Functionalized Nanocontainers as Dual Magnetic and Optical Probes for Molecular Imaging Applications

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A dual-probe optical and magnetic imaging system has been synthesized out of nanometer-sized zeolite L crystals. The zeolite channels contain the optically emitting green pyronine molecules which can be used as fluorescent labels for optical imaging. The surface has been modified by a gadolinium complex which introduces a probe for magnetic resonance imaging (MRI). The combination of the excellent three-dimensional spatial resolution of MRI with the high sensitivity of optical imaging should lead to a system which overcomes the shortcomings of each individual technology. The system has been characterized by fluorescence microscopy and nuclear magnetic resonance dispersion (NMRD) spectroscopy. The results obtained show that the probes open up interesting possibilities for imaging based on multiple read outs.

Introduction

Molecular imaging is the new frontier of medical diagnostics.[1] It aims at visualizing molecules or molecular events occurring on the cellular level which are the "signatures" of a given disease. It represents an outstanding breakthrough in the field of diagnostics currently provided in the clinical settings in terms of early diagnoses and in the efficient monitoring of therapeutic treatments. Among the available imaging modalities much attention has been devoted to magnetic resonance imaging, MRI, thanks to the superb anatomical resolution that can be attained. However MRI is a relatively insensitive technique and, therefore, requires large amounts of proton relaxation agents to affect the contrast which naturally occurs between different anatomical regions. To get a 50% contrast enhancement in a magnetic resonance, MR, image with the currently available commercial contrast agents a local concentration of ca. 50 μM, i.e., a number of ca. 10^9 Gd centers per cell, must be reached.[2]

Thus, the development of MR molecular imaging applications strongly relies on the search of tools that deliver a large number of imaging reporting units at the targeting site. For instance, Lanza and co-workers reported the visualization of integrin receptors (whose overexpression characterizes the tumor endothelium) by using functionalized microemulsion particles loaded with 10^5 Gd(III) complexes.[3,4]

There is an active search for nanosized carriers that can suitably target the diseased cells with their payload of imaging reporters. Much attention is currently devoted to phospholipid-based systems such as liposomes[5,6] and micelles.[7] These systems are readily taken up by cells as the phospholipids are the main constituents of cell's membrane. Thus, it is deemed of interest to design rigid, nanosized particles whose external surface is fully decorated by hydrophilic Gd(III) chelates to tackle applications not feasible with phospholipid-based systems. Recent examples are based on nanomaterials such as silica particles[8–10] and quantum-dots[11] coated with paramagnetic gadolinium complexes. Moreover, the particles may act themselves as carriers of drugs[12] or additional imaging probes to add further physiological information to the high spatial resolution delineated by MRI contrast agents.

This work presents the synthesis and spectroscopic characterization of novel nanozeolite L hybrid materials which can act as a dual optical and magnetic probe. The components responsible for imaging can be entrapped inside the zeolite

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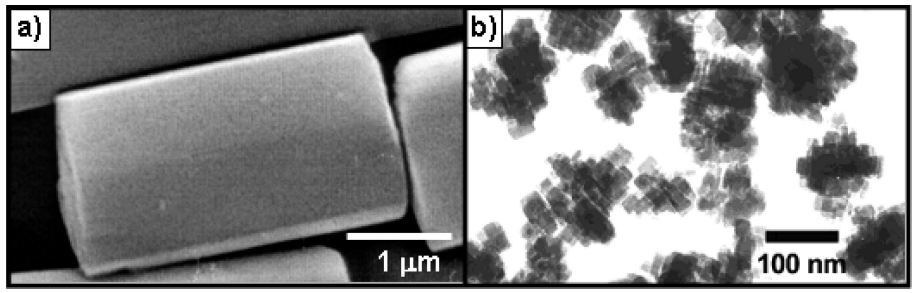

Figure 1. Zeolite L framework and morphology: (a) scanning electron microscopy (SEM) images of a single zeolite L crystal of about 3 μm length; (b) transmission electron microscopy (TEM) image of zeolite crystals with a length of about 30 nm.

