

**CONJUGATES OF LANTHANIDE CHELATES
AND PHENYLBORONATES FOR MOLECULAR
RECOGNITION OF TUMORS**

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CONJUGATES OF LANTHANIDE CHELATES AND PHENYLBORONATES FOR MOLECULAR RECOGNITION OF TUMORS

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In the memory of my grandfather

Stepan Grigorian

To my family

...I know what your trouble is. You are too serious. A clever face is not as yet an indication of cleverness. All silly things on Earth are done with a clever look on one's face. Smile, gentlemen! Smile!

Baron von Münchhausen

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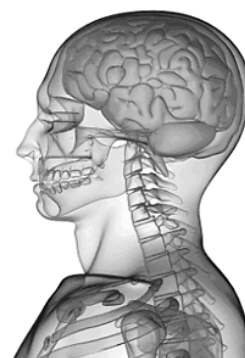
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IMAGING IN MEDICINE

CURRENT STATE • FUTURE

PERSPECTIVES

1



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MEDICAL DIAGNOSIS AND IMAGING TECHNIQUES

The beginning of the evolution of medical diagnosis can be fairly attributed to the Greek physician Hippocrates (400 BC) who proposed the concept of disease.¹ Qualitative approaches in the diagnosis predominated in medicine for a long time and only with the invention of the first thermometer and the pulse monitor by the Italian physician Santorius (1625) the quantitative aspect was brought into medical research. The introduction of the first light microscope by the Dutch naturalist Antoni van Leeuwenhoek (1725) entailed the discovery of many micro-biological substances and certainly opened a new era in medical science. But the most important milestone in the history of imaging science, beyond questioning, is the discovery of X-rays by the German physicist W. C. Röntgen in 1895,^{2,3} which can be considered as the beginning of the development of radiology.

The progress in medical sciences achieved during the last few decades, to a large extent, is due to the rapid developments in biomedical imaging that allow clinicians to examine the human body by combining information on anatomy, physiology and molecular events. The latter type of information is currently known as molecular imaging, which is defined as non-invasive *in vivo* characterization and measurement of biologic events at cellular and molecular level involved in normal and pathologic processes.⁴ The knowledge obtained in this research field will accelerate drug discovery, improve early detection of diseases as well as their prevention and therapy. Recently, the number of imaging systems and their applications has increased dramatically. Many comprehensive reviews on available imaging techniques appeared recently. Understanding of the advantages and limitations of each of them is the key towards the development of a highly complementary diagnostic system.

The multidisciplinary character of molecular imaging and the large variety of imaging modalities demand a good communication between different disciplines such as medicine, biology, chemistry, physics and computer sciences. In the introduction to this thesis a short overview of the imaging methods is given and the perspectives on the future developments of imaging probes and contrast media in combination with therapy are evaluated through the eyes of a chemist.

X-ray, Computed Tomography (CT)

More than 100 years after its discovery, X-ray imaging is still one of the fastest and easiest ways for clinicians to view the internal organs and structures of the body such as the gastrointestinal system and the skeleton. It can also be applied for high resolution diagnostic imaging of the breasts (mammography) and for imaging of thoracic activity including that of lungs and heart. The development of this conventional imaging technique has benefited from the progresses in digital technologies, optimization of the detection and improvement of image processing. Furthermore, the importance of the discovery of electromagnetic radiation is reflected in the rapid development of other derived imaging techniques such as Computed Tomography (CT), also known as CAT scanning (Computerized Axial Tomography). Similar to X-ray imaging, in CT the X-ray radiation passes through the body, where it is attenuated by the tissues resulting in a matrix of profiles of X-ray beams with different strength, which can provide a three-dimensional image. Invented by the British engineer G. Hounsfield, CT has developed since the early 1970s to one of the most powerful imaging techniques due to its unique ability to provide detailed information on almost all organs in the body, including soft tissues, bones and blood vessels, in one quick examination.

Ultrasound (US)

Ultrasound technology has started in the early 1940s and the first results on its medical application in brain investigation were already published in 1942.⁵ Thirty years later US became widely available in medicine, mainly thanks to the low costs of the equipment required. It is the most portable of all imaging systems and can be transported to the patient's bedside and the emergency room. Examinations are performed in real time and are able to visualize motions (>30 frames per second) with an in-plane resolution down to 40 μm and at 5 mm depth. The general disadvantages of this conventional imaging technique are well-known. First, the ultrasound propagation is attenuated by tissues in the body such as fat and bones, and by air in the lungs and second, image artifacts are a big problem. However, recent developments in hardware and targeted contrast agents encourage scientists to apply US for imaging of molecular processes *in vivo*.⁶

Nuclear Imaging (PET and SPECT)

The discovery of natural radionuclides in 1886 by the French physicist H. Becquerel has opened a new era in science. However, it took more than 50 years until radionuclides started to be applied in medicine for treatment, metabolic tracer studies, and later for nuclear imaging. A spectacular breakthrough in the development of nuclear medicine occurred in 1946 by the successful application of radioactive iodine for the treatment of thyroid cancer,⁷ whereas a more widespread medical application of radionuclides became possible after the introduction of a dosimetric approach.⁸ Today, nuclear imaging represents an effective diagnostic tool applied for visualization of almost all tissues in the body. The modern PET (Positron Emission Tomography) and SPECT (Single Photon Emission Computerized Tomography) are non-invasive nuclear imaging techniques based on tomographic reconstruction methods to obtain a three-dimensional representation of the distribution of radiolabels *in vivo*.⁹ In fact, the detectable radiolabel is used to indicate the fate of its unlabeled analogue without disturbing physiological processes.¹⁰ These labels are radioactive isotopes incorporated in PET or SPECT radiopharmaceuticals, administered at tracer doses and detected outside the body by photon-sensitive detectors. The differences in the physical properties of the radiopharmaceuticals applied in these two radio-imaging techniques determine their characteristics such as spatial resolution, specificity, required examination time, and sensitivity. In Table 1.1 a selection of commonly applied radionuclides is listed.

Table 1.1. Examples of PET and SPECT radionuclides.⁹

SPECT									
Nucleus	^{99m} Tc	¹²³ I	¹³¹ I	²⁰¹ Tl	⁶⁷ Ga	¹¹¹ In	¹³³ Xe	¹⁶⁶ Ho	¹⁷⁷ Lu ^a
T _{1/2}	6.0h	13.2h	8.0d	73h	78.3h	2.83d	5.3d	26h	6.7d
PET									
Nucleus	¹¹ C	¹³ N	⁶⁸ Ga	⁶⁴ Cu	¹⁵ O	¹⁸ F	⁸² Rb	¹⁵² Tb	¹⁶⁶ Ho ^a
T _{1/2}	20min	10min	68min	12.6h	122sec	110min	76sec	18.5h	26.6h

^{a)} applicable for therapy

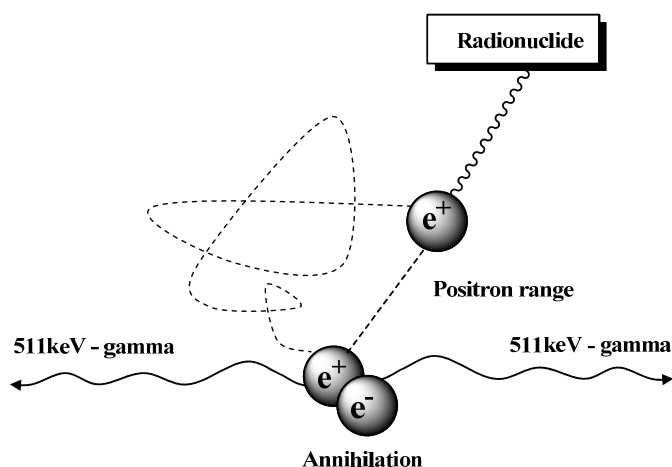


Figure 1.1. Schematic representation of PET imaging. The decay of the radionuclide is accompanied by the emission of a positron.

For PET, imaging agents with a short-living positron emitting radionuclide are applied. The emitted protons encounter electrons in the body and then annihilate. This annihilation produces two 511 keV γ -rays emitted simultaneously in opposite directions, which are detected by an array of surrounding detectors. The acquisition of a large number of coinciding annihilations provides information of the numerous events that can be translated into an image with information on the spatial distribution of the radioactivity.

Typically, two physics-related factors determine the spatial resolution of PET imaging: the positron range and the photon non-colinearity. The relatively short half-life time is a limiting factor that requires the presence of organic radiochemistry expertise and of an advanced infrastructure in the clinic. However, a useful feature of PET isotopes is their suitability to be incorporated into synthetic drugs by e.g. methylation or nucleophilic aromatic substitution reactions. It opens a lot of opportunities for PET to become a leading tool in the preclinical evaluation of novel drugs.

SPECT utilizes single photons with energies in the range of 140 keV. This single photon emitted from a radioactive isotope, travels through tissues and is detected by a gamma-radiation sensitive detector. Because only single photons are emitted from SPECT

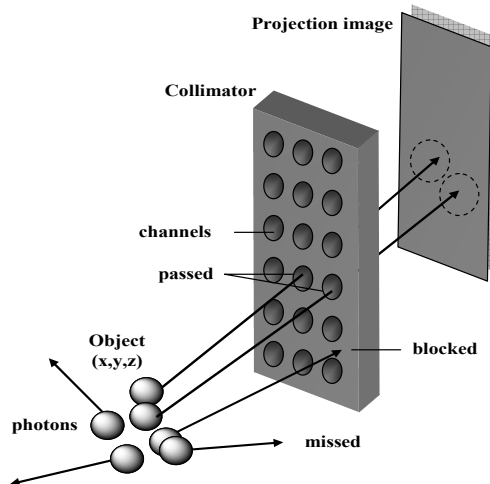


Figure 1.2. Schematic representation of SPECT imaging. A collimator forms an image of the object by letting through only rays in a particular direction.

radionuclides, localization of the radiation source is not possible without a collimator, a thick sheet of a heavy metal, such as lead, perforated by long thin channels (Figure 1.2). This unit physically absorbs the gamma rays except those emitted in a certain direction. Consequently, the spatial resolution possible with SPECT is dependent on the technological optimization of the collimator rather than on physical limitations of the method.¹¹ The sensitivity of SPECT is two or three orders of magnitude lower than in PET due to the need a collimator.¹² The radiopharmaceuticals applied for SPECT are relatively easy to produce since they possess a longer half-life time than PET agents. Therefore, SPECT is useful for imaging of biological processes *in vivo* that occur over hours or even days. Furthermore, it is possible to image two or more radiolabels simultaneously with SPECT using energy discrimination. One of the examples of such multiple probe imaging is a simultaneous study of myocardial perfusion and tissue viability using ^{99m}Tc and ^{201}Tl labeled probes.¹³

Optical imaging (OI)

Optical imaging is probably the most rapidly developing area in molecular imaging due to its high sensitivity and to technologies that make it possible to address a variety of questions in molecular oncology. It is based on the traveling of photons through tissues and the ability of certain molecules to absorb and emit light at particular wavelengths. This principle has been used in fluorescence microscopy since the 1850s and the first medical application was demonstrated already in 1924.¹⁴ Optical imaging of breast tissue dates back to 1929¹⁵ and was revived in the 1980s and 1990s when more powerful light sources and monitoring systems became available. Today, the progress in computer technology allows three-dimensional tomographic reconstruction of the images, image fusion and multi-channel imaging, which make this technique applicable for routine clinical investigations. The greatest challenge in OI technology is to overcome its limitation due to the relatively low depth of penetration in tissues. In fluorescence molecular tomography (FMT), a subject is rotated within an array of emitter/receiver devices. The recorded fluorescence is spatially encoded and therefore can be reconstructed tomographically. This approach allows high sensitivity (pM) and high resolution (1-2 mm) imaging, with a penetration depth up to 7-14 cm when appropriate fluorochromes are used.¹⁶ Furthermore, an optical modality has successfully been utilized in photodynamic therapy (PDT). This application involves the administration of a tumor localizing photosensitizer and its activation with light of a specific wave length. Then, tissue oxygen triggers a series of photochemical processes leading to cancer cell damage.¹⁷ In drug development, OI is an indispensable tool for the evaluation of the pharmacodynamics of a drug prior of its application in therapy.¹⁸

Magnetic Resonance Imaging (MRI)

In the 1940s, Bloch and Purcell discovered the ability of some nuclei to receive and transmit electromagnetic energy when placed in an external magnetic field. These properties were used in the concept of Magnetic Resonance Imaging (MRI) that was proposed 30 years later.¹⁹ The first prototypes of clinical scanners were tested already in the 1980s and today, MRI is applied in radiology as a non-invasive clinical imaging technique

on a large scale. The uniqueness of the method is its ability to produce high resolution 2D- and 3D-images of organs and soft tissues by means of NMR (Nuclear Magnetic Resonance) principles rather than by using harmful ionizing radiation. Generally, the strong NMR signal of tissue protons originating from water or fat is used for the creation of MR images. Clinical and preclinical MRI scanners have magnets that create an external magnetic field in the range of 1.5 – 9.4 and 11.7 T (Tesla) respectively, which corresponds to ^1H Larmor frequencies of 64 – 400 and 500 MHz. Furthermore, the scanner is equipped with small gradient coils that generate additional magnetic field gradients in the range of 20-100 mT/m along different axes. These gradients are employed for slice selection and phase- and frequency encoding. In this way protons at different locations respond to the gradients with resonances at the different chemical shifts. The set of resulting resonances can be translated into a three dimensional image. Basically, an MR image is a three dimensional representation of the intensities of the ^1H resonance of water in the body. The relaxation times can be exploited by the use of particular pulse sequences in combination with particular settings of the delay times.²⁰ In this way, for example T_1 - or T_2 -weighted images can be produced. The relaxation times of protons in tissues are strongly dependent on their physiological environment, and can drastically alter upon progressing of pathologies. This may be reflected in the MRI contrast. The contrast of MRI images can often be dramatically improved by the administration of a paramagnetic contrast agent (CA), which enhances locally the relaxation rates of the water protons. At present, about 40% of the MRI examinations are performed after the administration of a CA.

Since the intensity of an MRI signal is also strongly dependent on the strength of the external magnetic field, there is a tendency to move towards high field MRI instrumentation. For example, brain images obtained with an ultra high field MRI (7 T) have been published recently, which show many fine structures of the midbrain with a clarity and resolution never observed before.²¹ The relative low sensitivity of MRI remains its biggest drawback. Thus, besides technological improvement, current strategies of MRI research are focused on the development of exogenous contrast agents that shorten relaxation times and increase the contrast (see below).

Each of the available imaging methods has specific limitations that are extensively discussed in recent reviews.^{11,13,22,23} Table 1.2 lists the leading imaging technologies and compares their relative strengths and drawbacks.

Table 1.2. Evaluation of available imaging techniques.

Technique	Imaging Source	Resolution	Typical agents	Sensitivity CAs	Information	Price of instrument (€)
CT	X-rays	50 μm^a 0.3 mm ^b	Iodinated molecules	10^{-3} M	Anatomical, Physiological	~70.000
	Pros: No depth limit. Cons: Ionizing radiation.					
PET	β^- -radiation	1-2 mm ^a 5-7 mm ^b	^{18}F , ^{11}C labeled molecules ^c	10^{-11} - 10^{-12} M	Physiological, Molecular	>190.000
	Pros: Ideal for the study of pharmacodynamics, high sensitivity for detecting labeled probes <i>in vivo</i> , no depth limit. Cons: Expensive, low spatial resolution, ionizing radiation, the labeling of drugs is not easy					
SPECT	γ -radiation	1-2 mm ^a 10-14 mm ^b	$^{99\text{m}}\text{Tc}$, ^{111}In -labeled molecules ^c	10^{-9} - 10^{-10} M	Physiological, Molecular	~100.000
	Pros: Suitable for imaging of: long term processes, quantification of kinetic parameters, no depth limit. Cons: Lower sensitivity than PET, ionizing radiation,.					
MRI	Nuclear spin relaxation	10-100 μm^a 0.1 mm ^b	Paramagnetic Gd^{3+} chelates, Magnetic particles	10^{-3} - 10^{-5} M	Physiological, Molecular Anatomical	>200.000
	Pros: Excellent soft tissue resolution, no ionizing radiation, no limit in repeated studies unless contrast agent is applied, no depth limit. Cons: Poor sensitivity, expensive, not allowed for patients with pacemakers.					
OI	Near infrared fluorescence	1 μm^a 1-2mm ^b	Fluorochromes, Photoproteins	10^{-15} - 10^{-17} M	Physiological, Molecular	<70.000
	Pros: Highly sensitive, high resolution, quantitative imaging, gene expression. Cons: Expensive, depth limit in cm range.					
US	Cyclic sound 20-60kHz	50 μm^a	Microbubbles	1 bubble	Anatomical, Physiological	~70.000
	Pros: Cheap, portable, no ionizing radiation, vascular and interventional imaging. Cons: Depth limit in cm range, artifacts.					

^{a)} For high-resolution small-animal imaging systems. ^{b)} Clinical application. ^{c)} Commonly applied.

However, the information that can be obtained with the various techniques, is often complementary and, therefore, a combination of techniques may be advantageous. Multi-modality imaging offers opportunities not only to image pathologies but also to study interactions between novel drug systems and biological targets, metabolism, and morphological changes. Today, the challenge is to detect the smallest number of cells that develop in the organism at an early stage of disease. Conventional imaging techniques are unable to detect small tumors, whereas migration of metastases from the primary site often starts before the tumor can be located. Radionuclear imaging is highly sensitive but often lacks the anatomical information to localize the disease. Therefore, a correlation between structural and functional information is required, which can be achieved, for instance by

fusion of PET or SPECT images with MRI or CT images by means of software application. However, the time interval between the individual scans and repositioning of the patient in between are the drawbacks of this approach. To facilitate the process of correlation of imaging modalities and to simplify patient handling, a new class of diagnostic instrumentation has been developed that combines CT and radionuclide imaging in a simultaneous scan.²⁴ An example of this approach is the PET-CT system that became the most useful imaging device for early diagnosis of cancer and for monitoring of cancer therapy. Another example of a multi-modal imaging instrument is an MRI-PET imaging device. The high resolution imaging capability of MRI, which does not involve harmful radiation, in combination with the molecular information obtained from PET is very promising.²¹ Recently, the first fully integrated experimental set-up for MRI-PET has been introduced by Siemens. It has a PET module, consisting of a gamma-camera ring, which is integrated in the magnet, allowing the simultaneous acquisition of tomographic PET and MRI images.

CONTRAST MEDIA

The efficiency of most of the imaging techniques depends on agents that alter the image contrast upon their administration and distribution in tissues. Therefore, the development of contrast media is one of the important tasks of chemists contributing to the advances of medical diagnostics. A number of excellent reviews covering the developments and properties of imaging agents has been published recently.²⁵⁻²⁹ This section gives a selection of typical examples relevant for this thesis rather than a complete literature survey.

MRI Contrast Agents

The function of MRI contrast agents is to decrease the relaxation time of protons in tissues, increase the contrast and, in some cases even provide physiological or molecular information. In contrast to the agents used in nuclear imaging, it is not the agent that is observed in MRI, but the effect of its presence on the relaxation times of water protons in

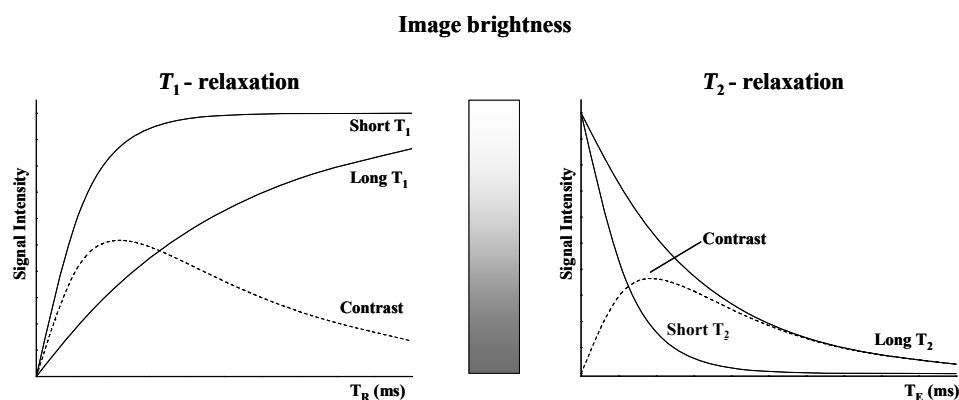


Figure 1.3. A schematic representation of T_1 and T_2 effects on the image brightness at the certain time intervals: T_R (repetition time) and T_E (echo-time) used in the common pulse sequences respectively.

tissues. Both, T_1 and T_2 weighted contrast can be used for the imaging and, accordingly, the contrast agents can be divided into two groups depending on whether they are able to decrease T_1 (resulting in positive contrast) or T_2 (resulting in negative contrast) relaxation times (see Figure 1.3).

Positive Contrast Agents (T_1)

Exogenous T_1 contrast agents contain a paramagnetic metal-ion, typically gadolinium(III), which has a very large magnetic moment due to the presence of 7 unpaired f -electrons. Their electronic relaxation provides fluctuating magnetic fields in neighboring nuclei, which results in enhancement of the NMR relaxation rates of these nuclei.^{30,31} In this way, the T_1 relaxation time of bulk water protons can be decreased dramatically due to interaction with the Gd-ion in the CA molecule resulting in an increased contrast. In the absence of CA, the differences in relaxation times exclusively depend on physiological differences between tissues. Since Gd(III) is extremely toxic even at low doses (10-20 $\mu\text{mol kg}^{-1}$), it has to be coordinated to an organic molecule; usually Gd(III) chelates have a very low toxicity.³² To avoid leaching of free Gd(III), it is important that these complexes are thermodynamically stable. Additionally, they should exhibit a high kinetic stability, meaning that transmetallation of Gd(III) in the complex by endogenous metal ions is

negligible. Complexes with poor kinetic stability have been shown to be able to exchange coordinated Gd(III)-ions with some biologically important metal ions such as Zn(II), Ca(II), or Cu(II).³³ The non-coordinated Gd(III)-ion formed can be hydrolyzed to insoluble Gd(OH)₃, which can accumulate in the skeleton, liver and skin, especially in patients with renal failure.³⁴ As shown recently, this accumulation of free Gd(III)-ion can cause a very severe syndrome - nephrogenic systematic fibrosis (NFS). There is no effective treatment for this newly described disease and its exact pathogenesis is not yet completely understood. Extensive studies on the kinetic and thermodynamic stability of the Gd(III)-complexes have shown that complexes of macrocyclic ligands such as DOTA, DO3A, HP-DO3A are remarkably more stable than those of linear chelates.^{35,36} These results indicate that the choice of the ligand for metal complexation is extremely important. Any structural changes on the potential CAs, like conjugation to a targeting group, or other alterations aimed to optimize their physical properties may not be at the expense of kinetic stability.

Most clinically approved T_1 contrast agents are derivatives of polyaminocarboxylates: Gd-DTPA (Magnevist), Gd-DOTA (Dotarem), Gd-DTPA-BMA (Omniscan), Gd-HP-DO3A (Prohance) (see Figure 1.4). The number of inner-sphere water molecules (q) directly coordinated to the Gd(III)-center is a very important determinant of relaxivity. Most commercially available CAs are monohydrated ($q=1$). Some agents with two water molecules in the first coordination sphere of the Ln(III) ion have been developed, for example Gd-HOPO and Gd-AAZTA. Other agents with higher hydration number have also been proposed, but such agents usually are thermodynamically less stable and may suffer from a “quenching” effect due to the interaction with HCO_3^- , PO_4^{3-} or proteins that are able to replace water by aspartate and glutamate residues.³⁷

Other parameters that govern the relaxivity process are shown in Figure 1.4. Briefly, the relaxation rate $R_1 (=1/T_1)$ evolves from diamagnetic (intrinsic tissue relaxation rate) and paramagnetic (Gd-based) contributions and is dependent on the concentration of the contrast agent according to Equation 1.1, where r_1 is the relaxivity ($\text{s}^{-1}\text{mM}^{-1}$) and $[\text{CA}]$ the concentration of the contrast agent (mM). The concentration needed for a contrast agent to achieve an efficient performance in MR imaging is in the range that can be easily tolerated by the body (0.1 mM).

$$R_1 = R_{1,\text{dia}} + r_1 [\text{CA}] \quad 1.1$$

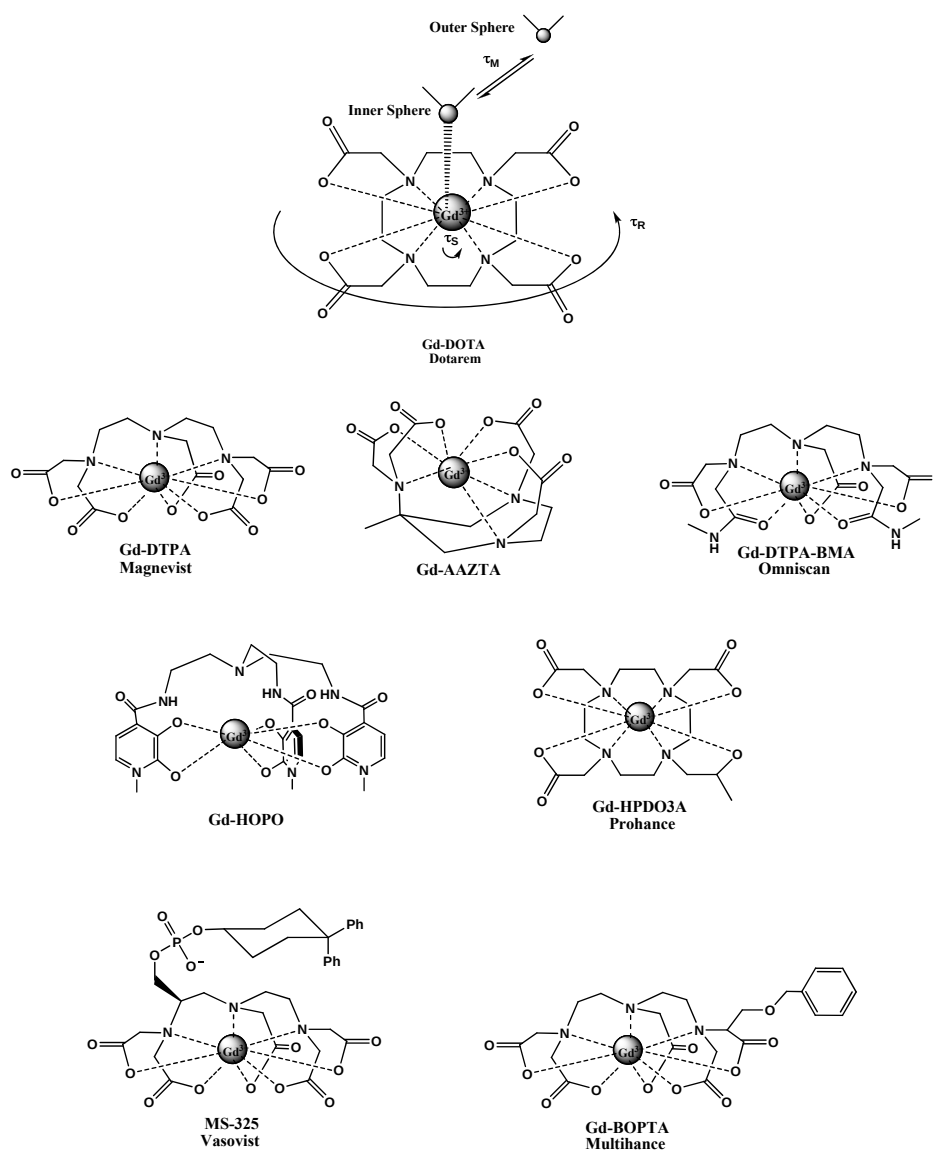


Figure 1.4. Examples of commercially available and clinically applied Gd-based CAs. Parameters governing the relaxivity are shown on the structure of Gd-DOTA: rotational correlation time (τ_R), water exchange time (τ_M), electronic relaxation time (τ_S). For the sake of clarity Gd(III)-coordinated water molecules have been omitted.

