Synthesis and Characterization of a Novel DTPA-like Gadolinium(III) Complex: A Potential Reagent for the Determination of Glycated Proteins by Water Proton NMR Relaxation Measurements

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The synthesis of a novel DTPA-like ligand **2** containing two boronic acid phenylamide substituents and its Gd(III) complex **3** is reported. The measurement of the proton relaxation rate over the proton Larmor frequency range from 0.01 to 50 MHz of an aqueous solution of this paramagnetic complex suggests that it contains only one water molecule in its inner coordination sphere, as was previously found for the parent DTPA complex. Competition trials in presence of variable amounts of $[Gd–EDTA]^-$ afford a K_f value of 2.0×10^{16}. Since the boronic functionalities form stable linkages with sin–diol moieties, the interaction of complex **3** with glycated albumin leads to the formation of a ternary albumin–**3** complex. The large size of this adduct is responsible for a lengthening of the molecular reorientational time τ_R, which in turn results in an increase of water proton relaxation rate (R_{1p}). The observed R_{1p} enhancement then provides a direct evaluation of the extent of glycation of the albumin specimen.

NMR spectroscopy has found limited applications in analytical chemistry because of its low sensitivity although in recent years the availability of high-field superconducting magnets has partially overcome this problem. This had led to several studies in clinical chemistry dealing with the analysis of biological fluids with the simultaneous determination of a number of low to medium molecular weight metabolites and drugs down to 10^{-4}–10^{-5} M concentration.[1] However, the NMR determination of high molecular weight species remains impossible.

An alternative route to the direct observation may be pursued through the use of suitable paramagnetic probes, whose effect on the relaxation properties of the solvent water protons is dependent upon the type of interactions with the analytes to be determined.

As a first test to probe this idea we chose the determination of glycated albumin which may be present in significant amounts in blood serum in presence of high levels of glucose.[2] The nonenzymatic glycation of proteins is a quite common process, and it is known to involve mainly ϵ-terminal amino groups of lysine residues.

As a paramagnetic probe we dealt with a suitable functionalized derivative of the DTPA–gadolinium complex. This latter species has found widespread application[3] as contrast agent in magnetic resonance imaging (MRI) for its ability to alter markedly the water relaxation properties and for its high thermodynamic stability ($K_f = 10^{22}$), which largely limits the formation of free Gd^{3+} ions. DTPA acts as an octadentate ligand leaving the ninth coordination site on the Gd^{3+} ion available for a water molecule which is involved in a fast (on the NMR time scale) exchange with the bulk water. This process is then responsible for transferring the high paramagnetism associated with the Gd^{3+}-ion (seven unpaired electrons) to the solvent protons.

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Experimental Procedure

Materials. Diethylenetriaminepentaacetic acid dianhydride, (3-aminophenyl)boronic acid hemisulfate salt, dimethyl sulfoxide, gadolinium chloride hexahydrate, and human serum albumin samples were purchased from Sigma, St. Louis, MO, analytical grade, and used without further purification. The extent of glycation of different HSA samples was determined by using the fructosamine assay.[4] Methanol, ethanol, potassium hydroxide, and sodium hydroxide were purchased from Carlo Erba, Milano, Italy, and were of analytical grade.

Synthesis of the Amide 2: 6-(Carboxymethyl)-3,9-bis((*N*-3-boroxyphenyl)carbamoyl)methyl)-3,6,9-triazaundecanedioic Acid. To 372 mg (2 mmol) of (3-aminophenyl)boronic acid sulfate salt was added 356 mg (1 mmol) of diethylenetriaminopentaacetic acid dianhydride in 5 mL of anhydrous dimethyl sulfoxide. The reaction mixture was stirred at room temperature for 90 min.

The solution was neutralized with 3 equiv of KOH dissolved in methanol, and then anhydrous ethanol was added (approximately twice of the DMSO solution volume) to precipitate the salt as white crystals. The precipitate was separated from the liquid phase by centrifugation, washed three times with anhydrous ethanol, and then dried under vacuum overnight.

