Lanthanide(m) Complexes of DOTA–Glycoconjugates: A Potential New Class of Lectin-Mediated Medical Imaging Agents


Abstract: The synthesis and characterization of a new class of DOTA (1,4,7,10-tetraakis(carboxymethyl)-1,4,7,10-tetraazacyclododecane) monoamido-linked glycoconjugates (glucose, lactose and galactose) of different valencies (mono, di and tetra) and their SmIII, EuIII and GdIII complexes are reported. The 1H NMR spectrum of EuIII–DOTALac2 shows the predominance of a single structural isomer of square antiprismatic geometry of the DOTA chelating moiety and fast rotation about the amide bond connected to the targeting glycodendrimer. The in vitro relaxivity of the GdIII–glycoconjugates was studied by 1H nuclear magnetic relaxation dispersion (NMRD), yielding parameters close to those reported for other DOTA monoamides. The known recognition of sugars by lectins makes these glycoconjugates good candidates for medical imaging agents (MRI and gamma scintigraphy).

Keywords: glycoconjugates • lanthanides • lectins • macrocycles • magnetic resonance imaging

Introduction

The number of suitable radiometals for use in nuclear medicine (both in diagnosis and in therapy) is considerable (γ-emitters like 111In or 67Ga; positron emitters as 68Ga or 64Cu, and particle emitters like 90Y, 177Lu (β-emitter) or 213Bi (α-emitter).[1–4] However, the central question in the development of new radiopharmaceutical agents is how to deliver them specifically to the cellular targets (receptors). Many strategies have been attempted and some are currently in clinical use. The general strategy in the field of gamma-imaging is based on the use of radio-labeled conjugates that bind to specific receptors that are overexpressed in certain regions or organs in the body. Specific carriers like monoclonal antibodies[5] and peptides[6] proved, so far, to be the most successful approaches. The moiety of the conjugate that encapsulates the radio metal is a bifunctional chelator which simultaneously presents a functionality (usually carboxylic and amine groups) suitable for covalent binding to the carrier agent.

Magnetic resonance imaging (MRI) is a diagnostic modality based on the enhancement of contrast given by paramagnetic contrast agents (CAs). Gadolinium(III) chelates demonstrated to be the most suitable paramagnetic CAs for MRI, due to the high paramagnetism of the GdIII ion (4f7) and to its slow electron spin relaxation. The efficiency of a MRI CA is based on its ability to selectively reduce the water proton relaxation times of normal and lesioned tissues in the body, and is expressed by its relaxivity, r1 (in units of mmol−1 s−1). The relaxivity is defined as the longitudinal relaxation rate enhancement of the water protons for 1 mmol concentration of paramagnetic compound.[7–8]

Nowadays molecular imaging is a field of fast rising interest.[9] The main requisites in molecular imaging using MRI are high relaxivity and high bioselectivity, in order to deliver...
as many paramagnetic species as possible to the receptors of interest and thus obtain images of the targeted cells.

For the detection of hepatic lesions there are a few hepatocyte-specific MRI CAs which are lipophilic or amphiphilic compounds: \([\text{Gd(BOPTA)(H}_2\text{O})]^{3+}\) (BOPTA = 4-carboxy-5,8,11-tris(carboxymethyl)-1-phenyl-2-oxa-5,8,11-triazatridecan-13-ic acid),[10] \((\text{Gd(EOB-DTPA)(H}_2\text{O})]^{3+}\) (EOB-DTPA = (5)-N-[2-[bis(carboxymethyl)amino]-3-(4-ethoxyphenyl)-propyl]-N-[2-[bis(carboxymethyl)amino]ethyl]glycine),[11] \((\text{Mn(dpdp)(H}_2\text{O})]\) (DPDP = N,N'-1,2-ethanediylbis-[N-[3-hydroxy-2-methyl-5-[phosphonoxyoxymethyl]-4- pyridyl]methyl]glycine),[12] superparamagnetic iron oxides,[13] liposomal[14] and micellar[15] magnetic systems.

The focus of our ongoing research plan is the synthesis of multivalent glycoconjugates of metal complexes as potential agents for medical imaging (MRI and scintigraphy). These compounds include two different moieties: a metal chelator capable of forming chelates with lanthanide(III) ions with high kinetic and thermodynamic stability, and a sugar moiety capable of interacting with high affinity and selectivity with endogenous lectins.[16] Lectins are a large group of carbohydrate binding proteins broadly found in nature (including plants, animals and lower organisms). The lectin-carbohydrate interaction controls many biological events (immune function, fertilization, infectious cycles, metastasis, etc.).[17] The effect of the valence of synthetic and natural glycoconjugates on the affinity to lectins has been fully demonstrated; although the physical nature of the phenomenon is still debatable.[18] A critical feature in the glycoconjugate-lectin interaction is the topology of both the glycoconjugate and lectin. In this respect, the relative orientation and spacing of the carbohydrate residues in the glycoconjugate in relation to the distribution of the Carbohydrate Recognition Domains (CRDs) on the lectins is of fundamental importance.[18,19] The hepatocyte cells on liver express lectins that recognize terminal \(\beta\)-galactosyl residues on desilylated glycoproteins- asialoglycoprotein receptor (ASGPR).[20] The targeting of endogenous lectins for drug delivery is an appealing concept.[21] The ASGPR can be targeted by attaching galactose residues to a desired carrier containing efficient reporter groups.[22–26] The pioneering work of Lee and others has demonstrated that the order of increasing affinity (in vivo and in vitro) of multivalent glycoconjugates for the ASGPR is tetra > tri > di > mono.[22]

The targeting of the ASGPR has been demonstrated both in a cell line and mice with a \(^{111}\text{In-radiolabelled galactopyranosyl conjugate of DOTA (1,4,7,10-tetrakis(carboxymethyl)-1,4,7,10-tetraazaacyclodecanec).}\)[27] \(^{99m}\text{Te-DTPA-GSA.}\) a conjugate of galactosylated serum albumin (GSA) with \(^{99m}\text{Te-DTPA (DTPA = 3,6,9-tri(carboxymethyl)-3,6,9-triazaundecan-1,11-diolic acid), is useful in SPECT (single photon emission computed tomography) hepatic imaging to assess ASGPR in mice[28] and used clinically in humans.[29,30] Monocrystalline iron oxide nanoparticles (MION) conjugated to the bovine plasma protein asialoefetuin (ASF), (MION-ASF),[31] arabinogalactan (AG)-coated ultra-small superparamagnetic iron oxide particles (USPIO), (AG-USPIO),[32,33] spin-labelled arabinogalactan,[34] and a Gd-DTPA conjugate of polysine (PL) derivatized with galactosyl groups (Gd-DTPA-gal-PL),[35] have also been developed as potential contrast agents for liver MRI by targeting the hepatocyte ASGPR and tested in cells and mice.