The CAs of the first generation distribute over the extracellular space of the body rather aselectively. A lot of research has been devoted to the optimization of these agents by adjustments of the chemical structures to tune the various parameters that govern the relaxivity.³⁸ Further improvements have been achieved with contrast agents that localize in particular organs. Examples are Gd-BOPTA (see Figure 1.4), which thanks to its hydrophobic character accumulates in the liver and the so-called blood pool agents, which have a prolonged residence time in the cardiovascular system. The latter agents are usually high molecular weight compounds, which can be achieved by covalent or non-covalent binding of low-molecular chelates to high molecular weight compounds. A very efficient agent of this class is MS-325 (see Figure 1.4), which has a high affinity for human serum albumin.³⁹ Besides MS-325, high molecular weight compounds are also used for this purpose, additionally having a long rotation correlation time (τ_R), which is beneficial to reach high relaxivities.

The research during the last years has been focused on the design of systems for molecular imaging (viz. agents that can also give diagnostic information on a cellular or a molecular level). Two types of molecular MRI CAs can be discriminated: (i) responsive agents and (ii) targeted agents.

Responsive contrast agents have relaxivities that are sensitive to physico-chemical properties of the microenvironment in the region of their distribution. It has been shown that *in vivo* changes such as enzyme activity, pH, metal ion concentration or oxygen pressure can be visualized by specially designed responsive contrast agents.^{25,38} For instance, the Gd(III) complex of a tetraamide derivative of DOTA (Gd-DOTA-4AmP) shows a relaxivity which is dependent on the pH. This pH dependence can be associated with the changes in hydration number of the metal complex in both, first and second coordination sphere as a function of the protonation state of the molecule.^{40,41} Upon conjugation of this agent to a PAMAM-dendrimer, the pH response to the relaxivity (Δr_1) has shown to be significantly enhanced compared to the original low molecular phosphonate-based Gd-DOTA-chelate.⁴² A sufficient pH response is observed in the range 6-8 and, therefore, these agents are particularly suitable for the detection of tumor sites,⁴³ which typically have relatively low extracellular pH values (pH < 6.9).

Targeted MRI CAs for molecular imaging, usually are designed for the detection of markers of a disease through molecular recognition. Often, it concerns the imaging of over-expressed receptors. The concentration of receptors on a cell surface is usually quite low (in the order of 10^4 - 10^5 receptors/cell). It has been estimated that 10^2 - 10^3 paramagnetic chelates per receptor are needed to generate a sufficient contrast, which can be translated to a local concentration of Gd^{3+} of 10-100 μM .⁴⁴⁻⁴⁷ It is reasonable to assume that occupation of a high number of receptors would disturb the metabolic equilibrium. To overcome this problem, many strategies of a high pay-load delivery of CA to the site of interest have been proposed, including the conjugation of Gd(III)-chelates to macromolecules (e.g. cyclodextrines, dendrimers, or inulin)⁴⁸ or encapsulation into apoferritin,⁴⁹ liposome vesicles⁵⁰ or by attachment to nanoparticles.⁵¹ A targeting vector present on the carrier molecule for recognition of specific epitopes on the cellular membrane is an efficient way of amplification of imaging signal. An example of strong signal amplification, using a targeting MRI CA, is the visualization of the angiogenesis marker, the endothelial $\alpha_v\beta_3$ integrin, by a biotinylated monoclonal antibody conjugated via avidin to Gd-containing nanoparticles characterized by $r_1=19.1 \text{ s}^{-1}mm^{-1}$ (94.400 Gd units per particle).⁵² In general, the targeting vectors applied are natural compounds or analogues; only a few examples of non-natural targeting vectors have been reported up to now. Recently, a bisphosphonate monoamide DOTA analogue has been demonstrated to be a potential bone imaging agent,⁵³ while phenylboronate derivatives of the same chelate have been proposed as good candidates for the recognition of tumors.⁵⁴

Recently, a new class of CAs based on the Chemical Exchange Saturation Transfer (CEST) has entered the MRI research field.⁵⁵ These agents are functioning by reducing the water proton signal through the transfer of magnetization from exchangeable protons on the agent to the bulk water protons. Ideally, the chemical shift difference between the exchanging sites is large and the exchange rate is slower than this difference ($k_{ex} \leq \Delta\omega$). Selective saturation of the resonance of the mobile protons by a specific radio frequency brings about the magnetization transfer from the CEST agent to the bulk water molecules resulting in a decrease of the bulk-water signal. A big advantage of this technique is that the CEST effect can be switched on and off at will by the selective presaturation. The effectiveness of these agents can be optimized by tuning both the exchange rate and the

chemical shift of the exchanging proton. Some paramagnetic compounds (PARACEST) can perfectly fulfill this by increasing the frequency differences.⁵⁶

Negative Contrast Agents (T_2)

T_2 contrast agents influence the signal intensity by shortening the transverse relaxation time. This decrease of the relaxation time can be ascribed to the large magnetic susceptibility in the presence of these agents that are able to induce local field gradients. The interest in this type of agents is growing simultaneously with the development of high field MRI, since the transverse relaxation time depends on the square of the magnetic field according to Equation 1.2, where f is the fraction Ln-complex/water, q is the number of coordinated water molecules, τ_M is the residence time of coordinated water molecules exchanging with the bulk and $\Delta\omega_M$ is the chemical shift of the coordinated water molecule. By contrast, T_1 contrast agents generally have a decreasing trend upon increase of the magnetic field strength above 1 T.

$$\frac{1}{T_2} = fq \frac{\tau_M \Delta\omega_M^2}{1 + \tau_M^2 \Delta\omega_M^2} \quad 1.2$$

Dysprosium(III) analogues of existing T_1 contrast agents have been demonstrated to be promising agents for the negative contrast applicable at high magnetic field.⁵⁷ However, the fine tuning of the molecular structure is crucial since the residence time of water in the complex (τ_M) needs to be balanced between 0.1 and 1 μ s in order to get an optimal R_2 , which can be defined in a similar way as R_1 :

$$R_2 = R_{2,\text{dia}} + r_2 [\text{CA}] \quad 1.3$$

Currently, the most common T_2 contrast agents are iron-containing superparamagnetic nanoparticles. These particles are magnetized in the external magnetic field of the MRI machine. The strong magnetic field gradients associated with these magnetized particles

effectively disturb the phase-coherence of the nuclear magnetization of the water protons, which results in a decrease of T_2 . Generally, the particles are coated by dextran or other polymers preventing agglomeration and offering the opportunity of functionalization of their surface with either specific targeted vectors for markers of e.g. apoptosis or inflammation⁵⁸ or fluorophores such as near infrared agents.⁵⁹ Depending on their size and their coating, the family of iron-oxide nanoparticles can be classified as: SPIO (superparamagnetic, 50-500 nm), USPIO (ultra small superparamagnetic, 4-50 nm), MION (monocrystalline) and cross-linked (CLIO).⁶⁰ Carbon nanotubes have recently been used for the assembly of multi-modality probes with iron-oxide as the reporter for MRI.⁶¹

Recently, it has been shown that paramagnetic Dy_2O_3 nanoparticles have potential as negative contrast agents, particularly for high field MRI.⁶² These particles showed only a small T_1 relaxation enhancement, whereas the T_2 relaxation rates were found to be dramatically enhanced.

The elements of the lanthanide series show great similarity in their chemical properties, whereas their physical and radiochemical properties differ significantly. This phenomenon is often exploited in research on contrast agents, by using one of the lanthanides as a probe for another one. For example, europium(III) is often applied as Gd-analogue to elucidate structural properties of new chelates using its luminescent properties,⁶³ while radioactive analogues such as ^{177}Lu or ^{160}Tb could be used to study targeting due to the high sensitivity of the gamma-counting method.⁵⁴

Other Imaging Modalities

Lanthanides are also utilized as imaging probes for optical imaging. The long excited state lifetimes of lanthanides (microseconds for IR-emitting Yb, Er and Nd and milliseconds for Eu and Tb complexes) allow their exploitation for time-resolved imaging. Furthermore, lanthanide emission bands are sharp compared to organic fluorophores; linewidths are typically smaller than 10 nm. Therefore, Ln(III)-complexes represent an important class of luminescent agents with unique excited state properties. A sensitizing chromophore fragment needs to be incorporated into the agent molecule to transfer the excited state energy to the encapsulated lanthanide-ion. In this way a more efficient excitation can be

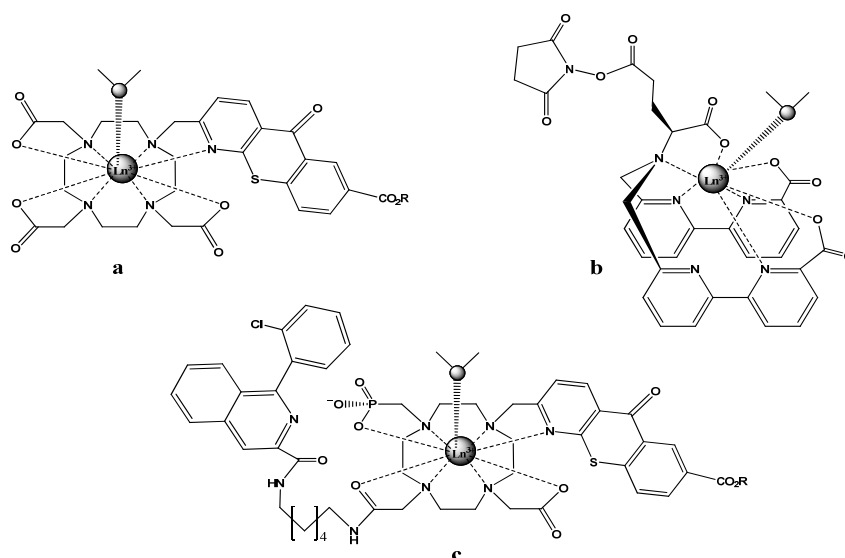


Figure 1.5. Examples of luminescent imaging probes (Ln=Eu, Tb)

achieved compared to the direct excitation of Ln^{+3} ($\epsilon \leq 1 \text{ M}^{-1}\text{cm}^{-1}$).⁶⁴ Recent examples of optical imaging complexes exploiting a functional group as an emissive ‘tag’ are depicted in Figure 1.5, where (c) was designed for the labeling of glioblastoma cells.⁶⁵

PET and SPECT imaging, in contrast to other imaging techniques, cannot be performed without an imaging agent. Many *in vivo* markers are known to be suitable for radiolabeling, however for the majority of targets there are still no suitable radioligands available. This gives a lot of opportunities for developing new ideas for radio-imaging agents with high *in vivo* affinity, metabolic stability and with the proper pharmacokinetics. PET can play an important role in pharmaceutical research since each of the main elements (carbon, nitrogen, oxygen) in biological molecules can be substituted by a radioisotope. However, ^{18}F -based radiopharmaceuticals are the most favorable for PET applications due to their low positron energy (0.64 MeV) and convenient half-lifetime (110 min). Sterically, fluorine is very similar to hydrogen and, therefore, it can substitute the latter in a biomolecule, without altering the biological behavior. The most important examples of this type of agents are 2- ^{18}F -fluoro-deoxy-D-glucose (^{18}F FDG) for studying e.g. the glucose

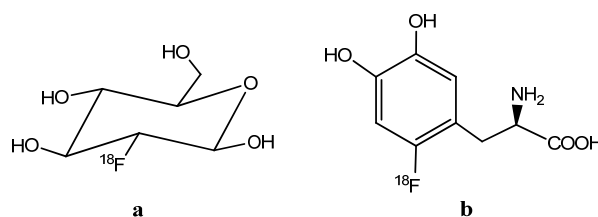


Figure 1.6. Molecular structures of [^{18}F]FDG (a) and [^{18}F]6-fluoro-L-DOPA (b).

metabolism, and [^{18}F]-fluoro-3,4-dihydroxyphenylalanine ([^{18}F]6-fluoro-L-DOPA) for imaging of the cerebral dopamine metabolism and neuroendocrine tumors⁶⁷ (Figure 1.6). Several strategies can be applied for the radiolabeling, including electrophilic and nucleophilic substitution and the recently developed ‘click’ chemistry pathways that seem to open possibilities for introducing ^{18}F -labeled ligands with various substituents.⁶⁸ An alternative way to introduce a radiolabel is by conjugating a targeting vector with a complex of a radioactive metal ion. The clinically applied ^{111}In -DTPA-derivatized octreotide (OctreoScan), the analogue of somatostatin for the *in vivo* localization of endocrine tumors, is a good example. A similar approach exploiting octreotide as targeting vector was used by Maecke et al., who used the conjugation with the NODAGA-chelate⁶⁹ to enable complexation of short life emitters such as ^{66}Ga and ^{68}Ga and later with DOTA-analogues⁷⁰ for the complexation of ^{57}Co (Figure 1.7). Several other peptide receptors that are overexpressed in tumors can be used for targeting. For example, bombesin receptors are overexpressed in prostate, breast and small cell lung cancer. Therefore, recently DOTA-conjugates of bombesin analogues have been developed for ^{177}Lu complexation. In all cases, the influence of the spacer between the metal chelating moiety and the peptide targeting vector seems to be crucial for the targeting properties and for the bio-distribution of the radiopharmaceutical.

Obviously, the increasing knowledge on targets and molecular events that are playing a role in pathologies is a driving force in the development of new targeting vectors and radiolabeling strategies. It cannot be stressed enough that successful molecular imaging requires probes with high affinity, specificity and *in vivo* stability. One of the most important criteria for the success of an imaging agent is its capability to overcome biological delivery problems. A highly specific agent is useless if it is not able to reach the

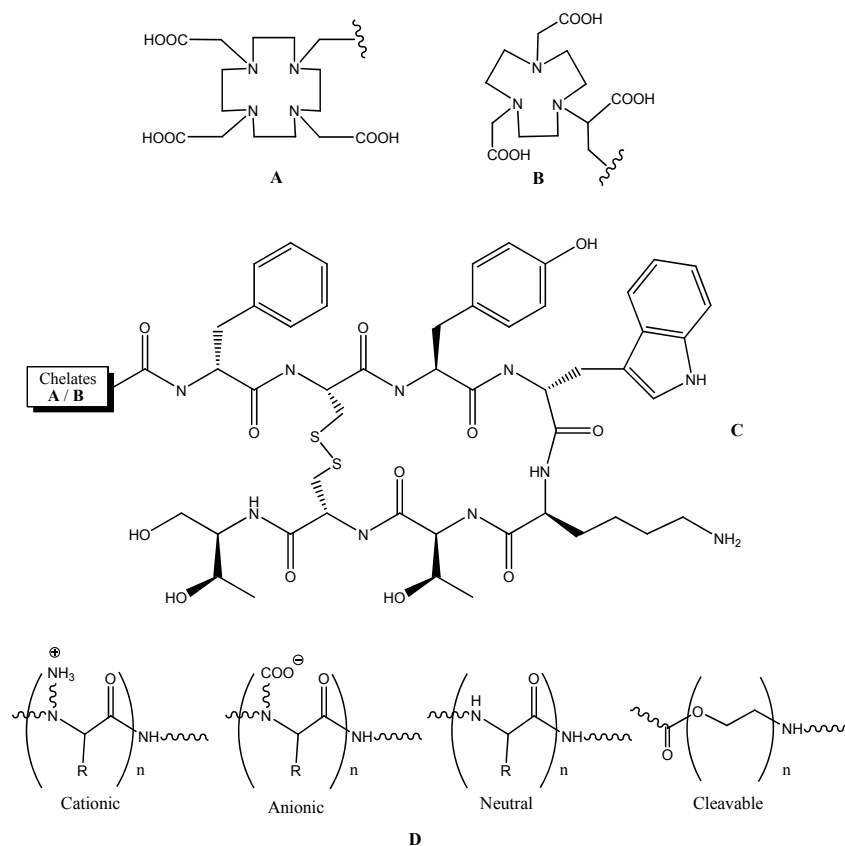


Figure 1.7. DOTA (A) and NODAGA (B) conjugated to octreotide (C). Examples of linkers for the modification of pharmacokinetics (D).

target due to biological barriers. An example of such a restriction is the blood-brain barrier (BBB), which can be an important issue, especially for nuclear imaging. From the chemical point of view the solution can be found in adjusting synthetic strategies considering factors such as molecular weight of the imaging agent or the drug (< 400-600 Da), the overall charge of the molecule and the amount of hydrogen bonds between the agent and water (<8-10).⁷¹

In the search to overcome delivery problems, liposomes have been considered as promising carriers for imaging and therapeutic agents for cancer treatment.^{72,73} They consist of a phospholipid bilayer and an aqueous core, are biocompatible and can carry a high pay-

load of contrast agent or drug. The incorporation of amphiphilic components like polyethylene glycol (PEG) into the membrane provides steric protection against blood clearance and macrophage uptake prolonging the circulation time and increasing the accumulation in tumors. Controlled size and lipid composition may facilitate specific *in vivo* delivery. The search for the lipid composition of liposomes suitable for temperature triggered release of their content by means of hyperthermia is of great interest for drug delivery.⁷⁴ Additionally, these particles can be labeled with markers for different imaging modalities, such as fluorophores,⁷⁵ Gd-loaded complexes for MRI,⁵⁰ or radionuclides.⁷⁶

The research in radiology in the past years was very much focused on the physical properties of imaging systems such as resolution, time, signal-to-noise improvements etc., which actually meant engineering control. Today, the imaging technology is highly developed and many of these problems have been solved. The new tendency of a multidisciplinary approach in molecular imaging research needs to be encouraged as this is the only solution to shorten the way of new pharmaceuticals and imaging agents from the lab bench to the clinical applications.

OUTLINE OF THE THESIS

This thesis describes a multidisciplinary search for optimized contrast agents for medical imaging. Considering MRI as a major high resolution diagnostic technique, this research is aimed to overcome the sensitivity problems by application of a targeting vector in combination with an imaging reporter. The work on the design of both modules for a contrast enhancement included the optimization of the physicochemical properties of the lanthanide(III) chelates as well as their recognition ability towards the overexpressed target on the tumor cells.

In the following two chapters ¹⁷O NMR methods for the determination of some of the physical parameters that govern MRI contrast are evaluated. Understanding of the properties of lanthanide ions and their coordination behavior in particular, is very important for the research aimed at the optimization of contrast agents. Similarly, the number of water molecules directly coordinated to the lanthanide and their exchange with the bulk water play a significant role for the efficiency of an MRI contrast agent. The optimization of these

parameters requires a careful tuning of the structure of the chelating molecule, taking into account the kinetic and thermodynamic stability, which are highly important factors for clinical applications. The research described in chapter 4 is focused on a study of pyridine based chelates for the complexation of lanthanides with extremely high water exchange using the ^{17}O NMR variable-temperature method.

Increase of the specificity of the agents is another approach to optimize the contrast. Therefore, the second part of the thesis deals with the development of a potential carbohydrate targeting agent for the visualization of tumors. A fundamental multinuclear NMR study described in chapter 5 deals with the reversible covalent binding between a phenylboronic targeting group and the diol function of sialic acid - a sugar overexpressed on the surface of cancerous cells. The feasibility of this idea of targeting by using this biomarker is confirmed in chapter 6 by the results of a series of incubation experiments with the human glioma cell line and a radioactive ^{160}Tb -DTPA-based complex bearing two phenylboronic targeting groups. Improvements on the stability of this complex were realized by substituting the backbone of the previously designed agent for a DOTA-structure. The research is concluded in chapter 7, which describes the optimization of the delivery of the novel targeting agent to the tumor site by thermo-sensitive liposomes with a carefully tuned transitional temperature for hyperthermia.

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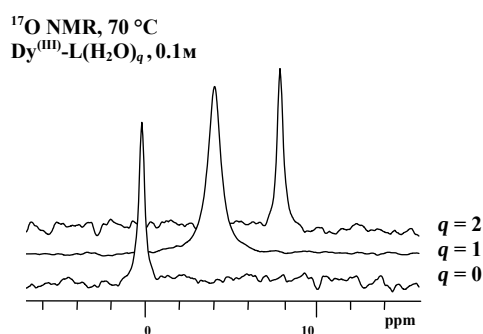
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HOW TO DETERMINE THE NUMBER OF INNER-SPHERE WATER MOLECULES IN LANTHANIDE(III) COMPLEXES BY ^{17}O NMR SPECTROSCOPY

2



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INTRODUCTION

The contrast of MR images can be dramatically improved by the administration of paramagnetic lanthanide(III) complexes as contrast agents (CAs) prior to the examination.¹ The most common metal ion used in MRI contrast agents is Gd(III), which is extremely efficient in enhancing the water relaxation rates, the parameters governing the contrast in the MR images. The chelate plays an important role as well, since it should provide high kinetic stability to the contrast agent, which is essential for low toxicity. Usually, sufficient kinetic stability is only achieved with chelates that are at least heptadentate. The coordination sphere is then completed with 1-2 water molecules.

The improvement of the image contrast is the result of the enhancement of longitudinal ($1/T_1$) or transverse ($1/T_2$) relaxation rates of the bulk water protons, which is achieved through their exchange with the metal coordinated water molecule(s). The number of inner-sphere water molecules is proportional to the relaxivity of the complex (the relaxation rate enhancement per mmol of Gd(III)) and, therefore, is an important parameter for the assessment of new potential MRI contrast agents.

Various methods of determination of the number of coordinated water molecules in Ln(III) complexes have been described in the literature, including NMR-relaxation,²⁻⁴ luminescence spectroscopy,^{5,6} and ^{17}O NMR chemical shift measurements.⁷ In this technical note, we will discuss some practical procedures for the latter method.

DISCUSSION

The presence of a water molecule in the inner-sphere of a paramagnetic lanthanide complex is obviously reflected in ^{17}O NMR data of water. When the exchange of water between the complex and the bulk is rapid on the ^{17}O NMR time scale, the chemical shift of a Ln(III)-bound water molecule (δ_m) is related to the observed shift (δ_{obs}) referred to that of pure water via Equation 2.1.

$$\delta_{\text{obs}} = \delta_{\text{w}} + q \cdot P_m \cdot \delta_m \quad 2.1$$

Here, δ_χ is the bulk magnetic susceptibility (BMS) shift due to the presence of the paramagnetic ion, q is the number of inner-sphere water molecules, and P_m the molar ratio Ln(III)/H₂O in the sample. It should be noted that the term δ_χ vanishes if the samples are measured in a standard NMR tube (one compartment) with frequency locking of the spectrometer, since then the observed shifts are automatically corrected for BMS effects. If the measurements are conducted with an unlocked spectrometer, δ_χ can be easily determined by adding a small amount of *tert*-butanol to the sample and measuring the ¹H NMR chemical shift difference between the methyl resonances of this sample and that of a sample with only water and a small amount of this reference.

The ¹⁷O chemical shift of a Ln(III)-bound water molecule (δ_m) can be dissected in contributions of three terms: the diamagnetic (δ_{dia}), contact (δ_{con}), and pseudocontact (δ_{pc}) shifts. Therefore, the lanthanide induced shift (Δ) of water upon its coordination to a Ln(III) cation can be derived from Equation 2.1 as:

$$\Delta = \frac{\delta_{obs} - \delta_\chi}{P_m} = q \cdot \delta_{dia} + q \cdot \delta_{con} + q \cdot \delta_{pc} \quad 2.2$$

The diamagnetic shift originates from the non-paramagnetic contributions, such as inductive and direct field effects as a result of interaction of the ¹⁷O nucleus with the lanthanide ion and can usually be neglected or obtained directly by measuring the corresponding diamagnetic La(III) and/or Lu(III) complexes. The contact shift is the result of through-bond interaction of unpaired electron-spin density of *f*-electrons of the metal to the oxygen of the bound water molecule, whereas the pseudocontact or dipolar shift is a through-space effect of the interaction between the magnetic moments of the unpaired electrons at the Ln(III) ion and the ¹⁷O nucleus. Both δ_{con} and δ_{pc} can be expressed as the product of a term that is a characteristic for the Ln(III) ion but independent of the complex structure ($\langle S_z \rangle$ and C^D , respectively) and a term that is independent of the Ln(III) ion but dependent on the complex structure (F and G , respectively) (see Equation 2.3). Values for $\langle S_z \rangle$ and C^D have been reported and are compiled in Table 2.1.

$$\Delta = q \cdot \delta_{dia} + q \cdot \langle S_z \rangle \cdot F + q \cdot C^D \cdot G \quad 2.3$$

Table 2.1. Values of $\langle S_z \rangle$,⁸ relative values of C^D as scaled to -100 for Dy⁹ and theoretical values of μ_{eff} ¹⁰ for the series of lanthanides at room temperature.

Ln	$\langle S_z \rangle$	C^D	μ_{eff}
Ce	-0.974	-6.3	2.56
Pr	-2.956	-11.0	3.62
Nd	-4.452	-4.2	3.68
Sm	0.224	-0.7	1.55-1.65
Eu	7.569	4.0	3.40-3.51
Gd	31.500	0	7.94
Tb	31.853	-86	9.7
Dy	28.565	-100	10.6
Ho	22.642	-39	10.6
Er	15.382	33	9.6
Tm	8.210	53	7.6
Yb	2.589	22	4.5

For Ln(III)-bound ^{17}O nuclei, the contact term in Equation 2.3 is the dominating¹¹ and also the most useful one for the determination of q , since F depends on the hyperfine coupling constant between the unpaired electrons of the Ln(III) ion and the ^{17}O nucleus of water ($A/\hbar$) according to Equation 2.4.

$$F = \frac{\beta}{3kT\gamma_{\text{O}}} \cdot \frac{A}{\hbar} \cdot 10^6 = \frac{6.172 \cdot 10^{-3}}{T} \cdot \frac{A}{\hbar} \quad 2.4$$

Here, β is the Bohr magneton, k is the Boltzmann constant, γ_{O} is the ^{17}O gyromagnetic ratio and T is the absolute temperature. Previously, we have demonstrated that F and thus $A/\hbar$ is almost independent on the structure of the Ln(III) complex under study and even on the nature of the oxygen atom;¹² the value of $A/\hbar$ is always in the range $-(3.9 \pm 0.3) \times 10^6 \text{ rad} \cdot \text{s}^{-1}$. The same values for $A/\hbar$ have been obtained by elaborate simultaneous fittings of ^{17}O NMR chemical shifts, longitudinal and transverse relaxation rates and ^1H NMRD profiles.¹³ Therefore, Equation 2.4 can be rewritten as:

$$F \approx \frac{-2.407 \cdot 10^4}{T} \quad 2.5$$

Consequently, q can be directly determined by dividing the contact contribution to Δ by $\langle S_z \rangle$ and the value of F as calculated with Equation 2.5.

For Gd(III), C^D (see Table 2.1) and, consequently also the pseudocontact contribution is zero. Therefore, for Gd(III) complexes the observed chemical shift can be used directly to evaluate q , provided that the exchange between Gd(III) bound water and bulk water is fast on the ^{17}O NMR time scale. In general, this situation exists for temperatures of about 80°C. Extensive line-broadening often makes accurate measurements on Gd(III) complexes difficult and, therefore, measurements on the corresponding Dy(III) complex may be favorable. The contact contribution to the ^{17}O chemical shifts of water bound to this lanthanide ion is usually more than 85%¹⁴ and, therefore, q can be determined in a good approximation also directly from the value of Δ neglecting the diamagnetic and pseudocontact terms in Equation 2.3. The accuracy of q values determined in this way is usually ± 0.2 and the results obtained are in good agreement with those of other methods, such as luminescence and the simultaneous fitting procedures mentioned above.¹⁵

The NMR method presented is very fast, since it requires the measurement of ^{17}O and ^1H spectra of only two samples, which takes in general less than 15 min. The concentration of the Ln(III) complex should be not less than 20 mM for sufficient accuracy, but the NMR measurements can be performed with very small quantities (e.g. samples of about 50 μL in a capillary), since sensitivity is never a problem.

