Articles

Evaluation of the Stability and Animal Biodistribution of Gadolinium(III) Benzylamine-Derivatized Diethylenetriaminepentaacetic Acid

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The need for a readily available Gd(III) bifunctional chelate for protein conjugation has led to the development of LDTPA (*N*,*N*-bis[2-[*N*′,*N*′-bis(carboxymethyl)amino]ethyl]-4-amino-L-phenylalanine). The benzylamine group is readily converted to the isothiocyanato group (SCN-LDTPA) by treatment of the lithium salt of LDTPA with thiophosgene. SCN-LDTPA was successfully conjugated to three proteins, BSA (bovine serum albumin), mannose BSA, and galactose BSA. All protein conjugates were labeled with $^{111}In^{3+}$ or $^{153}Gd^{3+}$. Competition of Gd-LDTPA with DTPA (diethylenetriaminepentaacetic acid) resulted in a log stability constant of 21.2. The thermodynamic stability constant of Gd-LDTPA was also measured. The log Gd(III) stability constant (log *K*) is 21.99, and the log protonation constants (pK_a's) are 10.16, 8.92, 5.35, 3.93, 2.71, and 1.89. Comparison of the thermodynamic stability constants for $Gd(LDTPA)^{2-}$ with other DTPA derivatives indicates that the stability of $Gd(LDTPA)^{2-}$ is similar to $Gd(DTPA)^{2-}$ (log K = 22.4), and higher than DTPA derivatives with one or more carboxylate arm(s) functionalized. The biodistribution of ^{153}Gd-LDTPA−protein conjugates is consistent with the *in vitro* stability measurements. By monitoring the bone accumulation of $^{153}Gd^{3+}$, ^{153}Gd-LDTPA−protein shows a higher *in vivo* stability than ^{153}Gd-DTPA−protein, the radiolabeled protein conjugate formed by the reaction of DTPA dianhydride with proteins.

The site-specific delivery of radioactive metal ions using chelate biomolecule conjugates has potential medicinal applications in radiodiagnostics,[1] radiotherapy,[2] and magnetic resonance imaging (MRI).[3] The increased interest in labeling proteins and antibodies with radiometals such as ^{111}In(III), ^{67}Ga(III), and ^{67}Cu(II) along with generating targeted gadolinium(III)-based MRI contrast agents has led to the development of bifunctional chelates (BFCs).[1] The development of BFC's for targeted gadolinium(III) MRI agents must consider the properties of Gd(III), in particular the importance of nine coordination sites for high complex stability. For MRI, one coordination site will be occupied by a water molecule, so the ideal BFC will contain eight coordinating groups.

Generating a ligand with one site available for protein or antibody conjugation while maintaining strong metal binding poses a significant challenge. Currently, several groups are preparing derivatives of DTPA (diethylenetriaminepentaacetic acid)[4] and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate)[5] for protein conjugation and targeted MRI. Most of these derivatives are prepared through multistep syntheses resulting in low yield recovery of the final product. This is particularly true for macrocyclic-based BFC's.[6] Unlike radiopharmaceuticals where a maximum of milligrams of material are administered, MRI requires much larger amounts; therefore, a large-scale, high-yield bifunctional chelate is needed. To avoid the synthetic difficulties associated with preparing BFC's, DTPA dianhydride, which is commercially available, has become the commonly used agent for Gd(III) BFC.[7] Recently, the use of DTPA dianhydride has been called into question due to the substantial cross-linking observed between the chelate and protein.[8] The cross-linking lowers the yield of the protein conjugate and limits the number of DTPA sites available for metal binding. Also, coordination through a carboxylate arm removes one coordination site on the chelate, compromising the stability of the metal complex. This is apparent since DTPA dianhydride−protein conjugates labeled with ^{153}Gd have been shown to dissociate the metal both *in vitro* and *in vivo*.[9,10] The need for a readily available DTPA derivative bifunctional chelate which only has one site for protein conjugation has lead to the development of LDTPA.[11]

Recently, the high-yield synthesis of *N*,*N*-bis[2-[*N*′,*N*′-bis(carboxymethyl)amino]ethyl]-4-amino-L-phenylalanine (LDTPA) was reported,[11] but stability and biological data were not given. LDTPA is a DTPA derivative functionalized at the methylene of the central carboxymethyl arm with a benzylamine group, as shown in Scheme 1. Since the derivatization occurs at the central amine, the binding conformation of LDTPA to Gd(III) is predicted to be similar to Gd-DTPA. In the continuing search for chelates for radiodiagnostics and MRI,[12] we have synthesized LDTPA, modified the benzylamine group for protein conjugation, and explored both the *in vivo* and *in vitro* stability.

Results

Synthesis and Lipophilicity. Although the synthesis of LDTPA has been reported,[11] it was necessary

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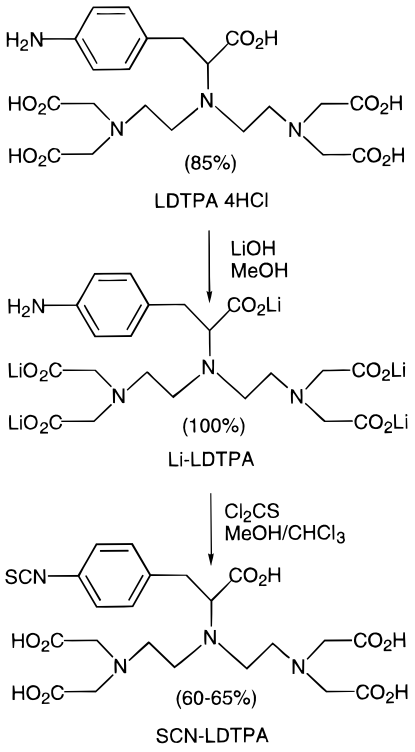
Scheme 1



to convert the aromatic amine to a more reactive group for protein conjugation. The isothiocyanate group is extremely reactive toward primary amines, like the ϵ-amine on lysine, and can be easily generated from the amine on LDTPA. Direct reaction of the tetrahydrochloride salt of LDTPA with thiophosgene[13] resulted in incomplete conversion to the isothiocyanate derivative. Converting the LDTPA hydrochloride salt to the lithium salt effectively neutralized the acid and increased the solubility of LDTPA in methanol. The direct addition of thiophosgene in chloroform to the neutralized LDTPA resulted in high-yield conversion of the aromatic amine to the isothiocyanate (Scheme 1).

The octanol/water partition coefficients of In(III)- and Gd(III)-LDTPA were determined to compare the lipophilicity of LDTPA to DTPA. The In-LDTPA log *P* is -0.88 ± 0.12, and the log *P* for Gd-LDTPA is -1.11 ± 0.09 compared to -2.8 for Gd-DTPA.[12] Both In-LDTPA and Gd-LDTPA are more lipophilic than the corresponding DTPA species due to the aromatic group on the chelate. The effect of the aromatic group is not sufficient to dramatically change the solubility of either compound, and both In-LDTPA and Gd-LDTPA are predicted to be negatively charged at neutral pH and are extremely water-soluble.

Stability. The *in vitro* stability of Gd-LDTPA was evaluated utilizing both qualitative and quantitative techniques. Competition and serum stability studies were performed to determine the relative stability of Gd-LDTPA in the presence of ligands competing for Gd(III). The protonation and thermodynamic log *K* constants were determined to allow for quantitative comparisons to previously reported gadolinium chelates.

The competition of ^{153}Gd-LDTPA with 1, 10, and 100 equiv of DTPA at pH 7.0 and 5.5 is shown in Figure 1. At neutral pH, ^{153}Gd-LDTPA demonstrates slow ^{153}Gd(III) exchange with DTPA. At 1 h, less than 5% ^{153}Gd-DTPA is observed at a 10-fold excess of DTPA to