In this paper we report the synthesis and characterization of a series of multivalent lanthanide(III)-glycoconjugates, based on DOTA-monoamide functionalized chelators (Figure 1). DOTA-like chelators are well known to form LnIII chelates of high thermodynamic and kinetic stability, which is of crucial importance for in vivo applications.[36]

The covalent binding of GdIII chelates to macro and biomolecules[37,38] and the formation of supramolecular assemblies[13] have been reported to slow down the tumbling rate of the paramagnetic chelates which leads then to higher relaxivities. In the present work, the in vitro relaxivity of the GdIII-glycoconjugates was measured and the parameters that influence relaxivity were determined. We hypothesised that the glycoconjugates-lectin association could substantially slow down the rotation and therefore enhance proton relaxivity of the GdIII-labelled glycoconjugates. This could be the basis of a new class of paramagnetic CAs targeted to lectins, both soluble and membrane-bound. In order to test this concept we studied the interaction of GdIII-glycoconjugates in vitro with the model lectin Ricinus communis agglutinin (RCA) through relaxometric measurements. To our knowledge this is the first time that the interaction between a GdIII-glycoconjugate and a lectin is experimentally investigated in the context of MRI.

**Results and Discussion**

**Synthesis of the ligands:** We devised the synthesis of a series of glycoconjugates of metal complexes, which includes various galactose (Gal), glucose (Glc) and lactose (Lac) derivatives of different valencies, namely the monoderivatives 1 (DOTAGal, DOTAGlc, DOTALac), the divalent glycoconjugates 2 (DOTAGal2, DOTAGlc2, DOTALac2) and the tetravalent glycoconjugate 3 (DOTAGal3) (Figure 1).

We have devised a convergent synthesis for these ligands. A metal chelator block and a sugar block suitably functionalised were synthesised separately. Subsequent coupling afforded the fully protected glycoconjugates, which were then deprotected to give the final compounds 1, 2 and 3.

The metal pro-chelator block 7 is a protected amino-functionalized DOTA monoamide derivative (Scheme 1).

![Image](image_url)

As sugar block we used a carboxylic acid-functionalised glycodendrimer, prepared as illustrated for generation 1 (G1) dendromers 10[37] (Scheme 2).

Generation 2 (G2) dendrimer 12 was prepared in a convergent way from diamine 9 and TFA salt 11a (Scheme 3). The synthesis of diamine 9 was accomplished by a modification of Roy’s original procedure[37] (Scheme 4).

The standard DCC/HOBt (DCC = dicyclohexylcarbodiimide; HOBt = 1-hydroxybenzotriazole) coupling procedure revealed successful for preparing fully protected monovalent 18, divalent 17 and tetravalent 16 glycoconjugates. A two-step deprotection, TFA/CH3Cl, followed by KOMe/EtOH, afforded the fully deprotected monovalent 1, divalent 2 and tetravalent 3 glycoconjugates (Schemes 5 and 6).
The characterization of intermediate fully protected compounds, especially glycoconjugates 16, 17 and 18 by \textsuperscript{1}H NMR spectroscopy proved difficult due to extensive broadening and overlapping of signals. The assignment of the \textsuperscript{1}H and \textsuperscript{13}C NMR spectra from fully deprotected compounds in D\textsubscript{2}O revealed even more challenging, as experienced by other groups working with different types of multivalent glycoconjugates\textsuperscript{[19,37]} High resolution mass spectrometry (HRMS) confirmed the identity of the final compounds.

NMR characterization of the glycoconjugate ligands in aqueous solution: The \textsuperscript{1}H NMR spectra of the DOTAGal (1a), DOTAGlc (1b), DOTALac (1c), DOTAGal\textsubscript{2} (2a),

\begin{equation*}
\text{Figure 1. Structure of monovalent 1, divalent 2 and tetravalent 3 glycoconjugate ligands.}
\end{equation*}

\begin{equation*}
\text{Scheme 1. a) BrCH\textsubscript{2}C(O)OEt, CH\textsubscript{2}Cl\textsubscript{2}; b) BrCH\textsubscript{2}C(O)OBa/K\textsubscript{2}CO\textsubscript{3}, CH\textsubscript{3}CN; c) neat H\textsubscript{2}N(CH\textsubscript{2})\textsubscript{2}NH\textsubscript{2}.}
\end{equation*}
DOTAGlc\(_2\) (2b), DOTALac\(_2\) (2c) and DOTAGal\(_4\) (3) ligands (10mM) in D\(_2\)O were obtained in the pH 0.90–9.50 range, at 25\(^\circ\)C, 60\(^\circ\)C and 80\(^\circ\)C and fully assigned using the DQF-COSY technique. The \(^1\)H shifts at pH 7.0 are listed in the Experimental section.