channels[13] or are at the surface of the zeolites.[14] On the particle coat we describe the functionalization with either the red emitting Eu−DOTA (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) or MRI contrast agent Gd−DOTA moiety. Ln−DOTA chelates have been chosen due to their high stability and therefore low toxicity. The toxicity of Gd(III) in its free ionic form can be overcome upon complexation to high binding constant ligands such as DOTA and DTPA.[15,16] Also, the synthetic versatility of changing the metal ion makes these systems very appealing materials, allowing for the rich physical properties of all the lanthanides to be exploited. The inorganic scaffolds, to which the Ln−DOTA is coupled, are zeolite L crystals. Zeolite L is a biocompatible cylindrically shaped crystalline aluminosilicate in which corner-sharing SiO_4 and AlO_4^- tetrahedra create a one-dimensional channel array. Such channels can be filled with fluorescent dyes or other species smaller than the channel entrance (0.71 nm).[13] Since these systems are synthesized from a bottom-up hydrothermal technique, their size and morphology may be strictly controlled depending on the reaction conditions.[17] Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have shown the systems studied herein to have dimensions of 3 μm for the big crystals and 30 nm for the individual small crystals (see Figure 1).

Results and Discussion

On the basis of the observation that a large number of labels implemented on a rigid slowly tumbling skeleton could have interesting properties for MRI, 30 nm zeolite L crystals have been used for this purpose. Furthermore, we have already demonstrated that molecular dyes can be entrapped inside the channels and that using the appropriate channel functionalization dioxygen cannot diffuse inside resulting in an enhanced stability of the guest molecules.[18] We have therefore designed a dual probe in which the known filled zeolite can be functionalized on the entire surface using chelating ligands able to strongly bind luminescent or paramagnetic ions. In order to produce a prototype system which can be characterized by fluorescence microscopy and to prove that the surface can be fully functionalized, empty zeolite L crystals with a length of about 3 μm have been used. These larger systems have identical structural and chemical properties to those of 30 nm length[19] but are, of course, easy to characterize with optical techniques.

The first step in the reaction involves filling of the zeolite channels with the molecular dyes, pyronine molecules, which was achieved upon exchanging the potassium cations. Then functionalization of the whole surface of the zeolites, with reactive groups, resulted in the desired chelating systems. This has been achieved employing 3-aminopropyl triethoxysilane (APES), which is known to react with free Si−OH groups on the zeolite surface[20] leading to a good coverage of the crystal. In the next step commercially available DOTA−NHS was coupled to the amino functionalized zeolite surface. Finally, the DOTA chelates on the whole surface of the zeolite L crystals were complexed with Eu(III) or Gd(III) in water at neutral pH. The samples were repeatedly washed in order to remove uncoordinated Eu(III) or Gd(III) ions and unbound complexes. A simplified scheme of the synthesis is depicted in Figure 2.

The samples containing the luminescent lanthanide Eu(III) have been characterized by fluorescence microscopy and emission spectroscopy, in order to ascertain whether a defined covalent bond was present between the chelating DOTA group and the ion. The intense linelike red emission of the europium complex comes from transitions within the partially filled 4f orbitals[21] providing us with a characteristic handle for this analysis. Because of the low sensitizing ability of DOTA resulting from its low absorbance efficiency, and consequently low europium emission, an external sensitizer, 4-bromophenyl-6-carboxy-2,2′-bipyridine,[22] available in our laboratories was added in order to absorb the light and sensitize the europium emission (Figure 3a). Since the DOTA ligand occupies eight coordination positions of the ion there is a coordination site available for at least one water molecule. The carboxylate group of the sensitizer, which is strongly bound to the lanthanide, will take the remaining