When the methyl ^1H resonance of *tert*-butanol is used as an internal reference during the measurements, the value of the BMS shift (δ_χ) is obtained as well. This opens the possibility to evaluate simultaneously the concentration of the lanthanide ion, c , in the sample through Equation 2.6.¹⁶

$$\delta_\chi = \frac{4\pi c s}{T} \left(\frac{\mu_{\text{eff}}}{2.84} \right)^2 \times 10^3 \quad 2.6$$

Here c is the concentration of the paramagnetic Ln(III) complex in $\text{mol}\cdot\text{l}^{-1}$, s is dependent on the shape and the position of the sample in the magnetic field ($s = 0, 1/3, -1/6$ for a sphere, cylinder parallel or perpendicular to the magnetic field, respectively), and μ_{eff} is the effective magnetic moment for the particular Ln(III)-ion (see Table 2.1).

The q values obtained by the methods described above are frequently not integer numbers but rather fractions. This can be due to inaccuracy of the measurement or to the existence of an equilibrium of complexes with different q values. UV-Vis spectrometric measurements of the ${}^7F_0 \rightarrow {}^5D_0$ transition for the corresponding Eu(III) and Tb(III) complexes can be applied for the estimation of the molar fractions of the co-existing complexes with different q .^{17,18} However, more insight can be obtained in this case by a separation of the various ${}^{17}\text{O}$ NMR shift contributions by means of NMR measurements on a series of lanthanide complexes of the same organic ligand.¹⁹ If a change in the number of inner-sphere water molecules occurs across the lanthanide series, this will be reflected in both a change of q and of G . This situation can be visualized by plotting the chemical shifts according to a linearized form of Equation 2.3 (see Equation 2.8).

$$\Delta_{para} = \Delta - q \cdot \delta_{dia} = q \cdot \langle S_z \rangle \cdot F + q \cdot G \cdot C^D \quad 2.7$$

$$\frac{\Delta_{para}}{C^D} = \frac{\Delta - q \cdot \delta_{dia}}{C^D} = \frac{q \cdot \langle S_z \rangle}{C^D} F + q \cdot G \quad 2.8$$

A change of q along the lanthanide series results in a break in such a curve because of the change of both the slope and the intercept upon going from light to heavier lanthanides. This is nicely demonstrated in Figure 2.1, where we have plotted the ${}^{17}\text{O}$ chemical shifts of water in samples of aqueous solutions of the triflates of the various lanthanides according to Equation 2.8. It is known that the triflates do not coordinate in the first coordination sphere of the Ln(III)-ions and, therefore, this system represents well the Ln(III) aquo ions.

Figure 2.1 shows a clear break in the curve between Nd(III) and Tb(III). The slopes of the lines for the light and the heavy Ln(III) ions have a ratio of 9 : 8, which is in agreement with a change of hydration number from 9 to 8 occurring upon going from La(III) to Lu(III) as shown by a neutron scattering first order difference study²⁰. The F value for both

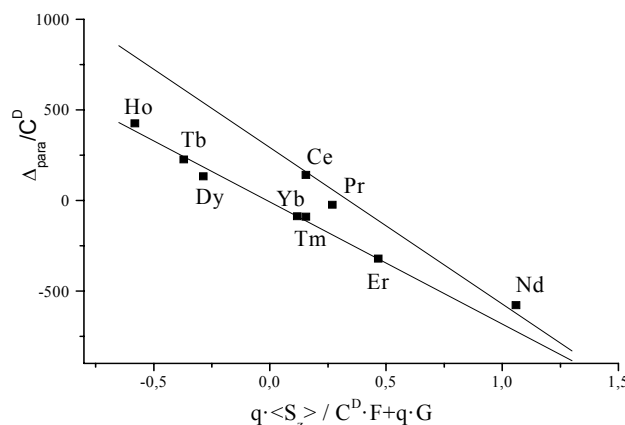


Figure 2.1. Plots of Δ_{para}/C^D vs. $q \cdot \langle S_z \rangle / C^D \cdot F + q \cdot G$ for the series of aqueous solutions of Ln(III) trifluoromethanesulfonates (triflates). The samples were measured at 54.1 MHz and 25 °C. The values for Gd(III) and Sm(III) are not included because of the small C^D of these lanthanides.

lines is -84 , which corresponds to $A/\hbar = 4.05 \times 10^6 \text{ rad s}^{-1}$, confirming that $A/\hbar$ is independent of the Ln(III) ion and its coordination.

The ^{17}O LIS (lanthanide induced shift) method for the determination of q can be conveniently combined with the in situ preparation of a Ln(III) complex by monitoring the ^{17}O NMR shift of water upon increase of the Ln/ligand ratio (ρ_L). Figure 2.2 displays the examples of preparations of Dy(III) complexes of two DTPA-derivatives.²¹ The position of break points in the linear decrease of these ^{17}O NMR titration curves indicates the formation of 1 : 1 (a) and 2 : 1 (b) Dy(III) complexes, respectively.

From the initial slopes in both curves and the equations described above, it can be calculated that the Dy(III) ions in the complexes Dy[cy(DTPA-en)] and Dy₂[cy(DTPA-en-DTPA-en)] have 1.2 and 0.8 water molecule in their first coordination sphere, respectively. After the equivalence points, a steep increase of the negative slopes is observed, which can be ascribed to the presence of free Dy(III), which has 8 water molecules in its first coordination sphere. After the titration, the amount of ligands required for back titration of the excess Dy(III) can be determined using the ρ_L values of the equivalence points.

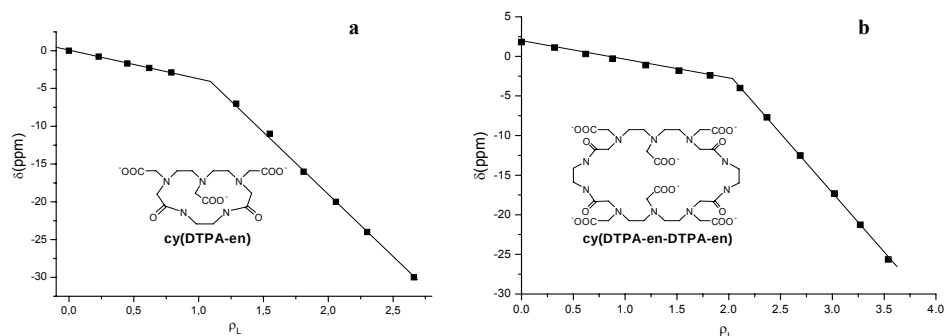


Figure 2.2. Dy(III) ^{17}O NMR induced shift vs. the molar ratio Dy(III)/ligand (ρ_L) for a 0.05 M solution of ligand in D_2O at 75 °C.

EXPERIMENTAL

Methods

Here we give an example of the determination of q along with the data treatment for the Dy(III)-DTPA complex. All chemicals were purchased from Aldrich. NMR spectra were measured on a Bruker Avance-400 spectrometer. ^{17}O NMR measurements were performed at 54.1 MHz in 5 mm sample tubes at 70 °C using 0.05 s of acquisition time and no acquisition delay. After collecting 128 scans, the ^{17}O chemical shifts were determined by fitting of the resonances with mixed Lorentzian-Gaussian curves. The measurements were performed with and without frequency locking of the spectrometer for samples in D_2O and H_2O , respectively.

A. Determination of q for a sample in D_2O as the solvent

19.6 mg (49.8 μmol) of DTPA was weighed directly into a 5 mm NMR tube and dissolved in 0.5744 g of D_2O , 17.8 mg (47.3 μmol) of $\text{DyCl}_3 \cdot 6\text{H}_2\text{O}$ was added to the same tube to yield the solution of the desired complex with 5% excess of ligand. A small amount of the

sample solution was used in the Xylenol Orange test to confirm the absence of free Dy(III) ion.²² The pH of the sample was measured using a Corning 125 pH meter with a calibrated micro-combination probe and corrected to 6.5 by addition of a freshly prepared solution of NaOH. The amount of water introduced during the pH adjustment was determined by weighing.

From the total amounts of Dy and water (including that of the used $\text{DyCl}_3 \cdot 6\text{H}_2\text{O}$ and the water introduced during the pH adjustments) P_m was determined to be 0.0011. The sample was placed in the spectrometer at 70 °C, and left for 10 minutes in order to reach the temperature. A ^{17}O NMR spectrum was recorded with the deuterium frequency lock and then the sample was substituted by a sample of pure D_2O , of which the ^{17}O NMR shift was measured with the same spectrometer settings. The difference in ^{17}O chemical shifts of the water resonances was -2.20 ppm (δ_{obs}). Thus, $\Delta = -2.20/P_m = -1988.6$ ppm (see Equation 2.2). At 70 °C, $F \approx -70.14$ ppm (see Equation 2.5) and therefore $q = \Delta / (\langle S \rangle \cdot F) = -1988.6 / (28.565 \times -70.14) = 0.99$ (see Equation 2.3).

B. Determination of q for a sample in H_2O as the solvent

The sample was prepared in the same way as for method A, but now with exclusively MQ-water as the solvent. Neat *tert*-butanol (10 μL) was added as an internal reference. After equilibration of the temperature at 70 °C in the spectrometer, the ^{17}O chemical shift of the water resonance was determined without frequency locking. Then the sample was substituted by a reference sample of pure H_2O and the ^{17}O NMR shift was measured with the same spectrometer settings. Subsequently, in order to determine the BMS, a coaxial-inner tube containing water and a drop of *tert*-butanol was inserted and a ^1H NMR spectrum was measured. Finally, the ^{17}O shift corrected for the BMS effect ($\delta_{\text{obs,corr}}$) was evaluated using Equation 2.9.

$$\delta_{\text{obs,corr}} = \delta_{\text{obs}}^{^{17}\text{O},\text{H}_2\text{O}} - (\delta_{\text{complex}}^{^1\text{H},t\text{-Bu}} - \delta_{\text{ref}}^{^1\text{H},t\text{-Bu}}) \quad 2.9$$

Here, $\delta_{obs}^{^{17}\text{O},\text{H}_2\text{O}}$ is the observed difference in the ^{17}O chemical shifts for water in the samples with complex and pure water, and the second term on the right-hand side is the BMS correction, which is the ^1H chemical shift difference between the methyl resonances from the outer- and inner-tube. The value of q as calculated from $\delta_{obs,corr}$ in the similar way as described under method A was 1.08.

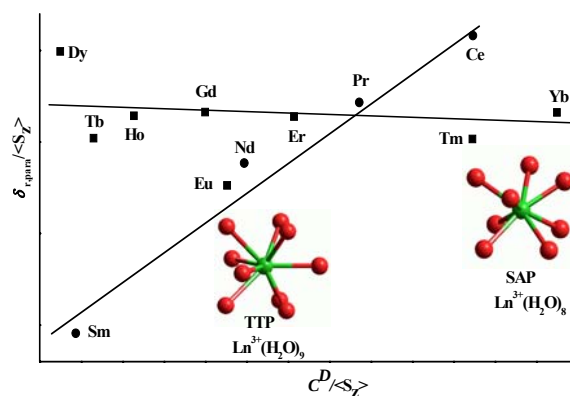
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THE STRUCTURE OF THE LANTHANIDE AQUO IONS AS STUDIED BY ^{17}O NMR SPECTROSCOPY AND DFT CALCULATIONS

3



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INTRODUCTION

Lanthanide(III) chelates have unique chemical and physical properties, which have been widely exploited in many fields including catalysis,¹ molecular imaging,² molecular therapy,³ and structural analysis.⁴ The electronic configuration of the Ln(III) ions is $[Xe]4f^n$, where $n = 0 - 14$. La(III) and Lu(III) have no unpaired electrons and are diamagnetic, whereas the other lanthanide(III) ions in this series have 1 – 7 unpaired electrons and are thus paramagnetic. As a result of the shielding of their 4*f* valence electrons by the 5*s* and 5*p* electrons, the 15 Ln(III) ions have very similar chemical behavior and the various Ln(III) complexes of a particular ligand are generally almost isostructural. However, the physical properties, such as the paramagnetic induced NMR shifts and relaxation rate enhancements that they induce in nuclei of bound ligands show substantial differences across the series. Hence, a large set of structure related physical data can be collected on a set of (almost) isostructural Ln(III) complexes and this has been utilized extensively for the elucidation of the solution structure of Ln(III) complexes.⁴

The coordination number of Ln(III) ions for one of the most simple ligands possible, water, has been the subject of some controversy in the past, but nowadays, it is generally accepted that the number of water molecules in the first coordination sphere of Ln(III)-aquo ions decreases from 9 to 8 with the decrease of the ionic radius of the Ln(III) ions across the series.^{5,6} Molecular dynamics simulations suggest that a distinct transition from 9- to 8-coordination takes place at Sm(III).⁷⁻⁹

Previously, we have shown that Ln(III) induced paramagnetic shifts of the water ¹⁷O resonance can be used to evaluate the Ln(III) solvation number in aqueous solutions of Ln(III) complexes.^{4,10-12} The ¹⁷O NMR method has successfully been applied for the determination of the number of Ln(III) bound water molecules in many lanthanide complexes. Lanthanide induced shifts for the Ln(III) aquo ions have been reported in the early days of ¹⁷O NMR by Lewis et al.¹³ and by Reuben and Fiat.¹⁴ These data were measured at low field instruments and, unfortunately, the accuracy of the chemical shifts did not allow a discrimination between 8- and 9-coordination in these complexes.

A large number of crystal structures containing $Ln(H_2O)_9^{3+}$ or $Ln(H_2O)_8^{3+}$ moieties have been reported in the literature. For example, lanthanide bromate nonahydrates have

been reported to have $[\text{Ln}(\text{H}_2\text{O})_9](\text{BrO}_3)_3$ stoichiometry.^{15,16} The lanthanide trifluoromethanesulfonates have similar molecular structures.¹⁷⁻¹⁹ It has been shown that the Ln(III) ion in these compounds is coordinated by nine water molecules with a tricapped trigonal prism (TTP) coordination polyhedron. Water deficiency was found for the smallest Ln(III) ions and the TTP polyhedron was stabilized by hydrogen bonding to the trifluoromethanesulfonate anion.¹⁹ $\text{Ln}(\text{H}_2\text{O})_8^{3+}$ ions have been observed in various crystal structures of more complicated systems. The most frequently observed Ln(III) coordination polyhedron in these structures is the square antiprism (SAP), but bicapped octahedrons and trigonal prisms occur as well.

Previously, we have shown, by ^{17}O NMR, that aqueous solutions of $\text{Dy}(\text{CF}_3\text{SO}_3)_3$ have 8 water molecules in the inner coordination sphere, whereas the CF_3SO_3^- anions do not bind directly to the Dy(III) ion but occur predominantly in the second coordination sphere.²⁰ In the present paper, we describe a ^{17}O NMR study on aqueous solutions of each of the Ln(III) trifluorosulfonates (except $\text{Ln} = \text{Pm}$). The experimental lanthanide induced chemical shifts are dissected into the various shift contributions and then the contact contribution is applied to obtain information about the number of inner-sphere water molecules in the various aquo ions, whereas the pseudocontact contribution is compared with values calculated for TTP and SAP geometries for Ln-aquo ions as obtained from density functional theory (DFT) calculations.

RESULTS AND DISCUSSION

To avoid any interference of internal standards, the ^{17}O NMR spectra of aqueous solutions of the lanthanide trifluoromethanesulfonates were recorded with respect to water as external reference using the substitution method. Using a high resolution liquids probe with cylindrical sample tubes would result in substantial bulk magnetic susceptibility (BMS) contributions to the Ln(III) induced shifts for the paramagnetic compounds ($\text{Ln} = \text{La}, \text{Lu}$).²¹ Therefore, it was decided to measure these shifts with the use of a solid state probe, in which the sample is spun under an angle of 54.7° (the magic angle) with the main magnetic field. Under those conditions, the BMS shift contribution vanishes.^{22,23}

The Ln(III) induced water ^{17}O NMR shifts observed appeared to be linearly dependent on the concentration of the Ln(III) triflate. Therefore, it may be concluded that the exchange of water between a Ln(III)-aquo ion and the bulk is fast on the ^{17}O NMR time scale under the conditions applied. The observed chemical shifts of the ^{17}O water resonance of the Ln-triflate samples (δ_{obs}) were converted into reduced chemical shifts (δ_r) with the use of Equation 3.1.

$$\delta_r = q\Delta = (\delta_{\text{obs}} - \delta_{\text{ref}}) / P_m \quad 3.1$$

Here, δ_{ref} is the chemical shift of pure water and P_m is the molar ratio Ln(III)/H₂O. The δ_r values obtained are compiled in Table 3.1. The δ_r values for the paramagnetic Ln(III) ions contain a diamagnetic contribution originating from, for example, inductive effects. As can be seen from Table 3.1, the shifts observed for the diamagnetic La(III) and Lu(III) aquo ions are relatively small. The diamagnetic contributions to the chemical shifts of the various paramagnetic Ln(III) aquo ions were estimated by interpolation between the chemical shifts for the La(III) and Lu(III) systems, assuming a linear relationship with the atomic number of the Ln(III) in question (Table 3.1).

The paramagnetic contribution to the reduced shifts, $\delta_{r,\text{para}}$ is linearly proportional to the hydration number of the Ln(III) ion (q) and the bound shift of a Ln(III) bound ^{17}O nucleus in a complex with stoichiometry $\text{Ln}(\text{H}_2\text{O})_q^{3+}$ (see Equation 3.2). These bound ^{17}O chemical shifts arise from two terms: the contact (Δ_{con})^{24,25} and pseudocontact or dipolar (Δ_{pc}) shifts.²⁶⁻²⁸

$$\delta_{r,\text{para}} = q\Delta - q\Delta_{\text{dia}} = q(\Delta_{\text{con}} + \Delta_{\text{pc}}) \quad 3.2$$

The contact shift is the result of a through-bond interaction of the unpaired electron-spin density of f -electrons of the lanthanide to the oxygen of the bound water molecule, whereas the pseudocontact or dipolar shift is a through-space effect of the interaction between the magnetic moments of the unpaired electrons at the Ln(III) ion and the ^{17}O nucleus. For a complex with effective axial symmetry, Δ_{con} and Δ_{pc} can be expressed as in Equations 3.3 and 3.4.

Table 3.1. Experimental and calculated reduced Ln(III) induced ^{17}O NMR water shifts (ppm) in 0.1 M aqueous solutions of Ln(III) triflates at 54 MHz and 25 °C.

Ln	Observed		Calculated		
	δ_t	δ_r	$\delta_{r,\text{dia}}$	$\delta_{r,\text{con}}$	$\delta_{r,\text{para}}$
La	287	287	287	0	0
Ce	728	1039	281	769	-11
Pr	2171	2588	274	2335	-20
Nd	3864	3788	268	3517	4
Sm	-30	78	255	-177	1
Eu	-6327	-5764	248	-5979	-34
Gd	-20819	-24639	242	-24881	0
Tb	-23322	-24125	235	-25160	799
Dy	-14124	-21378	229	-22563	956
Ho	-15134	-17287	222	-17884	375
Er	-10250	-12265	216	-12150	-331
Tm	-5884	-6767	209	-6485	-491
Yb	-1530	-2061	203	-2045	-219
Lu	196	196	196	0	0

$$\Delta_{con} = \langle S_z \rangle > \frac{\beta}{3kT\gamma_0} \frac{A}{\hbar} \times 10^6 = \langle S_z \rangle > F \quad 3.3$$

$$\Delta_{pc} = D(A_2^0 \langle r^2 \rangle)G \quad 3.4$$

Here, $\langle S_z \rangle$ is the spin expectation value along the direction of the magnetic field and is characteristic of the Ln(III) ion in question, β is the Bohr magneton, k is the Boltzmann constant, T the absolute temperature, γ_0 the ^{17}O gyromagnetic ratio, $A/\hbar$ the hyperfine coupling constant between the unpaired electrons of the Ln(III) ion and the ^{17}O nucleus of water, D is proportional to the magnetic susceptibility tensor of the Ln(III)-aquo ion and to the Bleaney's constant C^D , A_2^0 and $\langle r^2 \rangle$ are crystal field parameters, and G is a geometric factor of the Ln(III) complex. The latter is given by Equation 3.5.

$$G = \frac{3\cos^2\theta - 1}{r^3} \quad 3.5$$

Values of $\langle S_z \rangle$ and values for C^D have been tabulated in the literature.²⁴⁻²⁸ In Equation 3.5, r is the distance between the Ln(III) ion and the bound oxygen nucleus, whereas θ is the angle between the Ln-O vector and the main symmetry axis of the Ln(III) complex.

From Equations 3.3 – 3.5, it can be seen that both Δ_{con} and Δ_{pc} can be expressed as the sum of terms that are characteristic of the Ln(III) ion but independent of the nucleus under study ($\langle S_z \rangle$ and C^D , respectively) and terms that are independent of the Ln(III) ion but dependent on the nucleus under study (F and G). For a series of isostructural Ln(III) complexes, Equation 3.2 can be simplified as 3.6, which can be written in two linear forms (see equations 3.7 and 3.8).²⁹ Here, C^D is the Bleaney constant scaled to -100 for Dy(III) and K is a constant proportional to the crystal field parameters.

$$\delta_{r, \text{para}} = \langle S_z \rangle (qF) + C^D (qKG) \quad 3.6$$

$$\frac{\delta_{r, \text{para}}}{C^D} = \frac{\langle S_z \rangle}{C^D} (qF) + qKG \quad 3.7$$

$$\frac{\delta_{r, \text{para}}}{\langle S_z \rangle} = qF + \frac{C^D}{\langle S_z \rangle} (qKG) \quad 3.8$$

Plots of δ_r according to Equations 3.7 and 3.8 both show a break at Eu(III) (See Figures 3.1 and 3.2). Previously, we have demonstrated that for the Ln(III) bound ^{17}O nuclei, F and thus $A/\hbar$ is almost independent on the structure of the Ln(III) complex under study and even of the nature of the oxygen atom, the value of $A/\hbar$ being always in the range $(-3.9 \pm 0.3) \times 10^6 \text{ rad s}^{-1}$.¹⁰⁻¹² From the slopes of the lines in Figure 3.1, it can be concluded that upon going from Ce to Yb, a rather abrupt decrease of qF occurs at Eu. Since F is almost constant, this break must correspond to a decrease of q from 9 to 8 at Eu, which is in agreement with the results of a neutron scattering first-order difference study.^{5,6}

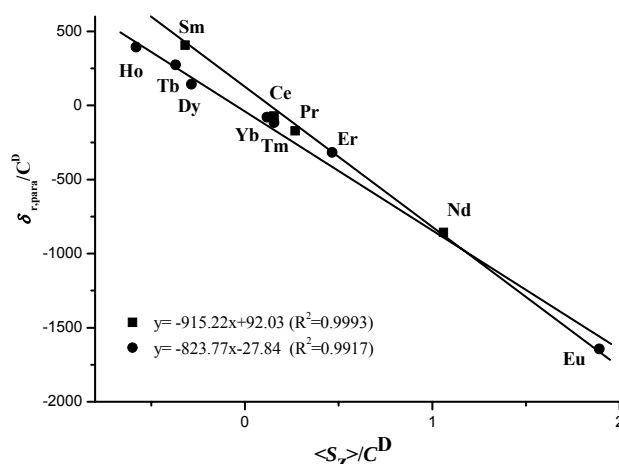


Figure 3.1. Plot according to Equation 3.7 of the paramagnetic contributions of ^{17}O NMR shifts of water in the system $\text{Ln(III)triflate-water}$.

The plot according to Equation 3.8 shows a clear break at Eu(III) as well (see Figure 3.2); such a large change in slope cannot be explained by a gradual change of G across the Ln(III) series as a result of the lanthanide contraction.³⁰ Thus, the change of q at Eu(III) must be accompanied by a change of G and/or the crystal field parameters.

Computation of geometries of $\text{Ln}(\text{H}_2\text{O})_8^{3+}$ and $\text{Ln}(\text{H}_2\text{O})_9^{3+}$

The geometries of the octa- and nonacoordinated Ln(III)-aquo ions in aqueous solution were evaluated with Density Functional Theory (DFT) calculations at the B3LYP/6-31+G(d) level. As there is not a good all-electron basis set for lanthanides, the effective core potential (ECP) of Dolg et al. and the related [5s4p3d]-GTO valence basis set were applied in these calculations.³¹ This ECP includes $46 + 4f^n$ electrons in the core, leaving the outermost 11 electrons to be treated explicitly, and it has been shown to provide reliable results for a wide range of lanthanide complexes.³² Compared to all-electron basis sets, ECPs account to some extent for relativistic effects, which are believed to become important for the elements from the fourth row of the periodic table. In these calculations

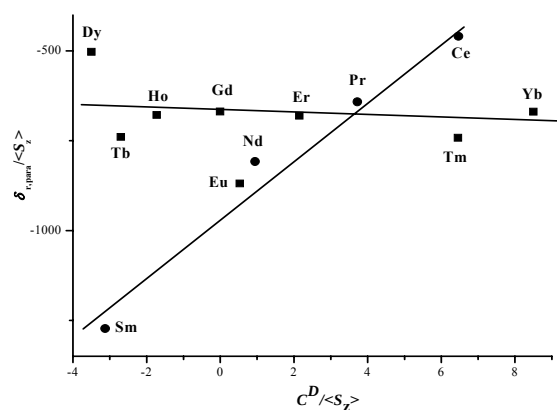


Figure 3.2. Plot according to Equation 3.8 of the paramagnetic contributions of ^{17}O NMR shifts of water in the system Ln(III)-triflate-water.

the surrounding solvent effects (water) were taken into account by using the polarized continuum model (PCM). The computed $\text{Ln}(\text{H}_2\text{O})_9^{3+}$ ions have a TTP coordination polyhedron with a slightly distorted D_3 symmetry, whereas the $\text{Ln}(\text{H}_2\text{O})_8^{3+}$ ions show SAP structure with a slightly distorted S_8 symmetry (see Figure 3.3). This is in agreement with quantum chemical calculations performed at the HF (Hartree-Fock) level on the Nd, Gd and Yb aquo ions.³⁴ Because of the optimization convergence difficulty in case of the C-PCM model, full convergence of the geometry optimizations was not reached.³⁵⁻³⁸ Thus, the calculated geometries must be considered as stable conformations for the chosen minimization algorithm rather than true energy minima.³⁶ However, it must be pointed out that geometry optimizations performed in vacuo (data not shown) provide calculated structures showing Ln-O bond distances considerably longer (ca. 0.05 Å) than those obtained in aqueous solution, as often observed when Ln complexes are studied in vacuo by means of quantum mechanical calculations.^{39,40} The shortening of the Ln-O bond distances observed in solution can be ascribed to a stronger water-ion interaction due to the solvent polarization effects that, increasing the dipole moment of the free water molecules, increase the water-ion interaction.³⁴ Thus, the surrounding solvent effects must be included in geometry optimization to obtain molecular structures in better agreement with solid state and solution experimental structures.

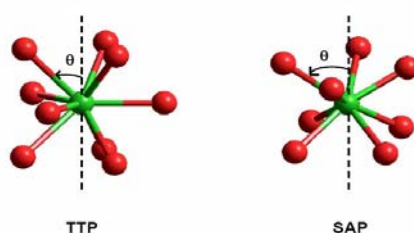


Figure 3.3. The coordination polyhedra of the Ln(III) aquo ions. The dotted lines give the location of the main symmetry axis.

In Table 3.2, the geometrical parameters defining the calculated TTP structures are compared with those of X-ray structures reviewed by Kepert.¹⁸ Additionally, the SAP structures are compared in Table 3.3 with averages of a series of X-ray structures containing $\text{Ln}(\text{H}_2\text{O})_8^{3+}$ ions in a SAP arrangement. The agreement between the calculated and the X-ray structures appears to be very good. The calculated structures for the $\text{Ln}(\text{H}_2\text{O})_9^{3+}$ systems show r_{ax} bond distances somewhat longer than the experimental ones ($0.05 - 0.08 \text{ \AA}$), while the calculated r_{eq} distances are slightly shorter than those found experimentally in the solid state ($0.03 - 0.07 \text{ \AA}$). It is normally found that DFT calculations based on the B3LYP model provide Ln-donor distances slightly longer than the experimental ones observed by X-ray crystallography.⁴¹ Thus, in the case of the $\text{Ln}(\text{H}_2\text{O})_9^{3+}$ systems our calculations appear to overestimate the interaction between the lanthanide and the equatorial water molecules. As a result, the calculated r_{ax} and r_{eq} distances differ only by $0.003\text{--}0.045 \text{ \AA}$, while the experimental ones differ by $0.09\text{--}0.20 \text{ \AA}$. However, the calculated r_{eq} distances are always longer than the r_{ax} ones, in agreement with the experimental evidence. The calculated structures for the $\text{Ln}(\text{H}_2\text{O})_8^{3+}$ systems show Ln-O distances somewhat longer than the solid state ones. The calculated structure of $\text{Nd}(\text{H}_2\text{O})_9^{3+}$ presents Nd-O distances that are in good agreement with those determined experimentally in solution from neutron diffraction studies ($2.50 \text{ \AA}$).⁶

Table 3.2. Comparison of the geometrical parameters of the TTP coordination polyhedra in $\text{Ln}(\text{H}_2\text{O})_9^{3+}$ obtained from DFT calculations with those observed in crystal structures.