In all cases, changing the pH did not affect the sugar proton resonances, but had a profound effect on the DOTA macrocyclic and pendant arm CH\(_2\) signals. The CH\(_2\) signals from all pendant arms except one remained sharp throughout the pH range, as well as the signals from the macrocyclic CH\(_2\) protons, which are very broad below pH 3, due to the rigidifying effect of the presence of internal hydrogen bonds. These signals sharpen considerably and shift to low frequencies as the pH increases above 3.0, due to successive selective deprotonation of some of the nitrogens and carboxylate oxygens, destroying most of those internal hydrogen bonds. These signals sharpen considerably and shift to low frequencies as the pH increases above 3.0, due to successive selective deprotonation of some of the nitrogens and carboxylate oxygens, destroying most of those internal hydrogen bonds. A temperature increase has a similar sharpening effect on those resonances.

The CH\(_2\) resonances of the bridging moieties are generally sharp, indicating flexibility, and have almost no pH dependence, with some exceptions. In DOTAX\(_2\) (X=Glc, Gal and Lac) and in DOTAGal\(_4\), the NCH\(_2\)C(O) proton signals shift somewhat (e.g. from 4.06 ppm at pH 1.1 to 4.12 ppm at pH 5.2, for DOTAGlc\(_2\)) but broaden and disappear at pH > 5.5, while some broadening is also observed for the NCH\(_2\) protons close to the branching protonated nitrogen atoms. This indicates that an interaction occurs at high pH between these positively charged nitrogens and the negatively charged deprotonated DOTA-monoamide pendant carboxylate groups.
NMR studies of some LnIII-glycoconjugates: The $^1$H NMR spectra of the paramagnetic complexes SmIII-DOTAGal, SmIII-DOTAGlc, SmIII-DOTALac, and EuIII-DOTALac were obtained in D$_2$O at 25°C (see Figure 2 for EuIII-DOTALac). For all the SmIII complexes and the EuIII complex, the protons of the sugar moieties and the bridging arms are hardly perturbed by the paramagnetic center, with very small broadenings and paramagnetic shifts smaller than 0.05 ppm (with the exception of the NCH$_2$CH$_2$ protons of the EuIII-DOTALac complex, with a paramagnetic shift of +0.30 ppm). This indicates that they have preferred solution conformations with the sugar and linker moieties extended away from the LnIII-binding chelate moiety, due to the $r^3$ and $r^3$ dependency, respectively, of the dominant dipolar contributions to the paramagnetic relaxation and shift induced by the LnIII ion, where $r$ is the LnIII-proton distance in the complex. However, the CH$_3$ resonances within the macrocyclic ring and pendant arms of the derivatized DOTA moiety are strongly shifted, and somewhat broadened. These features show close similarities with the parent DOTA and related complexes. These complexes can adopt two diastereomeric structures, the square antiprismatic (M) and the twisted square antiprismatic (m) structure, which differ in the layout of the coordinating arms relative to the macrocyclic ring, with a twist angle between the two planes formed by the four nitrogens and four oxygens in the coordination sphere of the LnIII of about 40° (M) and -20° (m). For the same LnIII complex, the paramagnetically shifted $^1$H resonances of the M isomer cover a spectral window about double of the m isomer. The EuIII-DOTA-Lac$_2$ complex shows two sets of signals corresponding to M- and m-type structures, with a relative intensity ratio of about 3:1 (Figure 2), similar to what has been found for other asymmetric DOTA derivatives. The paramagnetic centers are expected to cause a separate resonance for each of the protons of each isomer in the asymmetrically substituted amide ligand, with a total of 24. All of these are seen for the M isomer, in the range +33.5 to -16.6 ppm range, but only some for the m isomer, for example, at ppm -8.8, -7.2, -6.0, -1.2. A comparison of the relative shifts of the M isomer of EuIII-DOTA-Lac$_2$ and EuIII-DOTA allows the assignment of the resonances of this isomer to the types of protons present in the DOTA moiety of the glycoconjugate (ppm: -16.6, -15.5, -15.0, -14.8 for ac$_2$; -14.6, -12.5, -12.1, -11.4 for ax$_2$; -10.6, -7.9, -7.6, -6.8 for ac; -5.6, -4.8, -3.1, -2.9 for eq; -1.9, -0.1, 0.0, +1.9 for eq, +31.1, +31.4, +32.6, +33.5 for ax, Figure 2). In the present case, the orientation of the substituent did not lead to a doubling of these resonances as a consequence of the formation of two structural isomers, indicating that fast rotation about the amide bond occurs, similar to what has been found for other monosubstituted amide derivatives, but not in disubstituted ones. The signals are somewhat broadened at 25°C due to M—m isomeric exchange, but this exchange broadening effect is much more noticeable for the SmIII complexes.
where only six broad resonances are detectable at +7.2, +4.2, +1.6, +1.4, +1.2 and −2.1 ppm, which, when compared with those of SmIII-DOTA,[40] can be assigned, respectively, to the ac2, ax2, ac1, eq1, eq2 and ax1 protons of the M isomer. However, this broadening causes overlap of the four signals from each type of proton in the four chelate units, and does not allow observation of the m isomer.

NMRD studies of the GdIII-glycoconjugates and their binding to RCA: The proton relaxivity of the GdIII complexes describes the efficiency of the magnetic dipolar coupling between the water proton nuclei and the paramagnetic metal ion, therefore it is a direct measure of the efficacy of the complex as a contrast agent. The paramagnetic ion enhances the relaxation rates of the bulk water protons by long-range and short-range interactions (outer sphere and inner sphere relaxation, respectively). The inner sphere term is governed by the exchange rate of the inner sphere water molecule (kex), the rotational correlation time of the complex (τR), and the longitudinal and transverse electronic relaxation rates of the GdIII (1/T1e and 1/T2e). The outer sphere contribution to the overall proton relaxivity depends on the electron spin relaxation rates and the diffusion coefficient for the diffusion of a water proton away from a GdIII complex (see Appendix).[43]