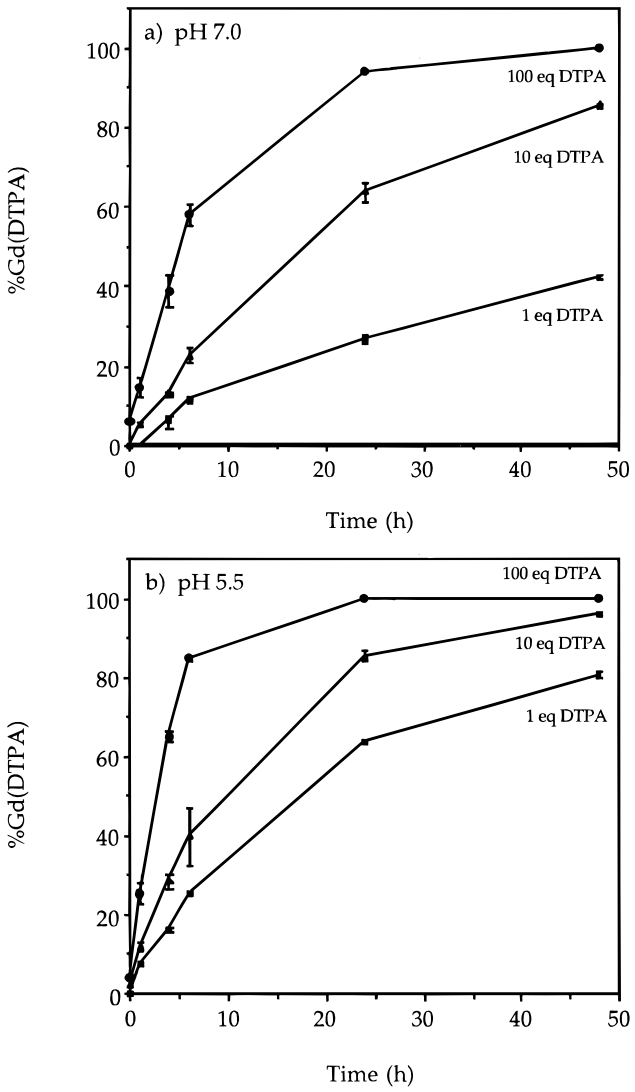


Figure 1. Competition of ^{153}Gd-LDTPA with DTPA at (a) pH 7.0 and (b) pH 5.5. A 1 mM solution of LDTPA was labeled with 50 μCi of ^{153}Gd(OAc)$_n^{3-n}$ in 0.4 M sodium acetate buffer at either pH 7.0 or pH 5.5. 1 mM DTPA (1 equiv), 10 mM DTPA (10 equiv), or 100 mM DTPA (100 equiv) was added in 0.4 M sodium acetate at either pH 7.0 or pH 5.5. Aliquots (1 μL) were removed at 1, 4, 6, 24, and 48 h. The %Gd(DTPA) formed was determined by radio-thin layer chromatography on silica developed with 1:1 methanol/10% aqueous ammonium acetate as described.

LDTPA. Even with a 100-fold excess of DTPA to LDTPA, only about 16% ^{153}Gd-DTPA was observed at 1 h. By 48 h, significant amounts of ^{153}Gd-DTPA were observed for all reactions. The approximate asymptotic flattening of the curves with time suggest that the reaction mixtures are slowly approaching equilibrium. At pH 5.5, both DTPA and LDTPA exchange ^{153}Gd(III) faster than at pH 7.0, probably due to the protonation of the metal complex. By 24 h, more then 50% of the gadolinium was found as ^{153}Gd-DTPA for all reaction mixtures. Both the 10-fold and 100-fold excess DTPA mixtures completely removed ^{153}Gd(III) from LDTPA by 48 h.

A similar study was performed competing ^{153}Gd-DTPA with 1, 10, and 100 equiv of LDTPA, demonstrating that LDTPA competes with DTPA for the metal ion at pH 7.0 or 5.5. Since LDTPA could compete with DTPA for the metal ion at neutral pH, it was predicted that the stability of ^{153}Gd-LDTPA is similar to ^{153}Gd-

DTPA. The increased release of ^{153}Gd(III) at pH 5.5 for both competition reactions suggests that both DTPA and LDTPA will release gadolinium in an acidic environment when challenged with a competing ligand.

The above results demonstrate the ability of LDTPA to compete with DTPA for Gd(III), and the developed experimental parameters were used to estimate the stability constant of Gd-LDTPA. A qualitative measurement of the log K stability constant for Gd(III) with LDTPA was obtained through a series of reaction mixtures at pH 7.4, as described under Experimental Section. All reactions were incubated at room temperature for 6 days, at which time the percentage of ^{153}Gd-DTPA and the percentage of ^{153}Gd-LDTPA were unchanged for all three reactions. Two equilibrium expressions can be written for the competition reactions, one for ^{153}Gd-LDTPA (eq 1) and one for ^{153}Gd-DTPA (eq 2). The division of the equilibrium expressions shown

$$^{153}\text{Gd} + \text{LDTPA} \rightleftharpoons {}^{153}\text{Gd-LDTPA};\ K_1 = [{}^{153}\text{Gd-LDTPA}]/[{}^{153}\text{Gd}][\text{LDTPA}] \quad (1)$$

$$^{153}\text{Gd} + \text{DTPA} \rightleftharpoons {}^{153}\text{Gd-DTPA};\ K_2 = [{}^{153}\text{Gd-DTPA}]/[{}^{153}\text{Gd}][\text{DTPA}] \quad (2)$$

in eq 1 by the expression shown in eq 2 gives an equation for the log K of ^{153}Gd-LDTPA in terms of concentrations. Since a tracer quantity of ^{153}Gd was used, the concentration of ^{153}Gd-chelate present at equilibrium was significantly less than the initial concentrations of the chelates; therefore, the concentrations of LDTPA and DTPA were assumed to remain unchanged at equilibrium. Since the concentration of LDTPA was equal to the concentration of DTPA and the concentrations remained unchanged, these terms have been removed from eq 3. At equilibrium, the

$$K_1 = ([{}^{153}\text{Gd-LDTPA}]/[{}^{153}\text{Gd-DTPA}]) \times K_2 \quad (3)$$

percentage of ^{153}Gd-LDTPA is 5.75 and the percentage of ^{153}Gd-DTPA is 94.25 for all three reaction mixtures. The stability constant for Gd-DTPA, K_2, is 2.88×10^{22} .[14] By substituting these values into eq 3, K_1 is 1.76×10^{21}; therefore, the estimated log stability constant for ^{153}Gd-LDTPA is 21.2.

The *in vitro* competition studies suggest that ^{153}Gd-LDTPA is a stable complex. To evaluate the stability of ^{153}Gd-LDTPA in biological media, serum stability studies were performed. Serum contains a mixture of proteins and small molecules which can potentially compete with LDTPA for Gd(III). Greater than 80% of ^{153}Gd-LDTPA was intact after 72 h, even at the lowest concentration (90 μM) tested. The low concentration of ^{153}Gd-LDTPA used is comparable to the concentration of transferrin (30 μM),[15] a known metal binding protein, in serum. Less than 2% decomposition was observed over 48 h for all of the concentrations of ^{153}Gd-LDTPA examined, indicating that ^{153}Gd-LDTPA is stable in serum.

The thermodynamic stability constants of various DTPA derivatives have been reported. To quantitatively compare the stability of Gd-LDTPA with other gadolinium chelates, the thermodynamic log stability constant and the protonation constants were measured. The addition of the benzylamine group to DTPA, to give LDTPA, resulted in a small decrease in the magnitude of the log formation constant of LDTPA with Gd^{3+} (log K = 21.99) compared with DTPA (log K = 22.4), as shown in Table 1. The successive log protonation constants (pK_a's) of LDTPA are 10.16, 8.92, 5.35, 3.93, 2.71, and 1.89. The first four represent protonations on the various amino groups, while the remaining two are carboxylate protonations. The value 5.35 is probably associated with the aromatic amino group. In addition to the normal Gd(III) complex GdL^{2-}, there exists a protonated chelate $GdHL^{-}$ of some importance below pH 6 and a diprotonated GdH_2L complex below pH 3. The $GdHL^{-}$ species contributes toward the more facile exchange of the Gd(III) tracer between DTPA and LDTPA at pH 5.5, as described above. The GdH_2L species is of no practical importance, but under physiological conditions, the fully formed GdL^{2-} species is the only one present. The species distribution indicates, at physiological pH, that the dominant species is $Gd(LDTPA)^{2-}$, just like $Gd(DTPA)^{2-}$. The stability constants and protonation constants for a series of DTPA derivatives are shown in Table 1. A linear relationship is observed between the sum of the pK_a's and the log K's, as shown in Figure 2. The one point below the line is the only compound represented whose

Table 1. Gadolinium(III) Stability Constants for Polyamino Carboxylate Derivatives (Structures Are Shown in Chart 1)

ligand[a]	log K	pK_a's	reference
LDTPA	22.0	10.16; 8.92; 5.35[b]; 3.93; 2.71; 1.89	this work
DTPA	22.4	10.48; 8.60; 4.28; 2.60; 2.00	14
BOPTA	22.6	10.71; 8.27; 4.35; 2.83; 2.07	20
DTPA-PA	19.7	9.9; 6.4; 3.8; 1.8; low	21
DTPA-PE	18.9	9.8; 6.6; 3.8; 1.8; low	21
Et-DTPA	17.8	10.44; 7.42; 4.00; 2.80	22
DTPA-BMA	16.9	9.37; 4.38; 3.31; 1.43	23

[a] Abbreviations: BOPTA, 4-carboxy-5,8,11-tris(carboxymethyl)-1-phenyl-2-oxa-5,8,11-triazatridecan-13-oic acid; DTPA-PA, diethylenetriaminepentaacetic monopropylamide; DTPA-PE, diethylenetriaminepentaacetic monopropylester; Et-DTPA, *N*-ethyliminobis(ethylenenitrilo)tetraacetic acid; DTPA-BMA, diethylenetriaminepentaacetic-*N*,*N*′-bis(*N*-methylacetamide). [b] Deprotonation of aromatic amine.

