concentrations see: e) C. Heiss, R. S. Phillips, *J. Chem. Soc. Perkin Trans. 1* **2000**, 2821 – 2825 [15 % (v/v) cosubstrate 2-propanol, 0.12 mol L^{-1} substrate]; f) A. Matsuyama, H. Yamamoto, N. Kawada, Y. Kobayashi, *J. Mol. Catal. B* **2001**, *11*, 513 – 521 (0.3 mol L^{-1} substrate, no details on the NAD^+/NADH recycling system); g) H. D. Simpson, D. A. Cowan, *Protein Pept. Lett.* **1997**, *4*, 25 – 31 [11 % (v/v) cosubstrate 2-propanol, 0.44 mol L^{-1} substrate].

[9] Enzyme purification showed that at least four NAD^+/NADH-dependent active fractions are present in the cells, of which only one had a stability towards acetone and 2-propanol comparable to that of the whole cells. The other NAD^+/NADH depending dehydrogenases were either unstable or could not use 2-propanol/acetone as cosubstrate. However, all dehydrogenases displayed the same stereopreference.

[10] Lyophilized cells can be stored at +4 °C for several months without noticeable loss of activity.

[11] For growth conditions see: W. Kroutil, M. Mischitz, K. Faber, *J. Chem. Soc. Perkin Trans. 1* **1997**, 3629 – 3636.

[12] All optimization experiments were performed at 30 °C.

[13] Whole lyophilized cells lost 50 % of their activity in 30 % 2-propanol after 35 h, in 50 % 2-propanol already after 3 h.

[14] Since the solubility of most substrates in aqueous buffer is limited, apparent substrate concentrations are used throughout this study which correspond to mol substrate/total volume of the system.

[15] The reason for the decrease of activity at high substrate concentration may be inhibition, deactivation, or limitations of the substrate transport (diffusion, transport into the cell). However, the reason was not clarified for whole cells because of the complexity of the system, but will be elucidated for the isolated enzyme.

[16] This corresponds to a productivity of 26 mmol product $h^{-1}L^{-1}$ employing 0.3 g whole lyophilized cells, or 2.3 g product g^{-1} cells. Literature values for g product g^{-1} whole cells for reductions are at 0.006 g g^{-1}[7d] or 0.02 g g^{-1}.[8c]

[17] The moderate isolated yield results from the limited half-life of whole cells at 50 % (v/v) 2-propanol and may be increased by either using more cells or by starting at a lower concentration of 2-propanol [e.g. 30 % (v/v)] and by continuously increasing it.

[18] The use of different aldehydes/ketones such as, octanal, propanal, isobutyraldehyde, cyclohexanone, cyclobutanone, cyclopentanone, octan-2-one, chloroacetone, and others for cofactor recycling has been reported, although no detailed data are available: see ref. [8a]; J. Grunwald, B. Wirz, M. P. Scollar, A. M. Klibanov, *J. Am. Chem. Soc.* **1986**, *108*, 6732 – 6734; K. Nakamura, Y. Inoue, A. Ohno, *Tetrahedron Lett.* **1994**, *35*, 4375 – 4376; A. M. Snijder-Lambers, E. N. Vulfson, H. J. Doddema, *Recl. Trav. Chim. Pays-Bas* **1991**, *100*, 226 – 230; C. Gorrebeeck, M. Spanoghe, D. Lanens, G. L. Lemière, R. A. Dommisse, J. A. Lepoivre, F. C. Alderweireldt, *Recl. Trav. Chim. Pays-Bas* **1991**, *110*, 231 – 235; H. Weenen, R. L. G. M. Boog, W. Apeldoorn, *Proceedings of the international conference of Bioflavour 95* (Dijon, France), **1995**, pp. 375 – 380.

[19] Whole lyophilized cells lost 50 % of their activity in 20 % acetone after 40 h.

[20] This corresponds to a productivity of 18 mmol product $h^{-1}L^{-1}$ employing 0.6 g whole lyophilized cells or 0.8 g product g^{-1} cells. A literature value for g product g^{-1} cells of a comparable reaction is 0.01 g product g^{-1} cells.[8a]

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Compartmentalization of a Gadolinium Complex in the Apoferritin Cavity: A Route To Obtain High Relaxivity Contrast Agents for Magnetic Resonance Imaging**