The proton relaxivities have been measured for the GdIII glycoconjugates of the three types of sugars at three different temperatures (25, 37 and 60°C) between 0.2 and 20 MHz proton Larmor frequency. They are all characteristic of low molecular weight GdIII complexes. As a representative example we show the nuclear magnetic relaxation dispersion (NMRD) profiles of the divalent glucose derivative GdIII-DOTAGlc2 (Figure 3). The NMRD curves of the two divalent derivatives of galactose and lactose, GdIII-DOTA-Gal2 and GdIII-DOTALac2, respectively, can be found in the supporting information. The NMRD profiles have been fitted to the usual Solomon-Bloembergen-Morgan theory that relates the paramagnetic relaxation rates to the microscopic parameters of the GdIII complex (for equations see Appendix). As it has
been pointed out previously, given the large number of factors influencing proton relaxivity, the parameters describing relaxivity cannot be determined exclusively from the often featureless NMRD profiles. Therefore an independent access to some of the parameters is usually required. Water exchange rates have been determined on numerous GdII chelates, including [Gd(DOTA)(H2O)]0, [Gd(DTPA)- (H2O)]0 and their different amide derivatives. As a relatively general rule, it was found that the replacement of each carboxylate coordinating group of the poly(amino carboxylate) ligand by an amide function results in a decrease of the water exchange rate by a factor of 3–4.[43] This decrease is independent of the substituents on the amide group. The glycoconjugates studied here are all monoamido derivatives of DOTA4. Therefore, based on the large body of available data on the water exchange on similar DOTA-amamides, in the analysis of the NMRD profiles we fixed the rate as well as the activation enthalpy of the water exchange to usual values (ks, 298 = 1.2 × 106 s–1 and ΔH* = 30.0 kJ mol–1). Moreover, the diffusion coefficient for the diffusion of a water proton away from a GdII chelate (Dcal) as well as its activation energy is not much dependent on the nature of low molecular weight complexes; hence in the fitting procedures these two parameters were also fixed to common values.[44] Consequently, in the analysis of the NMRD profiles we fitted the rotational correlation time, τR, its activation energy, E, and the parameters describing the electron spin relaxation, that is, the trace of the square of the transition zero-field-splitting (ZFS) tensor, Δ, and the correlation time for the modulation of the ZFS, τ. The parameters obtained for the three GdII chelates are presented in Table 1.

Table 1. Parameters obtained from the analysis of the NMRD profiles for the GdII-glycoconjugates.

<table>
<thead>
<tr>
<th></th>
<th>DOTAGal2 (2a)</th>
<th>DOTAGlc2 (2b)</th>
<th>DOTALac2 (2c)</th>
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<tbody>
<tr>
<td>k</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
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<tr>
<td>ΔH*</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
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<tr>
<td>τR</td>
<td>242 ± 6</td>
<td>261 ± 8</td>
<td>306 ± 10</td>
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<tr>
<td>E</td>
<td>28.3 ± 0.3</td>
<td>27.2 ± 0.2</td>
<td>29.9 ± 0.2</td>
</tr>
<tr>
<td>τ</td>
<td>35 ± 2</td>
<td>39 ± 3</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>Δ</td>
<td>0.10 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>0.12 ± 0.02</td>
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<tr>
<td>Dcal</td>
<td>2.0 × 10–10 m2 s–1</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>Ecal/kJ mol–1</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
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[a] Parameters in italic have been fixed in the fit.

The rotational correlation times calculated for the different glycoconjugates are all reasonable for small chelates, and as expected, increase with increasing molecular weight from the galactose and glucose derivatives to the larger size lactose conjugates.[43] As τR values dominate the high-field NMRD values, the relaxivities of these complexes at 20 MHz and 25°C are significantly higher than those previously found for smaller DOTA-monoamido complexes.[42,43,45] The parameters obtained for the electron spin relaxation of the GdII complexes are also within the usual range for similar GdII-DOTA monoamido chelates, with a significant increase of Δ relative to the more symmetric GdII-DOTA chelate, affecting the low-field NMRD values.[42,43,45] Although in the last few years it has become evident that the simplified model of electron spin relaxation used here is not fully adequate to describe GdII chelates,[47] the application of the novel theories requires EPR data in a large field range which was beyond the scope of the present study.

The proton relaxivities of these small molecular weight chelates are limited by fast rotation. This is clearly shown by the temperature dependence of the profiles: proton relaxivities increase when the temperature decreases, thus the rotation slows down.

These glycoconjugates are capable of binding to lectins. In a solid phase competitive assay (ELLA, enzyme-linked lectin assay) we were able to verify a mini glycoside cluster effect in the binding of these glycoconjugates to PNA (peanut agglutinin).[18,46]