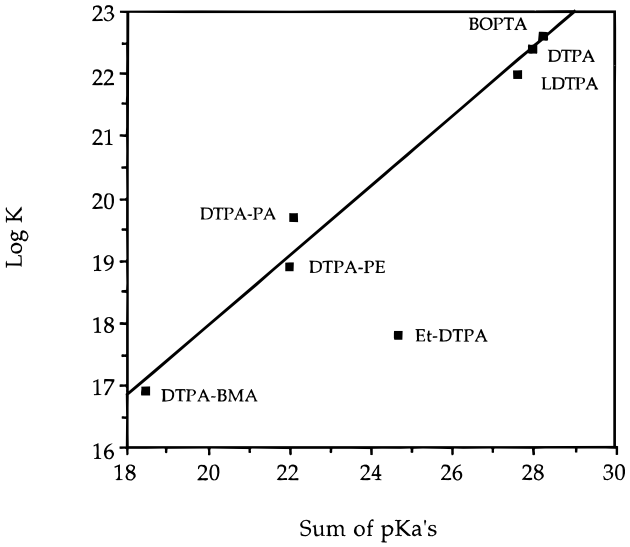


Figure 2. Relationship between the thermodynamic gadolinium DTPA derivatives stability constants (log K) and the sum of their protonation constants (pK_a's). The data were taken from Table 1, and the plotted line is the linear regression of the data points with Et-DTPA omitted.

Table 2. Characterization of LDTPA−Protein Conjugates[a]

protein	conjugate efficiency (chelate/protein)	sp act. (Ci/mmol) $^{111}In^{3+}$	sp act. (Ci/mmol) $^{153}Gd^{3+}$	labeling efficiency (%) $^{111}In^{3+}$	labeling efficiency (%) $^{153}Gd^{3+}$
BSA	1.2	50.6	4.1	>95	90
mannose BSA	0.6	55.1	1.6	>95	30−90
galactose BSA	0.3	49.3	1.8	>95	30−90

[a] All radiolabeled conjugates had greater than 95% radiochemical purity.

structure possesses nonconsecutively substituted carboxylate groups.

Conjugation. Conjugation of SCN-LDTPA to proteins with available lysine residues was readily achieved, but the efficiency was dependent on the protein. Bovine serum albumin (BSA), a nonspecific protein which does not participate in receptor recognition, showed the most efficient conjugation, with 1.2 mol of chelate per mole of protein. The targeted proteins, mannosylated BSA and galactosylated BSA, both had less than 1 mol of chelate per mole of protein. Mannosylated BSA is targeted to mannose receptors found on macrophages, while galactosylated BSA is targeted to asialgylcoprotein receptors found on hepatocytes. Each of these targeted proteins has 28−32 mol of saccharide per mole of BSA, suggesting that many available surface lysines are occupied.

Even though the conjugation efficiency was low, all the LDTPA−protein conjugates were readily labeled with either $^{111}In^{3+}$ or $^{153}Gd^{3+}$. A summary of the conjugation efficiencies, the specific activity of the radiolabeled conjugates, and the labeling efficiencies is given in Table 2.

Biodistribution and Clearance. The uptake and clearance, as well as the *in vivo* stability of ^{111}In-LDTPA−protein and ^{153}Gd-LDTPA−protein, were determined through biodistribution studies. Three different proteins, BSA, mannosylated BSA, and galactosylated BSA, were conjugated to LDTPA to explore how the stability of the metal-LDTPA chelate is affected by different tissue uptake. A summary of the ^{111}In-LDTPA−protein conjugate biodistribution data is shown in Table 3, and the ^{153}Gd-LDTPA−protein conjugate data are shown in Table 4.

The biodistributions of ^{111}In-LDTPA−protein conjugates, where the protein is either BSA, mannose BSA, or galactose BSA, are similar to the biodistributions of ^{111}In-DTPA−protein conjugates.[10] The biodistribution of ^{111}In-LDTPA−BSA indicates that the compound was initially circulating in the blood, but then cleared rapidly through the kidneys. ^{111}In-LDTPA−mannose BSA and ^{111}In-LDTPA−galactose BSA showed rapid accumulation in the liver with slow clearance over 24 h. The ^{111}In-LDTPA−mannose BSA cleared primarily through the kidneys, while ^{111}In-LDTPA−galactose BSA cleared primarily through the lower large intestine. Both kidney uptake and bone uptake appear to be less significant for ^{111}In-LDTPA−galactose BSA.

The biodistributions of ^{153}Gd-LDTPA−protein conjugates are summarized in Table 4. The distribution of ^{153}Gd-LDTPA−BSA was similar to ^{111}In-DTPA−BSA, with slow blood and body clearance. The Gd(III) compound clears the body through both the renal and hepatobiliary systems, as shown in Figure 3. The release of ^{153}Gd from the protein conjugate *in vivo* can be inferred by monitoring the accumulation of radioactivity in the bone. Since BSA is a nontargeted protein, a percentage of the bone accumulation will be due to blood circulating through the bone marrow. Independent studies in this group have shown that the percent of activity in the bone marrow due to the circulating blood is 15%.[16] Significantly less bone accumulation of ^{153}Gd was observed with ^{153}Gd-LDTPA−BSA at all time points, compared to ^{153}Gd-labeled DTPA dianhydride−BSA protein conjugate,[10] as shown in Figure 4.

Unlike ^{153}Gd-LDTPA−BSA, both ^{153}Gd-LDTPA−mannose BSA and ^{153}Gd-LDTPA−galactose BSA showed rapid clearance from the blood due to the presence of the saccharide moiety. ^{153}Gd-LDTPA−mannose BSA and ^{153}Gd-LDTPA−galactose BSA exhibited rapid uptake in the liver, due to receptor binding, followed by slow clearance. ^{153}Gd-LDTPA−mannose BSA and ^{153}Gd-LDTPA−galactose BSA showed different clearance pathways. The mannosylated protein conjugate cleared from the body primarily through the renal system while the galactosylated protein conjugate cleared primarily through the hepatobiliary system (Figure 3). The ^{153}Gd(III) uptake in the bone for ^{153}Gd-LDTPA−mannose BSA is comparable to that previously seen for ^{153}Gd-DTPA−mannose BSA[10] as shown in Figure 5. A significant decrease in bone accumulation was observed for ^{153}Gd-LDTPA−galactose BSA compared to ^{153}Gd-DTPA−galactose BSA[10] as shown in Figure 6.

Discussion

Synthesis of LDTPA, SCN-LDTPA, and LDTPA−Protein Conjugates. The published procedure for the synthesis of LDTPA[11] readily produces gram quantities of the tetrahydrochloride salt. High-purity material, for analytical use, can be easily obtained through generating the free base by passing the tetrahydrochloride salt through PVP resin. In order to couple LDTPA with proteins, it was necessary to convert the aromatic amino group to a more reactive functional group. Generally, either a bromoacetamide group[17] or an isothiocyanate group is used as a linking agent. The isothiocyanate group was chosen, since high conjugation efficiencies were previously obtained and the isothiocyanate can be easily stored.[18] The isothiocyanate group reacts specifically with the ϵ-amine on lysine, and the specificity of the reaction can allow for greater control over the conjugation product.[1]

For LDTPA to be a useful ligand for targeted MRI using protein conjugation, it must coordinate Gd(III). The labeling efficiency was evaluated with LDTPA coordinated to $^{153}Gd^{3+}$, with $^{111}In^{3+}$ being used for comparison. The slight increase in lipophilicity observed for LDTPA, due to the presence of the aromatic group, compared to DTPA, did not dramatically change the properties of the chelate. Both radiometals were quickly and easily coordinated to LDTPA using conditions similar to those used for DTPA labeling.[10]

The coupling of SCN-LDTPA to proteins with an available lysine residue was achieved under basic conditions, and unreacted chelate was removed by size-exclusion chromatography. A significant advantage of LDTPA over DTPA dianhydride is that there is only one reactive group, which limits the side reactions that can occur. In Scheme 2a, LDTPA reacts preferentially with

