedioic acid)[10] and $[Gd(EPTPA\text{-}bz\text{-}NO_2)]^{2-}$ (EPTPA-bz-NO_2 = 4-nitrobenzyl-3,6,10-tri(carboxymethyl)-3,6,10-triaza-dodecanedioic acid),[25] but is similar to that of [Gd(DO3ASQ)]. The substitution of an acetate arm with a squarate on the linear poly(aminocarboxylate) ligand has been shown to cause a significant effect on the exchange lifetime. The less negatively charged and less strongly coordinated group would be expected to loosen the ligand around the metal

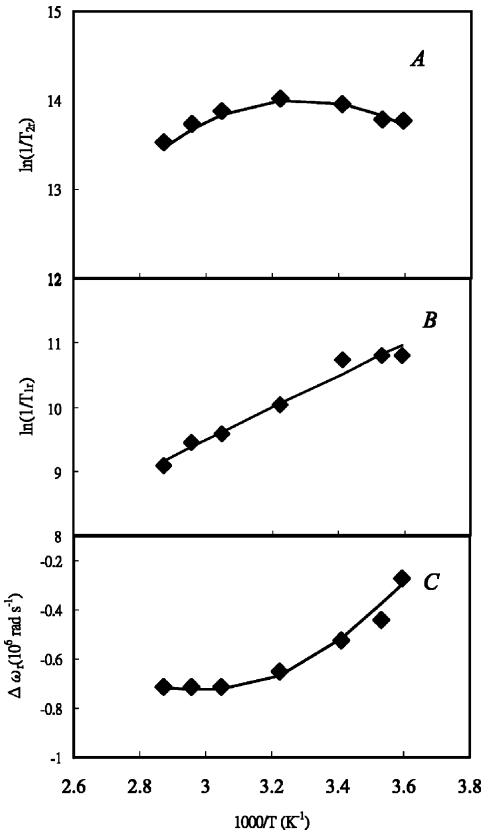


Fig. 4 Temperature dependence of the reduced ^{17}O relaxation rates and chemical shift for $[Gd(TTDASQ)]^-$ at 9.4 T. The lines are functions calculated from a simultaneous least-squares fit.

center, thus decreasing the crowding at the water binding site and hence possibly giving rise to a shorter gadolinium–inner-sphere water oxygen distance and a lower water-exchange rate. For small Gd(III) chelates, the k_{ex}^{298} values of $[Gd(TTDASQ)]^-$ and [Gd(DO3ASQ)] are significantly smaller than that of $[Gd(TTDA)]^{2-}$, but the relaxivity r_1 values of $[Gd(TTDASQ)]^-$ (5.2 dm^3 $mmol^{-1}$ s^{-1}) and [Gd(DO3ASQ)] (5.3 dm^3 $mmol^{-1}$ s^{-1}) are similar to those of $[Gd(TTDA)]^{2-}$ (4.9 dm^3 $mmol^{-1}$ s^{-1}), $[Gd(TTDA\text{-}BOM)]^{2-}$ (4.4 dm^3 $mmol^{-1}$ s^{-1}) and $[Gd(EPTPA\text{-}bz\text{-}NO_2)]^{2-}$ (4.7 dm^3 $mmol^{-1}$ s^{-1}) at 20 MHz and 25 ± 0.1 °C. It can be concluded that the mean residence lifetime τ_M does not contribute significantly to the relaxivity r_1 for the small molecule.[26]

At 298 K, the value of rotational correlation time (τ_R) for $[Gd(TTDASQ)]^-$ complex is also shown in Table 2. The τ_R values of $[Gd(TTDASQ)]^-$ (189 ps) is significantly higher than those of $[Gd(DTPA)]^{2-}$ (103 ps), $[Gd(TTDA)]^{2-}$ (104 ps) and $[Gd(TTDA\text{-}MA)]^-$ (103 ps). It is indicated that the introducing the squarate ring in TTDA increases the τ_R value and causes the higher relaxivity of the Gd(III) complex. Because the concentration of the $[Gd(TTDASQ)_3\text{–}pro_{19}]$ complex prepared in this study is below the detection limitation in ^{17}O NMR, the kinetic parameters of $[Gd(TTDASQ)_3\text{–}pro_{19}]$ complex such as τ_M and τ_R could not be obtained.