Under the best available experimental conditions, the concentration of the RCA120 was 0.5%, corresponding to a 0.04 mm concentration (more concentrated solutions are not commercially available), whereas the concentration of the GdII chelate was 1.0 mm (at lower concentrations of the paramagnetic species the relaxivity measurements become imprecise). Assuming an association constant of 1010 and two independent binding sites on the RCA molecule, and that the spacing of the sugar terminals on the divalent glycoconjugates does not allow the simultaneous clustering of both sugars on the same molecule of RCA, in the best case scenario we would have two Gd-glycoconjugate units per RCA molecule, that is, maximum 8% of all the GdII chelate would be bound to the protein. For all our systems we observed a slight increase (around 5%) in proton relaxivity in a solution of 0.5% RCA120. This relaxivity increase is not much higher than the error margin of the measurements, therefore we prefer not to interpret this result unambiguously in terms of lectin binding. Although the bound species very likely has a higher proton relaxivity due to slower rotation, its concentration is too low and therefore a substantial increase in relaxivity is difficult to observe. A clear effect of the glycoconjugate-lectin association on the in vitro proton relaxivity could only be observed with considerably higher concentrations of the lectin-bound GdII complex. The only possibility to increase the percentage of bound Gd-species, and therefore the relaxivity, is to have a higher concentration of lectin in the assay, however the lectin availability is a limiting factor.
The feasibility of in vivo MRI applications based on receptor binding in general depends on the concentration of the given receptor as well as on the reactivity of the receptor-bound species. Targeted receptors in cells in general are usually present at concentrations within the range 10^−9−10^−13 mol·g^−1 of tissue.\(^{[40]}\) The minimal concentration of a contrast agent that is detectable by MRI (the concentration necessary to observe a signal enhancement) has been estimated between 5 × 10^−10 mol·g^−1 for the low molecular weight Gd(DP03A) (\(r_1 = 3.7\) ms^−1·s^−1\)) and 1.9 × 10^−10 mol·g^−1 for a 6th generation, Gd(DTPA)-loaded dendrimer (\(r_1 = 5800\) ms\(^{−1}\)·s\(^{−1}\) per dendrimer) or 1.6 × 10^−11 mol·g^−1 for superparamagnetic iron oxide particles (\(r_2 = 72000\) ms\(^{−1}\)·s\(^{−1}\) per particle) (HP-D03A = 1,4,7-tris(carboxymethyl)-1,4,7,10-tetraazaacycloc-dodecane).\(^{[50]}\) In addition to receptor binding on the cell surface, receptor mediated endocytosis can lead to accumulation of the magnetic species inside the cell. With an efficient internalization mechanism, the concentration of the MRI contrast agent inside the cell can largely exceed what is expected solely by surface binding, thus it can help circumvent the problem of low MRI sensitivity. Preliminary results of biodistribution studies with Wistar rats using\(^{153}\)Sm-labeled glycoconjugates as potential candidates for lectin-mediated molecular imaging agents. The flexibility of the glycodendrimer moiety in solution is necessary to observe a signal enhancement (corresponding to a proton T2* value lower than expected from their molecular weight. However we envisaged that the lectin–glycoconjugate interaction could considerably slow down the tumbling rate and therefore increase the relaxivity of the Gd\(^{3+}\) chelates. Further studies are needed in order to unambiguously verify this effect. Our results open the way for the study of Gd\(^{3+}\)-glycoconjugates as potential candidates for lectin-mediated molecular imaging agents.

**Experimental Section**

**Materials and analysis:** Chemicals were purchased from Sigma-Aldrich and used without further purification. Solvents used were of reagent grade and purified by usual methods. Reactions were monitored by TLC on Kieselgel 60 F\(_{254}\) (Merck) on aluminium support. Detection was by examination under UV light (254 nm), by adsorption of iodine vapour and by charring with 10% sulphuric acid in ethanol. Flash chromatography was performed on Kieselgel 60 (Merck, 230–400 mesh). The relevant fractions from flash chromatography were pooled and concentrated under reduced pressure, \(-4^\circ\text{C}\). FAB mass spectra (positive mode) were recorded using a VG Autospec mass spectrometer with 3-nitrobenzyl alcohol (NBA) as matrix.

**NMR spectra:** \(^1\)H NMR (1D and 2D) and \(^13\)C NMR spectra were run on a Varian Unity Plus 300 NMR spectrometer, operating at 299.388 and 75.428 MHz, for \(^1\)H and \(^13\)C, respectively. Chemical shifts (\(\delta\)) are given in ppm relative to the CDCl\(_3\) solvent (\(\delta 7.27\)), \(^1\)C (77.36) as internal standard. For \(^1\)H and \(^13\)C NMR spectra recorded in D\(_2\)O, chemical shifts (\(\delta\)) are given in ppm, respectively, relative to TSP as internal reference (\(\delta 0.0\)) and tert-butanol as external reference (\(\delta 30.29\)). \(^1\)H and \(^13\)C NMR spectra were influenced by spin–spin coupling (Gd\(^{3+}\) ions present), and further stirred for 24 h. The white precipitate was filtered of and the residue was dried under vacuum to give a light yellow foam. This was purified by flash chromatography (100% CH\(_2\)Cl\(_2\) or CH\(_2\)Cl\(_2\)/MeOH/MeONa/H\(_2\)O 70:30:5:5) afforded the title compound (4.83 g, 79%) as a white syrup. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta = 1.27\) (s, \(J_{\text{H},\text{H}} = 7.2\) Hz, 3H; CH\(_3\)), 2.84 (m, 8H), 2.95 (m, 8H), 3.49 (m, 2H), 4.16 (q, \(J_{\text{C},\text{H}} = 7.2\) Hz, 3H; C(O)C\(_{\text{H}_3}\)), 40.03 (s, CH\(_3\)), 55.51 (s, C(O)OC\(_{\text{H}_3}\)), 135.34 (s, \(J_{\text{C},\text{H}} = 7.2\) Hz, 3H; CH\(_{\text{O}}\)); MS (FAB\(^+\), NBA): m/z: 639.8 (M\(^+\)K\(^+\)), 623 (100) \([M+Na]^+\); HRMS (FAB\(^+\), NBA): m/z: calculated for C\(_{37}\)H\(_{45}\)Na\(_2\)O\(_{12}\), found: 633.3996, \([M+Na]^+\).