Ln	Calculated				X-ray structures ¹⁷		
	r_{ax} (Å)	r_{eq} (Å)	θ_{ax} (°) ^a	$1000 \times G$	r_{ax} (Å)	r_{eq} (Å)	θ_{ax} (°)
Ce	2.575	2.578	44.21	1.683			
Pr	2.551	2.556	44.17	1.862	2.479	2.592	45.1
Nd	2.538	2.543	45.50	-0.947			
Sm	2.506	2.517	45.63	-1.121	2.460	2.550	47.2
Eu	2.491	2.504	45.68	-1.201			
Gd	2.475	2.493	45.72	-1.185	2.402	2.546	44.8
Tb	2.458	2.476	44.12	2.554			
Dy	2.444	2.466	44.40	2.040			
Ho	2.432	2.457	44.13	2.811	2.373	2.474	45.0
Er	2.420	2.447	44.29	2.518	2.370	2.520	45.5
Tm	2.409	2.452	45.91	-1.040			
Yb	2.399	2.444	46.04	-1.320	2.321	2.518	45.1

^a $\theta_{\text{eq}} = 90$ for all Ln(III) ions.

A good agreement is also found between the calculated geometries of $\text{Dy}(\text{H}_2\text{O})_8^{3+}$ and $\text{Yb}(\text{H}_2\text{O})_8^{3+}$ and the experimental Ln-O distances observed in solution from neutron diffraction experiments (2.38 and 2.32 Å, respectively).⁶

The G -values for the O nuclei in the various calculated structures were calculated by using Equation 3.5. For the TTP geometries, a weighted average of G -values for the equatorial and axial oxygen atoms was calculated. The values obtained are included in Tables 3.2 and 3.3. The six axial O-nuclei in the TTP polyhedron are in the positive shift cone ($\theta < 54.7^\circ$), whereas the three equatorial nuclei are in the negative shift cone ($54.7^\circ < \theta < 125.3^\circ$). After averaging, the G_{ax} and G_{eq} contributions nearly cancel, and consequently the G -values for this structure are small in an absolute sense. Slight changes in the calculated θ_{ax} values across the lanthanide series result in small positive or negative G -values depending on the particular lanthanide ion considered. The O-nuclei of the SAP polyhedron all have θ values of about 57° , which is in the negative shift cone ($\theta > 54.7^\circ$) close to the position where the sign of G changes. Consequently, the G -values for this structure are all negative and small in an absolute sense. The G -values are related to the slopes in Figure 3.2,

Table 3.3. Comparison of the geometrical parameters of the SAP coordination polyhedra in $\text{Ln}(\text{H}_2\text{O})_8^{3+}$ obtained from DFT calculations.

Ln	calculated			X-ray structures ^a	
	r (Å)	θ (°)	$1000 \times G$	r (Å)	θ (°)
Ce	2.537	57.44	-8.027		
Pr	2.516	57.38	-8.051		
Nd	2.500	57.31	-7.993	2.41 (0.04)	57.7 (0.8)
Sm	2.460	57.30	-8.358		
Eu	2.446	57.29	-8.478	2.40 (0.04)	56.7 (3.6)
Gd	2.433	57.44	-9.101	2.40 (0.02)	57.4 (2.2)
Tb	2.419	57.45	-9.294	2.38 (0.02)	57.3 (2.7)
Dy	2.405	57.48	-9.559	2.39 (0.02)	57 (3.3)
Ho	2.392	57.45	-9.612		
Er	2.378	57.52	-10.030		
Tm	2.368	57.27	-9.263		
Yb	2.355	57.42	-10.254	2.33 (0.03)	57.2 (1.3)

^a Averaged parameters from structures in Cambridge Structural Database. Standard deviations are given in parentheses.

and it can be seen that the slope obtained for the light lanthanide ions ($\text{Ln} = \text{Ce} - \text{Eu}$) is substantially different than that observed for the heavier ones, which is related, at least in part, to the different G -values of TTP and SAP geometries.

Fitting of the ^{17}O NMR data

In order to verify, whether the change in structure of the $\text{Ln}(\text{III})$ -aquo ions is also reflected in a change in crystal field parameters, the ^{17}O NMR data were fitted with Equation 3.6 using F and K as adjustable parameters. The data were split into two sets, one for Ce-Sm and another for Eu-Yb. The q values for these sets were fixed at 9 and 8, respectively. The best fit parameters obtained are given in Table 3.4 and the dissected chemical shifts of the various $\text{Ln}(\text{III})$ aquo ions as calculated with the best-fit parameters are included in Table 3.1. The values for F are the same within the accuracy range for the two sets of lanthanides; this value corresponds to a hyperfine coupling constant $A/\hbar = -4.2 \times 10^6 \text{ rad s}^{-1}$, which is in the generally observed range for a $\text{Ln}(\text{III})$ coordinated ^{17}O nucleus $((-3.9 \pm 0.3) \times 10^6 \text{ rad s}^{-1})$.¹⁰⁻¹²

Table 3.4. Best fit parameters obtained from fitting the ^{17}O NMR data to Equation 3.6.

Ln	F	K
Ce-Sm	-87 ± 2	2558 ± 567
Eu-Yb	-85 ± 5	244 ± 239

The best fit value of K for the heavier lanthanides (Eu–Yb) is an order of magnitude smaller than that of the lighter ones (Ce–Nd). This implies that the same holds for the crystal field parameters. A change of crystal field parameters going from a TTP to a SAP coordination should be expected, since the charge distribution around the Ln(III) ion in these polyhedra differ substantially. The sensitivity of the magnitude of the magnetic anisotropy of lanthanide complexes to the coordination environment has, for example, been demonstrated by Mironov et al. by means of numerical calculations on some model systems.⁴²

An alternative explanation for the very low G -values of the 8-coordinate Ln(III)-aquo ions would be that the angle θ is closer to 54.7° than predicted by the DFT calculations. It can be calculated that, if K was invariant across the lanthanide series, θ should be around 55° . In our opinion, a difference of about 2° with both the results of DFT calculations and X-ray structures of similar compounds is very unlikely.

CONCLUSIONS

The change of coordination number of the Ln(III) aquo ions across the lanthanide series (at Eu(III)) can be observed by ^{17}O NMR spectroscopy. Since the Ln–O hyperfine coupling constant is independent of the coordination polyhedron around the Ln(III) ion, the contact contribution to the Ln(III) induced shift is linearly proportional to the number of water molecules in the first coordination sphere of the Ln(III) ion. The change in hydration number at Eu(III) is also reflected in a change in pseudocontact shifts. Analysis of these shifts using DFT geometries optimized in aqueous solution indicates that the Ln(III)-bound oxygen atoms in $\text{Ln}(\text{H}_2\text{O})_9^{3+}$ are arranged in a TTP polyhedron and those in $\text{Ln}(\text{H}_2\text{O})_8^{3+}$ in a SAP polyhedron. This change in structure is reflected in a change of both G and the crystal field parameters.

EXPERIMENTAL

Lanthanide trifluoromethanesulfonates were purchased from Aldrich and 10% ^{17}O labeled water from Cortec, Paris, France. The NMR samples with a concentration of 0.1 M were prepared by dissolving the appropriate amount of the Ln(III) triflate under study in H_2O , which was acidified to pH 2 with diluted HCl. To each sample 20 μL of 10% ^{17}O labelled water was added. NMR spectra (^{17}O) were measured on a Bruker Avance 400 spectrometer using a solid state probe and 4 mm zirconia rotors, which were spun with a rate of 13 Hz. The spectra were recorded with a 90° pulse width (10 μs), a sweep width of 27100 Hz, 0.6 s acquisition time, and no acquisition delay. Peak positions were measured with respect to the resonance of pure water as external reference (substitution method). The reference sample was measured prior and after each measurement of a lanthanide triflate sample. Peak positions were determined by simulation of the resonances using Lorentzian peak shapes and the deconvolution program of the spectrometer. All fittings were performed using the computer program Micromath Scientist, version 2.0 (Salt Lake City, UT).

Computational details

All calculations were performed employing hybrid DFT with the B3LYP exchange-correlation functional,^{43,44} and the Gaussian 03 package (Revision C.01).⁴⁵ Full geometry optimizations of the $[\text{Ln}(\text{H}_2\text{O})_8]^{3+}$ and $[\text{Ln}(\text{H}_2\text{O})_9]^{3+}$ systems (Ln = Ce-Yb, except Pm) were performed in aqueous solution by using the quasi relativistic effective core potentials (ECPs) of Dolg et al. and the related [5s4p3d]-GTO valence basis sets for the lanthanides, and the 6-31+G(d) basis set for H and O atoms.³¹ Inclusion of solvent effects was performed using the integral equation formalism variant of the polarizable continuum model (IEF-PCM).⁴⁶⁻⁴⁹ In line with the united atom topological model (UATM),⁵⁰ the solute cavity is built as an envelope of spheres centered on atoms or atomic groups with appropriate radii. For both lanthanides³⁴ and water molecules (1.68 Å) the previously parameterized radii were used. During geometry optimization the non-electrostatic contributions to the energy and energy gradient, viz. cavitation, dispersion, and repulsion contributions, were omitted. Calculations were performed using an average area of 0.2 Å²

for all the finite elements (tesserae) used to build the solute cavities. Owing to the slow convergence of the optimizations,³⁸ they were stopped when the convergence parameters were about twice the default values.³⁶ For this reason frequency analysis was not performed to characterize the stationary points; thus, the final geometries correspond to stable conformations for the chosen minimization algorithm, rather than to true minima. Optimizations started from the molecular geometries optimized in vacuo, which were characterized by frequency analysis.

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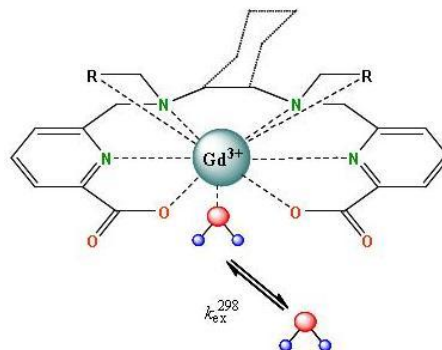
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LANTHANIDE (III) CHELATES CONTAINING PYRIDINE UNITS WITH EXTREMELY FAST WATER EXCHANGE

4



The contents of this chapter have been adapted from:

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INTRODUCTION

Stable chelates of trivalent Ln ions are gaining considerable interest because of their use in various biomedical applications, such as fluorescence imaging,¹ specific RNA-cleavage,² cancer radionuclide therapy,^{3,4} or magnetic resonance imaging (MRI).⁵ In particular, gadolinium complexes with poly(amino-carboxylate) ligands, such as $[\text{Gd}(\text{DTPA})(\text{H}_2\text{O})]^{2-}$ (DTPA = diethylenetriamine-*N,N,N',N'',N'''*-pentaacetate), $[\text{Gd}(\text{DTPA-BMA})(\text{H}_2\text{O})]$ (DTPA-BMA = DTPA-bis(methylamide), $[\text{Gd}(\text{DOTA})(\text{H}_2\text{O})]^{1-}$ (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate), and $[\text{Gd}(\text{HP-DO3A})(\text{H}_2\text{O})]$ (HP-DO3A = 10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-triacetate), attract considerable interest since they are commonly used as contrast agents (CA) in MRI.^{6,7} The spectacular evolution of this diagnostic technique into one of the most powerful modalities in clinical diagnostics has been largely assisted by the development of Gd(III)-based contrast agents. The image contrast upon administering of CA enhances by preferentially influencing the relaxation rate of the water protons in the target tissue. The efficiency of a CA is evaluated in terms of its relaxivity, which is defined as the relaxation rate enhancement per mM of the paramagnetic Gd(III)-ion (in $\text{s}^{-1}\text{mM}^{-1}$). The previously used complexes contain at least one Gd(III)-bound water molecule that exchanges with the bulk water of the surrounding tissues, resulting in an efficient mechanism for the longitudinal (T_1) and transverse (T_2) relaxation of water protons. The longitudinal relaxation rates of the bulk water protons are enhanced around the paramagnetic ion because of long-range interactions (outer-sphere relaxation) and short-range interactions (inner-sphere relaxation). The latter process is governed by four correlation times: the rotational correlation time of the complex (τ_R), the residence time of a water proton in the inner coordination sphere (τ_m), and the longitudinal ($1/T_{1e}$) and transverse electronic relaxation rates ($1/T_{2e}$) of the metal center.⁸ The theory predicts that high relaxivities at the imaging fields of 0.5 to 1.5 T may be observed for systems with a long rotational correlation time and relatively fast water exchange. For low molecular weight systems, the optimal value of τ_m is in the range of 30-40 ns.

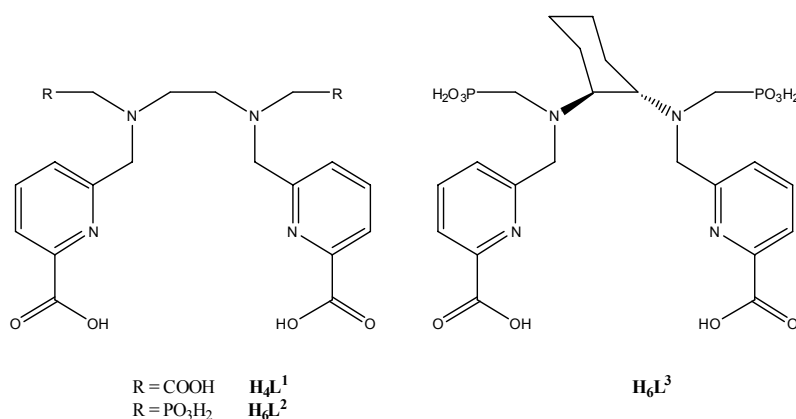


Figure 4.1. Structures of the ligands investigated in this study.

The modulation of the water exchange rate on Gd(III) complexes has been the subject of extensive studies. The current commercial Gd(III) chelates show water exchange rates that are an order of magnitude lower than the optimal value. Recently, it was shown that the phosphonate-containing analogues of the commercially used $[\text{Gd}(\text{DOTA})(\text{H}_2\text{O})]^-$ and $[\text{Gd}(\text{DTPA})(\text{H}_2\text{O})]^{2-}$ complexes have faster water exchange than the parent systems.^{9,10}

The presence of a bulky phosphonate group replacing a carboxylate in the framework of DOTA^{4-} or DTPA^{5-} causes an increased steric strain at the Gd(III)-coordinated water molecule and as a consequence an increase in the water exchange rate in their Gd(III) complexes. However, the water exchange rate on these systems is still largely inferior to that in $[\text{Gd}(\text{H}_2\text{O})_8]^{3+}$ ($k_{\text{ex}}^{298} = (8.04 \pm 0.8) \times 10^8 \text{ s}^{-1}$), which can be attributed to the easy rearrangement in the flexible coordination sphere of the latter complex. The Gd(III) aquo ion is eight-coordinate but very close to a hydration equilibrium between coordination numbers 8 and 9. From neutron diffraction measurements it is known that, as the ionic radius decreases in the lanthanide series, the coordination number of the aquo ions changes from 9 at the beginning to 8 at the end, with Sm(III) having an average coordination number of 8.5.¹¹ Similar results were obtained from ^{17}O NMR study on Ln(III) triflates, demonstrating a break in the contact contribution at Eu(III), reflecting a change in the number of water molecules in the first coordination sphere from 9 to 8 going from light to

heavier lanthanides (see Chapters 2 and 3 of this thesis).¹² By contrast, Gd(III) chelates have a much more rigid inner-sphere structure and its rearrangements require higher energies. On the basis of these considerations, faster water exchange is expected for Gd(III) complexes with a hydration equilibrium, especially when they possess a flexible inner-sphere structure.

In this work we report on three novel octadentate ligands containing pyridine units and carboxylate pendants, **L**¹ (=N,N'-Bis[(6-carboxy-2-pyridyl)methyl]ethane-1,2-diamine-*N,N'*-diacetic acid), and phosphonate pendants with a) ethane-1,2-diamine, **L**² (=N,N'-Bis[(6-carboxy-2-pyridyl)methyl]ethane-1,2-diamine-*N,N'*-methylenephosphonic acid) and b) cyclohexane-1,2-diamine, **L**³ (=N,N'-Bis[(6-carboxy-2-pyridyl)methyl]cyclohexane-(1*R*,2*R*)-diamine-*N,N'*-methylenephosphonic acid) backbones (Figure 4.1), which form stable complexes with Ln(III) ions in aqueous solutions. In these complexes, the Ln(III) ion is nine-coordinate, where a water molecule completes the inner coordination sphere.¹³

A modification of the chelate by substituting of carboxylate pendant by phosphate groups allows for the assessment of how small structural changes affect properties that are important with regard to MRI contrast agent application, such as hydration number, water exchange rate, or rotational dynamics of the complexes. Incorporation of a cyclohexyl unit in the ligand skeleton modulates the rigidity of the inner-sphere, which may have consequences on the water exchange for the Gd(III) complexes. Variable temperature ¹⁷O NMR measurements and nuclear magnetic relaxation dispersion (NMRD) investigations of the Gd(III) complexes were carried out to obtain parameters governing the relaxivities including rotation, electronic relaxation, and water exchange, while the temperature dependence on the UV-Vis spectra was used to confirm the equilibrium in the hydration number.

RESULTS AND DISCUSSION

The relaxivity describes the efficiency of magnetic dipolar coupling between the solvent nuclei and the paramagnetic metal ion, and represents a measure of the efficacy of the complex as a contrast agent. The relaxation rates of the bulk water protons in the vicinity of the paramagnetic ion are enhanced as a result of long- (outer-sphere relaxation) and short-

range (inner-sphere relaxation) interactions. As pointed out previously by Merbach et al.,¹⁴ it is difficult to calculate the parameters that determine the relaxivity of a given compound from nuclear magnetic resonance dispersion (NMRD) profiles without obtaining independent information of at least some of the most important parameters. Therefore, variable-temperature ¹⁷O NMR relaxation rates were measured on solutions of Gd(III) complexes of the chelates under study and on a diamagnetic reference solution. Such a combined study allows determining parameters describing water exchange and rotational dynamics. Furthermore, ¹⁷O NMR chemical shift measurements were included, because they give information on the hyperfine coupling constant ($A/\hbar$), which is directly dependent on the scalar coupling constant in case of fast water exchange. Additionally, UV-vis spectrophotometry was performed in order to confirm the hydration number of the **L**² and **L**³ ligands.

Assessment of the hydration state

The hydration state of the Ln(III)-complexes of **L**² and **L**³ was determined by a variable-temperature UV-vis study on aqueous solutions of the Eu(III)-analogues. The ⁵D₀ ← ⁷F₀ transition band of Eu(III) (577.5 – 581.1 nm) is very sensitive to the coordination environment and is often used to test the presence of different complex species.^{15,16} In the region of the ⁵D₀ ← ⁷F₀ transition, the spectrum of the Eu(III)-complex of **L**² shows two absorption bands at 578.0 and 579.4,¹⁷ while that of **L**³ shows signals at 579.0 and 579.6 nm (Figure 4.2). The intensity ratio of these bands changes with temperature. This temperature dependence can be explained in terms of a hydration equilibrium, where the band at lower energy is assigned to eight-coordinate, non-hydrated [Eu(L)]³⁻, while that at ca. 579 nm is attributed to nine-coordinate, monohydrated [Eu(L)(H₂O)]³⁻ (L= **L**², **L**³) (Equation 4.1).



$$K_{\text{Eu}} = \frac{[\text{Eu}(\text{L})(\text{H}_2\text{O})]}{[\text{Eu}(\text{L})]} \quad 4.2$$

$$\ln K_{\text{Eu}} = \frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT} \quad 4.3$$

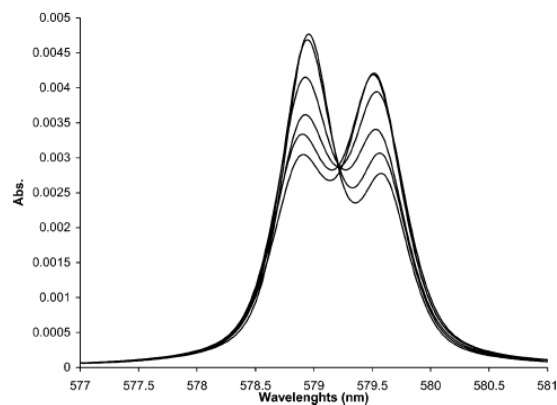


Figure 4.2. Variable-temperature UV-vis spectra of $[\text{Eu}(\text{L}^3)(\text{H}_2\text{O})_q]^{3-}$ ($q = 0, 1$; 20 mM; pH 5.5)

The ratio of the integrals of these two bands is related to the equilibrium constant K_{Eu} , which can be described by the Equation 4.1 since the concentration of the solvent is effectively constant. The reaction enthalpy, ΔH^0 , and the reaction entropy, ΔS^0 , for the equilibrium were obtained from the temperature dependence of K_{Eu} . The fit of the experimental data^{15,16} resulted in the values listed in Table 4.1 indicating that the Eu(III) complexes of L^2 and L^3 present similar average hydration numbers, with $q_{\text{ave}} = 0.64$ and 0.63 for L^2 and L^3 respectively, at 298 K.

Table 4.1. Thermodynamic parameters determined by UV-vis spectrophotometry for the hydration equilibrium in Equation 4.1.

Ligand	K_{Eu}^{298}	$\Delta H^0 (\text{kJ}\cdot\text{mol}^{-1})$	$\Delta S^0 (\text{J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1})$
L^2	1.8 ± 0.3	-11.6 ± 2	34 ± 5
L^3	1.7 ± 0.3	-13.5 ± 1	41 ± 4

Variable temperature ^{17}O NMR and NMRD measurements

The temperature dependence of the reduced longitudinal relaxation rates ($1/T_{1r}$), transversal relaxation rates ($1/T_{2r}$), and reduced chemical shifts $\Delta\omega_r$ for $\text{GdL}^1(\text{H}_2\text{O})^-$ is shown in Figure 4.2. The experimental ^{17}O NMR and NMRD data were simultaneously fitted with sets of equations usually used to describe variable-temperature ^{17}O NMR data, with Solomon-Bloembergen-Morgan equations, which describe the field dependency of the inner-sphere relaxivity, and with the Freed equation for the outer-sphere contribution to relaxivity.⁶ The full equation set used to fit the experimental data is summarized in the appendix of this chapter. However, it is useful to consider the simplified Equations 4.4 and 4.5 for the reduced relaxation rates, whereby the contribution to $1/T_{2r}$ of the chemical shift difference between the bound and the bulk water, $\Delta\omega_m$, has been neglected.

$$\frac{1}{T_{1r}} = \frac{1}{T_{1m} + \tau_m} \quad 4.4$$

$$\frac{1}{T_{2r}} = \frac{1}{T_{2m} + \tau_m} \quad 4.5$$

In these equations $1/T_{1m}$ and $1/T_{2m}$ are relaxation rates in the bound water. Since τ_m decreases, while T_{1m} and T_{2m} generally increase with increasing temperature, the sign of the temperature dependence of $1/T_{1r}$ and $1/T_{2r}$ will depend on which term dominates in the denominator of Equations 4.4 and 4.5.

The maximum observed in the temperature dependence of $1/T_{2r}$ (Figure 4.3) is characteristic of a change-over from the fast exchange limit at high temperature, whereby T_{2m} is the principal term in the denominator of Equation 4.13, to the slow exchange limit at low temperatures. Because $T_{1m} > T_{2m}$, the maximum observed in $1/T_{2r}$ is not observed for $1/T_{1r}$, which is within the fast exchange region for the whole range of experimentally available data from different temperatures.

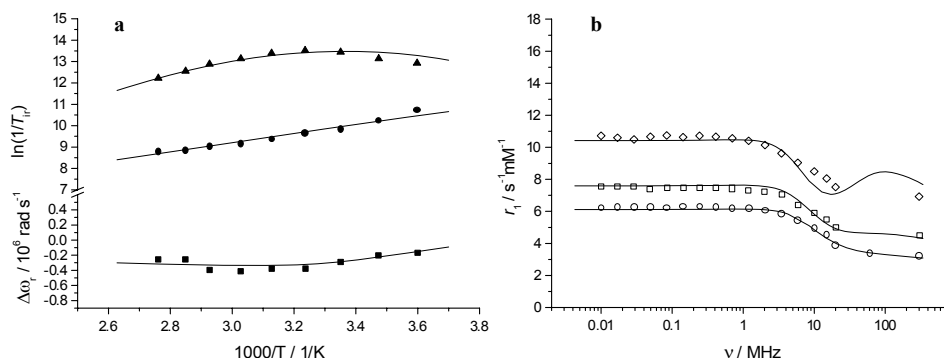


Figure 4.3. 0.153 M solution of $\text{GdL}^1(\text{H}_2\text{O})^-$, 25 °C, pH 6.38. a) Longitudinal (●), transverse (▲) relaxation rates and ^{17}O chemical shifts (■); b) NMRD profiles of $\text{GdL}^1(\text{H}_2\text{O})^-$, pH 6.6 at 5, 25 and 37 °C (from top to bottom).

The Gd(III) -complex of L^1 was investigated by water ^1H longitudinal relaxation time measurements at 5, 25 and 37 °C and magnetic field strengths varying between 2.347×10^{-4} and 7.05 T (NMRD). Longitudinal proton relaxation enhancements in NMRD studies are commonly expressed in relaxivities (r_1 , in $\text{s}^{-1}\text{mm}^{-1}$). The temperature dependence of the NMRD profile usually gives a good indication of the limiting parameters of the proton relaxivity. If the high field value of the relaxivity ($>10\text{MHz}$) does not decrease with increasing temperature, relaxivity is limited by slow water exchange, whereas in the opposite case, the fast rotation is the limiting factor, as it was indicated for the $[\text{GdL}^1(\text{H}_2\text{O})]^-$ complex (see Figure 4.3), which is usually the case for small Gd(III) chelates. The ^{17}O NMR and NMRD data were fitted simultaneously and the results, as compared in Table 4.2, seem to be in a good agreement with reported values for low molecular weight Gd -complexes.

The value obtained for the scalar coupling constant ($A/\hbar$), is smaller than those reported for other Gd(III) polyaminocarboxylate complexes that have one inner-sphere water molecule (typically $-3.8 \times 10^6 \text{ rad}\cdot\text{s}^{-1}$).¹⁴ The shift introduced by a Gd(III) -complex to the water ^{17}O resonance is proportional to q , if the exchange between Gd(III) -bound and the bulk water is rapid on the NMR timescale.⁸ Therefore, the small value obtained for $A/\hbar$ could reflect a q value lower than one, caused by the presence of a hydration equilibrium.

However, the possibility that the presence of the aromatic unit could affect the ligand backbone by an efficient spin delocalization with a consequent effect on $A/\hbar$ for the coordinated water molecule should not be excluded.