Silvio Aime,* Luca Frullano, and Simonetta Geninatti Crich

The search for high relaxivities continues to be a central item in the development of paramagnetic contrast agents (CA) for magnetic resonance imaging (MRI).[1–3] This class of diagnostic agents is mainly represented by highly stable Gd^{III} chelates since the Gd^{III} ion, with its seven unpaired electrons, provides both a very high magnetic moment and a long electronic relaxation time. The observed relaxivity r_1 for the free Gd^{III} chelates may be considered as the sum of three contributions arising from the water molecule(s) directly coordinated to the paramagnetic ion (inner-sphere term),[1, 4] from water molecules hydrogen bonded at the surface of the complex (second coordination sphere term),[5] and from water molecules diffusing in the proximity of the complex (outer-sphere term).[1, 4] By using the theory of paramagnetic relaxation, the design of improved systems has been pursued through the optimization of the determinants of each contribution to the overall relaxivity. However, it was found early on that the observed relaxivity could only be explained if further contributions are operative when interaction with the surface of the protein occurs. For example, the binding of GdDOTP (H_8DOTP = 1,4,7,10-tetrakis(methylenephosphonic acid)-1,4,7,10-tetraazacyclododecane), which does not contain any inner-sphere water molecules, to human serum albumin (HSA) causes a relaxation enhancement of approximately five times.[1, 6] It was straightforward to assign such additional contributions to water molecules and exchangeable protons on the surface of the protein in the proximity of the binding site(s) of the paramagnetic complex. Clearly the microenvironment of the paramagnetic chelate is highly relevant to the determination of the relaxation-enhancing capability of a given Gd^{III} chelate. We deemed it of interest to explore new routes for the attainment of high relaxivities by exploiting such "protein surface" effects. The aim was to design systems containing a large proteic surface for interaction with the paramagnetic complex that would affect a large number of hydration water molecules and mobile protons, which, in turn, would exchange with the bulk solvent and act as amplifiers of the presence of the CA. One way to deal with a large surface is to design a spherical compartment in which the CA is trapped while the water molecules are free

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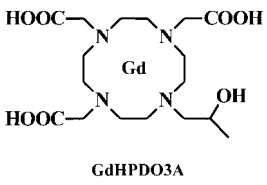
to move in and out. Herein we report the results obtained by using a naturally occurring host molecule: apoferritin.

Ferritin consists of a spherical protein shell (apoferritin) of 24 subunits with a central cavity which can accommodate up to 4500 iron atoms in the form of a ferric oxyhydroxide crystal.[7, 8] The central cavity is empty in apoferritin and has a diameter of 7–8 nm and an overall volume that can accommodate several contrast agent units. The access of external molecules to the inner cavity occurs through channels, formed at intersections of subunits, which are only 3–4 Å in diameter. Clearly water can diffuse through these channels, but molecules with the diameter (8–9 Å) of the currently used CA can not.[9, 10]

A procedure for trapping small molecules inside the interior of apoferritin was previously reported.[11] It consists first of the dissociation of apoferritin into subunits at pH 2 followed by its reforming at pH 7, thereby trapping the solution components within its interior (Figure 1). The loaded apoferritin is then separated from the molecules that are not trapped inside the shell by exhaustive dialysis. The best loading is obtained by using neutral molecules to reduce protein precipitation during the re-assembling process. Thus, among the available Gd^{III} chelates, GdHPDO3A, an extracellular contrast agent currently used in clinical practice, was chosen. The coordination cage is very tight ($\log K_f = 23.8$) and the complex displays one coordinated water molecule (at acidic and neutral pH values) in fast exchange with the bulk water.



GdHPDO3A

By following the procedure described above (Figure 1) it has been possible to entrap about 10 paramagnetic chelate units within the interior of apoferritin when the concentration of the protein and of GdHPDO3A were 1×10^{-5} M and 0.1 M, respectively. The stability of the inclusion compound appears quite good as the relaxation rate of an aqueous solution of apoferritin loaded with GdHPDO3A did not change over several hours at ambient temperature or over one week at 4 °C. Furthermore, control experiments were carried out on the native apoferritin to check whether the CA can diffuse through the channels or can bind to the protein surface. Solutions of apoferritin (1×10^{-5} M) were incubated with GdHPDO3A (0.1 M) at room temperature for two hours and then extensively dialyzed against 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffered saline. After this treatment, a relaxation rate of approximately 0.5 s^{-1} was measured, a value which is not too different from the diamagnetic contribution of the protein. This result indicates the absence of the paramagnetic complex in the solution and allows us to conclude that, under these conditions, it can not diffuse into the interior of the protein cavity nor bind to the outer surface.