**Synthesis of organically alkylated cyclen (5):** A solution of ethyl bromoacetate (3.0 g, 18.0 mmol) in CH\(_2\)Cl\(_2\) (30 mL) was added dropwise over 30 min to an ice-cooled solution of cyclen (4.08 g, 23.7 mmol) in CH\(_2\)Cl\(_2\) (50 mL). After 2 h the reaction mixture was allowed to reach room temperature and further stirred for 24 h. The white precipitate was filtered off and the residue was dissolved under reduced pressure to give a yellow solid. Purification by flash chromatography (100% CH\(_2\)Cl\(_2\) or CH\(_2\)Cl\(_2\)/MeOH/MeONa/H\(_2\)O 70:30:5:5) afforded the title compound (4.18 g, 68%) as a white foam. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta = 1.15\) (t, \(J_{\text{H},\text{H}} = 7.2\) Hz, 3H; CH\(_3\)), 2.84 (m, 8H), 2.95 (m, 8H), 3.49 (m, 2H), 4.16 (q, \(J_{\text{C},\text{H}} = 7.2\) Hz, 3H; CH\(_{\text{O}}\)); MS (FAB\(^+\), NBA): m/z: 699.8 (M\(^+\)K\(^+\)), 683 (100) \([M+Na]^+\); HRMS (FAB\(^+\), NBA): m/z: calculated for C\(_{37}\)H\(_{45}\)Na\(_2\)O\(_{12}\), found: 633.3981, \([M+Na]^+\).
The product was used on the next reaction without further purification. The oil was extracted and dried under reduced pressure to give a light red oil. The product was purified by flash chromatography with (100% CHCl₃ → 10% MeOH/CH₂Cl₂) to give the title compound as a colourless oil (2.50 g, 81% yield over two steps). ¹H NMR (300 MHz, CDCl₃): δ = 1.36 (s, 9H; Bu), 1.64 (q, J = 6.5 Hz, 4H; NCH₂CH₂), 1.99, 2.05, 2.06, 2.16 (s, 4 × 6H, OAc), 2.51 (m, 8H; overlapping signals from NCH₂ and SCH₂CH₂), 2.91–2.06 (m, 4H; SCH₂), 3.14 (s, 2H; NCH₂(OCO)), 3.35 (appq, J = 5.4 Hz, 4H; NCH₂CH₂CH₂), 3.95 (appt, J = 6.3 Hz, 2H; H-6a), 4.14 (m, 4H; NCHR₂), 4.5 (J = 9.9 Hz, 2H; H-1), 5.05 (dd, J = 9.9, 3.3 Hz, 2H; H-3), 5.21 (appq, J = 9.3 Hz, 2H; H-2), 5.44 (d, J = 3.3 Hz, 2H; H-4), 7.02 (brm, 2H; CO(NH)₂); MS (FAB⁺, NBA): m/z: [M⁺] = 1082 (100) [M⁺]; HRMS (FAB⁺, NBA): m/z: calc: for C₆H₁₂N₂O₂S₄Na, 1105.9166, found 1105.9180 [M⁺].

Synthesis of fully protected monovalent glycoconjugates 18a-c: Typical procedure illustrated for 18a: A round-bottomed flask was charged with ethylenediamine-functionalised cyclen (0.375 g, 0.61 mmol), peracetylated galactosyloxypropionic acid 8a (0.292 g, 0.67 mmol) and HOBt (0.100 g, 0.67 mmol) in CHCl₃ (20 mL). The reaction was initiated by adding dropwise a solution of DCC (0.146 g, 0.75 mmol) and DMAP (0.088 g, 0.75 mmol) in CH₂Cl₂ (5 mL) and concentrated under reduced pressure to give a white foam. The product was purified by flash chromatography (100% CHCl₃ → 20% EtOAc/CH₂Cl₂) to give the title compound as a white foam (1.58 g, 83% yield). ¹H NMR (300 MHz, CDCl₃): δ = 1.36 (s, 9H; Bu), 1.64 (q, J = 6.5 Hz, 4H; NCH₂CH₂), 1.99, 2.05, 2.06, 2.16 (s, 4 × 6H, OAc), 2.51 (m, 8H; overlapping signals from NCH₂ and SCH₂CH₂), 2.91–2.06 (m, 4H; SCH₂), 3.14 (s, 2H; NCH₂(OCO)), 3.35 (appq, J = 5.4 Hz, 4H; NCH₂CH₂CH₂), 3.95 (appt, J = 6.3 Hz, 2H; H-6a), 4.14 (m, 4H; NCHR₂), 4.5 (J = 9.9 Hz, 2H; H-1), 5.05 (dd, J = 9.9, 3.3 Hz, 2H; H-3), 5.21 (appq, J = 9.3 Hz, 2H; H-2), 5.44 (d, J = 3.3 Hz, 2H; H-4), 7.02 (brm, 2H; CO(NH)₂); MS (FAB⁺, NBA): m/z: [M⁺] = 1082 (100) [M⁺]; HRMS (FAB⁺, NBA): m/z: calc: for C₁₀H₁₆N₂O₃Na, 1130.9365, found 1130.9385 [M⁺].

Synthesis of tetravalent peracetylated thioglalactoside 12: A solution of divalent thioglalactoside 10a (2.83 g, 1.25 mmol) was stirred overnight with CH₃CN/THF (3:1, 20 mL). The solvent was removed under reduced pressure to give a light yellow foam, which was redisolved in CHCl₃ (20 mL) and the solvent was removed under reduced pressure. This procedure was repeated several times and the compound was further dried under vacuum to give 11a as a thick light yellow foam. ¹H NMR analysis revealed the disappearance of the signal at δ = 1.47 assigned to the tert-butyl group. No further purification or characterisation was carried out (we assumed a 100% yield on the deprotection reaction). The product was solved in ice-cooled acetone (10 mL) and stirred (pH ~ 5) and treated with H₂SO₄ (pH ~ 1). The precipitate dicyclohexylurea (DCU) was removed by filtration, and the solvent was removed under reduced pressure. The resulting oil was redissolved in ethyl acetate (20 mL) and washed with NaHCO₃ (sat. sol.; 3 × 20 mL) and brine (30 mL). The solvent was removed under reduced pressure, and the residue was purified by dry flash chromatography (100% CHCl₃ → 20% EtOAc/CH₂Cl₂) to give the title compound as a white foam (1.25 g, 81%). ¹H NMR (300 MHz, CDCl₃, selected signals: δ = 1.46 (s, 9H; Bu), 1.64 (q, J = 6.5 Hz, 4H; NCH₂CH₂), 1.99, 2.05, 2.06, 2.16 (s, 4 × 6H, OAc), 2.51 (m, 8H; overlapping signals from NCH₂ and SCH₂CH₂), 2.91–2.06 (m, 4H; SCH₂), 3.14 (s, 2H; NCH₂(OCO)), 3.35 (appq, J = 5.4 Hz, 4H; NCH₂CH₂CH₂), 3.95 (appt, J = 6.3 Hz, 2H; H-6a), 4.14 (m, 4H; NCHR₂), 4.5 (J = 9.9 Hz, 2H; H-1), 5.05 (dd, J = 9.9, 3.3 Hz, 2H; H-3), 5.21 (appq, J = 9.3 Hz, 2H; H-2), 5.44 (d, J = 3.3 Hz, 2H; H-4), 7.02 (brm, 2H; CO(NH)₂); MS (FAB⁺, NBA): m/z: [M⁺] = 1658.5739, found 1658.5671 [M⁺].