In the ligands L^2 and L^3 , phosphonate groups have a tendency to induce a second hydration sphere around the metal complexes due to their charge and important structural ordering effects.¹⁸ By remaining in the proximity of the paramagnetic Gd(III) center for a non-negligible time, these second-sphere water molecules may represent a significant contribution to the overall proton relaxivity. The second-sphere term also contributes to the longitudinal ^{17}O NMR relaxation, which is influenced by dipolar and quadrupolar contributions, both related to τ_R .¹⁹

The second-sphere water feels the rotation of the chelate and, consequently, the T_1 values will be affected by the presence of second sphere water molecules. In contrast to T_1 , the ^{17}O NMR, T_2 relaxation is mainly governed by the scalar mechanism and therefore remains insensitive to τ_R and, thus to the presence of a second hydration shell. The data of ^1H and ^{17}O NMR $\text{Gd}L^2(\text{H}_2\text{O})_q$ and $\text{Gd}L^3(\text{H}_2\text{O})_q$ were analyzed simultaneously with ^1H NMR relaxation rates. The hydration state at each particular temperature has been calculated from the equilibrium constant as determined by UV–Vis spectrophotometry for the Eu(III) analogue.²⁰ A second-sphere contribution was considered for both hydrated and non-hydrated species. The data have been fitted to the common Solomon-Bloembergen-Morgan theory relating the observed paramagnetic relaxation rates to microscopic parameters. Altogether, a very high number of parameters (21) are involved in the fit, with several of them in connection to ^{17}O and ^1H NMR data. The number of second sphere water molecules was fixed to three per complex, based on quantum mechanical calculations.¹³ These calculations showed that three second-sphere water molecules are in close proximity of the Gd(III)-ion with Gd–O distance of ca. 4.1 Å, whereas the others are relatively distant (more than 5.3 Å). The distances between O and H atoms were also fixed based on these calculations to 2.54 and 3.1 Å respectively. Four parameters describe the relaxation contribution of the second-sphere water. The exchange rate of the second-sphere water $k_{ex,2nd}^{298}$, was fixed to $2 \times 10^{10} \text{ s}^{-1}$, based on the simulations by Borel et al.,²¹ while the activation energy ($\Delta H_{2nd}^\ddagger$) was set to $20 \text{ kJ}\cdot\text{mol}^{-1}$. The distances of the second-sphere O and H atoms from the Gd-ion were fixed to 4.1 and 3.5 Å respectively.

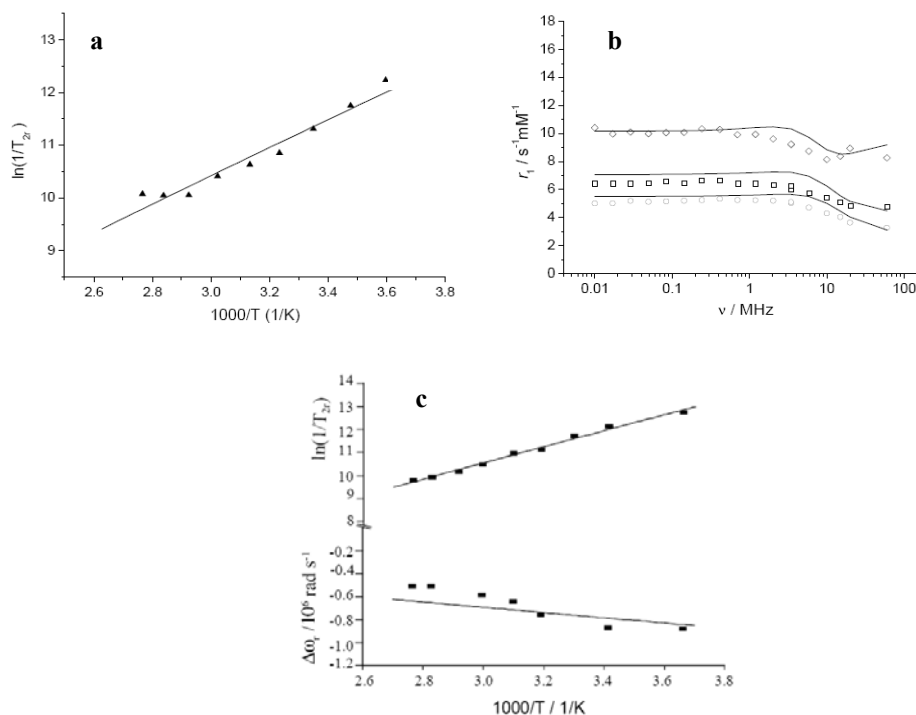


Figure 4.4. a) Transverse ($\blacktriangle$) ^{17}O relaxation rates at 7.05 T, pH 7.09; b) ^1H NMRD profiles at 5, 25 and 37 °C (from top to bottom), pH = 6.94 of $\text{GdL}^2(\text{H}_2\text{O})_q$; c) ^{17}O transverse relaxation rates and ^{17}O chemical shifts for $\text{GdL}^2(\text{H}_2\text{O})_q$ at 9.4 T, pH 9.2.

The activation energy of the correlation time for the modulation of the zero-field splitting, E_v , had to be fixed to a value of $1 \text{ kJ}\cdot\text{mol}^{-1}$; otherwise, unrealistically low values were obtained. In the simultaneous fit of the 7.05 T ^{17}O NMR and NMRD data for $\text{GdL}^2(\text{H}_2\text{O})_q$, the value of the hyperfine coupling constant ($A/\hbar$) was fixed to $-3.5 \times 10^6 \text{ rad}\cdot\text{s}^{-1}$.

The empirical constant describing the outer-sphere contribution of the ^{17}O NMR chemical shift, C_{os} , was fixed to 0. The rotational dynamics of the complex was evaluated using different rotational correlation times for the motion of the Gd inner-sphere water O- and Gd inner-sphere water H-vector (τ_{RH} and τ_{RO}), as observed by ^{17}O NMR and ^1H NMRD, respectively.²² The quadrupolar coupling constant, $\chi(1+\eta^2/3)^{1/2}$, was fixed to 7.58 MHz, the value of pure water.

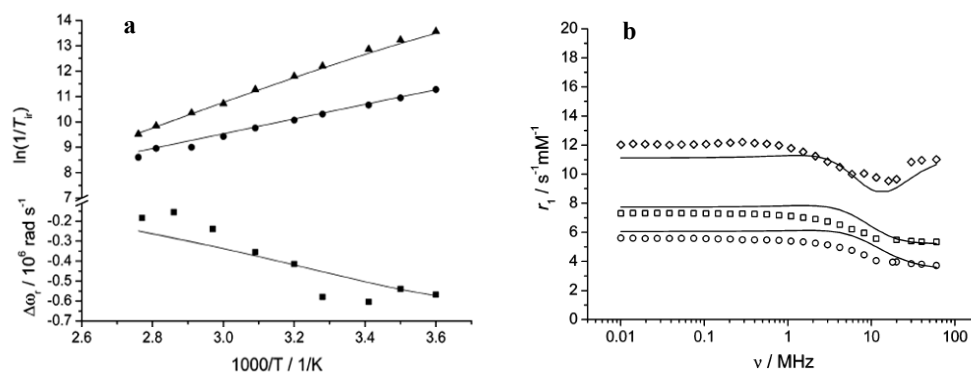


Figure 4.5. a) Reduced longitudinal (●) and transverse (▲) ^{17}O NMR relaxation rates and ^{17}O chemical shifts (■) of $\text{GdL}^3(\text{H}_2\text{O})_q$ solution at 9.4 T; b) ^1H NMRD profiles of $\text{GdL}^3(\text{H}_2\text{O})_q$, pH 6.55 at 5, 25, and 37 °C (from top to bottom), the lines represent simultaneous fit of ^{17}O NMR and NMRD as explained in the text.

The best least-squares fits were obtained using the parameters listed in Table 4.2. The diffusion coefficient (D_{GdH}^{298}), and its activation energy (E_{DGdH}) were calculated to be $(24 \pm 2) \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1}$ and $26 \pm 2 \text{ kJ} \cdot \text{mol}^{-1}$ for $\text{GdL}^2(\text{H}_2\text{O})_q$ and $(24 \pm 2) \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1}$ and $32 \pm 2 \text{ kJ} \cdot \text{mol}^{-1}$ for $\text{GdL}^3(\text{H}_2\text{O})_q$. The experimental data and the fitted curves for both complexes are presented in Figures 4.4 and 4.5.

The parameters characterizing water exchange of the chelates in study are shown and compared to those of GdDTPA and $\text{Gd}(\text{H}_2\text{O})_8^{3+}$ in Table 4.2. The rate of water exchange varies by 2 orders of magnitude between the acetate derivative $\text{GdL}^1(\text{H}_2\text{O})$ and its phosphonate analogue $\text{GdL}^2(\text{H}_2\text{O})_q$, with an intermediate k_{ex}^{298} value for $\text{GdL}^3(\text{H}_2\text{O})_q$. Although, k_{ex}^{298} calculated here for $\text{GdL}^2(\text{H}_2\text{O})_q$ by using an experimentally determined scalar coupling constant is slightly lower than in the preliminary study,¹⁷ it remains an extremely high value for a Gd(III) chelate, being comparable for the Gd(III)-aquo ion. This very fast exchange is related to the presence of a hydration equilibrium for the chelate and, more importantly, to the very flexible inner coordination sphere around the metal ion.

Table 4.2. Parameters obtained from the simultaneous analysis of ^{17}O NMR and NMRD data.

Parameter	$\text{GdL}^1(\text{H}_2\text{O})$	$\text{GdL}^2(\text{H}_2\text{O})_q$	$\text{GdL}^3(\text{H}_2\text{O})_q$	$\text{GdDTPA}(\text{H}_2\text{O})^a$	$\text{Gd}(\text{H}_2\text{O})_8$
q^{298}	1	0.64	0.63	1	8
k_{ex}^{298} [10^6 s^{-1}]	5.0 ± 0.6	700 ± 80	149 ± 12	3.3 ± 0.2	800
$\Delta H^\ddagger$ [$\text{kJ} \cdot \text{mol}^{-1}$]	40.1 ± 2.4	22 ± 2	32 ± 2	51.6 ± 1.4	15.3
$\Delta S^\ddagger$ [$\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$]	+17.8	-3 ± 6	$+18.9 \pm 6$	+53.0	-23.1
$A/\hbar$ [$10^6 \text{ rad} \cdot \text{s}^{-1}$]	-2.3 ± 0.8	-3.5 ± 0.3	-3.5 ± 0.3	-3.8 ± 0.2	-5.3
τ_{RO}^{298} [ps]	55 ± 10	155 ± 10	145 ± 13^c	58 ± 11^b	41
E_r [$\text{kJ} \cdot \text{mol}^{-1}$]	17.9 ± 1.6	27.1 ± 0.8	22.0 ± 0.6	17.3 ± 0.8	15.0
τ_V^{298} [ps]	12.6 ± 0.8	22 ± 2	18 ± 2	25 ± 1	7.3
Δ^2 [10^{20} s^{-2}]	1.2 ± 0.1	0.82 ± 0.09	0.95 ± 0.10	0.46 ± 0.02	1.19

^a Reference 14. ^b the high fitted values obtained for the quadrupolar coupling constant explain the low τ_{RO} values.

^c $\tau_{\text{RH}}/\tau_{\text{RO}} = 0.70 \pm 0.05$

Other Gd(III) complexes that also present a hydration equilibrium do not necessarily have such extreme water exchange rates. For instance, GdDO3A and GdDO2A⁺ both have differently hydrated species in aqueous solution ($q_{\text{ave}} = 1.8$ and 2.8, respectively), but they show only a limited increase of the water exchange rate as compared to monohydrated GdDOTA.²³ In addition to the decreased negative charge, which has a “slowing” effect in dissociatively activated mechanisms, the flexibility of the inner-sphere of these macrocyclic complexes is the main factor for the limited increase in k_{ex}^{298} . The inner-sphere structure is always less rigid for complexes of non-macrocyclic ligands. The introduction of a cyclohexyl ring in the amine backbone in **L**³ rigidifies the structure and, as consequence, slows down the water exchange by a factor of 5 on the Gd(III) complex.

CONCLUSION

In this work three new pyridine containing octadentate ligands are presented as promising chelating agents for Ln(III). The corresponding Gd(III) complexes are nine-coordinate, whereby in **L**¹ a water molecule completes the metal-ion coordination sphere. This induces a relaxivity in solution of the complex at the imaging fields ($5.0 \text{ s}^{-1} \text{ mM}^{-1}$ at 20 MHz and 25 °C) that is somewhat larger than those reported for the standard CA's (4.74 and 4.69 s^{-1}

mm⁻¹ at 20 MHz and 25 °C for [Gd(DOTA)(H₂O)]⁻ and [Gd(DTPA)(H₂O)]²⁻, respectively). Modification of the ligand backbone by substituting the two carboxylates by phosphonate groups (**L**²) increased the steric strain at the metal-ion coordination sphere resulting in an equilibrium between hydrated and non-hydrated species and, consequently to a very fast water exchange ($k_{ex}^{298}=7.0\times10^8\text{s}^{-1}$) comparable to the reported [Gd(H₂O)₈]³⁺ ($k_{ex}^{298}=8.0\times10^8\text{s}^{-1}$). The flexibility of the organic ligand also has an important influence on the water exchange rate. Increasing the rigidity of the ligand by introducing a cyclohexyl moiety in the backbone structure decreases the water exchange rate by a factor of 5 ($k_{ex}^{298}=1.5\times10^8\text{s}^{-1}$) in the **L**³ ligand. Thus, on the basis of structural considerations it is possible to control the water exchange rate of the inner-sphere water molecule, an important parameter to be optimized in order to obtain new, more efficient MRI contrast agents.

EXPERIMENTAL

Sample preparation

The Ln(III) complexes of **L**¹, **L**² and **L**³ were prepared by mixing equimolar amounts of GdCl₃·6H₂O and the ligand in H₂O; pH values were adjusted with the aid of dilute solutions of NH₄OH (**L**²) or NaOH (**L**¹ and **L**³) and HCl. The concentrations and pH values of the solutions used for ¹⁷O NMR variable temperature measurements were: Gd(III)**L**¹ 0.153 M, pH 6.8; Gd(III)**L**² 0.095 M, pH 7.09; Gd(III)**L**³ 0.020 M, pH 5.73 and for NMRD profiles: Gd(III)**L**¹ 1.5 mM, pH 6.60; Gd(III)**L**² 4.87 mM, pH 6.94; Gd(III)**L**³ 1.0 mM, pH 6.55.

UV-Vis spectroscopy

UV-visible spectra on solution of the Eu(III) complexes of **L**² and **L**³ were recorded in the temperature range 15.5-84.7 °C on a Perkin-Elmer Lambda 19 Spectrometer, in the thermostatable cells with a 5-cm optical length (λ=576.5-581.5 nm).

¹⁷O NMR measurements

Variable-temperature ¹⁷O NMR longitudinal (T_1) and transverse (T_2) relaxation time measurements on aqueous solutions of the Gd(III) complexes were obtained on a Varian INOVA-300 spectrometer operating at 7.05 T, 40.7 MHz or on a Bruker ARX 400 spectrometer, 9.4 T, 54.2 MHz. They were referenced to an acidified water solution (HClO₄, pH 3.3, 0 ppm). Longitudinal ¹H and ¹⁷O NMR relaxation times (T_1) were measured by the inversion-recovery pulse sequence,²⁴ and the transverse relaxation times (T_2) were obtained by the Carr-Purcell-Meiboom-Gill spin-echo technique.²⁵ The difference between the chemical shifts of protons of *tert*-butyl alcohol in the paramagnetic sample and in pure water was used to correct the ¹⁷O NMR shift for the contribution of the bulk magnetic susceptibility.²⁶ To improve sensitivity in ¹⁷O NMR, 10 µl of ¹⁷O-enriched water (10% H₂¹⁷O, Cortec) was added to each sample.

NMRD

The $1/T_1$ NMRD profiles of the Gd(III) complexes were recorded at, 5, 25 and 37 °C with a Stellar field cycling system covering a range of magnetic fields from 2.3474×10^{-4} to 0.35 T, corresponding to a proton Larmor frequency range of 0.01-10 MHz. Additional points were obtained on Bruker Minispec PC-20 (20 MHz), mq 30 (30 MHz), mq 40 (40 MHz), and mg 60 (60 MHz) relaxometers respectively.

Data analysis

Experimental variable-temperature ¹⁷O NMR and NMRD data were fit with a computer program written by É. Tóth and L. Helm (Swiss Federal Institute of Technology Lausanne, Switzerland) and Micromath Scientist version 2.0 (Salt Lake City, UT, USA). The reported errors correspond to one standard deviation obtained by statistical analysis.

ACKNOWLEDGEMENT

Thanks are due to Dr. Carlos Platas-Iglesias for bringing this research topic under attention of the author.

APPENDIX

From the measured ^{17}O NMR relaxation rates and angular frequencies of the $[\text{Gd}(\text{L})(\text{H}_2\text{O})]^-$ solution ($1/T_1$, $1/T_2$, and ω) and of the acidified water reference ($1/T_{1A}$, $1/T_{2A}$, and ω_A), the reduced relaxation rates and chemical shifts, $1/T_{1r}$, $1/T_{2r}$, and ω_r can be calculated, which may be written as Equations 4.6 – 4.7,²⁷⁻³⁰ where $1/T_{1m}$, $1/T_{2m}$ are the relaxation rates in the bound water, $\Delta\omega_m$ is the chemical shift difference between the bound and bulk water (in the absence of a paramagnetic interaction with the bulk water), P_m is the mole fraction of bound water, and τ_m is the residence time of water molecules in the inner-coordination sphere. The total outer-sphere contribution to the reduced relaxation rates and chemical shift are represented by $1/T_{1os}$, $1/T_{2os}$, and $\Delta\omega_{os}$.

$$\frac{1}{T_{1r}} = \frac{1}{P_m} \left[\frac{1}{T_1} - \frac{1}{T_{1A}} \right] = \frac{1}{T_{1m} + \tau_m} + \frac{1}{T_{1os}} \quad 4.6$$

$$\frac{1}{T_{2r}} = \frac{1}{P_m} \left[\frac{1}{T_2} - \frac{1}{T_{2A}} \right] = \frac{1}{\tau_m} \frac{T_{2m}^{-2} + \tau_m^{-1} T_{2m}^{-1} + \Delta\omega_m^2}{(\tau_m^{-1} + T_{2m}^{-1})^2 + \Delta\omega_m^2} + \frac{1}{T_{2os}} \quad 4.7$$

$$\Delta\omega_r = \frac{1}{P_m} (\omega - \omega_A) = \frac{\Delta\omega_m}{(1 + \tau_m T_{2m}^{-1})^2 + \tau_m^2 \Delta\omega_m^2} + \Delta\omega_{os} \quad 4.8$$

As shown in previous investigations,^{31,32} the outer sphere contributions to the ^{17}O relaxation rates are negligible, therefore Equations 4.6 and 4.7 were simplified (see Equations 4.3 and 4.4) and $\Delta\omega_{os}$ was neglected in this study (Equation 4.8).

The inner-sphere contribution to $\Delta\omega_r$ is given by the chemical shift of the bound water molecules, which is determined by the hyperfine interaction between the Gd^{3+} electron spin and the ^{17}O nucleus via Equation 4.9,³³ where g_L is the isotropic Landé g -factor ($g_L = 2.0$ for Gd^{3+}), S is the electron spin ($S = 7/2$ for Gd^{3+}), B is the magnetic field and $A/\hbar$ is the hyperfine or scalar coupling constant.

$$\Delta\omega_m = \frac{g_L\mu_B S(S+1)B}{3k_B T} \times \frac{A}{\hbar} \quad 4.9$$

The ^{17}O longitudinal relaxation rates in Gd^{3+} solutions are dominated by the dipole-dipole and quadrupolar mechanisms and, when the extreme narrowing conditions are met, may be expressed to a good approximation as in Equation 4.10, where $\gamma_S = g_L\mu_B/\hbar$ is the electron gyromagnetic ratio ($\gamma_S = 1.76 \times 10^{11} \text{ rad s}^{-1} \text{ T}^{-1}$ for $g_L = 2.0$), γ_I is the nuclear gyromagnetic ratio ($\gamma_I = -3.626 \times 10^7 \text{ rad s}^{-1} \text{ T}^{-1}$ for ^{17}O), r_{GdO} is the distance between the electron charge and the ^{17}O nucleus (the metal-oxygen distance in the point dipole approximation), τ_R is the rotational correlation time for the Gd–O vector, $\tau_{\text{di}}^{-1} = \tau_m^{-1} + T_{\text{ie}}^{-1} + \tau_R^{-1}$, I is the nuclear spin ($I = 5/2$ for ^{17}O), χ is the quadrupolar coupling constant, and η is the asymmetry parameter of the electrical field gradient at the oxygen nucleus.^{31, 32}

The rotational correlation time, τ_R , is assumed to obey simple exponential temperature dependence as in Equation 4.11, where τ_R^{298} is the correlation time at 298.15 K and E_R is the activation energy.

$$\frac{1}{T_{1m}} = \left[\frac{1}{15} \left(\frac{\mu_0}{4\pi} \right)^2 \frac{\hbar^2 \gamma_I^2 \gamma_S^2}{r_{\text{GdO}}^6} S(S+1) \right] \times \left[6\tau_{d1} + 14 \frac{\tau_{d2}}{1 + \omega_S^2 \tau_{d2}^2} \right] + \frac{3\pi^2}{10} \frac{2I+3}{I^2(2I-1)} \chi^2 (1 + \eta^2/3) \tau_R \quad 4.10$$

$$\tau_R = \tau_R^{298} \exp \left[\frac{E_R}{R} \left(\frac{1}{T} - \frac{1}{298.15} \right) \right] \quad 4.11$$

$$\frac{1}{T_{2m}} = \frac{S(S+1)}{3} \left(\frac{A}{\hbar} \right)^2 \left(\tau_{1s} + \frac{\tau_{2s}}{1 + \omega_S^2 \tau_{2s}^2} \right) \quad 4.12$$

The ^{17}O transverse relaxation rates in Gd^{3+} bound water molecules are dominated by the scalar relaxation mechanism³⁴ and are given to a very good approximation by Equation 4.12, where $\tau_{\text{is}}^{-1} = \tau_m^{-1} + T_{\text{ie}}^{-1}$. The residence time (or exchange rate, k_{ex}) of water molecules in the inner-sphere is assumed to obey the Eyring equation as written in Equation 4.13, where $\Delta S^\ddagger$ and $\Delta H^\ddagger$ are the entropy and enthalpy of activation for the exchange process and k_{ex}^{298} is the exchange rate at 298.15 K. The electronic rates ($1/T_{\text{ie}}$) were approximated using

Equations 4.15 and 4.16, where ω_s is the Larmor frequency, Δ^2 is the trace of the square of the ZFS tensor, and τ_v is the correlation time for the modulation of the ZFS. We assume that this correlation time has Arrhenius behavior (Equation 4.14).

$$\frac{1}{\tau_m} = k_{ex} = \frac{k_B T}{h} \exp\left[\frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{RT}\right] = \frac{k_{ex}^{298} T}{298.15} \exp\left[\frac{\Delta H^\ddagger}{R} \left(\frac{1}{298.15} - \frac{1}{T}\right)\right] \quad 4.13$$

$$\tau_v = \tau_v^{298} \exp\left[\frac{E_v}{R} \left(\frac{1}{T} - \frac{1}{298.15}\right)\right] \quad 4.14$$

$$\left(\frac{1}{T_{1e}}\right) = 1/25 \Delta^2 \tau_v [4S(S+1) - 3] \times \left(\frac{1}{1 + \omega_s^2 \tau_v^2} + \frac{4}{1 + 4\omega_s^2 \tau_v^2}\right) \quad 4.15$$

$$\left(\frac{1}{T_{2e}}\right) = \Delta^2 \tau_v \left(\frac{5.26}{1 + 0.372\omega_s^2 \tau_v^2} + \frac{7.18}{1 + 1.24\omega_s^2 \tau_v^2}\right) \quad 4.16$$

The measured longitudinal proton relaxation rate, R_1^{obs} is the sum of a paramagnetic and a diamagnetic contribution as expressed in Equation 4.17, where r_1 is the proton relaxivity, which can be divided into inner- and outer-sphere term as $r_1 = r_{1IS} + r_{1OS}$. The inner-sphere term is given in Equation 4.18, where q is the number of inner-sphere water molecules, which was calculated for each particular temperature on the basis of the temperature dependency of the hydration equilibrium constant as determined for the corresponding Eu(III)-analogue by UV-vis. For $[\text{Gd}(\text{L}^1)(\text{H}_2\text{O})]^-$ q was taken as 1.

$$R_1^{obs} = R_1^d + R_1^p = R_1^d + r_1[\text{Gd}^{3+}] \quad 4.17$$

$$r_{1IS} = \left(\frac{q}{1000 \times 55.55}\right) \frac{1}{T_{1m} + \tau_m} \quad 4.18$$

The outer-sphere contribution to the relaxivity arising from diffusing water molecules in vicinity of the complex for a 1 mM solution can be described by Equations 4.19 and 4.20, where N_A is the Avogadro constant, J_{OS} is its associated spectral density function, $j = 1, 2$, a_{GdH} is the distance of closest approach of a water molecule to Gd^{3+} , and the correlation time τ_{GdH} corresponds with a_{GdH}^2/D_{GdH} .

$$r_{iOS} = \frac{32N_A\pi}{405} \left(\frac{\mu_0}{4\pi} \right)^2 \frac{\gamma_I^2 \gamma_S^{22} \hbar^2}{a_{GdH} D_{GdH}} S(S+1) [3J_{OS}(\omega_I; T_{1e}) + 7J_{OS}(\omega_S; T_{2e})] \quad 4.19$$

$$J_{OS}(\omega, T_{je}) = \text{Re} \left\{ \frac{1 + \frac{1}{4} \left[i\omega\tau_{GdH} + \left(\frac{\tau_{GdH}}{T_{je}} \right) \right]^{\frac{1}{2}}}{1 + \left[i\omega\tau_{GdH} + \left(\frac{\tau_{GdH}}{T_{je}} \right) \right]^{\frac{1}{2}} + \frac{4}{9} \left[i\omega\tau_{GdH} + \left(\frac{\tau_{GdH}}{T_{je}} \right) \right] + \frac{1}{9} \left[i\omega\tau_{GdH} + \left(\frac{\tau_{GdH}}{T_{je}} \right) \right]^{\frac{3}{2}}} \right\} \quad 4.20$$

The diffusion coefficient for the diffusion of water protons away from the Gd(III) complex D_{GdH} was assumed to follow an exponential behavior versus the inverse of the temperature, with an activation energy E_{GdH} , as given in Equation 4.21.

$$D_{GdH} = D_{GdH}^{298} \exp \left\{ \frac{E_{GdH}}{R} \left(\frac{1}{298.15} - \frac{1}{T} \right) \right\} \quad 4.21$$

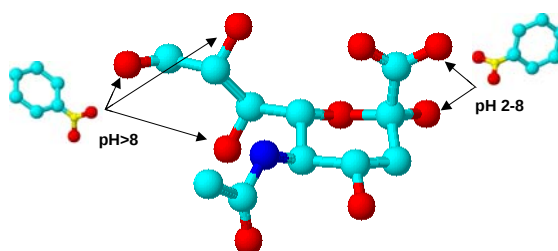
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MOLECULAR RECOGNITION OF SIALIC ACID END GROUPS BY PHENYLBORONATES

5



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INTRODUCTION

The contrast in MR images can be greatly improved by the use of contrast agents (CAs).¹ However, most of the currently available MRI CAs distribute rather unselectively over the extracellular space. If it was possible to accumulate CAs in the diseased tissue by means of molecular recognition, a much better contrast could be achieved. This has become feasible thanks to the increased knowledge of receptors, which allows the design of MRI CAs that respond to, for example, functional groups that are overexpressed due to disease.² Tumors are known to have cell surfaces on which sialic acids are overexpressed. Sialic acids consist of the nine-carbon amino sugar neuraminic acid (Neu).³ The most common member of this family is 5-acetylneuraminic acid (**4**, Figure 5.1).