The tripotassium salt of **2** was characterized by elemental analysis and ^{13}C NMR spectroscopy in D_2O solution at pH = 7. ^{13}C spectral data (ppm relative to TMS): 52.5 (t, 2), 54.2 (t, 2), 57.3 (t, 1), 60.5 (t, 2), 61.1 (t, 2), 124.2 (d, 2), 127.5 (d, 2), 130.0 (d, 2), 131.5 (d, 2), 137.1 (s, 2), 130.6 (s, 2), 173.8 (s, 1), 174.5 (s, 2), 180.5 (s, 2).

Synthesis of the Gd^{3+} Complex 3 and of the Diamagnetic La^{3+} Analog. Equimolar amounts of **2** (3 K^+ salt) and $GdCl_3 \cdot 6H_2O$ were mixed in water and the pH of the solution was brought to 7.3 with NaOH. The measurement of the solvent proton relaxation rate ($R_{1p} = 4.6$ dm^3 mol^{-1} s^{-1}) was used as an analytical probe to assess the formation of the complex. The observation that the addition of a slight excess of ligand did not affect the observed solvent relaxation rate was taken as an evidence that no free Gd^{3+} ions were present.

The La complex has been obtained by mixing equimolar amounts of the tripotassium salt of **2** and $LaCl_3 \cdot 6H_2O$ at pH = 7.3.

NMR Measurements. High resolution ^1H, ^{11}B, and ^{13}C NMR spectra were recorded on a JEOL EX-400 Fourier Transform spectrometer,

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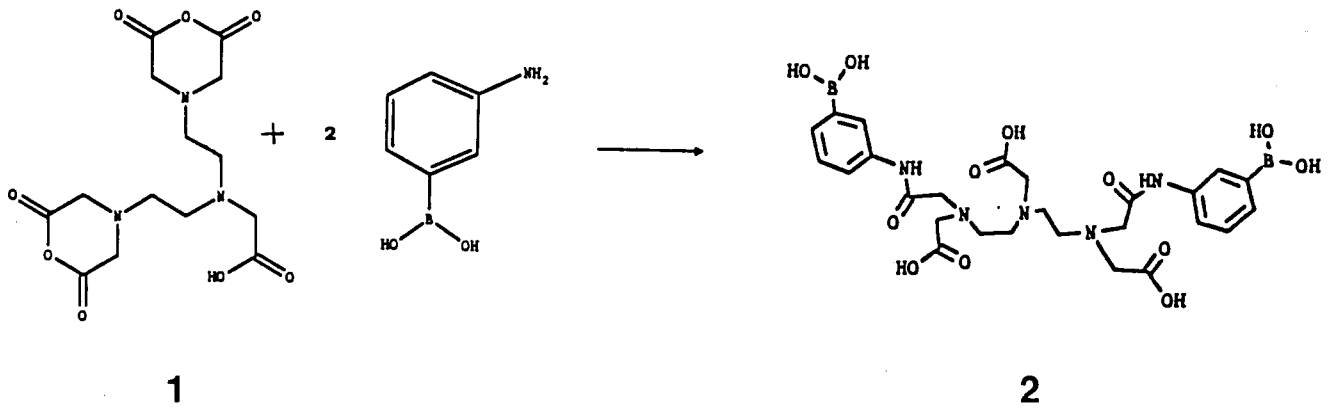
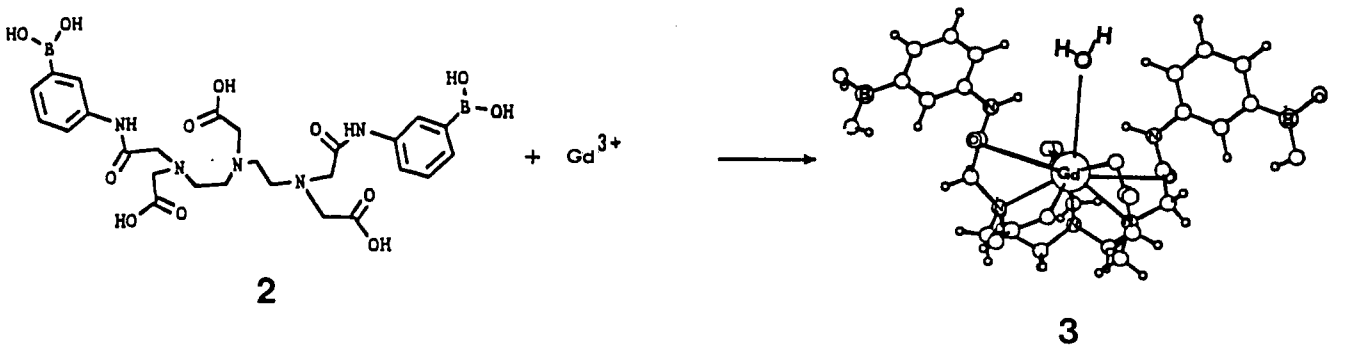
Scheme I