Table 3. Biodistribution of ^{111}In-LDTPA−Protein in Mature, Female Sprague−Dawley Rats (Four Animals per Time Point)[a]

	time after injection (h)			
organ	1	4	24	48
BSA				
blood	61.3 ± 15.6	53.8 ± 4.2	10.8 ± 1.7	3.33 ±0.43
liver	15.4 ± 1.5	17.0 ± 1.1	21.1 ± 2.4	19.6 ± 3.3
kidney	3.15 ± 0.12	3.58 ± 0.23	3.63 ± 0.27	3.61 ± 0.54
bone	14.8 ± 2.6	19.1 ± 1.1	24.9 ± 1.0	25.9 ± 3.8
colon	0.86 ± 0.18	1.63 ± 0.20	2.87 ± 0.49	2.76 ± 0.39
mannose BSA				
blood	0.255 ± 0.046	0.149 ± 0.016	0.129 ± 0.027	0.141 ± 0.019
liver	89.2 ± 2.0	87.6 ± 2.1	75.2 ± 8.0	61.0 ± 7.6
kidney	0.144 ± 0.033	0.244 ± 0.018	0.336 ± 0.047	0.465 ± 0.046
bone	10.5 ± 0.5	10.1 ± 1.4	8.9 ± 0.9	9.84 ± 1.57
colon	0.0179 ± 0.004	0.156 ± 0.071	0.732 ± 0.128	0.542 ± 0.096
galactose BSA				
blood	0.0826 ± 0.004	0.0462 ± 0.005	0.0385 ± 0.008	0.0267 ± 0.005
liver	99.7 ± 14.2	104 ± 9	65.4 ± 10.4	41.9 ± 1.8
kidney	0.0446 ± 0.002	0.0688 ± 0.015	0.112 ± 0.008	0.140 ± 0.012
bone	0.227 ± 0.049	0.329 ± 0.120	0.379 ± 0.085	0.290 ± 0.064
colon	0.0098 ± 0.002	4.80 ± 2.80	9.40 ± 0.64	5.52 ± 0.62

[a] Data are expressed as % ID/organ.

Table 4. Biodistribution of ^{153}Gd-LDTPA−Protein in Mature, Female Sprague−Dawley Rats (Four Animals per Time Point)[a]

	time after injection (h)				
organ	1	4	24	48	96
BSA					
blood	83.4 ± 12.1	57.0 ± 4.4	12.5 ± 5.2	3.37 ± 0.56	0.418 ± 0.085
liver	14.2 ± 0.5	18.6 ± 2.1	20.0 ± 2.2	17.4 ± 3.5	13.8 ± 1.9
kidney	3.06 ± 0.36	3.69 ± 0.11	3.71 ± 0.57	2.83 ± 0.32	2.73 ± 0.31
bone marrow	1.19 ± 0.25	1.75 ± 0.21	3.07 ± 1.56	2.46 ± 0.67	2.50 ± 0.23
bone	18.1 ± 1.3	22.3 ± 1.3	31.8 ± 6.7	35.9 ± 4.3	44.6 ± 4.4
colon	0.952 ± 0.154	2.30 ± 0.14	2.83 ± 0.67	2.37 ± 0.28	2.40 ± 0.36
mannose BSA					
blood	0.405 ± 0.056	0.131 ± 0.011	0.063 ± 0.017	0.040 ± 0.015	0.032 ± 0.012
liver	70.2 ± 0.4	61.1 ± 7.7	47.0 ± 5.9	35.0 ± 13.7	24.4 ± 3.3
kidney	0.387 ± 0.140	0.542 ± 0.146	0.353 ± 0.022	0.379 ± 0.072	0.405 ± 0.082
bone marrow	2.61 ± 0.27	2.33 ± 0.38	2.13 ± 0.31	1.77 ± 0.2	1.23 ± 0.22
bone	15.6 ± 3.1	16.4 ± 2.3	16.7 ± 0.5	20.4 ± 1.6	26.0 ± 5.0
colon	0.089 ± 0.009	0.327 ± 0.059	0.403 ± 0.146	0.210 ± 0.044	0.241 ± 0.065
galactose BSA					
blood	0.207 ± 0.056	0.045 ± 0.027	0.020 ± 0.011	0.037 ± 0.029	0.015 ± 0.009
liver	91.3 ± 14.6	86.1 ± 12.1	60.9 ± 5.2	34.8 ± 24.2	32.1 ± 3.3
kidney	0.114 ± 0.031	0.105 ± 0.015	0.140 ± 0.026	0.145 ± 0.021	0.193 ± 0.042
bone marrow	0.031 ± 0.025	0.109 ± 0.073	0.040 ± 0.019	0.061 ± 0.043	0.079 ± 0.030
bone	3.59 ± 0.40	3.77 ± 0.38	7.01 ± 1.3	8.70 ± 0.57	9.55 ± 1.00
colon	0.032 ± 0.005	4.09 ± 1.31	6.07 ± 1.70	35.3 ± 1.5	1.35 ± 0.34

[a] Data are expressed as % ID/Organ. The bone marrow was extracted from the femur.

the ϵ-amine on the lysine residue forming a thiourea linkage to yield the conjugated product. The most common method of coupling DTPA to a protein or antibody for $^{111}In^{3+}$ labeling is to use the cyclic dianhydride of DTPA (Scheme 2b),[7] which results in significant side reactions.[8] DTPA dianhydride has two reactive groups as shown in Scheme 2b. When conjugation occurs, either one or both of the anhydrides can react with lysine residues, resulting in several possible products. If both anhydrides react, significant quantities of the protein or antibody are lost. The use of LDTPA for protein and antibody conjugations currently employing DTPA dianhydride will offer a significant advantage in the preparation of ^{111}In-radiopharmaceuticals by increasing the yield and simplifying the purification of the conjugated products.

Although SCN-LDTPA preferentially reacts with the lysine ϵ-amine residue on the protein, the efficiency of the conjugation was low (Table 2). The low conjugation efficiencies can be improved by using single amino acid residues, like L-lysine, or the synthetic peptide poly(L-lysine).[19] Preparation of LDTPA−L−lysine was readily achieved at pH 9.0 with a greater than 50% yield of isolated product. Conjugation of SCN-LDTPA to poly-(L-lysine) resulted in greater than 50 chelates per polylysine.[19] This suggests that the low efficiency observed for the conjugation of SCN-LDTPA to BSA, mannose BSA, and galactose BSA is a function of the protein and not the bifunctional chelate. It is important to remember that the environment surrounding the lysine residue will affect the chemical reactivity; therefore, the protein structure plays a significant role in the conjugation reaction.[1] Both mannose BSA and galactose BSA have 28−32 mol of saccharide conjugated to the BSA backbone, greatly affecting the electrostatics and sterics of the molecule. Most of the readily available sites are already used, and the remaining sites are either blocked by the saccharides or buried in the interior of the protein.

Stability. The competition of ^{153}Gd-LDTPA with DTPA demonstrates the kinetic stability of the complex. The metal exchange rate can be affected by the pH and the concentration of the competing ligand. At pH 7.0, ^{153}Gd-DTPA and ^{153}Gd-LDTPA appear to have similar kinetic stability as determined by the slow formation rate of ^{153}Gd-DTPA from Figure 1 at 1 equiv of DTPA.

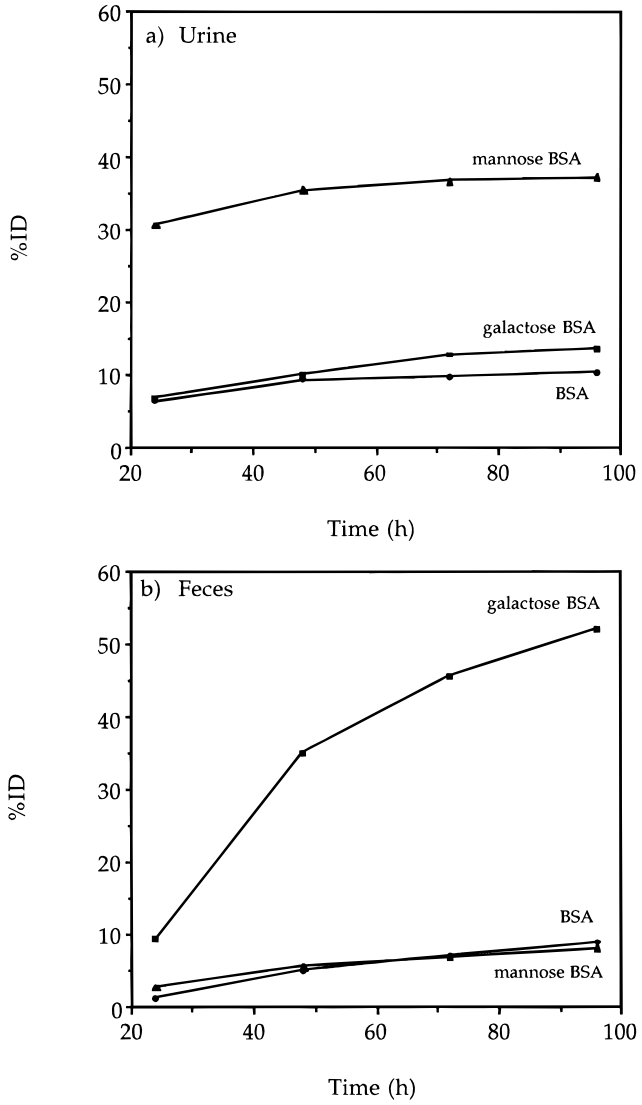


Figure 3. Percent injected dose (%ID) excreted in (a) the urine and (b) the feces for ^{153}Gd-LDTPA−protein conjugates. Urine and feces were collected at 24, 48, 72, and 96 h postinjection, and the activity was assayed on a γ well counter.