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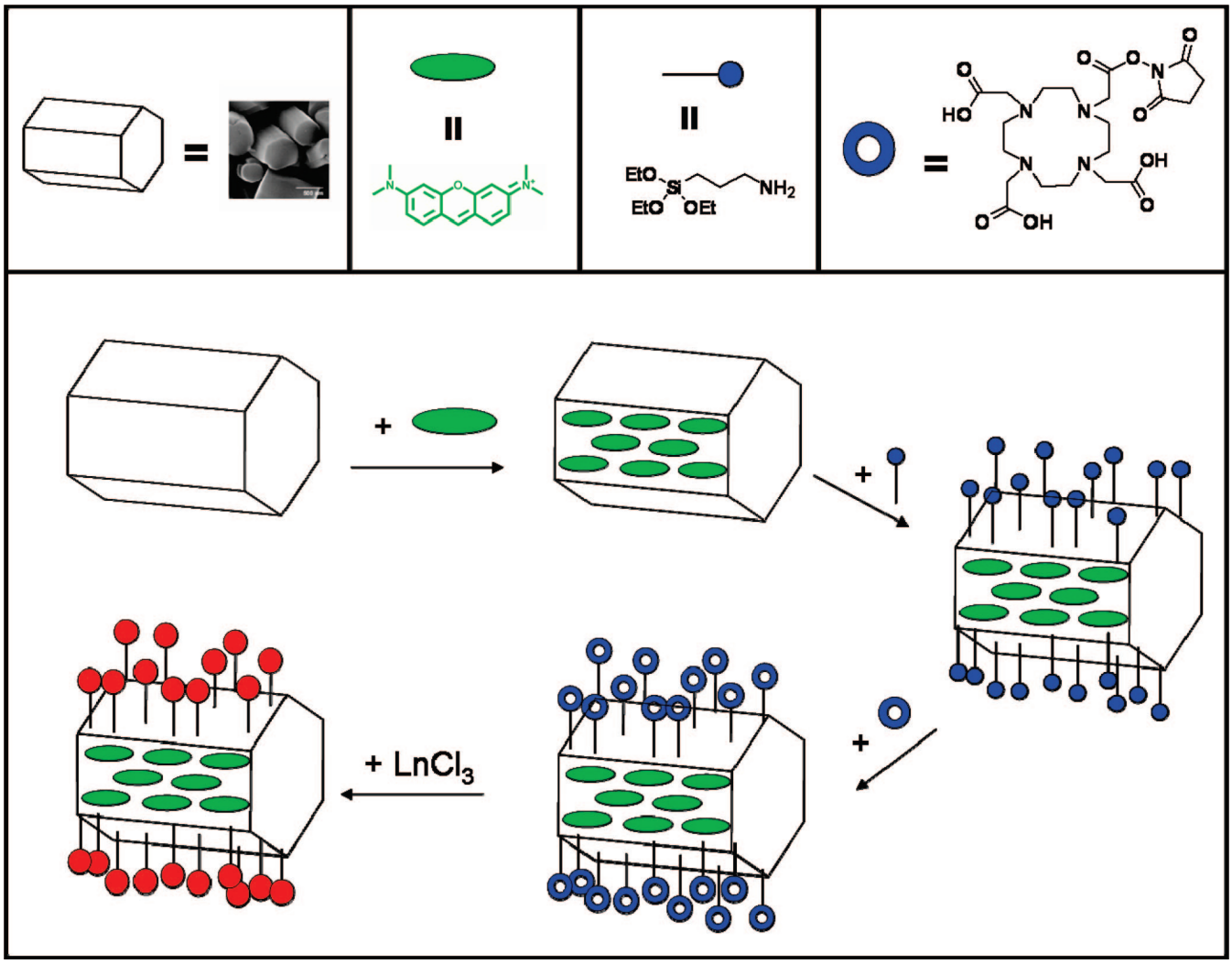

Figure 2. Synthesis of Ln−DOTA functionalized zeolite L crystals. In the first step pyronine molecules are introduced into the channels via ion exchange. In the second step a linker (APES) is bound to the entire surface of the zeolite L crystals. In the third step the chelating ligand (DOTA) is covalently attached to the back side of the linker. The final step involves complexation of the lanthanide ions to the surface of the zeolite L crystals. The lanthanide DOTA complexes are represented as red balls.

free coordination position.[23] In the fluorescence microscopy images, the presence of the Eu−DOTA covering the whole surface of the zeolite L crystals of 2 μm length can be seen (Figure 3b). The absence of a europium signal in the washing solution (Figure 3a) proves that the Eu ions are strongly bound to the surface of the zeolite, through the DOTA group, and are not leaching. A model Eu−DOTA compound with the same sensitizer was prepared and measured in the same conditions. It showed the characteristic europium emission bands, with identical features of the complex anchored to the zeolite surface (see Figure 3a).