1 2

Scheme II



2 3

operating at a field strength of 9.4 T (proton Larmor frequency = 400 MHz) and a temperature of 298 K. Proton solvent longitudinal relaxation times were measured at 20 MHz and 298 K on a Stelar SpinMaster spectrometer (Stelar, Mede(PV), Italy) by means of the inversion–recovery technique (16 experiments, four scans). The reproducibility in T_1 measurements was ±0.4%. The temperature was controlled by a JEOL air flow heater, equipped with a copper–constantan thermocouple; the actual temperature in the probehead was measured with a Fluke 52 k/j digital thermometer, with an uncertainty of 0.5 K.

The $1/T_1$ NMRD profiles of water protons were measured at 298 K over a continum of magnetic fields from 0.00024 to 1.2 T (corresponding to 0.01 ÷ 50 MHz proton Larmor frequency) on the Koenig–Brown relaxometer installed at the Department of Chemistry of the University of Florence. The relaxometer works under complete computer control with an absolute uncertainty in $1/T_1$ of ±1%.

Results and Discussion

To recognize the glycated protein we chose the boronic functionality, whose ability to form a stable linkage with *cis*-diol moieties is well established, and an affinity chromatography method based on boronic functionalized resin is currently used among others in clinical practice to determine glycated hemoglobin.[5]

The synthesis of the DTPA ligand functionalized with boronic acid was carried on by reacting the DTPA anhydride **1**[6] with (3-aminophenyl)boronic acid (Scheme I). The bis(boronic acid) DTPA ligand **2** (after salification with 3 equiv of K^+) was then reacted with an equimolar amount of $GdCl_3 \cdot 6H_2O$ to afford the corresponding Gd–chelate complex **3** (Scheme II).

For these Gadolinium complexes, the measurement of the water proton relaxation rates of their aqueous solutions provides a useful tool to their characterization. In fact, according to the Solomon–Bloembergen–Morgan[7] theory, it is well established that

$$R_{1,p} = T_{1,obs}^{-1} - T_{1,dia}^{-1} = \frac{Nq}{55.56} \frac{1}{T_{1M} + \tau_M} \quad (1)$$

$$T_{1M}^{-1} = \frac{2}{15} \frac{\gamma_I^2 g^2 \beta^2 S(S+1)}{r^6} \left[\frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{7\tau_c}{1 + \omega_I^2 \tau_c^2} \right] \quad (2)$$

$$\frac{1}{\tau_c} = \frac{1}{\tau_R} + \frac{1}{\tau_M} + \frac{1}{\tau_s} \quad (3)$$