At pH 5.5, both ^{153}Gd-LDTPA and ^{153}Gd-DTPA have much lower kinetic stability. Previous studies have looked at the effect of pH on the decomposition of ^{153}Gd-mxDTPA−BSA, the radiolabeled protein conjugate formed by coupling 2-[[4-(isothiocyanato)benzyl]-6-methyl]diethylenetriaminepentaacetic acid (mxDTPA, Chart 1) with BSA, and ^{153}Gd-DTPA−BSA.[10] For mxDTPA, the aromatic isothiocyanato group and a methyl group are on the alkane backbone of DTPA, and the material is a mixture of four optical isomers.[4] Like LDTPA, mxDTPA only has one site available for protein conjugation. Both ^{153}Gd-mxDTPA−BSA and ^{153}Gd-DTPA−BSA showed significant dissociation of the radiometal at pH below 6.0 with a large excess of DTPA. This observation is consistent with the dissociation seen for ^{153}Gd-LDTPA and suggests that the LDTPA complex will not be stable in an acidic environment. Further, the concentration of the competing ligand will affect the rate of Gd(III) dissociation. If the competing ligand has a high affinity for Gd(III), low concentrations will be necessary to remove the metal from either LDTPA or DTPA relative to initial concentrations.

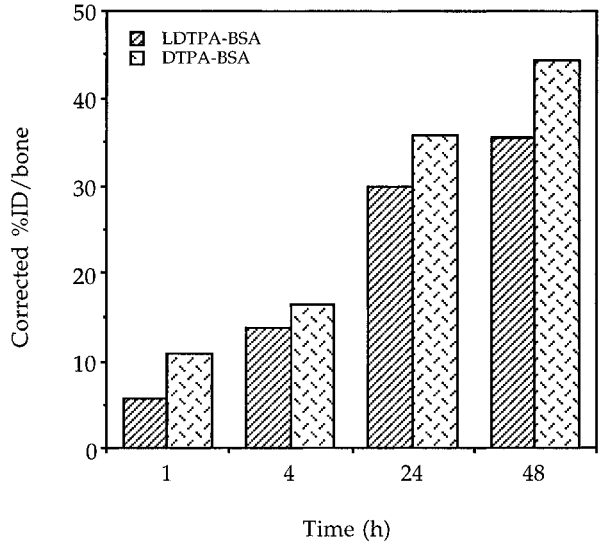


Figure 4. Comparison of the ^{153}Gd bone uptake between ^{153}Gd-LDTPA−BSA and ^{153}Gd-DTPA−BSA (data from reference 10). The corrected percent injected dose per bone was calculated by subtracting 15% of the %ID/blood from the experimental %ID/bone.

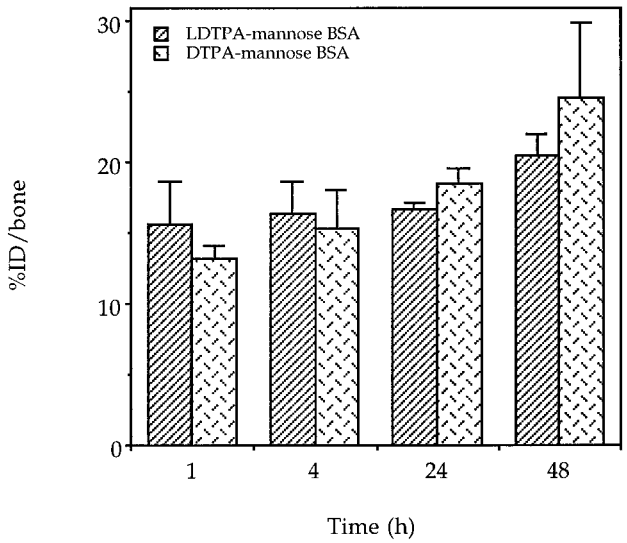


Figure 5. Comparison of the ^{153}Gd bone uptake between ^{153}Gd-LDTPA−mannose BSA and ^{153}Gd-DTPA−mannose BSA (data from reference 10).

The dissociation of ^{153}Gd(III) from LDTPA in the presence of biological ligands was evaluated through *in vitro* serum stability studies. The pH of serum is approximately neutral, so little decomposition was expected. Several concentrations of ^{153}Gd-LDTPA were incubated with freshly prepared rat serum. Naturally occurring albumin may weakly associate metals and has a concentration of about 0.7 mM.[15] No dissociation of ^{153}Gd-LDTPA was noted at either 0.9 mM or 0.45 mM up to 48 h, concentrations where competition with albumin would be apparent. Only at low concentrations of ^{153}Gd-LDTPA (90 μM) and greater than 48 h was any dissociation observed. The low concentration of ^{153}Gd-LDTPA is the same order of magnitude as the concentration of transferrin in the serum (30 μM).[15] Since little dissociation was observed, ^{153}Gd-LDTPA is expected to remain intact in the bloodstream for several days. In terms of targeted tissue uptake, ^{153}Gd-LDTPA−protein conjugates are predicted to arrive at the target tissue with the Gd(III) coordinated.

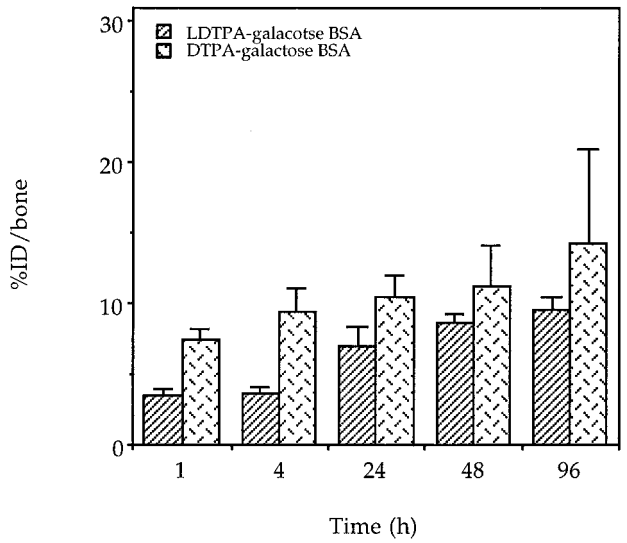
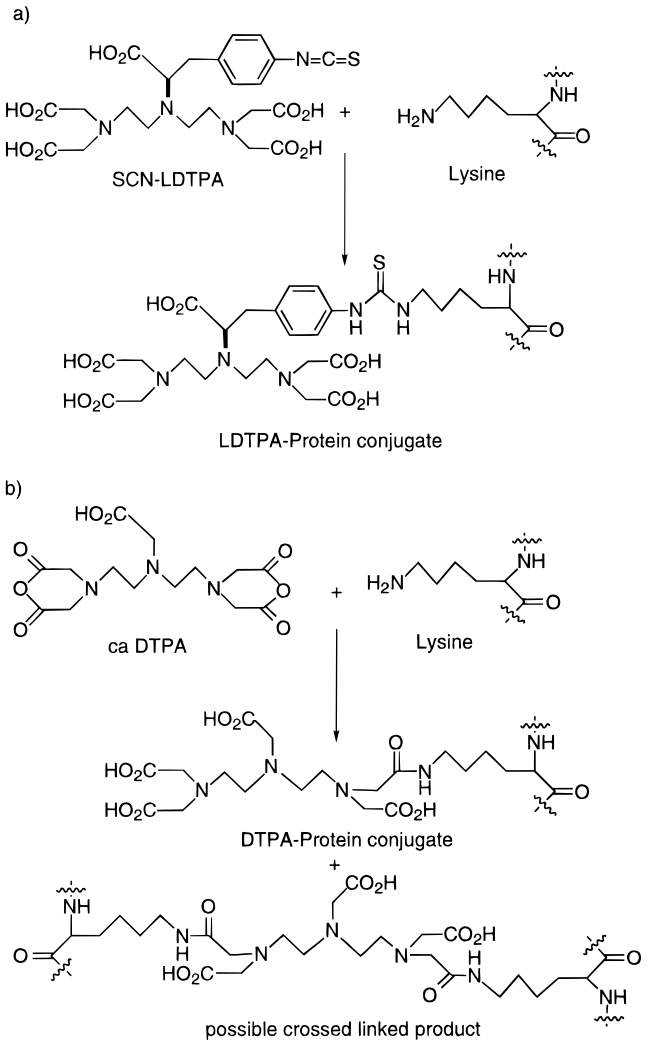


Figure 6. Comparison of the ^{153}Gd bone uptake between ^{153}Gd-LDTPA−galactose BSA and ^{153}Gd-DTPA−galactose BSA (data taken from reference 10).