After the successful characterization of the empty large zeolite the synthesis was repeated using zeolite L crystals of only 30 nm lengths that have been filled with pyronine molecules via ion exchange. The synthesis was performed as described above. For characterization, emission spectroscopy was used, resulting in emission spectra that are showing the europium as well as the pyronine emission in an EtOH suspension (see Figure 3c).

The 30 nm Gd−DOTA zeolite crystals were synthesized, using identical conditions described for the Eu(III) complexes, and have been characterized using NMR spectroscopy. The size distribution of the synthesized Gd(III) nanozeolites was analyzed with dynamic light scattering and shows the 30 nm crystals to be aggregated in groups of 4−5 particles with a diameter of about 150−200 nm (see the Supporting Information) in water. This is similar to that reported in the literature for the free zeolites.[24] A rough estimate of the number of Gd−DOTA on the 30 nm zeolite has been obtained by calculating the number of zeolite in 10 mg of sample with a known Gd(III) concentration of 0.538 mM (see the Experimental Section). The calculation resulted in about 370 units Gd−DOTA per zeolite crystal.

Relaxometric Characterization of the Gd Functionalized Zeolites. The relaxivity of the zeolite clusters per Gd(III) ion has been measured at 25 °C and at 20 MHz by measuring the relaxation rate of water protons upon increasing the concentration of the Gd−DOTA functionalized

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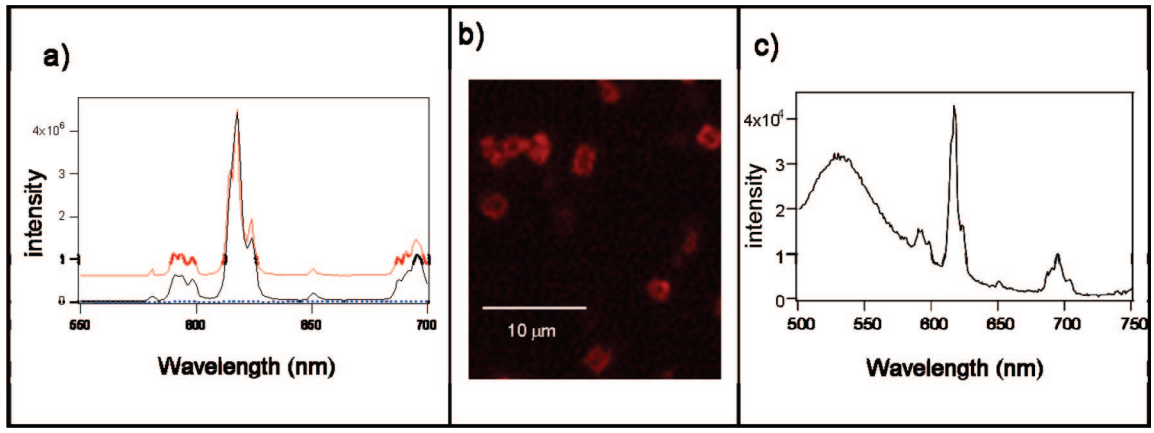

Figure 3. Characterization of the systems: (a) emission spectrum of the Eu−DOTA reference compound (red line, spectrum offset for clarity), emission spectrum of Eu−DOTA functionalized zeolite L 3 μm (black line), and of the last washing solution (dotted line). All the spectra were taken in EtOH suspension exciting at 300 nm; (b) microscope image of the 1−2 μm Eu−DOTA functionalized zeolite; (c) emission spectrum of Eu−DOTA functionalized zeolite L 30 nm filled with pyronine, excited at 340 nm in EtOH suspension.

zeolite L in water suspension. A relaxivity value of 30 mM^{-1} s^{-1} (referred to the Gd concentration) has been obtained. It is more than 6 times larger than the value of the parent Gd−DOTA complex, and it endows the zeolite L particle with an overall relaxivity of ca. 11 000 mM^{-1} s^{-1}. By keeping in mind that the MR visualization of a cell requires a number (N) of Gd(III) chelates given by $N = 10^9$/relaxivity, the Gd−DOTA loaded zeolite L systems should be able to detect cells when 10^4-10^5 particles have accumulated on the target cells.