$$\frac{1}{\tau_s} = \frac{1}{\tau_{s0}} \left[\frac{1}{1 + \omega_s^2 \tau_v^2} + \frac{4}{4 + 4\omega_s^2 \tau_v^2} \right] \quad (4)$$

where R_{1p} is the paramagnetic contribution to the longitudinal relaxation rates, T_{1M} is the longitudinal relaxation time of water protons present in the first coordination sphere of the metal, $T_{1,obs}$ is the observed T_1 value and $T_{1,dia}$ is the diamagnetic contribution, i.e. in the absence of the paramagnetic complex. N is the molar concentration of the paramagnetic complex, and q is the number of coordinated water molecules; r is the distance between the protons of the coordinated water molecule and the paramagnetic metal ion. τ_M is the exchange lifetime of water molecules in the first coordination sphere, τ_s is the electronic relaxation time, τ_R is the reorientational correlation time. γ_I is the gyromagnetic ratio of the proton, and ω_I is its Larmor frequency; ω_S is the electron Larmor frequency, β is the Bohr magnetron and g is the electronic g factor. S is the electronic spin quantum number of the paramagnetic ion. Equation 4, called the Bloembergen–Morgan equation, describes the frequency dependence of the electronic relaxation time τ_s as a function of its zero field value $\tau_{s,0}$ and of a correlation time τ_v.

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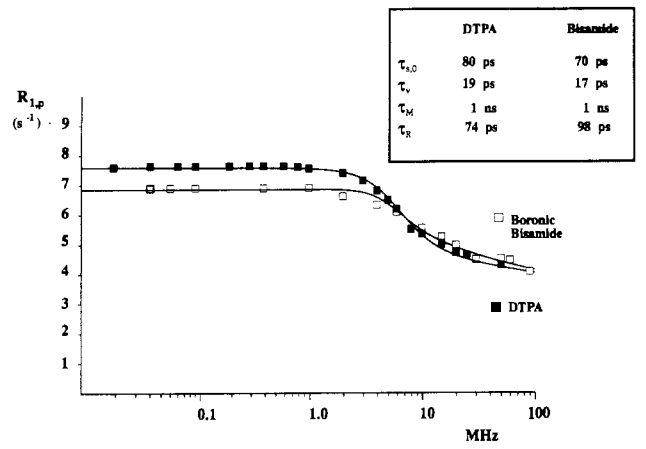

Figure 1. $1/T_1$ NMRD profiles of aqueous solutions of Gd–DTPA and **3** at pH = 7.3 and at 298 K. The solid lines through the experimental data are calculated with the parameters reported in the insert.

A number of observations on polyaminocarboxylate complexes of Gd^{3+} have definitively established[8] that, at magnetic fields higher than 0.25 T, R_{1p} values are essentially determined by the molecular reorientational time τ_R and by the q value.

An aqueous 1 mM solution of complex **3** at 25 °C and at a Larmor frequency of 20 MHz showed a solvent proton relaxation rate (R_{1p}) of 4.6 $dm^3\ mmol^{-1}\ s^{-1}$. This value is quite similar to the values found for other closely related DTPA–bisamide Gd^{3+} complexes[9] as well for the parent DTPA gadolinium complex (R_{1p} = 4.3 $dm^3\ mmol^{-1}\ s^{-1}$) itself to suggest that the octadenticity of the ligand is maintained and the number of water molecules in the inner coordination sphere is not increased.

To further assess the stoichiometry proposed for **3** we compared its $1/T_1$ NMRD profile with that of the parent Gd–DTPA complex (1 mM, 298 K, pH = 7.3) (Figure 1). The experimental data were fitted by eqs 1–4 using $\tau_{s,0}$, τ_v, τ_M, and τ_R as adjustable parameters and assuming a single coordinated water molecule (q = 1) and a proton–metal distance of 3.1 Å. The outer sphere contribution was calculated from Freed's equation[8] as previously done for a number of small-sized complexes.[3a] The main differences between Gd–DTPA and **3** deal with $\tau_{s,0}$ and τ_R. The electronic relaxation time $\tau_{s,0}$ is slightly longer for the parent Gd–DTPA complex, and it is responsible for the higher relaxivity at lower magnetig field strengths. On the other hand the increased molecular weight of **3** causes an elongation of the molecular reorientational time τ_R, which dominates the molecular correlation time τ_c at higher fields resulting then in an higher relaxivity in the high frequency observation range.