Scheme 2



The thermodynamic formation constant for LDTPA with gadolinium and the LDTPA protonation constants are compared to DTPA, as well as other DTPA derivatives, in Table 1. The log K for LDTPA with Gd(III) of 22.0 is only 0.4 log unit below that for DTPA. The similarity of the log K and the pK_a's between LDTPA and DTPA suggests that functionalization which does not interfere with the coordinating groups does not significantly change the log K. Further evidence of this is seen by comparing LDTPA to BOPTA (Chart 1).[20] Both LDTPA and BOPTA are functionalized off of a methylene in the central carboxymethyl arm. This effectively leaves all three amines as well as the five carboxylated groups free to coordinate with the metal. Unlike LDTPA, BOPTA is a mixture of two isomers which were not resolved, and further derivatization of BOPTA is required for protein conjugation.

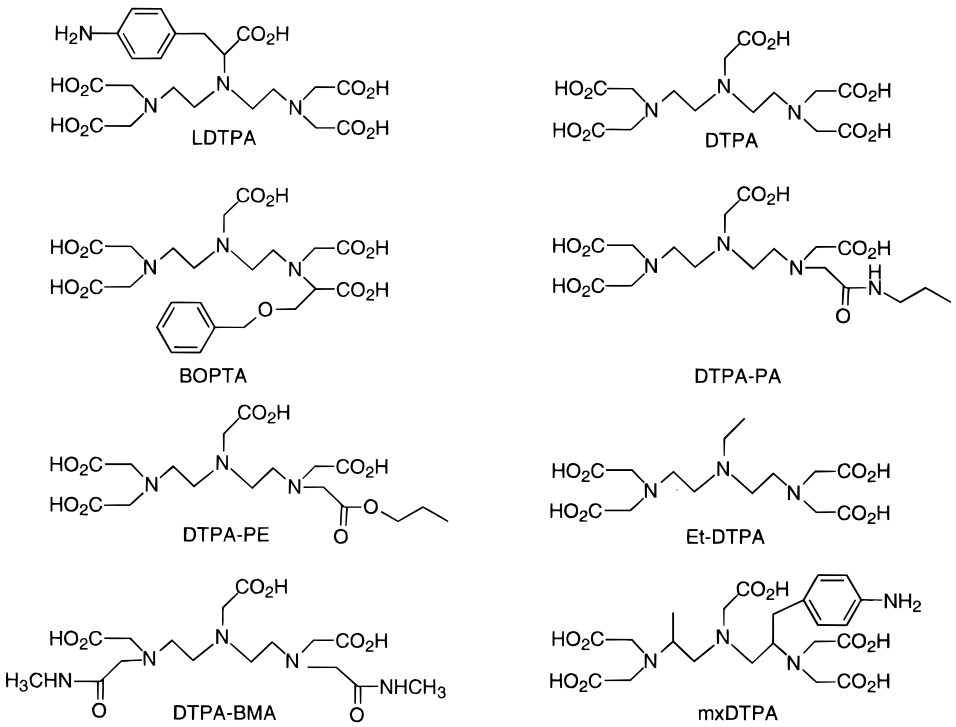
It can be clearly seen from Table 1 that functionalization through a carboxylate group dramatically lowers the stability of the Gd(III) complex. Both DTPA-PA (Chart 1),[21] with a propylamide group instead of a carboxylate, and DTPA-PE (Chart 1),[21] with a propyl ester group instead of a carboxylate, have lower Gd(III) stability constants by 10^2−10^3 than DTPA or LDTPA. The lower stability constants are the result of both esters and amides acting as weak ligands for Gd(III) compared to carboxylates. Complete removal of a carboxylate group and replacement with a noncoordinating group, as in Et-DTPA (Chart 1),[22] lower the stability of the corresponding Gd(III) complex by 10^4. Finally, functionalization of two carboxylated groups is highly unfavorable, as illustrated by DTPA-BMA (Chart 1).[23]

Derivatization of DTPA not only affects the stability constant but also affects the protonation constants. A plot of the sum of the protonation constants versus the thermodynamic log K's, shown in Figure 2, results in a linear relationship, as first noted by Sherry et al.[21] With the exception of Et-DTPA, the stability of the Gd(III) complex is proportional to the basicity of the backbone amines. Comparing LDTPA to DTPA, the pK_a's of the backbone amines are similar: for LDTPA, 10.16, 8.92, and 3.93, while the pK_a's of the backbone in DTPA are 10.48, 8.60, and 4.28. The additional aromatic amino group in LDTPA makes it slightly more basic, but this group is not involved in the coordination of the metal. Direct functionalization of one or two carboxylate groups lowers the first two pK_a's by anywhere from 10 to 10^3. The DTPA derivative Et-DTPA behaves differently from the other species because this derivative breaks the consecutive carboxylate structure, thus weakening the chelate effects. The central nitrogen has lost an anionic carboxylate which dramatically lowers the Gd(III) stability constant through lower electrostatic attraction and lowered coordination. Such ethyl groups also possess hydrophobic character and tend to disfavor cationic combination with the charged metal ion.

The above discussion offers some insight into the expected stability of protein conjugates *in vivo*. If the bifunctional chelate is conjugated in a manner which modifies a carboxylate group, the *in vitro* Gd(III) stability of the protein conjugate is predicted to decrease. This is exactly the situation when DTPA dianhydride is conjugated to proteins or polypeptides (Scheme 2b). As will be discussed below, these *in vitro* arguments also hold true for *in vivo* results.

Biological Activity. The biodistributions of ^{111}In- or ^{153}Gd-LDTPA−protein conjugates were performed to evaluate the *in vivo* stability, to determine the tissue uptake, and to determine the subsequent clearance of the compounds. Previous studies demonstrated that

Chart 1



^{111}In-DTPA glycoprotein conjugates are metabolized to ^{111}In-DTPA−lysine and the metabolite is stable *in vivo*.[24] The biodistributions of ^{111}In-LDTPA−proteins and ^{153}Gd-LDTPA−proteins will be discussed from several different points of view. First, the ^{111}In-LDTPA−protein conjugate data are compared to the previous studies. Next, since ^{153}Gd(III) has been shown to dissociate from DTPA−proteins,[10] the release of Gd(III) from the LDTPA−protein conjugates will be evaluated by comparing the ^{153}Gd bone uptake with the ^{111}In bone uptake. The efficiency and stability of the LDTPA conjugates versus the DTPA dianhydride conjugates will also be evaluated by comparing bone uptake.

The biodistribution of ^{111}In-LDTPA−protein conjugates shown in Table 3 is consistent with the previous data for ^{111}In-DTPA−protein conjugates.[10] The initial blood clearance and tissue uptake are determined by the conjugated protein. BSA is a nontargeted protein which slowly clears the blood through the liver and kidneys. Mannosylated BSA, targeted to the mannose receptors found on macrophages throughout the rat but predominantly in the liver,[25] quickly clears the blood and accumulates in the liver. Galactosylated BSA is targeted to asialoglycoprotein receptors found on hepatocytes in the liver,[25] and quickly clears the blood and accumulates exclusively in the liver. It is interesting that the bone distribution of ^{111}In-LDTPA−mannose BSA and ^{111}In-LDTPA−galactose BSA remains unchanged through the course of the experiment, but the ^{111}In-LDTPA−BSA bone distribution increases by 40% over 48 h. A similar trend was observed for ^{111}In-DTPA−BSA[9] with a 30% increase in bone activity in 48 h while the ^{111}In-DTPA−mannose BSA bone activity remained unchanged. The higher rate of bone uptake for ^{111}In-chelate−BSA is probably due to the slower blood clearance of BSA compared to the targeted proteins. With a higher percentage of radiolabeled BSA circulating, there is a greater probability of metabolism, and subsequent deposit of the radiolabel in the bone.

The initial tissue uptake and clearance of ^{153}Gd-LDTPA−protein conjugates (Table 4) show the same pattern as the ^{111}In(III) studies. BSA slowly clears the blood; mannose BSA and galactose BSA quickly clear the blood by 1 h and accumulate in the liver. The ^{153}Gd-LDTPA−protein conjugates are slowly excreted from the animal, as shown in Figure 3. Less than 20% of the injected dose is accounted for in the excretion for ^{153}Gd-LDTPA−BSA at 96 h, while the remaining activity is in the bone. Mannose BSA clears somewhat better via the renal system with approximately 40% clearance in 96 h. The remaining activity is split equally between the liver and the bone. Galactose BSA clear the most efficiently, with 60% removal by 96 h, mostly through the hepatobiliary system. The majority of the remaining activity is in the liver.