Heparin affinity assay and the effect of heparin on the relaxivity of the $[Gd(TTDASQ)_3\text{–}pro_{19}]$

The binding affinity of free protamine and $[(TTDASQ)_3\text{–}pro_{19}]$ with gradient eluent has been deposited as ESI† (Fig. 4S). Fig. 4S(A) shows that free protamine has a strong affinity for heparin; hence, the retention time is nearly 20 min if the eluent was a gradient 0–2 mmol dm^{-3} NaCl. On the other hand, Fig. 4S(B) shows that the retention of $[(TTDASQ)_3\text{–}pro_{19}]$ is similar to that of free protamine if a gradient 0–2 mol dm^{-3} NaCl is used as eluent. This result shows that $[(TTDASQ)_3\text{–}pro_{19}]$ has a high affinity for heparin.

The binding interaction of $[Gd(TTDASQ)_3\text{–}pro_{19}]$ and heparin is assessed by measuring the water proton relaxation rates of solutions containing the paramagnetic complex and increasing concentrations of the heparin. In addition to obtaining the binding strength, K_A (by assuming one binding site on the protein), these measurements also provide a direct assessment of the bound relaxivity of the $[Gd(TTDASQ)_3\text{–}pro_{19}]$–heparin conjugate ($r_1^b$).

Titration of 0.08 mmol dm^{-3} aqueous solutions of the $[Gd(TTDASQ)_3\text{–}pro_{19}]$ with heparin at pH 7.4 and 25.0 ± 0.1 °C in 50 mmol dm^{-3} HEPES buffer solution is shown in Fig. 5. Each value in this titration is given by the sum of three contributions in eqn (4)[27–29]

$$R_{1obs} = R_{1p}^F[Gd(TTDASQ)_3\text{–}pro_{19}] + R_{1p}^B\{[Gd(TTDASQ)_3\text{–}pro_{19}]\text{–heparin}\} + R_{1d} \quad (4)$$

where R_{1d} is the diamagnetic contribution, R_{1p}^F and R_{1p}^B are the relaxivity of the complex and the macromolecular $[Gd(TTDASQ)_3\text{–}pro_{19}]$–heparin adduct at 25.0 ± 0.1 °C, respectively. For the

Table 2 Kinetic and NMR parameters of $[Gd(TTDASQ)]^-$, $[Gd(DTPA)]^{2-}$, $[Gd(EPTPA\text{-}bz\text{-}NO_2)]^{2-}$, $[Gd(TTDA)]^{2-}$, $[Gd(TTDA\text{-}BOM)]^{2-}$, [Gd(DO3ASQ)] and $[Gd(TTDA\text{-}MA)]^-$ as obtained from the simultaneous fit of ^{17}O NMR data

Parameter	TTDASQ	DTPA[b]	EPTPA-bz-NO_2[b]	TTDA[c]	TTDA-BOM[c]	DO3ASQ[d]	TTDA-MA[e]
$10^{-6}k_{ex}^{298}/s^{-1}$	6.4 ± 1.2	4.1 ± 0.3	150 ± 40	146 ± 17	117 ± 8	7.5	37.8 ± 2.4
$\Delta H^{\ddagger}/kJ\ mol^{-1}$	37.4 ± 1.1	52.0 ± 1.4	22.1 ± 1.8	23.1 ± 0.5	18.7 ± 0.7	41	28.6 ± 0.9
$\Delta S^{\ddagger}/J\ mol^{-1}\ K^{-1}$	11.1 ± 1.8	56.2 ± 5.0	−9.1 ± 5.0	−11.1 ± 3.1	−27.6 ± 2.3		−3.98 ± 2
$10^{-6}A/\hbar/rad\ s^{-1}$	−3.8 ± 0.3	−3.8 ± 0.2	−3.2 ± 0.2	−3.2 ± 0.3	−3.1 ± 0.2	*−3.8*	−3.9 ± 0.2
τ_R^{298}/ps	189 ± 11	103 ± 10	122 ± 12	104 ± 12	119 ± 8	78 ± 2	103 ± 11
C_{os}	*0*[a]	0.18 ± 0.04	*0.1*	*0*	0.1		0
$E_R/kJ\ mol^{-1}$	21.1 ± 0.9	18 ± 2	19.0 ± 1.7	24.8 ± 1.5	20.9 ± 2.8		17.9 ± 0.7
Method	^{17}O	^{17}O	^{17}O, EPR, NMRD	^{17}O	^{17}O	^{17}O–R_{2p},NMRD	^{17}O

[a] Italicized parameters fixed in the fitting procedure. [b] Taken from ref. 25. [c] Taken from ref. 11. [d] Taken from ref. 2. [e] Taken from ref. 10.