The formation constant of this complex has been determined through the measurement of solvent proton relaxation rates of solutions containing the ligand **2** and variable amounts of Gd–EDTA ($K_f = 1.1 \times 10^{17}$).

In fact, in such a solution a competitive reaction for sequestering Gd^{3+} ions takes place

$$\text{Gd–EDTA} + \mathbf{2} \Leftrightarrow \mathbf{3} + \text{EDTA}$$

whose equilibrium constant is clearly related to the ratio between the K_f's of the two Gd^{3+} complexes:

$$K = K_{f,3}/K_{f,\text{Gd-EDTA}}$$

The proton relaxation rate of this solution is given by

$$R_{1,obs} = {R_{1p}}^{3}[\mathbf{3}] + {R_{1p}}^{\text{Gd-EDTA}}[\text{Gd–EDTA}] + R_{1,w}$$

where [GdEDTA] is equal to (C_0 – [**3**]) and C_0 is the total Gd^{3+} concentration.

Now, setting R_{1p} equal to 7.5 and 4.6 $dm^3\ mmol^{-1}\ s^{-1}$ for Gd–EDTA and **3**, respectively, the determination of $K_{f,3}$ is straightforward. This measurement was repeated over five concentration values of **2** keeping constant the concentration of Gd–EDTA (5 mM). The K_f value determined through this procedure is (2.0 ± 0.3) × 10^{16}. The remarkable difference between this value and the corresponding one of Gd–DTPA (8.0×10^{22}) is clearly related to the decreased ability of the CO groups in the amido functionalities to coordinate the Gd^{3+} ion in respect to the carboxylic functionalities.

In presence of glycated proteins, the reaction shown in Scheme III takes place.[5] As this adduct is formed, a marked enhancement of the proton relaxation rates is observed, as a consequence of the increased τ_R. Such a proton relaxation enhancement associated with the interaction between a paramagnetic metal ion and a macromolecule is well known.[10,11]

Interestingly, this approach has been also suggested to improve the effectiveness of the DTPA–Gd complex in MRI through its covalent conjugation to bovine serum albumin.[12]

The formation of the ternary albumin–**3** complex has been assessed also through the comparison of the $1/T_1$ NMRD profiles of **3** in the presence and in the absence of glycated albumin (Figure 2). The relaxivity values of the solution of **3** containing glycated albumin are higher at any field strength, showing a small, but detectable, relaxivity peak (typical of paramagnetic macromolecular complexes[8]) at 20–30 MHz. Although we were unable to get a good fit of the experimental data to values calculated through eqs 1–4 and the outer-sphere contribution[8] (likely because of the inadequacies of the available theory for the latter contribution), the observed behavior clearly supports the occurrence of a protein bound complex.

Further evidence for the formation of a boronic ester between complex **3** and glucidic moieties on the macromolecules has been gained through ^{11}B NMR spectroscopy once the analog diamagnetic La^{3+} complex of ligand **2** is considered.

The ^{11}B NMR spectrum of an aqueous solution of this complex shows a broad absorption centered at 27 ppm from BF_4^- taken as reference; as an aliquot of glycated albumin is added to this solution (to raise a 1:1 ratio between the number of boronic groups and the sugar moieties), this ^{11}B signal disappears and a new even more broadened signal appears at ~5 ppm. The latter signal is assigned to the formation of the boronic ester group on the basis of similar behavior reported in studies dealing with borate esters of polyhydroxy polycarboxylates and polyols.[13]

In order to assess the potential utility of this approach to measure out the correct proportions of glycated protein in real samples, we measured the proton relaxation times of solutions of **3** (0.56 mM) containing variable amounts HSA at different degrees of glycation (Figure 1) as measured by the fructosamine method.