Synthesis of bis-amine (9): Dowex1-X2-100 resin (30 mL, wet resin, HO⁻) was added to a solution of compound 15 (3.10 g, 7.1 mmol) in MeOH/H₂O (50/20 mL). The reaction mixture was shaken at room temperature for 72 h. The resin was filtered off, washed with water and methanol, and the filtrate was concentrated under reduced pressure to give a yellow oil. The compound was purified by flash chromatography (100% CHCl₃ → 10% MeOH/CH₂Cl₂) to give the title compound as a colourless oil (1.21 g, 69%). ¹H NMR (300 MHz, CDCl₃): δ = 1.47 (s, 9H; Bu), 1.67 (q, J = 6.5 Hz, 4H; NCH₂CH₂), 3.12 (s, 2H; NCH₂(OCO)), 3.59 (appq, J = 6.3 Hz, 4H; NCH₂CH₂(O), 8.40 (brt, 2H), 11.47 (O(CO)F₃); ¹³C NMR (75.6 MHz, CDCl₃): δ = 25.54 (NCH₂), 27.75 (CH₃), 38.07 (NCH₂CH₃), 51.83 (NCH₃), 55.76 (NCH₂(OCO)), 81.98 (OC(CH₃)₃), 115.89 (q, J = 287 Hz, CF₃), 157.34 (q, J = 37.0 Hz, NHC(O)(CF₃)), 171.18 (C(O)O)Bu).
1H NMR analysis revealed the disappearance of the signal at a 14.77 ppm assigned to the tert-butyl group. The compound was carried forward without further purification or characterization (we assumed a 90% yield on the basis of the reaction times to afford the crude product in ice-cooled CH2Cl2 (5 mL) and titrated (pH paper) to pH 9-10 with DIPA. To this reaction mixture was added a solution of ethylenediamine-functionalized cyclen 7 (0.147 g of 0.24 mmol) in CH2Cl2 (5 mL). After solution (10.0 g of 0.53 mmol) in CH2Cl2 (5 mL) was added dropwise. The resulting mixture was stirred for 15 min on an ice bath, allowed to reach room temperature and further stirred overnight. The precipitated DCU was filtered of and the solvent was removed under reduced pressure. The resulting oil was dissolved in ethyl acetate (50 mL) and washed with saturated KHCO3 (3×50 mL) and brine (50 mL). The solvent was removed under reduced pressure, and the residue was purified by flash chromatography (100% CHCl3→10% EtOH/CHCl3) to give the title compound as a white foam (0.53 g, 90%).

1H NMR (300 MHz, CDCl3) selected signals: 0.82 (s, 3H, Bu), 2.32–2.36 (t, 2H, OCH2), 4.32 (s, 4H, NCH2CH2C6H4), 2.62 (m, 4H, SCH2C6H4), 3.27 (m, 4H, NCH2CH2NH), 3.82 (d, J=10.2, 7.8 Hz, 2H, H-7), 4.16 (d, J=12.3, 2.0 Hz, H-2), 2.87 (d, J=12.3, 4.5 Hz, 2H, H-6b), 4.09 (d, J=10.2, 7.8 Hz, 2H, H-1), 4.98 (t, J=10.2, 7.8 Hz, 2H, H-5), 5.02 (t, J=9.9 Hz, 1H, H-1), 7.33 (brs, 2H, H-8), 7.59 (brs, 2H, H-9), 7.77 (brs, 1H, H-7); MS (FAB+): m/z (%) 154 (100) [M]+; HRMS (FAB+): m/z: calculated for C14H12D5NO2S 262.0438, found 262.0438 [M]+.

1H NMR analysis revealed the disappearance of the signal at 14.77 ppm assigned to the tert-butyl group. The compound was carried forward without further purification or characterization (we assumed a 90% yield on the basis of the reaction times to afford the crude product in ice-cooled CH2Cl2 (5 mL) and titrated (pH paper) to pH 9-10 with DIPA. To this reaction mixture was added a solution of ethylenediamine-functionalized cyclen 7 (0.147 g of 0.24 mmol) in CH2Cl2 (5 mL). After solution (10.0 g of 0.53 mmol) in CH2Cl2 (5 mL) was added dropwise. The resulting mixture was stirred for 15 min on an ice bath, allowed to reach room temperature and further stirred overnight. The precipitated DCU was filtered of and the solvent was removed under reduced pressure. The resulting oil was dissolved in ethyl acetate (50 mL) and washed with saturated KHCO3 (3×50 mL) and brine (50 mL). The solvent was removed under reduced pressure, and the residue was purified by flash chromatography (100% CHCl3→10% EtOH/CHCl3) to give the title compound as a white foam (0.53 g, 90%).
J. P. André, J. A. Martins et al.

\[ J = 6.6 \, \text{Hz}, \, 4\H; \, \text{SCH}-CH_2, \, 2.96 \, (\, J = 6.6 \, \text{Hz}, \, 4\H; \, \text{SCH}_2), \, 3.76 \, (m, \, 2\H; \, \text{DOTA amide NCH}_2\text{CO}), \, 3.32 \, (s, \, 6\H; \, \text{DOTA acetate NC(O)}) \]

\[ \delta = 23.83, \, 26.40, \, 35.38, \, 38.31, \, 39.07, \, 46.80, \, 48.83, \, 48.98, \, 50.42, \, 52.11, \, 52.71, \, 56.43, \, 57.60, \, 60.41, \, 61.18, \, 68.69, \, 71.06, \, 72.03, \, 72.64, \, 75.51, \, 75.91, \, 78.37, \, 78.79, \, 85.53 \, (C-1), \, 103.05, \, 167.97, \, 172.92, \, 174.70, \, 178.80; \, \text{MS (FAB*, NBA): m/z (\%):} \]

\[ 1443 \, (5) \, [\text{M}+H]^+; \, 1442 \, (7) \, [\text{M}]^+; \, 1341 \, (17), \, 1321 \, (5); \, \text{HRMS (FAB*, NBA): m/z:} \]

\[ \text{calcd for C}_{18}\text{H}_{30}\text{N}_{10}\text{O}_{6}, \, 1442.6018; \, \text{found} \, 1442.6017 \, [\text{M}+H]^+ \].