To determine if the ^{153}Gd(III) bone accumulation is due to deposition of the radiometal cation in the bone matrix, the bone marrow was harvested from the femur.[26] Gd(III) is known to bind tightly to calcium binding proteins,[27] and as can be seen in Table 4, the majority of the ^{153}Gd activity is associated with the bone, suggesting it is deposited in the matrix.

The bone uptake for ^{153}Gd-LDTPA−protein conjugates is generally higher than the corresponding ^{111}In(III) conjugates. By comparing the percentage injected dose per bone for ^{111}In-LDTPA−BSA to the corresponding ^{153}Gd(III) species, an increased bone accumulation over the ^{111}In(III) species at 48 h is observed. The dramatic differences in bone accumulation between the ^{111}In(III) and ^{153}Gd(III) species become more apparent for the receptor-specific proteins. The bone accumulation of ^{153}Gd-LDTPA−mannose BSA is 50% higher than the corresponding ^{111}In(III) species, and ^{153}Gd-LDTPA−galactose BSA is 97% greater. The difference between BSA and the glycoproteins suggests that uptake in a specific tissue greatly affects the stability of the Gd(III) chelate. Previously, In(III) and Gd(III) chelate glycoproteins have been shown to ac-

cumulate in the lysosome.[10,24] The *in vitro* competition studies described above indicate that the stability of Gd-LDTPA will be compromised below pH 5.5. The lysosomal pH is well below 5.5,[28] and the ligand will be protonated, increasing the probability of releasing $^{153}Gd^{3+}$ to a better receptor.

In evaluating LDTPA as a bifunctional chelator, it is useful to compare the biological stability with the most commonly conjugated ligand, DTPA dianhydride.[10] In Figures 4 through 6, the percentage injected dose per bone is plotted for ^{153}Gd-LDTPA−BSA and ^{153}Gd-DTPA−BSA (Figure 4) and for mannose BSA (Figure 5) and galactose BSA (Figure 6). The LDTPA− and DTPA−mannose BSA protein conjugates behave similarly at early time points, but by 48 h, DTPA−mannose BSA releases more ^{153}Gd(III) than the LDTPA compound. At early time points for BSA and galactose BSA, the LDTPA conjugates show significantly lower bone accumulation than the corresponding DTPA conjugate. After 24 h, both LDTPA and DTPA have significantly increased bone accumulation compared to earlier time points, but the bone uptake from ^{153}Gd-LDTPA−BSA is still lower. This suggests that the stability of the gadolinium complex plays an important role in the complex stability at early time points. At later times, biological processes take over. As the radiolabeled conjugates are incorporated into specific tissues, the body begins to metabolize the species resulting in the release of ^{153}Gd(III).

Like LDTPA, mxDTPA (Chart 1) is a bifunctional chelate which is functionalized to leave the five carboxylate arms free to coordinate to gadolinium(III).[4] The reported biodistributions for ^{153}Gd-mxDTPA−proteins[10] show similar trends to those reported here for LDTPA. Unlike LDTPA, mxDTPA is difficult to synthesize and contains more than one isomer, and a thermodynamic stability constant has not been reported.[4]

The biological stability of ^{153}Gd-LDTPA−protein conjugates is greater than that of the widely used DTPA dianhydride. Some insight can be obtained to why this difference exists by looking at the thermodynamic stability constants. When DTPA dianhydride is conjugated to the ϵ-amine of lysine, one of the carboxylate groups is transformed to an amide (Scheme 2b). From Table 1, the log K for DTPA-PA drops by 10^2, and the basicity of the backbone nitrogens is also lowered. Before the ^{153}Gd-chelate protein conjugates are incorporated into a specific tissue, the DTPA−protein is at a disadvantage. Figure 4 clearly shows that the predicted drop in the stability for DTPA−protein is evident *in vivo*. At 1 h postinjection, ^{153}Gd-LDTPA−BSA bone uptake is approximately half that of DTPA−BSA. Incorporation of ^{153}Gd-chelate−glycoprotein into the liver will also have a greater effect on the DTPA conjugate. Since the pK_a's of DTPA-PA are lower, protonation will occur at a lower pH. In the lysosome, the pH is 4.7−4.8,[28] effectively protonating at least one of the carboxylate groups on both DTPA−glycoprotein and LDTPA−glycoprotein. The drop in stability for the protonated DTPA glycoprotein is predicted to be similar to that of DTPA-BMA (Table 1), a DTPA derivative with two carboxylate groups modified leading to a 10^5 drop in the log K. For protonated LDTPA−glycoproteins, only one carboxylate group has been modified, so a 10^2 drop in stability is expected. The predicted decrease in Gd(III) stability of the ligand glycoproteins can be seen *in vivo* by looking at the later time points. In all cases, LDTPA−glycoproteins show less Gd(III) bone accumulation than the DTPA−glycoproteins.

Summary

LDTPA has been shown to be a good ligand for both In(III) and Gd(III). The high-yield synthesis and easy preparation of SCN-LDTPA make this ligand an improved agent for protein conjugation to Gd(III). SCN-LDTPA can be conjugated to proteins without side reactions, offering a synthetic advantage over the currently used BFC, DTPA dianhydride. The thermodynamic stability constant suggests that ^{153}Gd-LDTPA will initially be stable *in vivo*, and the biodistribution confirmed this. Further studies to develop LDTPA as a targeted MRI contrast agent are in progress.

Experimental Section

Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium acetate, and ammonium acetate were purchased from Fluka (Buchs, Switzerland). Ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), bovine serum albumin (BSA), and bicine were purchased from Sigma Chemical Co. (St. Louis, MO). Mannose BSA and galactose BSA were purchased from E-Y Labs (San Mateo, CA). $^{111}InCl_3$ was provided by Mallinckrodt Inc. (St. Louis, MO), and $^{153}GdCl_3$ was purchased from either Amersham (Arlington Heights, IL) or Isotope Products (Burbank, CA). Indium acetate was purchased from Aesar Johnson Matthey Inc. (Seabrook, NH). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI), and water was purified through a Waters-Millipore purification system before use. Sprague-Dawley rats were purchased from Sasco (Omaha, NE), and all animal experiments were performed in compliance with the Washington University Animal Care Committee guidelines.

Biospin-6 prepacked size-exclusion columns were purchased from BioRad (Richmond, CA). A Pharmacia FPLC system with a UV detector was fit with a Superose 12 HR 10/30 gel filtration column from Pharmacia. Silica gel 60 F-254 thin-layer chromatography plates were from EM Science.

***N,N*-Bis[2-[*N,N*-bis(carboxymethyl)amino]ethyl]-4-amino-L-phenylalanine (LDTPA).** The tetrahydrochloride salt of LDTPA was prepared by the literature method[10] and converted to the free base by passage through PVP resin (25% cross-linked). Typically, 250 mg of LDTPA·4HCl (0.39 mol) was dissolved in 500 μL of water and applied to a PVP resin column previously activated with 5% ammonium hydroxide followed by washing with water. The product was eluted with water; the UV-active fractions were combined and concentrated *in vacuo* to a yellow oil. Addition of methanol precipitated the free base as a pale yellow solid (116 mg, 60%). Anal. Calcd for $C_{21}H_{30}N_4O_{10}\cdot 1.5H_2O$: C, 47.96; H, 6.33; N, 10.66. Found: C, 48.08, H, 6.59, N, 10.39.

***N,N*-Bis[2-[*N,N*-bis(carboxymethyl)amino]ethyl]-4-isothiocyanato-L-phenylalanine Lithium Salt.** The isothiocyanate derivative of LDTPA was prepared from the lithium salt by the method of Keana and Mann.[29] The lithium salt of LDTPA was prepared by the addition of $LiOH\cdot H_2O$ (60 mg, 1.4 mmol, 9 equiv) in 2 mL of water to a methanol solution of the tetrahydrochloride salt of LDTPA (100 mg, 0.16 mmol, in 10 mL of methanol). The solution was stirred for 3 h, filtered through Celite, and evaporated to dryness. Residual lithium hydroxide was removed by dissolving the residue in methanol (3 × 5 mL), filtering, and evaporating. The pale yellow solid (83 mg, 0.16 mmol) was redissolved in 5 mL of methanol, and thiophosgene (15 mL, 0.19 mmol) in 10 mL of chloroform was added. The pale yellow solution was stirred at room temperature for 1 h and evaporated to dryness to recover a tan residue. The residue was redissolved in methanol and precipitated with dry acetone (53 mg, 60%). ^{1}H NMR (D_2O): δ 2.75 (m, 5), 3.05 (m, 6), 3.30 (t, 2), 3.55 (br s, 6), 7.14 (m, 4).