The good linearity found between R_1 and albumin fructosamine concentration is very promising to support such proton relaxation enhancement approach as a new method for the determination of glycated proteins.

Apparently the presence of two potential sites of interaction on **3** does not cause any complication as the observed R_1 values are linearly proportional to the extent of glycation only. Furthermore, this observation allows us to rule out in these experiments any contribution from the albumin concentration itself. In fact, the collection of points in Figure 3 contains data

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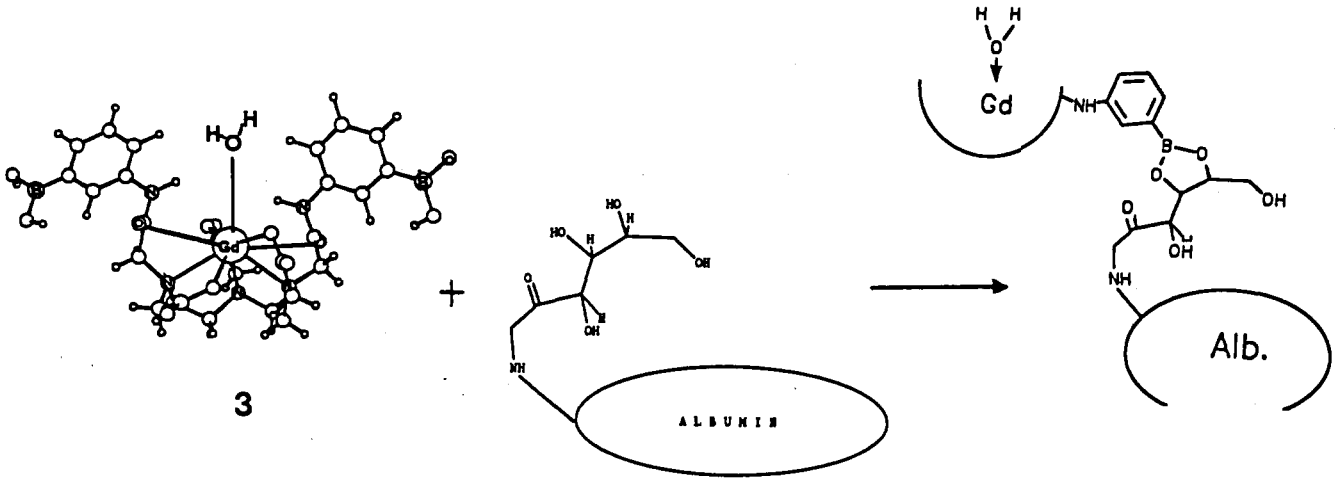
Scheme III



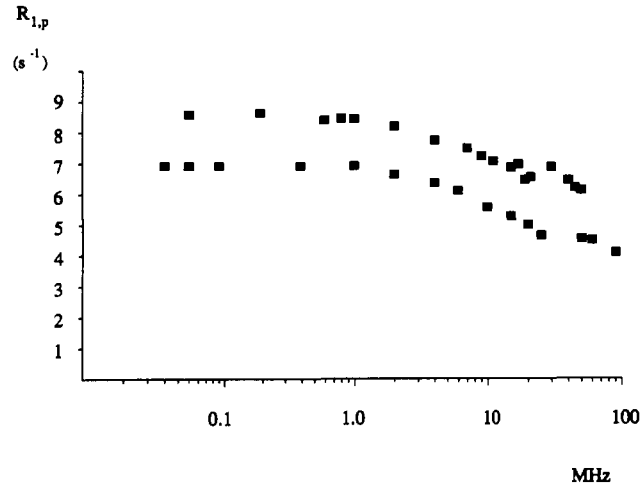


Figure 2. Comparison of $1/T_1$ NMRD profiles of 3 in the presence (upper trace) and in the absence (lower trace) of glycated albumin, at pH = 7.3 and 298 K. Original data were obtained on 0.5 mM solutions of 3. The lower trace refers to a 1 mM albumin solution corresponding to a fructosamine concentration of 0.36 mM.