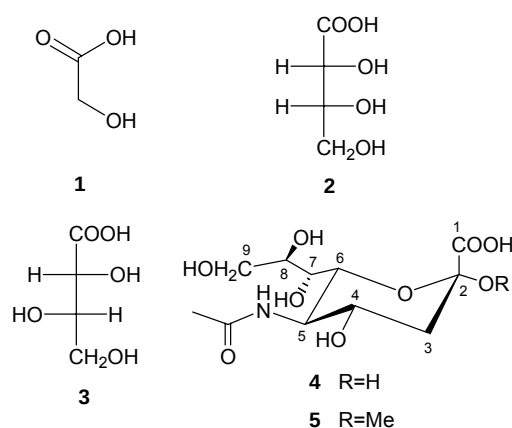


Figure 5.1. Structures of the polyols investigated in this study. **(1)** Glycolic acid, **(2)** D-Erythronic acid, **(3)** L-Threonic Acid, **(4)** 5-acetylneuraminic acid (Neu5Ac), **(5)** 2- α -O-methyl Neu5Ac.

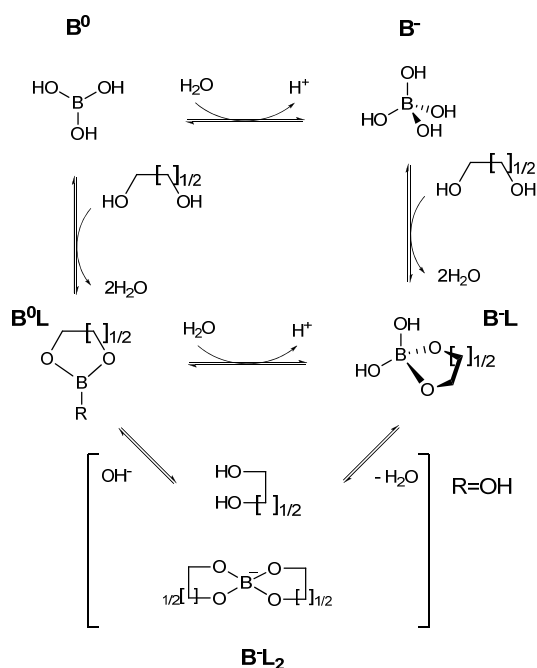


Figure 5.2. Ester formation between 1,2- or 1,3-diols and boric acid ($R=OH$) or phenylboronic acid ($R=Ph$).

The importance of the sialic acid level as a diagnostic and prognostic indicator for different diseases has prompted the quest for specific targeting agents. Different approaches have been undertaken for this purpose. Bertozzi and coworkers have exploited the sialosides biosynthetic pathway to express a modified sialic acid, serving as an anchor for covalent binding of an aminoxy-substituted Gd-[DTPA]-complex.⁴ The disadvantage of such a covalent targeting is that sialic acid catabolism occurs in lysosomes where the pH is relatively low (ca. 4-5). Under these conditions metal ions may dissociate from the chelate and exert toxic effects.

Currently, the only promising class of synthetic sugar receptors known is based on arylboronic acids. The ability of boric and boronic acids to form stable esters with polyhydroxy compounds is well known.^{5,6} Boric acid can form cyclic esters with 1,2- or 1,3-diols in 1:1 or 1:2 ratio; the possible equilibria between boric acid, borate and the corresponding esters are summarized in Figure 5.2.⁷ The esters formed by borate anions,

with the boron in its tetragonal hybridization, are always much more stable than the trigonal ones formed by boric acid. Phenylboronic acid (PBA) behaves similarly to boric acid, although evidently it can only form esters with a 1:1 stoichiometry (Figure 5.2). The presence of the aryl group also allows the design of more complex structures that can act as saccharide receptors.⁸

Some examples of receptors for sialic acid based on arylboronic acids have been reported in the literature. Shinkai et al. have developed a fluorescent sensor bearing a PBA moiety that is apt to interact with diols and the positively charged 1,10-phenanthroline-Zn²⁺ chelate moiety for carboxylate binding.⁹ The two-site binding of sialic acid to the receptor was demonstrated by the fact that the association constant was much lower in the absence of Zn²⁺.

Patterson et al. have explored carbohydrate receptors based on poly(allylamine) (PAA) polymer substituted with both fluorescent arylboronic acid and carboxylic acid functions.¹⁰ PAA with a 2% loading of the fluorescent arylboronic acid shows a response to sialic acid that is much higher than that for glucose, and slightly higher than that for fructose. Kataoka et al. have demonstrated that the PBA moiety in the copolymer of 3-acrylamidophenylboronic acid with *N,N*-dimethylacrylamide acts as an inhibitor for the cellular binding of an *N*-acetylneuraminic acid-specific lectin on lymphocytes.¹¹ The binding of PBA to the sialic acid moiety was studied by ¹¹B, ¹H, ¹³C, and ¹⁵N NMR spectroscopy with 3-(propionamido)phenylboronic acid as a model.¹² This compound shows an unusual pH dependence of the conditional stability constant of its esters with sialic acid, which was attributed to the formation of a trigonal boronic acid ester stabilized by an intramolecular B–N or B–O bond between the amide group of Neu5Ac and the boron atom.

The design of new targeting CAs capable of recognizing diseased tissue requires insight in the binding modes of PBA to sialic acid, which is overexpressed on the cell surface. Therefore we decided to study the interactions between PBA and the α -hydroxycarboxylic acids glycolic acid (**1**), erythronic acid (**2**) and threonic acid (**3**) as models for the various relevant functional groups in sialic acid (see Figure 5.1). With the insight obtained it is possible to understand the binding of PBA to Neu5Ac (**4**) and its 2- α -methyl derivative (**5**). The latter compound serves as a model for Neu5Ac bound to a neighboring sugar unit in glycoproteins.

RESULTS AND DISCUSSION

Glycolic acid

Glycolic acid (**1**, HG) is the simplest α -hydroxycarboxylic acid and its interaction with PBA was studied to gain insight into the binding of PBA to the α -hydroxycarboxylic acid moiety in Neu5Ac (C(1)OOH and C(2)OH). The ^{11}B spectrum of a mixture of glycolic acid (0.1 M) and PBA (0.1 M) displays a resonance for the equilibrium between free B^0 and B^- (Figure 5.3). In addition, a second resonance at $\delta = -9.9$ ppm is observed between pH 3 and 9 (see Figure 5.4). The chemical shift of this resonance is typical for a PBA ester, which has a tetragonal boron atom ($\text{B}^-\text{G}^{\text{carboxylate}}$, Figure 5.3). The exchange between this species and B^0/B^- is slow on the ^{11}B NMR time scale. The concentration of $\text{B}^-\text{G}^{\text{carboxylate}}$ reaches a maximum at pH 6; at pH > 8, its concentration is much lower, and at pH > 10 it is no longer observable. The maximum in concentration of this species occurs when the pH value of the sample is between the pK_a of glycolic acid (4.21 ± 0.02) and that of PBA (10.05 ± 0.03) in the same solvent. This indicates that the ester concerned is formed by reaction of B^0 with L^- (Figure 5.3). A similar species has been observed in the system glycolic acid – boric acid.⁷ The proposed binding mode of glycolic acid to the boron atom in the B:L ester was supported by measuring the ^{17}O NMR spectrum of a sample, in which the carboxylate group of glycolic acid was 10% enriched in ^{17}O . The spectrum of a 0.8 M solution of the ^{17}O -enriched glycolic acid in the absence of PBA at -15°C shows a single relatively sharp resonance at $\delta = 253$ ppm. After the addition of one equivalent of PBA at pH 7.3 two broad resonances (at $\delta = 264$ and 391 ppm) became visible, signaling the loss of symmetry of the carboxyl group upon binding to boron (Figure 5.5). A similar behavior has been observed upon coordination of glycolic acid to Al^{3+} .¹³ The signal at $\delta = 391$ ppm can be assigned to the carbonyl oxygen of the B-bound carboxylate. The other carbonyl oxygen atom is most likely coinciding with the resonance of the free carboxylate at about $\delta = 250$ ppm.¹⁴

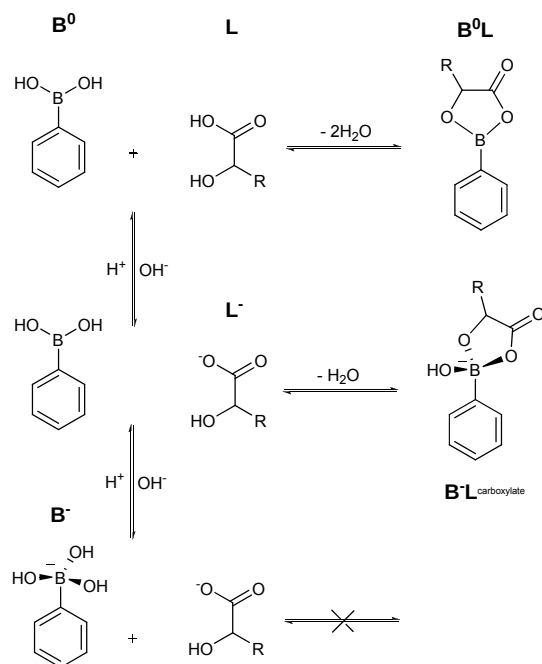


Figure 5.3. Possible reactions between an α -hydroxyacid (glycolic acid $R=H$) and phenylboronic acid.

The formation constant (K_f) of the B^-G ester, corresponding to the equilibrium shown in Equation 5.1, can be defined by Equation 5.3, whereas the acidity constant (K_{Gly}) of HG is given by Equation 5.2. The ^{13}C and ^{11}B chemical shifts for the species that are in rapid exchange on the NMR time scale can be described by Equations 5.6 and 5.7, respectively. Here, χ_n is the molar fraction of species n and δ_n is its intrinsic chemical shift. The molar fractions of free PBA ($B^0 + B^-$) and of the ester with glyconate (B^-G) were determined from the integrals in the ^{11}B NMR spectra.



$$K_{Gly} = [G^-][H^+]/[HG] \quad 5.2$$

$$K_f = [B^-G]/[B^0][G^-] \quad 5.3$$

$$C_{tB} = [B^0] + [B^-] + [B^-G] \quad 5.4$$

$$C_{tGly} = [HG] + [G^-] + [B^-G] \quad 5.5$$

$$\delta_i^C = \sum_n \chi_n^C \delta_n^C \quad 5.6$$

$$\delta_i^B = \sum_n \chi_n^B \delta_n^B \quad 5.7$$

The value of K_f was evaluated to be 1.6 ± 0.2 by a simultaneous fitting of the ^{13}C and ^{11}B NMR shifts and the molar fractions of the boron species as obtained from the integrals, with Equations 5.2 – 5.7. To facilitate a comparison of the various compounds discussed, we also defined a conditional stability constant at pH 7.4, K_f^c (see Equation 5.8). For glycolic acid K_f^c equals K_f .

$$K_f^c = [B^-G] / ([B^0] + [B^-])([G^-] + [HG]) \quad 5.8$$

Erythronic and threonic acid

Erythronic acid (**2**, HE) and threonic acid (**3**, HT) (see Figure 5.1) were investigated because they contain both, an α -hydroxycarboxylic acid and a 1,2,3 triol motif in their structures. Both moieties occur in Neu5Ac and are potential binding sites for PBA. It should be noted that the vicinal-diol unit at C7/C8 in Neu5Ac has the *erythro* configuration. The ^{11}B NMR spectra of a mixture of equimolar amounts of erythronic or threonic acid (0.1 M) and PBA (0.1 M) as a function of pH are very similar for both sugar acids. Two signals are present in the entire pH range investigated, corresponding to free PBA (B^0/B^-) and to a convolution of the resonances of the various possible PBA – substrate esters (Figure 5.6).

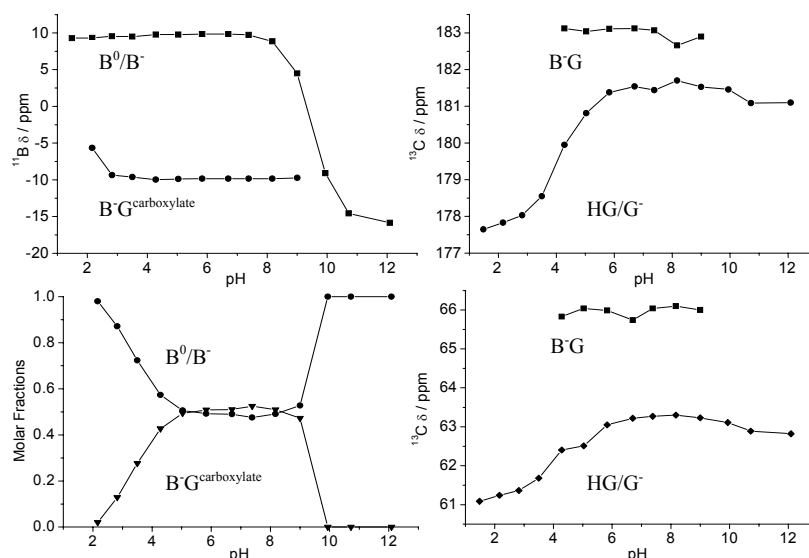


Figure 5.4. pH dependence of the ^{11}B resonances (top left), molar fractions calculated from the integration of the ^{11}B NMR resonances (bottom left) and pH dependence of the ^{13}C resonances in the carbonyl (top right) and aliphatic area (bottom right) for a mixture of glycolic acid (1 M) and phenylboronic acid (1 M).

In both cases the chemical shift of the bound species, in the pH range up to 9, is about $\delta = -10$ ppm, which is similar to the value for the species $\text{B}^-\text{L}^{\text{carboxylate}}$ observed in the PBA-glycolate system. Therefore, it may be concluded that also here an α -hydroxycarboxylate ester ($\text{B}^-\text{T}^{\text{carboxylate}}$ or $\text{B}^-\text{E}^{\text{carboxylate}}$) with the boronate moiety bound to C1-C2 is the predominant ester at pH 2–9.

Above pH 9, a shift of about 2 ppm towards lower frequencies is observed, indicating the disruption of the interaction at C1 and C2, which is consistent with the behavior of glycolate. However, now a new ester is formed that is in fast exchange with $\text{B}^-\text{L}^{\text{carboxylate}}$ ($\text{L}=\text{T}$ or E) on the ^{11}B NMR time scale. In this high pH region, only interactions with the exocyclic triol moiety (at C2, C3 and C4) need to be considered. Three binding modes of B^- may be envisaged: 1) to C2 and C3, 2) to C3 and C4, and 3) to C2 and C4.

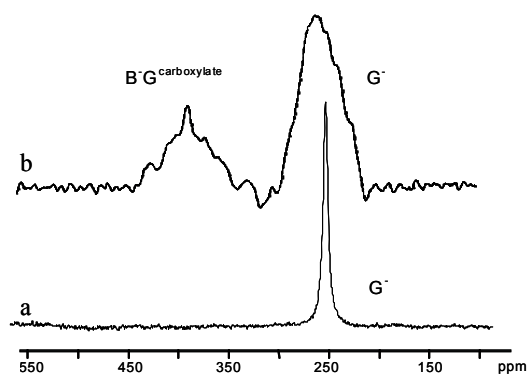


Figure 5.5. ^{17}O NMR spectrum of a solution of (a) glycolic acid (0.8 M) and (b) mixture of glycolic acid (0.8 M) and PBA (0.8 M) in methanol/water (1:2 v/v, 10% D_2O) at pH 7.3 (40.7 MHz, -15°C).

The former two cases lead to five-membered boronate esters, whereas the latter gives a six-membered ring. It has been shown that five- and six-membered phenylboronate esters have chemical shifts of about $\delta = -12$ ppm and $\delta = -15$ ppm, respectively.¹⁵ From the resonance at $\delta = -12$ ppm at pH > 10 in the present case, it may be concluded that binding to vicinal diol functions occurs to a considerable extent. The presence of six-membered esters cannot be excluded, however, because the resonance for this species would have a chemical shift of about $\delta = -15$ ppm and, therefore, may be obscured by the signal for free B^0/B^- .

Between pH 2 to 9, the ^{13}C NMR spectra of 0.1 M erythronate or threonate in the presence of an equimolar amount of PBA are both similar to the spectra observed for the system HG-PBA (Figure 5.7). Two broad resonances are observed for the carboxylate group (C1), showing that for this nucleus the exchange between the boronate ester and the free acid is relatively slow on the NMR time scale. Broad and averaged signals are observed for all the other carbons, and only for the C2 of threonic acid is a partial splitting visible. These spectra are consistent with the formation of a $\text{B}^-\text{L}^{\text{carboxylate}}$ ester at C1 and C2 under these conditions.

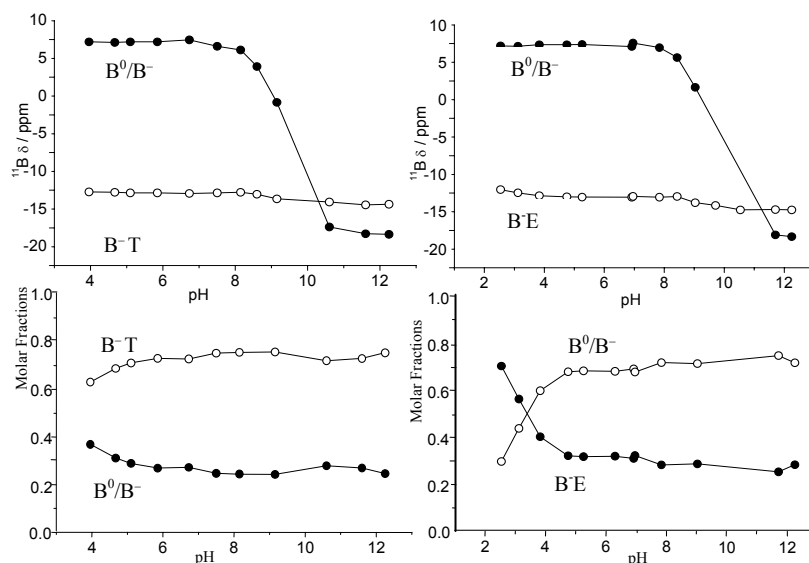


Figure 5.6. pH dependence of the ^{11}B NMR shifts of mixtures of threonic (top left) or erythronic acid (top right) (about 1 M) and PBA (0.9 M) along with the molar fractions determined by the ^{11}B integrations (threonic acid - bottom left; erythronic acid -bottom right).

The spectra of erythronic and threonic acid in the presence of PBA become different above pH 9. In this region the spectra of erythronic acid display, besides resonances belonging to free PBA (B^-) and free erythronic acid (E^-), resonances corresponding to two pairs of $\text{B}^-\text{L}^{\text{diol}}$ esters. Based on previous studies⁷ on the interaction between H_3BO_3 and various poly(hydroxyl)carboxylates, these resonances can be attributed to the pair of five-membered esters formed by interaction of PBA at C3 and C4.

In these phenylboronate esters the B atom is chiral and, consequently, each of the interactions mentioned with the chiral α -hydroxycarboxylates results in two diastereoisomers. The major species observed at this pH can be assigned to the ester formed at the C3 and C4. Formation of an ester at C2 and C3 can be excluded because of their unfavorable *erythro* configuration.

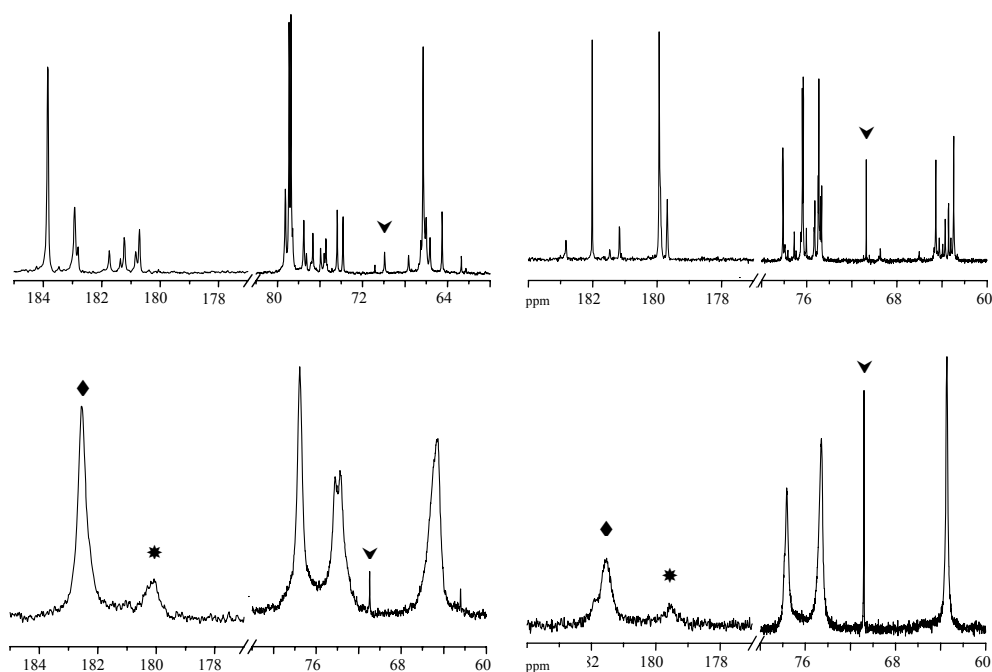


Figure 5.7. ^{13}C NMR spectra of a mixture of threonic acid (1 M) and phenylboronic acid (0.8 M) at pH 12.5 (top left) and 8.6 (bottom left), and of a mixture of erythronic acid (1 M) and phenylboronic acid (0.8 M) at pH 12.3 (top right) and 8.3 (bottom right) (* = free acid; ♦ = ester; ▼ = *t*-BuOH).

Upon interaction at positions C2 and C3, the two terminal groups would assume a sterically unfavorable position and, moreover, the negatively charged boron atom would be relatively close to the carboxylate anion.

Besides these sets of signals, two sets of signals of relatively low intensity are visible, suggesting the presence of a small quantity of the six-membered ester originating from the interaction at the positions C2 and C4 and of the five-membered ester formed at positions C2 and C3. For the sample of threonic acid and PBA, besides the resonances belonging to free PBA (B^-) and free threonic acid (L^-), six sets of relatively sharp resonances were observed above pH 9. These resonances can be attributed to three pairs of diastereomeric $\text{B}^- \text{L}^{\text{diol}}$ esters, originating from the interactions at C2/C3, C3/C4 and C2/C4. The major species is most likely one of the diastereoisomers of the ester formed with the diol C2 and C3, which has a *threo* configuration.

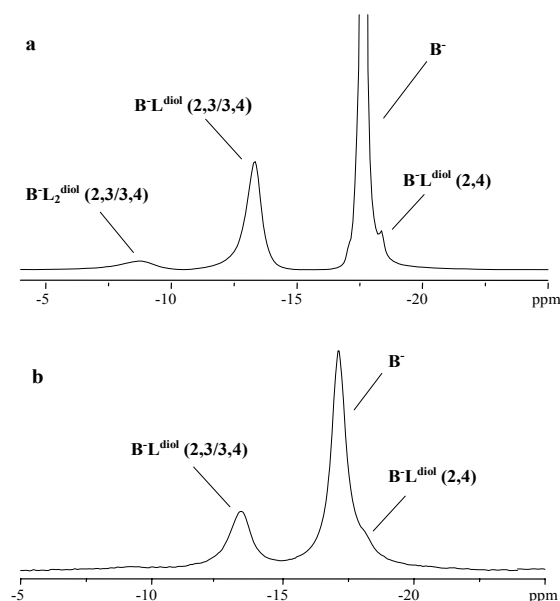


Figure 5.8. ^{11}B NMR spectra of a solution of threonic acid (1.0 M, spectrum **a**) and H_3BO_3 (2.2 M), and of a solution of erythronic acid (1.1 M, spectrum **b**) and H_3BO_3 (2.0 M) in methanol/water (1:2 v/v, 10% D_2O).

The integrals of the ^{11}B resonances in the spectra of threonic or erythronic acid and PBA were used to calculate the conditional stability constant, K_f^c , at pH 7.4, as defined in Equation 5.8 for glycolic acid. For threonic acid we determined K_f^c to be $11.4 \pm 1.5 \text{ M}^{-1}$ and for erythronic acid $4.4 \pm 0.5 \text{ M}^{-1}$. The higher formation constant for threonic acid with respect to erythronic acid reflects the presence of a more favorable *threo* configuration at C2/C3 in the former compound.

The nature of the species observed at basic pH in the mixture PBA and threonic acid (1:1) was confirmed by the study of the ^{11}B NMR spectra of a mixture of boric acid and threonic or erythronic acid (Figure 5.8). Borate and its esters have lower molecular volumes than the corresponding phenylboronate derivatives and, therefore, also lower rotational correlation times. Since the relaxation of ^{11}B is dominated by the quadrupolar mechanism, the ^{11}B relaxation rates of the boronate species are lower than those of the phenylboronate ones and, consequently, the linewidths of ^{11}B NMR resonances of the borate esters are

smaller than those of corresponding PBA esters, which facilitates the identification of the different species. The ^{11}B NMR spectrum of the threonic acid – boric acid mixture at pH 11 shows four peaks at $\delta = -8.8$ (2.7%), $\delta = -13.7$ (24.6%), $\delta = -17.7$ (70.8%) and $\delta = -18.4$ ppm (1.9%) (Figure 5.8). From the chemical shift increments derived by van Duin et al.,⁷ these four resonances can be assigned to the B^0L_2 species ($\delta = -8.8$ ppm), the five-membered

$\text{B}^0\text{L}^{\text{diol}}$ species ($\delta = -13.7$ ppm, interaction at C2 /C3 and C3/C4), the six-membered $\text{B}^0\text{L}^{\text{diol}}$ species ($\delta = -18.4$ ppm, interaction at C3/C4), and to the free borate anion ($\delta = -17.7$ ppm).

The spectrum of the erythronic acid – boric acid mixture shows slightly broader resonances but the pattern is similar to that observed for threonic acid. Here also, the resonance at $\delta = 13.4$ ppm (25.3%) is indicative of a five-membered $\text{B}^0\text{L}^{\text{diol}}$ species due to interaction at C2 and C3 and/or C3 and C4, and the resonance at $\delta = -17.1$ ppm (54.8%) can be assigned to the free borate anion. The shoulder observed on this peak at $\delta = -17.7$ ppm (19.2%) suggests the presence of six-membered $\text{B}^0\text{L}^{\text{diol}}$ ester. In this case the amount of B^0L_2 is negligible. A lower percentage of the bound species in the case of erythronic acid with respect to threonic acid is found in the form of five-membered esters, which reflects once again the presence of the unfavorable *erythro* configuration at C2/C3.

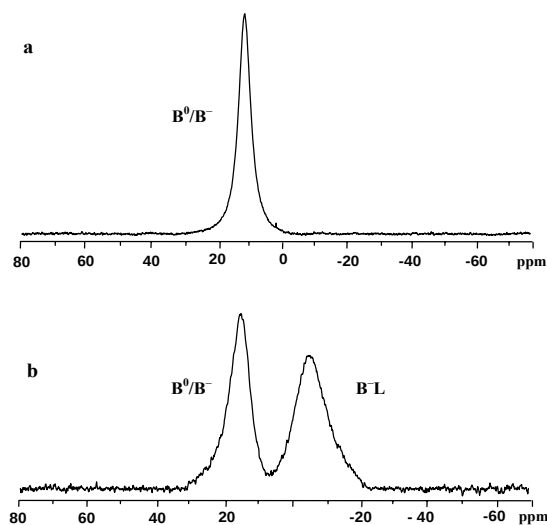


Figure 5.9. ^{11}B NMR spectra of (a) α -methyl Neu5Ac (5) (0.05 M) and PBA (1:2.5) at pH 5.4 and (b) Neu5Ac (0.6 M) and PBA (1:1) at pH 4.2 in methanol/water (1:2 v/v, 10% D_2O).

N-Acetylneuraminic acid

From the results described above, it can be expected that *N*-acetylneuraminic acid (Neu5Ac) may interact with PBA through the α -hydroxyacid unit (C1/C2) and/or the glycerol moiety (C7–C9). The glycerol moiety of Neu5Ac has an *erythro* configuration between C7 and C8,¹⁶ which is unfavorable for binding of PBA.