To get more insight into the determinants of the observed relaxivity, the water proton relaxation rates have been measured over an extended range of frequencies (0.01−80 MHz, at pH = 7, and 25 and 37 °C, Figure 4A). The prominent characteristic of these nuclear magnetic resonance dispersion (NMRD) profiles is represented by the relaxivity peak centered at 20 MHz typical of Gd(III) chelates bound to slowly moving systems. The relaxivity peak is more pronounced in the higher temperature profile and suggests a quenching effect due to the long exchange lifetime of the coordinated water. This feature has been confirmed by measuring the relaxivity as function of the temperature at 20 MHz (Figure 4B). The observed behavior is fully consistent with the occurrence of long exchange lifetimes of the coordinated water molecule, and this is expected for the neutral Gd−DOTA monoamide complexes.[25,26] Further considerations may be done looking at the pH dependency of the relaxivity of the system (Figure 4C), as a relaxivity variation centered around pH = 6 is clearly detected. Tentatively, one may surmise that the observed behavior can be associated to the protonation/deprotonation step of unreacted amino groups of the triethoxysilane moieties on the surface of zeolite L.

It has been reported[27,28] that the dissociation constants of acidic and basic groups grafted on silica surfaces often differ from those of their monomer analogues in solution. So it may be reasonable to think that in the zeolite grafted system the apparent pK value of the APES amino group may be

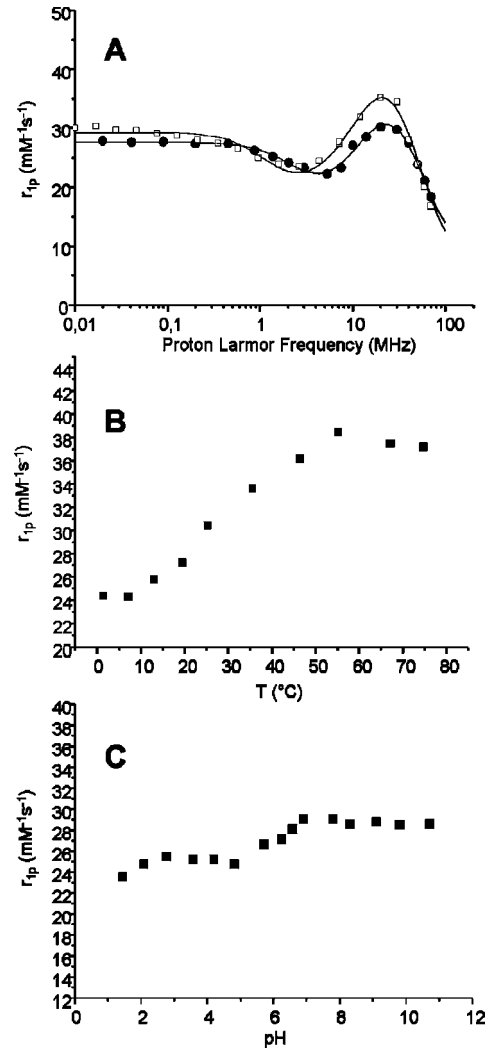

Figure 4. (A) 1/T1 NMRD profiles of a 1 mM solution of Gd−DOTA−zeolite L measured at 25 °C (●) and 37 °C (□); (B) temperature dependence of water proton relaxivity of Gd−DOTA−zeolite L measured at 20 MHz (0.47 T) and neutral pH; (C) pH dependence of water proton relaxivity of Gd−DOTA−zeolite L measured at 20 MHz (0.47 T) and 25 °C.