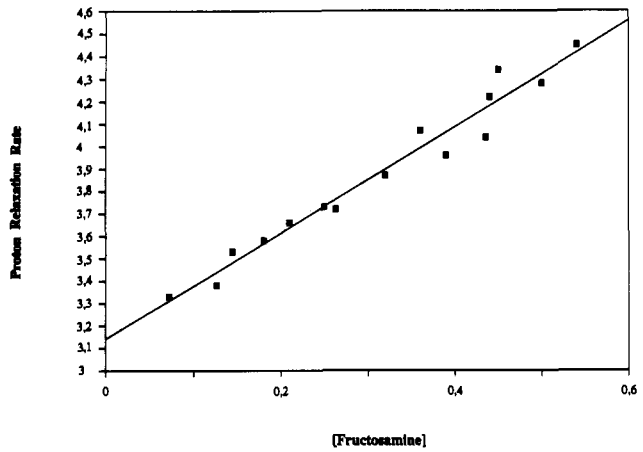


Figure 3. Water proton relaxation rates of 0.56 mM solution of 3 as a function of the millimolar concentration of glycidic groups on human serum albumin as determined by the fructosamine method.

from albumin samples at different degree of glycation, ranging from 0.37 to 2.2 hexose/albumin.

The slope of the straight line in Figure 1 affords an R_{1p} value for the ternary albumin–3 complex of 6.9 dm^3 $mmol^{-1}$ s^{-1}, which looks smaller than expected for such large adducts. A possible explanation of the observed behavior may be based on the large amplitude motions of the glucosidic moieties which cause a decrease of the effective reorientational time at the metal center with respect to the large value of the albumin molecule.[14]

In principle the effectiveness of this method may be limited by the presence of free sugars containing sin–diol moieties. To get an evaluation of such a possibility, we report that the observed R_1 value of a 0.16 mM solution of the complex 3 containing 15 mg/mL of glycated albumin (0.5 mM as fructosamine concentration) at pH = 7.4 decreases linearly from 1.38 to 1.19 s^{-1} as the concentration of added sorbitol increases from 0 to 30 mM. In this context we have also measured the effect of free sorbitol on R_{1p} of 3 in absence of protein and found that for a 1 mM solution it changes from 4.6 to 5.1 dm^3 $mmol^{-1}$ s^{-1} as the sugar concentration increases from 0 to 50 mM. This means that errors not higher than few percents may be expected when interferent species are present at ordinary concentrations in blood serum.

Since the observed differences in T_1 values are rather small, the practical applicability of this approach must be based on the accuracy of the measurement. On testing the reproducibility over a large number of measurements carried out on our Stelar SpinMaster spectrometer, we found that the T_1 value for a given specimen brings an experimental error of ±0.4%.

This means that the method may be able to measure out concentrations of glycated protein with an accuracy of ±1%.

Conclusions

We believe that the results reported in this paper introduce a novel area of development of paramagnetic Gd(III) complexes in addition to their well established role as contrast agents for magnetic resonance imaging.[3a,15]

The use of functionalized paramagnetic complexes may allow the quick determination of a variety of macromolecular substrates through the detection of the increase of solvent water proton relaxation rates as the result of the formation of slowly tumbling macromolecule–complex adducts.[16,17] Interestingly, the proposed procedure represents the NMR counterpart of the ESR free radical assay technique[18] based on the detection of changes in the line width of nitroxide resonance caused by the interaction of the labeled reagent with macromolecules.

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