The pH dependence of the ^{11}B NMR spectra of a solution of 0.6 M Neu5Ac in the presence of an equimolar amount of PBA is similar to that observed for erythronic or threonic acid. Two resonances were observed, which could be assigned to the free PBA (B/B^0) and to a possible ester (B^-L), respectively. At $\text{pH} < 8$, the BL^- resonance is at about $\delta = -10$ ppm. The ^{11}B NMR shift data of the glycolic, threonic, and erythronic acid interacting with PBA have shown that this chemical shift is typical for a $\text{B}^-\text{L}^{\text{carboxylate}}$ species. Therefore, it can be concluded that, at $\text{pH} < 8$, the α -hydroxycarboxylate moiety at C1/C2 is involved in the binding of PBA. Around pH 9 this ^{11}B NMR resonance shifts to about $\delta = -12$ ppm, which shows that the PBA unit moves to the glycerol moiety at C7–C9. The integrals of the ^{11}B NMR resonances at pH 7.4 were used to calculate the conditional stability constant, K_f^c , defined similarly to Equation 5.8, for glycolic acid. The values of K_f^c obtained for Neu5Ac are compared with those for glycolic, threonic and erythronic acid in Table 5.1.

The value of the conditional stability constant for Neu5Ac is much higher than that for erythronic acid under the same conditions. This may be rationalized by the increased distance between the boronate and the carboxylate anion that results in lower charge repulsion between the two groups.

The importance of the α -hydroxycarboxylate moiety for the binding is confirmed by an investigation of the interaction of PBA with the 2- α -O-methyl derivative of *N*-acetylneuraminic acid (**5**). In this case the methyl group blocks the interaction of the boronic function at the positions C1 and C2 leaving the glycerol tail as the only possible interaction site. As result, at $\text{pH} < 8$ no interaction is observable, while in the case of Neu5Ac an interaction with PBA is detected above pH 2 (Figure 5.9). At pH values higher than 8 the binding to the glycerol tail of sialic acids becomes important; however, the broad ^{11}B resonances prevent the observation of separate peaks for the various esters formed upon

Table 5.1. Conditional stability constants for the interaction between the investigated polyols and phenylboronic acid at pH 7.4 and 25 °C.

	K_f^c (M ⁻¹)
Glycolic acid	1.6 ± 0.2
Erythronic acid	4.4 ± 0.5
Threonic acid	11.4 ± 1.5
Sialic acid (Neu5Ac)	11.6 ± 1.9

interaction. The use of H₃BO₃, which gives sharper resonances, is therefore helpful for understanding the binding modes of the boronate function to **5**. At pH 11 the ¹¹B spectrum of a solution of H₃BO₃ (instead of PBA) and **5** shows three resonances at $\delta = -13.9$ (14.3%), $\delta = -16.8$ (79.3%) and $\delta = -18.1$ (6.3%) ppm. The peak at $\delta = -16.8$ ppm can be assigned to free B⁰/B⁻ (in fast exchange), whereas chemical shifts of $\delta = -13.9$ and $\delta = -18.1$ ppm are characteristic for five- and six-membered borate esters, respectively.

The ¹³C NMR spectrum of **5** in the presence of an excess of PBA reveals, besides the signals for free **5**, three sets of resonances (two of which have relatively high intensity), thereby suggesting that at least two major and one minor phenylboronate ester occur in this solution (Figure 5.10). Unfortunately, the complexity of both ¹³C and ¹H NMR spectra does not allow a complete assignment and evaluation of coupling constants. Therefore, these parameters cannot be used to discriminate between the various types of boronate esters. However, from the results of the ¹¹B NMR study on the borate esters (see above), it may be expected that both five- and six-membered esters are present in the sample with PBA as well. Since the B-atom in these PBA esters is chiral, three pairs of diastereoisomers can be envisaged. An inspection of molecular models shows that some of these isomers have severe steric interactions. Semi-empirical calculations show that, in particular, the PBA esters at C7/C8 and C7/C9 with the B atom in the *S* configuration are highly strained. These calculations were performed without taking into account counterions or solvation effects. Therefore, the relative energies should only be considered as a crude measure of the stabilities. Similar calculations on the borate esters are qualitatively in agreement with the stabilities as determined by ¹¹B NMR. The results are compiled in Table 5.2.

Table 5.2. Relative energies of Neu5Ac-PBA/H₃BO₃ complexes obtained from the semi-empirical calculations, compared to the complex with the lowest energy.

Complex	B-Binding site	ΔE [kJ]
Neu5Ac-Borate complex	8-9	0
	7-9	10.8
	7-8	21.4
Neu5Ac-PBA complex	8-9 R	0
	8-9 S	8.4
	7-9 R	16.7
	7-9 S	35.2
	7-8 R	20.9
	7-8 S	33.5

Based on the results of these calculations, we conclude that the two major sets of peaks that are observed in the ¹³C NMR spectrum can be assigned to the two diastereoisomeric five-membered esters with the boronate function attached to C8 and C9, whereas the minor compound is the *R*-isomer of the six-membered ester at positions C7–C9.

The pattern of the interaction between PBA and Neu5Ac that emerges from our investigations differs substantially from that reported by Kataoka et al. for 3-(propionamido)phenylboronic acid and Neu5A.¹¹ The occurrence of a relatively strong interaction at pH < 7 can, in our view, be ascribed to an interaction on the α -hydroxycarboxylate moiety present at positions C1 and C2 of Neu5Ac. At pH > 7, the glycerol tail of Neu5Ac becomes involved in the binding, and the sole interaction site at pH > 10. Kataoka et al. ascribed the high K_f^c observed at pH < 7 to a stabilization of the trigonal boronic function bound at C7 and C8 of Neu5Ac. This interaction seemed to be supported by ¹⁵N, ¹¹B, and ¹H NMR chemical shifts of the functions concerned and by 2D ROESY NMR spectra of the Neu5Ac esters or another PBA derivative reported by Strongin et al.¹⁷ An inspection of molecular models, however, suggests that both the B–N and B–O bonds would result in severe steric strain. For example, the separation of the B and the N atoms in this compound is significantly larger (3.5 Å) than the optimal length for a typical B–N bond, which is 1.6–1.7 Å.¹⁸ The presence of a dative bond also does not agree with the ¹¹B chemical shift, which was reported to be $\delta = -11.1$ ppm (pH 10), whereas a value of between $\delta = -4$ and -7 ppm would be expected for a B-atom involved in a dative bond.¹⁸

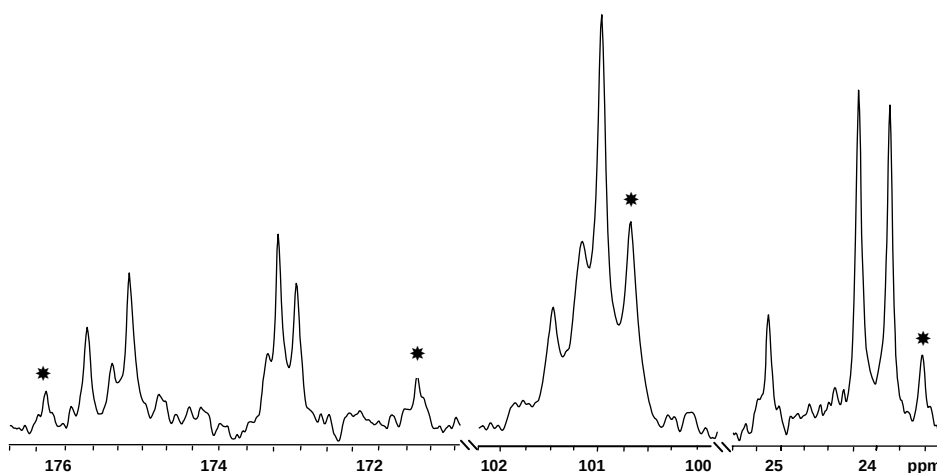


Figure 5.10. Fragments of the ^{13}C spectrum of a mixture of α -methyl Neu5Ac (0.6 M) and PBA (1 M) at pH 11.5 (* = free Neu5Ac).

Similar arguments hold for the ROESY data. The distances between the NH protons and various other protons in the PBA ester are strongly dependent on the rotations of the glycerol side-chain of Neu5Ac and cannot be directly related to the PBA derivative in question. All NMR spectroscopic data can equally well be interpreted by structures described above for the PBA esters of Neu5Ac. The phenyl moiety in these compounds may give rise to substantial induced chemical shifts in the nuclei in its proximity. These shifts will be strongly dependent on the position of the phenyl group with respect to these nuclei. The main binding sites of Neu5Ac for PBA are schematically summarized in Figure 5.11.

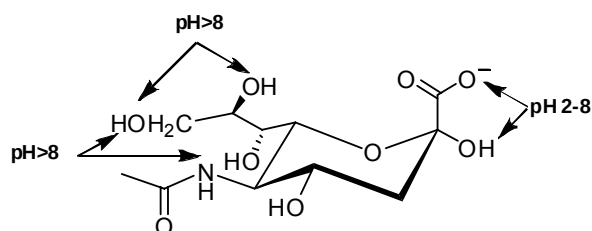


Figure 5.11. Schematic representation of the possible binding sites for PBA on Neu5Ac.

CONCLUSIONS

From the results of the present study it can be concluded that phenylboronic acid forms esters with Neu5Ac between pH 2 and 12. At low pH (2–8), the α -hydroxycarboxylate moiety at C1/C2 is involved in the binding, whereas at pH > 8, the binding of PBA takes place at the glycerol side chain to give a five-membered ester at C8 and C9. The formation of another five-membered complex at C7 and C8 is limited by the unfavorable *erythro* configuration of the glycerol tail. Molecular modeling studies confirm these conclusions, and show the lowest energies for the above-mentioned ester. The insight into the binding of PBA by Neu5Ac obtained in this study may be useful for the design of artificial receptors for sialic acid moieties in glycoproteins. On the cell surface sialic acids represent the terminal sugar residue of a glycan chain. That is, they are linked via C2 to position 3 or 6 of the penultimate sugar or to position 8 of another sialic acid molecule.²⁰ Consequently, the carboxyl group is not available for interaction with the PBA-receptor and, at physiological pH, it is the carrier of negative charge on the sugar molecule ($pK_a=2.2$). The experiment with the 2-O- α -methyl derivative of sialic acid demonstrates that interaction with PBA occurs, even without carboxylic group participation.

More selective and more effective artificial receptors for sialic acid residues in glycoproteins, therefore, should contain both a PBA unit and a group that is able to recognize the negatively charged COO^- group of sialic acid. Recently, we have successfully applied these principles in the design of a potential sialic acid targeting MRI contrast agents.²¹

EXPERIMENTAL

Compounds

D₂O was obtained from ARC Laboratories BV (Amsterdam, The Netherlands); ¹⁷O-enriched water (25% ¹⁷O) from A. Matheson, USA Company (Miamisburg, Ohio) and the water used for the preparation of samples was purified with a Milli-Q filtration system. Glycolic acid was purchased from Acros (Geel, Belgium) and *N*-acetylneuraminic acid

(Neu5Ac) was purchased from Rose Scientific Ltd (Edmonton, Canada). For some experiments Neu5Ac was converted into the methyl ester of 2- α -O-methyl-5-acetylneuraminic acid according to a published procedure.¹⁵ The structure was confirmed by ^{13}C NMR (75.48 MHz, D_2O , 25 °C, *t*BuOH, APT): δ = 174.85 (CO), 170.85 (CO), 100.38 (C), 72.27 (CH), 71.40 (CH), 70.19 (CH), 67.71 (CH), 65.30 (CH_2), 53.80 (CH), 53.17 (CH_3), 51.66 (CH_3), 41.58 (CH_2), 22.75 (CH_3). During the ^{11}B NMR titration the ester group was hydrolyzed at high pH giving **5** in situ, which was confirmed by the shift of the CH_3 peak at 53.17 in the ^{13}C NMR spectrum to 49.14 ppm. Threonic and erythronic acids were obtained from Aldrich as calcium salts and were converted into the corresponding water-soluble sodium salts using a Dowex 50W $\times$ 8 cation exchange resin. ^{17}O -enrichment of glycolic acid was achieved by stirring of a 0.81 M aqueous solution of glycolic acid containing 4% of ^{17}O -labeled water at 80 °C and pH $\approx$ 4 for a period of 16 h followed by freeze-drying of the solution. ^{17}O NMR, 40.67 MHz, D_2O , δ = 253 ppm, ref. H_2O , pH 5.

NMR spectroscopy

All NMR measurements were performed on Varian INOVA-300 and VXR-400S spectrometers at 25 °C unless stated otherwise using 5 mm sample tubes. All NMR spectra were recorded using a water/methanol mixture (v/v 2:1, 10% D_2O) as the solvent. ^{13}C NMR spectra were measured at 75.48 MHz and *t*BuOH was used as an internal reference with the methyl peak of the standard set at 31.2 ppm. ^{11}B NMR spectra were measured at 128.33 or at 96.3 MHz, using a 0.1 M solution of boric acid in D_2O (δ =0.00) as external standard. The conversion to the $\text{BF}_3\cdot\text{Et}_2\text{O}$ scale is as follows: δ (H_3BO_3 scale) = δ ($\text{BF}_3\cdot\text{Et}_2\text{O}$ scale) – 18.7. About 240 scans were collected using a delay and an acquisition time both of 1 s. ^{17}O NMR spectra were recorded operating at 40.67 MHz. A spectral window of 29996 Hz and an acquisition delay 0 s were applied. ^{17}O NMR spectra of mixtures of ^{17}O enriched glycolic acid and PBA were acquired at –15 °C in order to lower the exchange rates on the ^{17}O NMR time scale. The peak positions and the integrations of the resonances in ^{11}B , ^{17}O and quantitative ^{13}C spectra were determined by fitting the observed signals with Lorentzian line functions.

Determination of stability constants

NMR-titration experiments were used to determine both the pK_a values of the studied compounds and the apparent stability constant (K_f) of the complexes. The pH values of the samples were measured at ambient temperature using a Corning 125 pH meter with a calibrated micro-combination probe purchased from Aldrich Chemical Company. The pH values of the solutions were adjusted using 1M solutions of NaOH and HCl. The reported values are uncorrected for isotope effects and the presence of methanol. It has been reported that corrections for the solvent systems selected are negligible.¹⁶

Computer calculations were performed using the Micromath Scientist program, version 2.0 (Salt Lake City, Utah, U.S.A).

Molecular modeling

Calculations were performed using the HyperChem 7.5 Professional program. The MM+ force field was used to optimize the conformations of the possible complexes of Neu5Ac and PBA and to calculate the energies (given in kJmol^{-1}). The conformations obtained were re-optimized using AM1 semi-empirical quantum mechanics. The default options (Restricted Hartree-Fock (RHF) spin pairing) were used with the total charge -2 and spin multiplicity of 1. Molecular dynamics at 1000 K and searches with the conformational search tool of the HyperChem software were performed to obtain the various conformational minima.

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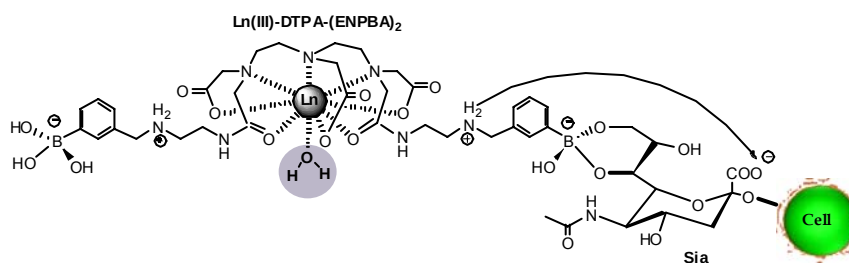
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PHENYLBORONATE ^{160}Tb COMPLEXES FOR MOLECULAR RECOGNITION OF GLYCOPROTEINS EXPRESSED ON TUMOR CELLS

6



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INTRODUCTION

Molecular recognition of biological processes at a cellular level as a way to identify pathologies in terms of molecular abnormalities offers opportunities for development of early diagnosis and therapy of cancer. Imaging modalities including PET, SPECT, and MRI can be used for molecular imaging. In the latter technique contrast agents (CAs) can be used to enhance the inherent contrast based on endogenous differences in water content, relaxation times and diffusion characteristics of soft tissues.¹⁻⁴ This is achieved by using paramagnetic agents with a high relaxivity, such as complexes of Gd(III) and DTPA- or DOTA-derivatives or iron oxide-particles. CAs can accumulate in tumor tissues after extravasation through leaky blood vessels, which are typical for tumors^{7,8} through endocytosis^{5,6}. Nowadays, several tissue-specific MRI CAs have been developed and reached the market.⁹ However, tumor-targeted agents belong to the less-developed area and are thus a subject of great interest. Defining molecular tumor specific biomarkers and the design of suitable agents for their recognition is an important approach in molecular imaging. Whatever the biomarker for molecular recognition might be - a molecule on the cell surface, a specific receptor, an enzyme or protein - its interaction with a specific extracellular agent should lead to a sufficient delivery of the imaging probe¹⁰ without initiating undesirable physiological interferences.¹¹ Thus, for imaging purposes targeting efficiency predominantly relies on the over-expression of the targeted receptor.

In this study sialic acid (Sia) has been exploited as a biomarker for cancer recognition since it is known to be over-expressed on the cell surface as a terminal group on the oligosaccharide chains of glycolipids and glycoproteins.^{12,13} This phenomenon is related to the growing and spreading of the tumor. Being 1000 × higher expressed on tumor cells as compared to normal cells, the 9-carbon sugar Sia is a promising target for an artificial targeting ligand that contains a phenylboronic acid moiety (PBA). A previous NMR study on the interaction between PBA and various sugars, including Sia, demonstrated that reversible covalent binding occurs on the diol functions through the formation of five- and mainly six-membered ring esters at physiological pH (Figure 6.1).^{14,15} The conditional stability constant of the PBA-Sia ester was determined to be 11.6 M⁻¹ in homogeneous solution.¹⁵

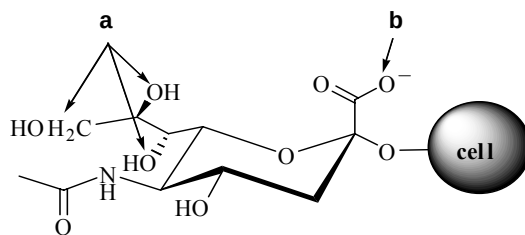


Figure 6.1. Binding-sites of a Sia-terminal end-group on a glycoprotein: formation of PBA-Sia esters (a) and electrostatic interaction with NH_2^+ (b).

Some preliminary results of *in vitro* experiments with a C6 rat glioma cell line and DTPA – PBA conjugates have been reported previously.¹⁶ Here, we report the results of a more detailed study on the exploitation of conjugates of a PBA-moiety with lanthanide complexes of DTPA-derivatives for the targeting of tumor cells. A human glioma cell line, derived from a most common brain tumor, was used as a model for tumor tissue. The membranes of these cells are, as any other cancer cell, covered by gangliosides, a class of sialic acid containing glycolipids that are negatively charged at physiological pH.¹⁷ The molecular formulas of the compounds studied are depicted schematically in Figure 6.2. The $\text{Ln(III)-DTPA-(PBA)}_2$ complex was designed for interaction of the PBA moieties with Sia, whereas $\text{Ln(III)-DTPA-(EN)}_2$ has two positively charged ammonium groups at physiological pH, which may interact electrostatically with the negatively charged carboxylate functions of Sia at the cell surface. In $\text{Ln(III)-DTPA-(ENPBA)}_2$ both targeting features are present in one molecule: PBA units for binding Sia-diol functions and ammonium groups for electrostatic interaction with carboxylate groups of Sia (see Figures 6.2 and 6.3). The efficacy of this approach is reflected in the higher conditional stability constant of the adduct between $\text{Gd(III)-DTPA-(ENPBA)}_2$ and Sia (50.4 M^{-1}) as measured in homogeneous solution at pH 7.4¹⁸ and is exploited in this study.

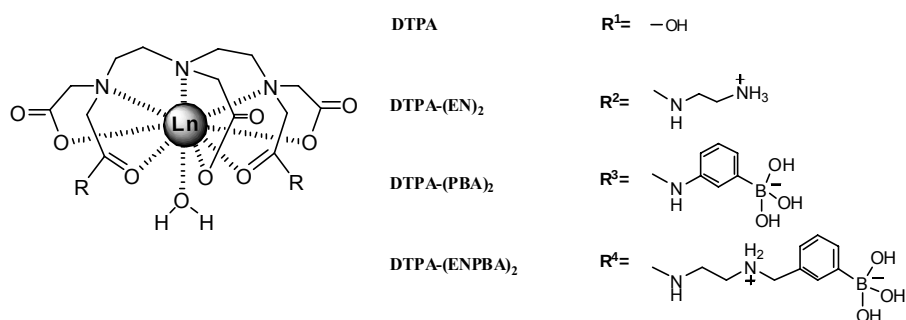


Figure 6.2. Schematic representation of the lanthanide complexes discussed.

RESULTS AND DISCUSSION

The interaction of ^{160}Tb complexes of DTPA, DTPA-(EN) $_2$, DTPA-(PBA) $_2$, and DTPA-(ENPBA) $_2$ (see Figure 6.2) with human glioma cells were studied in PBS medium. Under the conditions applied the cells remained vital during incubation for at least 4 h. This time interval was sufficient to reach the steady state of equilibrium between the bound and free complex on the cell surface. ^{160}Tb was a model lanthanide selected for these studies because of its high specific activity of 65 GBq mol^{-1} and its convenient half-life time (72.1 days) permitting experiments without concern of losing too much activity during the incubation period.

The binding of the conjugates during the interaction experiments is expressed as a function of time using a concentration of 0.1 mM of the complexes in the incubation medium (Figure 6.4). As expected, the parent system, Tb(III)-DTPA, which has no targeting group, did not interact with the cells. A small increase of cell associated radioactivity was observed after incubation with Tb(III)-DTPA-(EN) $_2$, which can be attributed exclusively to the electrostatic affinity of the complex towards the negatively charged cell surface. Higher levels of activity were observed after the incubation with Tb(III)-DTPA-(PBA) $_2$ due to covalent binding of PBA-function with Sia. Finally a significantly higher level of activity was reached upon incubation with Tb(III)-DTPA-(ENPBA) $_2$, which indicates that a two-site binding occurs through PBA ester formation of

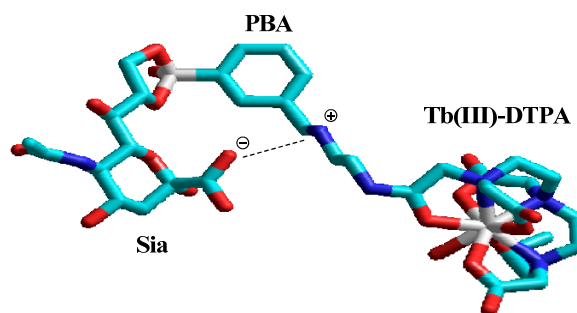


Figure 6.3. HyperChem model of interaction between Tb(III)-DTPA-(ENPBA)₂ and Sia on the surface of a cancer cell. The positively charged ammonium group of the complex is attracted by the negatively charged carboxylate of Sia stabilizing the Sia-Tb(III)-DTPA-(ENPBA)₂ adduct.

vicinal diol functions of Sia and an electrostatic interaction between a positively charged ammonium function of the complex and the negatively charged carboxylate of Sia. The binding sites for this interaction are shown schematically in Figure 6.1. Besides, modeling with Hyperchem demonstrated that the simultaneous occurrence of these interactions is feasible from a steric point of view (see Figure 6.3).

The amount of Sia end groups on the membranes of the cells in this particular cell line, as determined by the thiobarbituric acid assay, is $(2.3 \pm 0.5) \times 10^{-15}$ moles per cell, which is in agreement with the values reported in the literature.^{20,21} From the amount of cells used in the experiments, the Sia-level of the active binding sites can be estimated to be about 1.2 nmol/mg protein. Figure 6.4 shows that the levels of cell-associated activity of the cells incubated with Tb(III)-DTPA-(ENPBA)₂ reaches saturation after about 4 h at a level corresponding to a molar ratio complex/Sia that is slightly higher than 0.5. A complete saturation of the Sia-sites in these incubations apparently is not reached by the end of the experiment despite the large excess of targeting agent in the medium (2×10^{-8} mol) with respect to the amount of Sia available on the cell surfaces (6×10^{-10} mol).

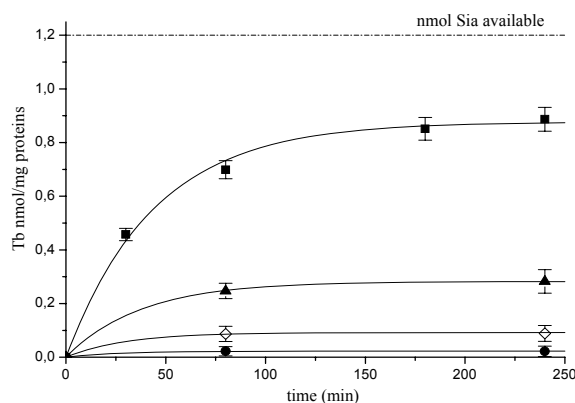


Figure 6.4. Interaction kinetics of 0.1 mM solutions of Tb(III)-DTPA-(ENPBA)₂ (■), Tb(III)-DTPA-(PBA)₂ (▲), Tb(III)-DTPA-(EN)₂ (◇) and Tb(III)-DTPA (●) with 2.5×10^5 human glioma brain tumor cells (U-251 MG) for 4 h at 37 °C in PBS medium.

Incubation of the cells with a higher concentration of Tb(III)-DTPA-(EN)₂ (1 mM) did not result in an increase in the level of saturation of this complex on the cell surface (see Figure 6.5). However, after a 4 h incubation with 1 mM Tb(III)-DTPA-(PBA)₂, the activity observed after 4 h of incubation increased up to a level corresponding to a molar ratio of complex/Sia of 1. This suggests that each Tb(III)-DTPA-(PBA)₂ molecule is binding to the Sia end group through only one of its PBA functions and that all Sia end groups on the cell membrane are occupied by the targeting agent. Surprisingly, with a 1 mM solution of Tb(III)-DTPA-(ENPBA)₂ levels of cell association activity after 4 h were substantially higher than those observed with a 0.1 mM solution and the obtained levels even exceed a 1:1 binding of Tb(III)-DTPA-(ENPBA)₂ by all Sia molecules on the cell surface by far (see Figure 6.5).

The selectivity of targeting complexes Tb(III)-DTPA-(PBA)₂ and Tb(III)-DTPA-(ENPBA)₂ towards Sia was confirmed by incubation of cells twice for 1 h with neuraminidase enzyme prior to the standard interaction experiment. In this way the terminal Sia-residues from gangliosides on the cell surface were removed¹⁹ and accordingly the cells became no longer recognizable for these complexes. After this treatment the cell-associated amount of activity after incubation with these complexes dropped by 90% (see Table 6.1).

Table 6.1. Association of Tb by human glioma cells upon incubation with a medium containing 0.1 mM Tb(III)-complex at 37 °C for 200 min with and without pre-treatment.

Tb(III)-compound	Pre-treatment of cells	Absorbed Tb (nmol/mg protein) ^a
Tb(III)-DTPA-(ENPBA) ₂	None	0.89 (0.12)
	Neuraminidase	0.11 (0.08)
	PBA	0.23 (0.07)
Tb(III)-DTPA-(PBA) ₂	none	0.28 (0.08)
	Neuraminidase	0.03 (0.01)
	PBA	0.07 (0.01)
TbCl ₃	none	0.12 (0.04)

^aValues are means (±SD) of two independent experiments each performed in quintuplicate.

Pre-treatment of cells with a medium containing an excess of PBA also resulted in a strongly decreased binding of Tb(III)-DTPA-(PBA)₂ and Tb(III)-DTPA-(ENPBA)₂ (see Table 6.1). Apparently, the PBA irreversibly blocks the Sia functions confirming the essential role that these functions play in the recognition of the glioma cells by Tb(III)-DTPA-(PBA)₂ and Tb(III)-DTPA-(ENPBA)₂.