**Preparation of Ln\textsuperscript{III}–glycoconjugates for NMR:** The Ln\textsuperscript{III}–glycoconjugates were prepared by adding a slight excess (1.1 equiv.) of an aqueous solution of LnCl\textsubscript{3} to an aqueous solution of the glycoconjugate. The solution was slowly adjusted to pH 5 withaq KOH and stirred at 70°C for 8 h. The final pH was adjusted to 7 with KOH (aq) and any precipitate was filtered off. The solution was concentrated and purified by gel filtration with Sephadex G10, eluting with water. The relevant fractions were pooled and freeze dried to afford the Ln\textsuperscript{III} complexes.

**Appendix**

**NMR:** The measured proton relaxivities (normalized to 1 mm Gd\textsuperscript{III} concentration) contain both inner and outer sphere contributions as in Equation (1):

\[ r_1 = r_{1a} + r_{1s} \]

(1)

The inner sphere term is given by Equation (2), where \( q \) is the number of inner sphere water molecules.

\[ r_{1s} = \frac{1}{1000} \times \frac{q \times 55.55 \times 1}{T_{1wa}+T_{1ss}} \]

(2)

The longitudinal relaxation rate of inner sphere protons, \( 1/T_{1s} \), is expressed in Equation (3):

\[ \frac{1}{T_{1s}} = 2 \left( \frac{n_s}{n_t} \right) T_{1wa} \left( S + 1 \right) + \frac{3\alpha_m}{1 + \alpha_m} + \frac{7\alpha_m}{1 + 7\alpha_m} \]

(3)

Here \( r_{1wa} \) is the effective distance between the Gd\textsuperscript{III} electron spin and the water protons, \( \alpha_m \) is the proton resonance frequency and \( r_{1wa} \) is given by Equation (4), where \( r_{1wa} \) is the rotational correlation time of the Gd\textsuperscript{III} ion. The linear vector:

\[ \frac{1}{T_{1wa}} = \frac{1}{T_{1s}} + \frac{1}{T_{1a}} + \frac{1}{T_{1m}} \]

(4)

The rotational correlation time is assumed to have simple exponential temperature dependence with an \( E_a \) activation energy, see Equation (5):

\[ r_{1m} = r_{1m}^{298} \exp \left( \frac{E_a}{R} \left( \frac{1}{T} \right) \right) \]

(5)

The electron spin relaxation rates, \( 1/T_{1a} \) and \( 1/T_{1m} \) for metal ions in solution with S > 2\( \frac{1}{2} \) are mainly governed by a transient zero-field-splitting mechanism (ZFS).\textsuperscript{[13]} In Equations 6 and 7 \( \Lambda^* \) is the trace of the square of the transient zero-field-splitting tensor, \( \tau_r \) is the correlation time for the modulation of the ZFS with the activation energy \( E_a \) and \( \alpha_m \) is the electron spin Larmor frequency as in Equation (8):

\[ \left( \frac{1}{T_{1a}} \right)^{zss} = \frac{1}{2\Lambda^*} \left( \frac{4\alpha_m}{S + 1} - 1 \right) \frac{1}{1 + \alpha_m} + \frac{4}{1 + 4\alpha_m} \]

(6)

\[ \left( \frac{1}{T_{1m}} \right)^{zss} = \Lambda^* \left( \alpha_m \frac{5.26}{1 + 0.372\alpha_m} + \frac{7.18}{1 + 1.24\alpha_m} \right) \]

(7)
The outer sphere contribution is described by Equation (9), where \( N_A \) is the Avogadro constant, and \( J_a \) is a spectral density function, see Equation (10):

\[
r_{os} = \frac{32 N_A \pi}{405 \chi} \left( \frac{\mu_0}{4\pi} \right) \sqrt{\frac{2}{3}} \text{Re} \left[ \frac{1}{(\nu \Gamma_{\text{Gd}} + \frac{4\pi}{\nu T_1})^{n} + \frac{\nu}{(\nu \Gamma_{\text{Gd}} + \frac{4\pi}{\nu T_1})^{n}} + \frac{1}{(\nu \Gamma_{\text{Gd}} + \frac{4\pi}{\nu T_1})^{n}} + \frac{1}{(\nu \Gamma_{\text{Gd}} + \frac{4\pi}{\nu T_1})^{n}} \right]
\]

with \( n = 2 \) for \( J_a \).

A value of 3.6 ppm was used for \( \nu \text{Gd} \). For the temperature dependence of the diffusion coefficient for the diffusion of a water proton away from a Gd\(^{3+}\) complex, \( D_{\text{Gd}} \), we assume an exponential temperature dependence, with an activation energy \( \Delta E_{\text{Gd}} \) as in Equation (11):

\[
D_{\text{Gd}} = D_{\text{Gd}}^{0}\exp \left( \frac{\Delta E_{\text{Gd}}}{k_B T} \right)
\]

Acknowledgment

This work was performed within the framework of the EU COST Action D18 “Lanthane chemistry for diagnosis and therapy.” The work was supported by the Foundation of Science and Technology (F.C.T.), Portugal (project POCTI/QUI/47005/2002) and FEDER. J.P. André would like to acknowledge Prof. H. R. Mäcke for the support he received during his sabbatical leave. É. Töth and A. E. Merbach acknowledge the Swiss National Science Foundation and the Swiss Federal Office for Education and Science for financial support. The authors are grateful to Lino Burai for his help in the NMRD measurements.


Received: July 8, 2004
Published online: October 7, 2004