FT-IR ν(NCS) 2100 cm^{-1}. Further characterization was hindered by the reactivity of the compound.

Partition Coefficients. To determine the lipophilicity of radiometal-labeled LDTPA, the octanol/water partition coefficient was measured. Typically, 4.6 μCi $^{111}In(LDTPA)^{2-}$ or $^{153}Gd(LDTPA)^{2-}$, generated by the addition of LDTPA to $^{111}In(OAc)_n^{3-n}$ or $^{153}Gd(OAc)_n^{3-n}$ in 0.4 M sodium acetate, pH 5.5, followed by incubation at room temperature for 45 min, was added to 1.00 mL of water and vigorously mixed with 1.00 mL of octanol. The two layers were separated by centrifugation, and the aqueous layer was removed. A fresh 1.00 mL water aliquot was added to the octanol layer, and the mixture was mixed vigorously followed by separation by centrifugation. The aqueous layer was removed, and both layers were weighed and counted. A final 1.00 mL water aliquot was added to the octanol layer, and the above process was repeated. The partition coefficient was calculated from five independent experiments.

Stability of $^{153}Gd(LDPTA)^{2-}$. Qualitatively, the stability of $^{153}Gd(LDPTA)^{2-}$ can be evaluated by competition of the complex with a known Gd(III) chelate like DTPA. The radiolabeled $^{153}Gd(LDPTA)^{2-}$ was generated by incubating LDTPA with $^{153}Gd(OAc)_n^{3-n}$ at pH 7.4 for 45 min. The purity of the complex was checked by radio-thin-layer chromatography [R_f of $^{153}Gd(LDPTA)^{2-}$ is 0.74], and 1, 10, or 100 equiv of DTPA was added relative to the LDTPA concentration. Aliquots were removed at predetermined time points and monitored by radio-thin-layer chromatography on silica developed with 1:1 methanol/10% aqueous ammonium acetate. Each reaction was performed in duplicate with less than 3% deviation observed between the two measurements. The % Gd-(chelate) was determined by comparison of the observed R_f's to a series of standards. The R_f of $^{153}Gd(DTPA)^{2-}$ is 0.64, and the R_f of $^{153}Gd(OAc)_n^{3-n}$ is 0.0.

To obtain an estimate of the $^{153}Gd(LDPTA)^{2-}$ stability constant, three reaction mixtures were prepared as follows: $^{153}Gd(LDPTA)^{2-}$ was added to DTPA; $^{153}Gd(DTPA)^{2-}$ was added to LDTPA; LDTPA and DTPA were added to $^{153}Gd(OAc)_n^{3-n}$. The reactions were incubated at room temperature at pH 7.4 adjusted with 0.4 M sodium acetate buffer, for 6 days. The same percentages of $^{153}Gd(LDPTA)^{2-}$ and $^{153}Gd(DTPA)^{2-}$ were obtained for each reaction mixture, as determined by radio-thin-layer chromatography.

The *in vitro* stability of $^{153}Gd(LDTPA)^{2-}$ was further evaluated by examining a biological fluid. Various concentrations of $^{153}Gd(LDTPA)^{2-}$ were incubated with freshly isolated rat serum in a water-jacketed oven at 37 °C with a 5% CO_2 atmosphere for 72 h. Either 0.9 mM, 0.45 mM, or 0.09 mM $^{153}Gd(LDTPA)^{2-}$ was added to 95 μL of serum. Control reactions containing only $^{153}Gd(LDTPA)^{2-}$ were run simultaneously under identical reaction conditions. Aliquots were removed at 1, 4, 24, 48, and 72 h and analyzed by radio-thin-layer chromatography on silica 60 plates developed with 1:1 methanol/10% aqueous ammonium acetate. All peaks at R_f less than 0.2 were assigned to protein-bound $^{153}Gd^{3+}$.

Determination of the Protonation Constants of LDTPA and Its Gd^{3+} Formation Constants. A Corning Model 150 pH meter was connected to Sargent−Welch extension electrodes in a sealed, thermostated (Fisher Scientific Model 901 constant-temperature bath), stirred, and argon-protected glass titration cell equipped with a 10 mL capacity Metrohm Piston burete. The meter was calibrated to read experimental −log $[H^+]$ directly with dilute standard HCl in 0.100 M KCl at 25.00(5) °C.

The protonation constant was measured by charging the above titration cell with 56.3 mg of LDTPA, 5.000 mL of 1.000 M KCl and 45.00 mL of doubly distilled water. After some time to allow for thermal equilibrium and argon protection, 0.100 mL aliquots of 0.0999 M standard, CO_2-free, KOH (prepared from Baker Dilute-it ampules) were added sequentially while recording the pH until the reading was >11.0. These data were used to calculate protonation constants with the program BEST,[30] and the computed log protonation constants were found to be 10.16, 8.92, 5.35, 3.93, 2.71, and 1.89 [standard deviation in pH for all (N = 63) data = 0.0035].

The gadolinium(III) stability constant was measured using a similar solution containing an equimolar concentration of $GdCl_3$ and titrated to pH 11. With the help of the program BEST, two protonation complexes ($GdHL^-$ and GdH_2L^0) as well as the normal GdL^{2-} complexes were found. For GdL^{2-}, the log formation constant is 21.99, and its successive log protonation constants to form $GdHL^-$ and GdH_2L^0 are 4.80 and 2.13, respectively [standard deviation in pH for the equilibrium (N = 48) data = 0.0016].

Coupling of SCN-LDTPA to BSA and Glycoproteins. SCN-LDTPA was conjugated to either BSA, mannosylated BSA, or galactosylated BSA by modification of the method reported by Franano et al.[10] Typically, the bifunctional chelate SCN-LDTPA (0.4 mg, 7.8 × 10^{-4} mmol, 10 equiv) in 5 μL of 0.1 M bicine buffer, pH 9, was added to the protein (5 mg, 7.6 × 10^{-5} mmol, 1 equiv) in 200 μL of bicine buffer. The total reaction volume was adjusted to 400 μL, and incubated at room temperature for 24 h. The protein conjugate was separated from excess ligand by FPLC on a Superose 12 gel filtration column eluted with 0.1 M sodium acetate, 0.15 M sodium chloride, pH 5.5. The desired product was concentrated by diafiltration using a Centricon 30 concentrator with a molecular weight cutoff of 30 000. Generally, 40−50% of the protein was recovered by mass, and the conjugation efficiency was determined by isotopic dilution. For each reaction, 10 mg of protein conjugate was incubated with 5 mCi of $^{111}In(OAc)_n^{3-n}$ and varying concentrations of cold $In(OAc)_n^{3-n}$. Nonspecific bound metal was removed by the addition of 10 mM EDTA followed by an additional 30 min incubation. The percentage $^{111}In^{3+}$-bound protein conjugate was determined by radio-thin-layer chromatography.[18]

LDTPA−Protein Conjugate Labeling. The bifunctional chelate LDTPA and the resulting protein conjugates were radiolabeled with either $^{111}In^{3+}$ or $^{153}Gd^{3+}$. For radiometal labeling, both $^{111}InCl_3$ and $^{153}GdCl_3$ were converted to the corresponding acetates by the addition of 0.4 M sodium acetate, pH 5.5. In a typical reaction, 400 μg of protein conjugate was incubated with either 340 μCi of $^{111}In(OAc)_n^{3-n}$ in pH 5.5 acetate buffer or 30 μCi of $^{153}Gd(OAc)_n^{3-n}$ in pH 7.4 acetate buffer for 45 min. The radiolabeled conjugate was purified by gel filtration through a Biospin-6 column to remove low molecular weight impurities. The purified radiolabeled protein conjugate was analyzed for radiochemical purity by FPLC on a Superose 12 size-exclusion column eluted with 0.1 M sodium acetate, 0.15 M sodium chloride, pH 5.5.

Biodistribution. $^{111}In^{3+}$- or $^{153}Gd^{3+}$-labeled protein conjugates were evaluated in mature, female Sprague−Dawley rats. For each, 19 μg of protein conjugate was injected, and four rats were sacrificed at predetermined time points. For $^{111}In^{3+}$, samples of blood, liver, bone, kidney, and lower large intestines (colon) were removed. For $^{153}Gd^{3+}$, the same organs were removed including the bone marrow.[26] The urine and feces were collected, and the percentage injected dose was calculated to determine the clearance of ^{153}Gd-LDTPA−protein conjugates. The percent injected dose per organ (%ID/organ) was calculated for these organs as described previously.[31]

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Supporting Information Available: Plot of Gd-LDTPA formed from the competition of Gd-DTPA with LDTPA, plot of the serum stability of Gd-LDTPA, speciation curve for LDTPA, and additional biodistribution data (6 pages). Ordering information is given on any current masthead page.

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