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around 6, i.e., 4−5 units lower than the bulk solution values. The close proximity between unreacted amino groups and the lanthanide chelates may prompt the formation of H-bonding interactions involving oxygen atoms of the coordination cage of the lanthanide(III) ion. The formation of H-bonds is stronger with protonated (pH < 5) than with deprotonated (pH > 7) amino groups and may cause a release of negative charge from the chelating moiety with a resulting increase of the residual positive charge at the metal center. This, in turn, yields to an increased enthalpy of the Gd−water bond and an increase of the exchange lifetime. Thus, the lower relaxivity measured at pH < 5 should be ascribed to a further lengthening of the exchange lifetime of the coordinated water molecule. Quantitative analysis of the NMRD profiles at neutral pH shows that the exchange lifetime (τ_M) passes from ca. 638 to ca. 437 ns upon increasing the temperature from 25 to 37 °C. The NMRD fitting yields a τ_R value of ca. 1.2×10^8 s at 25 °C which is slightly shorter than expected and outlines the occurrence of a relative mobility along the linker moiety. The other parameters that have been obtained from the analysis of the NMRD profiles are fully consistent with those expected for Gd−DOTA monoamide complexes.[29] The combination of visible emitting dye filled zeolite with gadolinium surface functionalization results in a dual optical and magnetic probe which may have biomedical application with the appropriate near-infrared dyes.

Conclusions

In conclusion, we have shown that biocompatible zeolite L crystals can be functionalized on the surface to obtain highly densely packed Gd(III)− or Eu(III)−DOTA complexes. Furthermore, we have exploited the use of the channels for entrapping molecular dyes which could be used for optical imaging. The combination of the fluorescent dyes inside the channels and the surface coverage with a paramagnetic species leads to a composite material which combines optical and magnetic properties for dual imaging. Due to the high number of channels of the 30 nm zeolites, several hundred molecules of different nature can be permanently or temporarily encapsulated within the crystal system. We are currently exploiting the possibility to use other types of probes for combining different techniques. We are also working on different surfactants to reduce the crystal aggregations and therefore to have nanocontainers of only 30 nm. Furthermore, due to the small size of the nanomaterials, in vivo application are envisaged, and work is in progress toward this direction.

Experimental Section

Photophysics. Steady-state emission spectra were recorded on a Spex Fluorolog 1681 equipped with a Xe arc lamp, a Hamamatsu R928 photomultiplier tube, and double excitation and emission monochromators. Emission spectra were corrected for source intensity and detector response by standard correction curves. The emission was detected at a right angle. Fluorescence microscopy images were taken of zeolite L crystals having a length of about 3 μm. Fluorescence microscopy was performed with an Olympus BX 41 microscope equipped with an Hg high-pressure lamp and the appropriate filters. Scanning electron microscopy was performed with a LEO 1530 VP. The TEM image of the nanozeolites is courteously given by Professor G. Calzaferri, University of Bern.

Water Proton Relaxivity Measurements. The longitudinal water proton relaxation rate was measured by using a Stelar Spinmaster (Stelar, Mede, Pavia, Italy) spectrometer operating at 20 MHz, by the standard inversion−recovery technique. The temperature was controlled with a Stelar VTC-91 air-flow heater equipped with a copper constantan thermocouple (uncertainty 0.1 °C). The proton 1/T1 NMRD profiles were measured over a continuum of magnetic field strength from 0.00024 to 0.47 T (corresponding to 0.01−20 MHz proton Larmor frequency) on a Stelar field-cycling relaxometer. The relaxometer works under complete computer control with an absolute uncertainty in 1/T1 of ±1%. Data points from 0.47 T (20 MHz) to 1.7 T (70 MHz) were collected on a Stelar Spinmaster spectrometer working at variable field. The Gd(III) concentration of Gd−DOTA−zeolite L solutions, for the relaxometric characterization, was determined by mineralizing a given quantity of sample solution by the addition of 37% HCl at 120 °C overnight: from the measure of the observed relaxation rate ($R1^{obs}$) of the acidic solution, knowing the relaxivity (r_{1p}) of Gd(III) aquaion in acidic conditions (13.5 mM^{-1} s^{-1}), it was possible to calculate the exact Gd(III) concentration (this method was calibrated using standard ICP solutions, and the accuracy was determined to be 1%).

Synthesis. The ligand DOTA NHS was obtained from Macrocyclics (U.S.A.) and used as received. All other reagents were obtained from Sigma-Aldrich and used as received. Pure zeolite L crystals were synthesized and characterized as described in ref 18. The potassium exchanged form was used for all experiments.