The relaxivity shown by each Gd^{III} complex included in the apoferritin at 20 MHz and 298 K is very high (about 80 ± 5 $mM^{-1}s^{-1}$). This value is almost 20 times higher than the r_1 value of the free GdHPDO3A in water (4.2 $mM^{-1}s^{-1}$),[12] and represents the highest value reported so far for systems containing one coordinated water molecule. One may argue that the observed relaxation enhancement might result from the dissociation of Gd^{III} ions during the preparative work-up and its successive coordination to the protein. This possibility has been ruled out on the basis of the following observations: 1) the proton relaxation rate does not increase, as would have been expected if Gd^{III} ions were released, when an aqueous solution containing the apoferritin and GdHPDO3A is maintained at pH 2 for more than one hour; 2) the relaxation rate of a solution of $GdCl_3$ (1×10^{-4} M) at pH 2 does not change upon the addition of apoferritin ($1x10^{-5}$ M). This observation suggests that the carboxylic groups on the protein

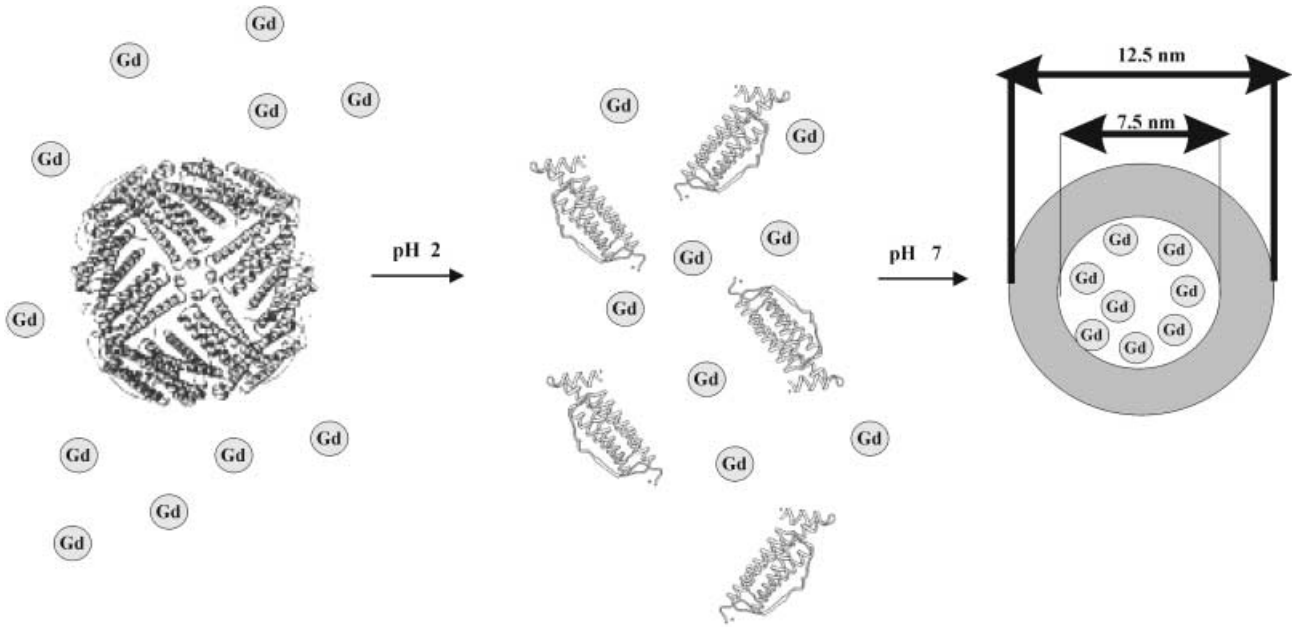


Figure 1. Schematic representation of the dissociation of apoferritin into subunits at pH 2, followed by its reforming at pH 7. In this way the solution components are trapped within its interior.

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surface are protonated at pH 2 and are, therefore, unable to coordinate the Gd^{3+} ions; 3) the high-resolution ^1H NMR spectrum of EuHPDO3A recorded at pH 2 is preserved upon its entrapment within the interior of apoferritin. The Eu^{III} complex was used because the long electronic relaxation time of the Gd^{III} ion induces severe line broadening of the resonances, which prevents the recording of high-resolution spectra. On the other hand, when the pH value of a solution containing GdHPDO3A-loaded apoferritin is lowered to pH 2, the T_1 value measured after one hour is that expected for aqueous solutions of GdHPDO3A at that concentration.