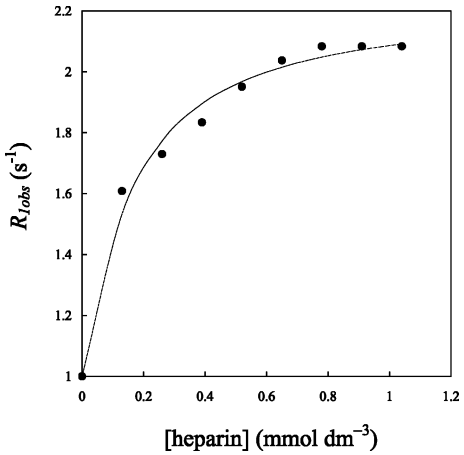


Fig. 5 Plot of the longitudinal water proton relaxation rate of a 0.08 mmol dm^{-3} [Gd(TTDASQ)$_3$–pro$_{19}$] solution as a function of heparin concentration at 20 MHz, 25 °C, 50 mmol dm^{-3} HEPES and pH 7.4.

following equilibrium (eqn (5)):

$$[\text{Gd(TTDASQ)}_3\text{–pro}_{19}] + \text{heparin} \underset{}{\overset{K_A}{\rightleftharpoons}} \{[\text{Gd(TTDASQ)}_3\text{–pro}_{19}]\text{–heparin}\} \quad (5)$$

it is possible to obtain the binding strength (K_A, eqn (6)) and thus R_{1p}^B by use of eqn (4).

$$K_A = [[\text{Gd(TTDASQ)}_3\text{–pro}_{19}]\text{–heparin}]/[\text{Gd(TTDASQ)}_3\text{–pro}_{19}]\,[\text{heparin}] \quad (6)$$

The binding constant (K_A) is 7.7 × 10^3 dm^3 mol^{-1} for the [Gd(TTDASQ)$_3$–pro$_{19}$]–heparin adduct. This result is consistent with the heparin affinity assay, which shows the higher affinity for the [Gd(TTDASQ)$_3$–pro$_{19}$] conjugate. The bound relaxivity (r_1^b) of the [Gd(TTDASQ)$_3$–pro$_{19}$]–heparin adduct is 30.9 dm^3 mol^{-1} s^{-1} which is significantly lower than that of the [Gd-TREN-HOPO-TAM-PEG-5000]–HSA adduct (74 dm^3 mol^{-1} s^{-1}) {TREN-HOPO = tris[(3-hydroxy-1-methyl-2-oxo-1,2-didehydropyridine-4-carboxamido)ethyl]amine}.[3] The difference in bound relaxivity of these adducts may be ascribed to the q value of [Gd(TTDASQ)$_3$–pro$_{19}$] ($q = 1$) and [Gd-TREN-HOPO-TAM-PEG-5000] ($q = 2$).[3]

Conclusion

The kinetic stability of [Gd(TTDASQ)$_3$–pro$_{19}$] toward transmetallation with Zn(II) is significantly higher than that of [Gd(DTPA-BMA)], but is lower than those of [Gd(DTPA)]$^{2-}$ and [Gd(TTDA-BOM)]$^{2-}$. From the analysis of the ^{17}O NMR relaxometric properties, the water exchange rate of [Gd(TTDASQ)]$^-$ is similar to that of [Gd(DO3ASQ)], but is higher than that of [Gd(DTPA)]$^{2-}$. Moreover, the binding affinity assay showed [Gd(TTDASQ)$_3$–pro$_{19}$] has a strong affinity for heparin. Therefore, [Gd(TTDASQ)$_3$–pro$_{19}$] may potentially be used as a targeted contrast agent for MRI.

Acknowledgements

We are grateful to the National Science Council of the Republic of China for financial support under Contract No. NSC 94–2627-M-037-001. This research was also supported in part by grants from National Health Research Institutes under Contract No. NHRI-EX-94-9424EI.

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