Loading of Pyronine. An amount of 40 mg of zeolite L crystals was suspended in 1.5 mL of H_2O, and 0.5 mL of an aqueous pyronine solution (0.5 M) was added. After stirring for 24 h the sample was repeatedly washed by centrifugation until the overstanding solution did not show any pyronine emission.

Amino Functionalization of Zeolite L 3 μm. An amount of 40 mg of zeolite L crystals, 1 μm, was suspended in dry toluene (4 mL), and 30 μL of APES was added. The suspension was sonicated for 30 s and stirred for 4 h at 105 °C. After cooling down to room temperature, the sample was centrifuged and the overstanding solution was pipetted off. The sample was washed twice with toluene.

Amino Functionalization of Zeolite L 30 nm. An amount of 40 mg of zeolite L crystals, 30 nm, was suspended in dry toluene (2 mL), and 30 μL of APES was added. The suspension was sonicated for 30 s and stirred for 4 h at 105 °C. After cooling down to room temperature, Et_2O (6 mL) was added and the sample was centrifuged for 30 min at 4000 rpm. After pipetting the overstanding solution off, the sample was washed with MeOH once and then dried in the oven at 75 °C overnight.

Reaction of NH_2 Groups on the Surface of Zeolite L 3 μm with DOTA−NHS. Amounts of 4 mg of zeolite L crystals and 6 mg (0.0072 mmol) of DOTA−NHS were suspended in 1 mL of dry DMF, and 16 μL of DIPEA was added. After stirring overnight, the sample was transferred into a round-bottom flask and the solvent was removed on the rotary evaporator. Afterward, the sample was transferred back into a centrifuge tube, using H_2O (ca. 2 mL) as solvent.

Reaction of NH_2 Groups on the Surface of Zeolite L 30 nm with DOTA−NHS. Amounts of 30 mg of zeolite L crystals and 94 mg (0.113 mmol) of DOTA−NHS were suspended in 1 mL of

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dry DMF, and 150 μL of DIPEA was added. After stirring overnight the crystals became soluble. After addition of 5 mL of Et_2O the sample precipitated and was centrifuged. The sample was washed with Et_2O twice and dried in the oven overnight.

Complexation of Eu(III) Ions with DOTA at the Zeolite L Crystals. To the DOTA functionalized zeolite L samples in H_2O 5.2 mg of $EuCl_3 \cdot 6H_2O$ (0.014 mmol) was added. After addition of 3 drops of 1 N NaOH the mixture was stirred overnight. After centrifugation and removal of the overstanding solution the sample was washed 10 times with EtOH until the washing solution did not show a europium signal in the emission spectroscopy after addition of a sensitizer.

Complexation of Gd(III) Ions with DOTA at the Zeolite L Crystals and Coverage Calculation. The DOTA functionalized zeolite L sample was suspended in 2 mL of H_2O, and 54.9 mg of $GdCl_3 \cdot 6H_2O$ (0.148 mmol) was added. After addition of a few drops of 1 N NaOH the mixture was stirred overnight. After centrifugation and removal of the overstanding solution the sample was washed with EtOH and dried in the oven at 75 °C. Eventual excess of uncomplexed Gd(III) ions has been removed by 24 h dialysis of the Gd−DOTA−zeolite L system against water.

Gd−DOTA−zeolite was dissolved in distilled water (10 mg/mL), and the Gd(III) concentration was determined to be 0.538 mM. So the number of Gd(III) for 10 mg of sample is (0.538 mmol/L)(1 L/1000 mL)(6.023×10^{20}/mmol) = 3.24×10^{17}.

The weight of one zeolite crystal (length 30 nm, diameter 15 nm) is $[(0.267)(15^2)(30)(2880)]/[(0.75)(6.023 \times 10^{23})] = 1.15 \times 10^{-17}$ g.

The number of zeolite crystals in 10 mg is (0.01 g)/(1.15×10^{-17} g) = 8.70×10^{14}.

The number of Gd(III) per zeolite crystal is then (3.24×10^{17})/(8.70×10^{14}) = 372.

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Supporting Information Available: Additional figures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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