The $1/T_1$ nuclear magnetic relaxation dispersion (NMRD) profile (Figure 2), recorded over a wide range of Larmor frequencies (0.01 – 20 MHz), shows that the GdHPDO3A-loaded apoferritin system displays high relaxivities at any field

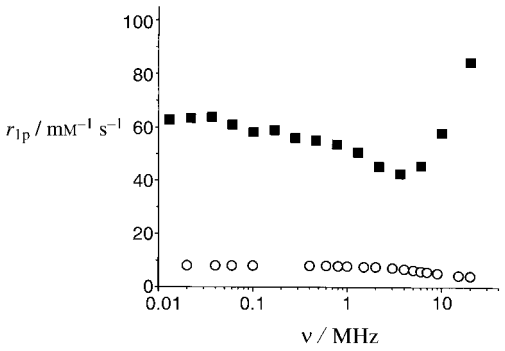

Figure 2. $1/T_1$ NMRD profiles (0.01 – 20 MHz) of a 1 mM solution of Gd-loaded apoferritin (■) and of GdHPDO3A (○) at pH 7 and 25 °C. ν = proton Larmor frequency, r_{1p} = relaxivity.

strength with a relaxivity hump at higher fields, as is expected for macromolecular paramagnetic adducts endowed with slow molecular reorientational times. Thus, the inclusion of several GdHPDO3A units per apoferritin cavity allows multiple interaction pathways to be established between the paramagnetic moieties, the water molecules (freely moving within the cavity or hydrogen bonded to the inner surface of the protein), and exchangeable protons, and thus yield an overall highly relaxing sink. The resulting relaxation scheme is basically the same as that occurring upon formation of protein/complex adducts, but it is now highly amplified by having a spherical surface.

Besides representing a good model for the attainment of high relaxivities, the Gd-loaded apoferritin system may have several novel applications. For example, it may be functionalized with suitable functionalities (antibody, Fab, other recognition synthons) to bind specific targeting molecules that act as reporters of given pathological states. Interestingly, an analogous approach was suggested some years ago by Hainfeld[13] for the delivery of ^{235}U atoms to tumors. Apoferritin was loaded with an average of about 800 atoms per molecule and then Fab antibody fragments were covalently attached to the uranium-loaded apoferritin. The resulting targeting system should provide significant advantages over boronated antibodies to meet the requirements for clinical neutron-capture therapy.

Experimental Section

GdHPDO3A was kindly provided by Bracco S.p.A. Apoferritin and all other chemicals were purchased from Sigma – Aldrich (St. Louis, MO, USA).

The loading of paramagnetic chelates in the apoferritin cavity was carried out by lowering the pH value of a 1×10^{-5} M protein solution (1 mL) to pH 2 using 1M HCl and maintaining this low pH value for about 15 min. After this time, when the dissociation of the apoferritin into its subunits was completed, the GdHPDO3A complex was added to the solution and the pH value was adjusted to 7.4 using 1M NaOH. The resulting solution was stirred at room temperature for about 2 h and then exhaustively dialyzed against HEPES-buffered saline (HEPES 5 mM, NaCl 0.1M, pH 7.4) to remove complexes not trapped inside the protein shell. The solution was then centrifuged. This operation is necessary to eliminate precipitated protein that may form during the experimental work-up. The final protein concentration was determined by the Lowry total protein micromethod assay (Sigma diagnostic). The residual GdHPDO3A concentrations were determined by inductively coupled plasma – atomic emission spectroscopy (ICP-AES). A single ICP-AES measurement represents the average of five replicates from the same sample (previously digested in nitric acid). The average value for the residual GdHPDO3A concentration was determined to be 0.0615 ± 0.005 mM from the results from four different preparations of GdHPDO3A-loaded apoferritin.

The longitudinal water proton relaxation rate was measured on a Stelar Spinmaster spectrometer (Stelar, Mede (PV) Italy) operating at 20 MHz by means of the standard inversion – recovery technique (16 experiments, 2 scans). A typical 90° pulse width was 3.5 μs and the reproducibility of the T_1 data was ± 0.5 %. The temperature was controlled with a Stelar VTC-91 air-flow heater equipped with a copper-constant thermocouple (uncertainty of ± 0.1 °C). The NMRD profile was measured over a magnetic field strength range of 0.00024 to 0.24 T (corresponding to 0.01 – 10 MHz) proton Larmor frequency) on a Spinmaster FFC, fast field cycling NMR relaxometer, (Stelar, Mede (PV) Italy) installed at the LIMA laboratory (Bioindustry park, Ivrea (TO), Italy). High-resolution ^1H spectra of the Eu complex were measured in D_2O with a JEOL EX-400 (400 MHz) spectrometer.

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