To obtain a better understanding of the surprisingly high levels of cell-bound Tb observed after incubation of the cells with 1 mM Tb(III)-DTPA-(ENPBA)₂, the concentration dependence of the interaction between this complex and the cell surface was studied in more detail. Two series of experiments were carried out at 37 and 4 °C, in which the concentration of Tb(III)-DTPA-(ENPBA)₂ in the medium was varied between 0.01 and 1.5 mM. In both cases the cells were incubated for 200 min with a solution of Tb(III)-DTPA-(ENPBA)₂ in PBS. The amount of cell-associated Tb taken as a function of the concentration of Tb(III)-DTPA-(ENPBA)₂ in the incubation medium is displayed in Figure 6.6. Upon increase of the concentration of the Tb(III)-complex in the medium at 37 °C, the amount of Tb absorbed seems to reach a plateau at about 1.2 nmol Tb/mg proteins, corresponding with a molar ratio Tb/Sia end groups of one. However, a further increase of the concentration leads to a steep increase of the amount of Tb retained by the cells up to about 5.5 nmol Tb/mg protein. Similar experiments at 4 °C show saturation with the Tb(III)-complex at 1.6 nmol/mg protein, which corresponds to 1.1 mol Tb per Sia group. It may be expected that all active cellular processes, including internalization, are

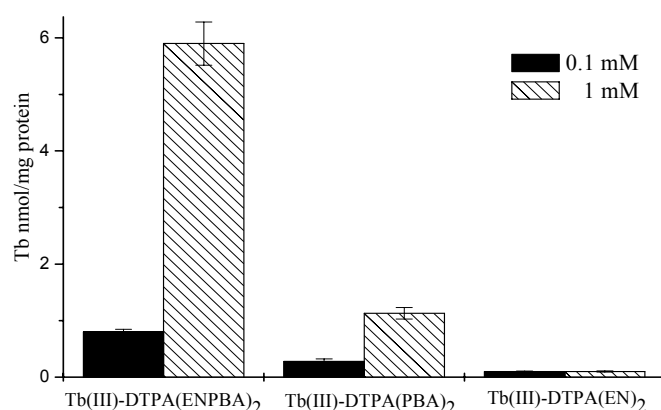


Figure 6.5. The effect of complex concentration in the incubation medium on the binding with the cell surface after 4 h incubation at 37 °C. The amount of Sia available on the cell membrane is 1.2 nmol/mg protein.

disabled at 4 °C, and therefore this experiment indicates that at low temperature 1 mol Tb/Sia end group is adsorbed on the cell membrane. The difference between the curves for 37 and 4 °C gives rise to the question whether internalization occurs, and if so, whether the cells are taking up intact Tb(III)-complex or free Tb³⁺ after its dissociation from Tb(III)-DTPA-(ENPBA)₂.

Aime and co-workers already demonstrated the poor stability of the DTPA-BMA chelates in cell experiments.²² Even though the concentrations used in the present study were much lower than in the experiments described by these authors, the kinetic stability of the Tb(III)-DTPA-(ENPBA)₂ complex deserves a closer look.

To establish whether the Tb(III)-DTPA-(ENPBA)₂ complex remains intact upon interaction with the cells, the amounts of Tb and B retained by the cells after incubation with non-radioactive Tb(III)-DTPA-(ENPBA)₂ were determined simultaneously by ICP on lysates of the cells. As can be seen in Figure 6.7, the amounts of Tb and B are about equal, regardless of the concentration of Tb(III)-DTPA-(ENPBA)₂ in the incubation medium. The amounts of cell-associated Tb observed by ICP were in agreement with those in the

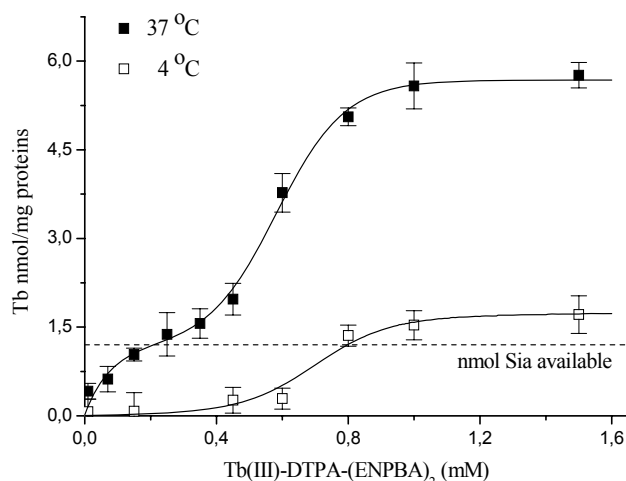


Figure 6.6. Binding of ^{160}Tb -label by human glioma tumor cells as observed upon incubation with various concentrations of $\text{Tb(III)-DTPA-(ENPBA)}_2$ for 200 minutes, 5×10^5 cells used.

experiments with the radioactive complex. Since for the intact $\text{Tb(III)-DTPA-(ENPBA)}_2$ complex, the Tb/B ratio is $\frac{1}{2}$, this experiment indicates that a significant amount of the Tb -complex has been dissociated into free Tb and free DTPA-(ENPBA)_2 . Apparently, the latter can be washed off from the cell surface relatively easily, which can be rationalized by considering the overall charges of the intact complex (neutral) with respect to the dissociated ligand (-3). Since the cell membrane is negatively charged, the interaction with the free ligand will be destabilized by electrostatic repulsion. Removal of free ligand, DTPA-(ENPBA)_2 , from the cell surface then allows binding of new Tb -containing complexes and thus a relatively high level of cell-associated Tb compared to B.

After a standard incubation procedure with the cells and $1 \text{ mM } ^{159}\text{Tb(III)-DTPA-(ENPBA)}_2$ complex in the medium for 200 min, cells were treated twice with a medium containing glycine (pH 2.8). This treatment ensures the removal of all acid releasable cell-surface bound species. The ICP measurement after lysing the cells indicated that virtually all B-containing species were removed from the cells by the glycine treatment (see Table 6.2), which shows that the internalization of the intact complex or the free ligand is

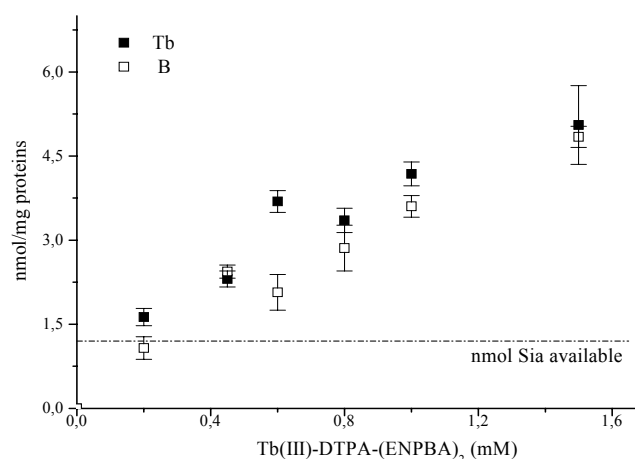


Figure 6.7. ICP determination of Tb and B content taken up by the cells after incubation with varying concentrations of $^{159}\text{Tb(III)-DTPA-(ENPBA)}_2$ for 4 h at 37 °C.

negligible. The amount of Tb in this lysate, however, was considerable (1.31 nmol/mg protein), which means that free Tb has been internalized and/or remains firmly associated to the cell membranes, for example to the phosphate groups of the phospholipids. The latter possibility cannot be excluded since the glycine treatment is not sufficient for the removal of free metal from phosphates.

The affinity of free terbium towards the cells was investigated by incubating cells with a PBS medium containing 0.1 mM TbCl_3 . Even after incubation for 200 min the amount of Tb accumulated by the cells was very low (0.12 nmol/mg protein, see Table 6.1). Tb^{3+} most probably precipitates as phosphate under those conditions, due to the presence of a buffer in the medium, PBS. It can, therefore be excluded that Tb-phosphates or free Tb^{3+} , formed by dissociation of $\text{Tb(III)-DTPA-(ENPBA)}_2$ in the medium are retained by the cells under the conditions applied in the interaction experiments.

To evaluate the kinetic stability of the Tb-DTPA-(ENPBA)_2 in the PBS medium, ^1H relaxivity measurements were performed on a 20 mM solution of $^{159}\text{Tb(III)-DTPA-(ENPBA)}_2$ in the presence of an amount of phosphates comparable to that present in the PBS medium used for the cell experiments. Because of the relatively low sensitivity of

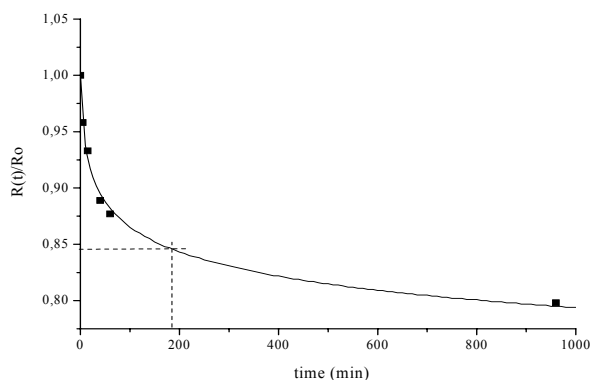


Figure 6.8. Decrease of the longitudinal ^1H relaxation rate ($R_1(t)$) as a function of time of $\text{Tb(III)-DTPA-(ENPBA)}_2$ (20 mM) in an aqueous solution at 25 °C, in the presence of phosphates (9 mM).

NMR, the concentrations employed had to be an order of magnitude higher than in the cell experiments. The longitudinal relaxation rate observed shows a decrease as a function of time (see Figure 6.8), which can be explained by dissociation of Tb^{3+} from the complex followed by precipitation as phosphate. After 200 min, which corresponds to the total incubation time of the cell incubation experiments, 85% of the complex is still intact. Since the concentrations applied in the cell experiments were much lower it is very unlikely that, under those conditions, the kinetic instability of the $\text{Tb(III)-DTPA-(ENPBA)}_2$ complex can account for the large amount of “free” Tb retained by the cells. It can be concluded that the dissociation of $\text{Tb(III)-DTPA-(ENPBA)}_2$ observed in the cell experiments is most likely taking place at the cell membrane. Apparently, dissociation only occurs upon strong interaction of a Ln-complex with the cell membrane as is reflected in low amounts (< 1 mol Tb/ mol Sia) ^{160}Tb observed on the cells after incubation with 1 mM solutions of $^{160}\text{Tb(III)-DTPA-(PBA)}_2$, $^{160}\text{Tb(III)-DTPA-(EN)}_2$, and $^{160}\text{Tb(III)-DTPA}$ (see also Figure 6.5).

The ICP measurements show that internalization of intact $\text{Tb(III)-DTPA-(ENPBA)}_2$ does not occur, whereas that of Tb^{3+} takes place to a limited extent. The large difference in the levels of cell bound Tb observed between experiments with radiolabeled complex at 37 and at 4 °C (see Figure 6.5) cannot be ascribed exclusively to internalization. Possibly, the kinetics of the borate ester formation, and the subsequent dissociation of the metal ion from the cell bound complex play a role as well.

Table 6.2. ICP measurements of cells incubated with 1 mM $^{159}\text{Tb(III)-DTPA(ENPBA)}_2$ at 37 °C, 200 min.

Processing ^a	nmol/mg protein ^b	
	Tb	B
Normal	4.98 (0.70)	4.84 (0.19)
Glycine-treatment	1.31 (0.38)	0.23 (0.02)

^aSee experimental part for the standard incubation procedure.^bExperimental values are means ($\pm$ SD) of two independent experiments each performed in triplicate.

CONCLUSIONS

This in vitro investigation of the affinity of PBA-containing DTPA-derivatives towards cancer cells with over-expressed sialic acid on the membrane demonstrates a significant level of binding. The selectivity of PBA-Sia binding was proven by two approaches: removing or pre-saturating of the target Sia or by using precursors of the complexes that lack the targeting vector (PBA). In both cases the absence of binding expressed in γ -activity of complexed terbium was clearly demonstrated by the specificity of the binding. From the complexes under study, $\text{Tb(III)-DTPA-(ENPBA)}_2$ showed the highest level of cell binding, which may be ascribed to synergism of the covalent binding of Sia by the PBA moiety and the electrostatic interaction between the positively charged ammonium group and the negatively charged cell surface. Subsequent to the binding, at the cell surface a transfer of the metal ion from $\text{Tb(III)-DTPA-(ENPBA)}_2$ complex takes place, possibly to the phosphate groups of the cell membrane. Internalization of the intact complex or of the free ligand, DTPA-(ENPBA)_2 does not take place, whereas the experiments suggest that if internalization of free Tb occurs, it happens only to a minor extent under the conditions applied.

The results demonstrate that recognition of tumor cells by PBA targeting functions is possible. However, the low kinetic stability of the DTPA-bisamide unit at the cell surface of the presently studied complex is a disadvantage, particularly for applications as MRI contrast agent, where relatively high concentrations are required. On the other hand, the transfer of the metal ion to the cells after recognition of Sia may be applicable in radiotherapy for the delivery of radioactive metals to tumor cells.

EXPERIMENTAL

Materials and methods

All chemicals were purchased from Aldrich and used without further purification. Ligands DTPA-(EN)₂, DTPA-(ENPBA)₂, and DTPA-(PBA)₂ were synthesized according to published procedures.¹⁶⁻¹⁸ A final purification step was performed with a Millipore stirred cell ultra-filtration system using an Amicon Cellulose Acetate membrane with 500 NMWL cut-off. An aqueous solution of labeled ¹⁶⁰TbCl₃ was prepared by thermal neutron activation of 10 mg of a 20 mM solution of ¹⁵⁹TbCl₃·6H₂O in a nuclear reactor (thermal neutron flux = $4.49 \times 10^{12} \text{ cm}^{-2}\text{s}^{-1}$, irradiation time = 5 h). The obtained solution (specific activity $A = 65 \text{ GBq mol}^{-1}$) was used without further purification. Complexations were carried out by mixing aqueous solutions of the metal salt and of the ligand with the latter in 5% excess at pH 7.2-7.4. The obtained solutions were kept a couple of hours at room temperature prior to use. The efficiency of this method was checked by HPLC using a Na⁺-chelex-column. The radioactivity of ¹⁶⁰Tb in the samples after incubation experiments was determined by measuring the intensity of emitted γ -rays using a NaI(Tl)-scintillation detector based γ -ray counter (Wallac). ICP measurements were performed with a Perkin Elmer Optical Emission Optima 4300 DV Spectrometer. The measurements of the terbium and boron contents in cell suspensions were calibrated by standard solutions of Tb(NO₃)₃ and H₃BO₃ respectively. The initial ratio between Tb and B in the Tb(III)-DTPA-(ENPBA)₂ complex was confirmed by ICP analysis to be 1:2. ¹H NMR measurements on the Tb(III)-DTPA-(ENPBA)₂ complex were performed with a Varian Inova-300 spectrometer operating at 300 MHz. Longitudinal relaxation rates were measured with the inversion recovery pulse sequence. The thiobarbituric acid assay was performed on a Varian Cary 3 Bio-UV-visible spectrophotometer operating at a wavelength of 532 nm and the protein assay on a Biotek Powerwave XS microplate reader at 750 nm.

Cell culture

Human glioma brain tumor cells, U-251 MG, were grown in Eagle Minimal Essential Medium (MEM) in an incubator at 37 °C in humidified air containing 5% CO₂. MEM was supplemented with 10% heat-inactivated fetal bovine serum (FBS), non-essential amino acids (0.1mM), 18 mM NaHCO₃, 1 mM Na-pyruvate, 2 mM L-glutamine, and antibiotics (penicillin/streptomycin). Prior to the experiments, cells were detached from the bottom of the culture flask by adding a 0.25% solution of trypsin in Phosphate Buffered Saline Solution (PBS), counted under a microscope and seeded into a 96- or 6-well multiplate at a density of 105 cells/ml. After incubation for 24 h, the medium was removed; the cells were washed once with PBS and then used directly for incubations. The amount of protein in each well was determined by the Bio-Rad DC protein assay using bovine serum albumin as a standard.^{23,24} The amount of Sia on the surface of the cells in study was determined by the Warren thiobarbituric acid assay.²⁵ To determine the amount of living cells during the experiments, 200 µl of cell suspension was transferred to a mixture of 50 µl 0.4% trypan-blue solution in 150 µl PBS. The final solution was counted under a microscope using a Neubauer counting chamber. Vital cells appeared spherical and colorless, whereas damaged cells appeared blue due to the uptake of trypan-blue. The result of the viability tests was expressed as the percentage of the total amount of cells that was vital.

In vitro cell interaction experiments

To the cells in a 96-well multi-plate, 200 µl of solution with the desired concentration of radioactive ¹⁶⁰Tb-complexes of the chelate under study in PBS were added in quintuplicate and incubated at 37 °C for various time intervals. After a given incubation time, the medium was removed and the cells were washed three times with ice-cold PBS. The removable wells were disconnected from the plate, placed into plastic tubes and the radioactivity was measured in a γ-counter. The radioactivity of the incubation medium, after removal from the cells was counted as well to check the mass balance. Various amounts of the Tb(III)-DTPA-(ENPBA)₂ complex in PBS medium were added to the cells

to study the interaction as a function of concentration of the complex in the medium. In those experiments, the cells were incubated for 200 min at two temperatures (either 37 or 4 °C) and, after the same washing procedure as described above, γ -counting was performed. For the interaction study by means of ICP, a 6-wells plate was used for the incubation of the cells with non-radioactive $^{159}\text{Tb(III)-DTPA-(ENPBA)}_2$ for 200 min (37 and 4 °C). After incubation at either 37 or 4 °C, the cells were washed with ice-cold PBS and then lysed by addition of 500 μl Triton solution (1%). Of the suspension obtained, 50 μl was used for the protein assay and 400 μl for ICP-analysis. In experiments to distinguish between bound and internalized complex, the cells were treated twice with a 0.05 M solution of glycine (pH 2.8) for 5 min to remove cell bound conjugates. After washing and lysis of the cells according to the procedure described above, ICP analysis was performed. Some experiments were carried out with cells of which Sia was removed from the surface by treatment with the enzyme neuraminidase (from *Vibrio cholera*) with a specific activity of 348 units/mg proteins following a published procedure.²⁶ The cells (10^5 per well) were incubated two times for 60 min in a medium containing 0.16 units of neuraminidase per well. After washing the cells twice with ice-cold PBS, interaction experiments were performed according to the general procedure described above. The cell viability was checked by the trypan-blue test. Every experiment was repeated independently at least three times.

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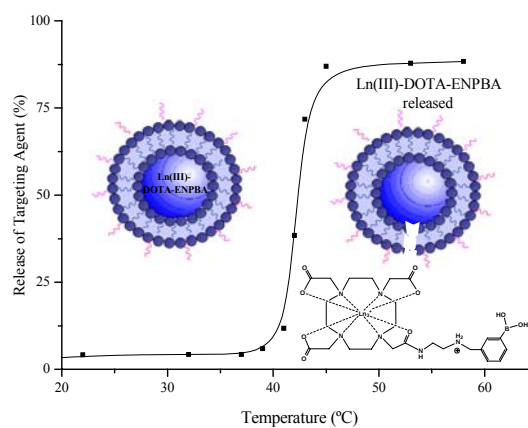
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A THERMOSENSITIVE LIPOSOME- ENTRAPPED DOTA-ENPBA TARGETING AGENT FOR DIAGNOSIS AND THERAPY OF TUMORS

7



INTRODUCTION

Tumor-targeted contrast agents and/or therapeutics are currently among the most promising approaches in cancer therapy. The research in this field is focused on the various aspects of tumor specific drug delivery and image-guided therapy. Recent developments in molecular imaging techniques make it possible to visualize pathologies already at early stages. Still, the search for a highly sensitive and tumor-selective contrast agent is encouraged by the need for real-time noninvasive monitoring of drug delivery and distribution, as well as quantification of the treatment efficiency, which will bring cancer therapy on a completely new level of personalized medicine.¹ An efficient drug delivery to the tumor site is limited by many factors. It is commonly recognized that, when for instance chemotherapy is applied, it is extremely difficult to reach therapeutic levels of the drug at the tumor sites without affecting healthy tissues. This urges for the development of novel approaches that aim at decreasing drug toxicity and increasing drug concentrations in the tumor. Several promising strategies for improved drug delivery have emerged recently.² One of them is the use of targeting agents, such as antibodies, aptamers or peptides with affinity to certain over-expressed epitopes on the cell surface of tumor tissues.³ Another approach is encapsulation of the drug into carriers such as liposomes, which are nanoscale biocompatible vesicles, composed of a phospholipid bilayer enclosing an aqueous compartment.⁴ The success of the use of liposomes for drug delivery to solid tumors is based on the enhanced permeability and retention (EPR) of macromolecules in tumor tissue in combination with the small size long-circulation capacity of liposomal drug carriers.^{5,6} Briefly, the fast growing tumor tissue is associated with non-effective lymphatic drainage and formation of neovasculature, composed of poorly-aligned endothelial cells with abnormal form and architecture, which leads to the enhanced accumulation of liposomes with a diameter smaller than 400 nm in the tumor.^{7,8} During blood circulation liposomes become coated with plasma proteins after which they are recognized by reticuloendothelial cells and removed by phagocytosis, especially in the liver and the spleen.^{9,10} Modification of the surface of the bilayer with lipid-grafted PEG (poly(ethyleneglycol)) extends the blood circulation half-life time significantly due to the formation of a hydrated shield around the liposomes, which provides a repulsive steric barrier against protein molecules

and inhibits macrophage uptake. Due to the prolonged presence in blood circulation, chances for extravasation into tumor vascular beds are higher.¹¹ Even though these conventional liposomes have been clinically evaluated for a variety of diseases,¹² the biggest challenge today remains to achieve an effective controlled release of the encapsulated drug from kinetically stable long circulating liposomes.

The concept of thermo-sensitive liposomes (TSL) first proposed in the late 1970s,¹³ and studied extensively after, offers possibilities for controlled drug delivery by means of local hyperthermia. By tuning the formulation of the lipids composing the bilayer it is possible to control its transition from gel-to-liquid phase at clinically applicable temperatures.¹⁴ Using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, $T_m = 41.5\text{ }^{\circ}\text{C}$) as the primary lipid makes the membrane highly leaky to small water-soluble molecules at a convenient phase-transition temperature. To increase the stability of the membrane and to prevent pre-leakage of the drug, a lipid with a longer aliphatic chain and, consequently a higher T_m is often included in the formulation, e.g. 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC, $T_m = 56.0\text{ }^{\circ}\text{C}$).¹⁵

Despite the significant advances that have been made in liposomal drug delivery, the problem that still remains to be solved is how to achieve an effective retention of the drug at the tumor site after it has been released from the liposomes. The penetration and initial accumulation of encapsulated small drug molecules is very efficient, but their rapid wash out and transfer back to plasma is a weak point.¹⁶

In our previous work we have demonstrated *in vitro* recognition of tumor cells by a phenylboronic acid (PBA) targeting vector conjugated to a DTPA-bis-amide molecule via a positively charged ethylenediamine bridge (EN). DTPA was used to chelate lanthanides (Ln) to the targeting vector.¹⁷ Sialic acid (Sia), a nine-carbon sugar over-expressed on the surface of tumor cells as the terminal group on glycan chains, has been exploited for molecular recognition of tumors.¹⁸ This was demonstrated using a human glioma cell line. It was shown that the tumor targeting can be ascribed to the formation of a reversible covalent binding between Sia's diol-function and the boronate function of PBA. However, two problems were faced during this study: i) a poor stability of $\text{Ln(III)-DTPA-(ENPBA)}_2$ leading to the leakage of free Ln(III)-ions, and ii) a competitive binding of the PBA-vector to other sugar moieties in plasma and to erythrocytes, which also contain Sia on their

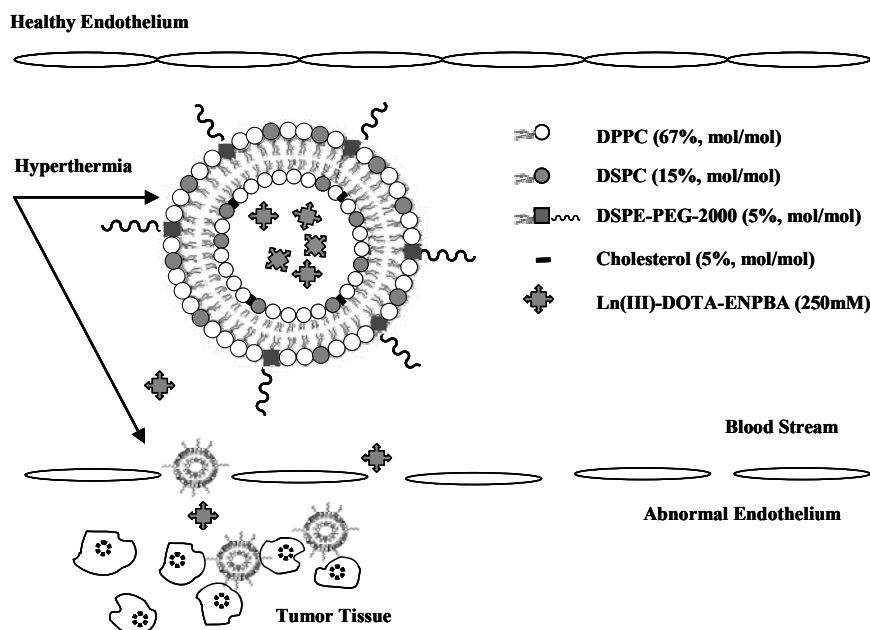


Figure 7.1. Schematic representation of temperature-triggered targeting of tumor cells by contrast agent encapsulated into TSL.

surface. The non-coordinated Ln(III)-ion formed can be hydrolyzed to insoluble $\text{Ln}(\text{OH})_3$, which can accumulate in the skeleton, liver and skin, especially in patients with renal failure.¹⁹ Thermodynamic and kinetic stability of lanthanide-based systems *in vivo* is an important point of concern that should be addressed during the design of new contrast/targeting agents.²⁰ Therefore, in the present study, we have modified the structure of the targeting agent by replacing the DTPA-bis-amide moiety by its DOTA analogue. The latter ligand is known to form lanthanide complexes with an extremely high thermodynamic and kinetic stability. Encapsulation of the designed complex into thermo-sensitive liposomes is expected to offer protection of the targeting group against binding to sugar moieties in the blood stream and to result in increased accumulation in tumors. A subsequent application of mild hyperthermia will lead to the release of the targeting agent, which will then immediately bind to the tumor cell surface. Figure 7.1 schematically represents the concept of the targeting by means of TSL combined with recognition of the

tumor by the metal complex. The formulation of the lipids was carefully tuned to $T_m = 41.5$ °C by measuring the fluorescence signal of temperature triggered release of encapsulated calcein, which possesses self-quenching properties when entrapped in liposomes at elevated concentrations. The same formulation was used for the preparation of liposomes containing Ln(III)-DOTA-ENPBA targeting agent. About 70% of encapsulated material was released in the temperature range of 41-43 °C as determined by ICP. The *in vivo* behavior of liposomes and their temperature-induced content release was studied by intravital confocal laser scanning microscopy in a human BLM melanoma xenograft grown in a dorsal skin-fold window chamber implanted in nude mice during and after application of local hyperthermia.

RESULTS AND DISCUSSION

Synthesis

The targeting agent used in this study was prepared following the synthetic route shown in Figure 7.2. The targeting PBA function was attached to the DOTA-chelator by a reductive amination reaction between the free amino-group of *t*-butyl protected ethylenediamine-functionalized DOTA (**3**) and the aldehyde group of 3-formylphenylboronic acid. The imine formation was monitored by the decrease of the intensity of the ^1H NMR resonance of the aldehyde function at 9.89 ppm as well as by the increase of the intensity of the imine resonance at 8.24 ppm. After complete conversion, the Schiff base was reduced by addition of NaBH_4 to the reaction mixture. The formed boric acid was converted into its tetramethyl ester, which was removed together with the solvents by evaporation under reduced pressure. In the last step of the synthesis, the phenylboronate-functionalized DOTA was deprotected and the title compound (**5**) was purified by cation-exchange chromatography.

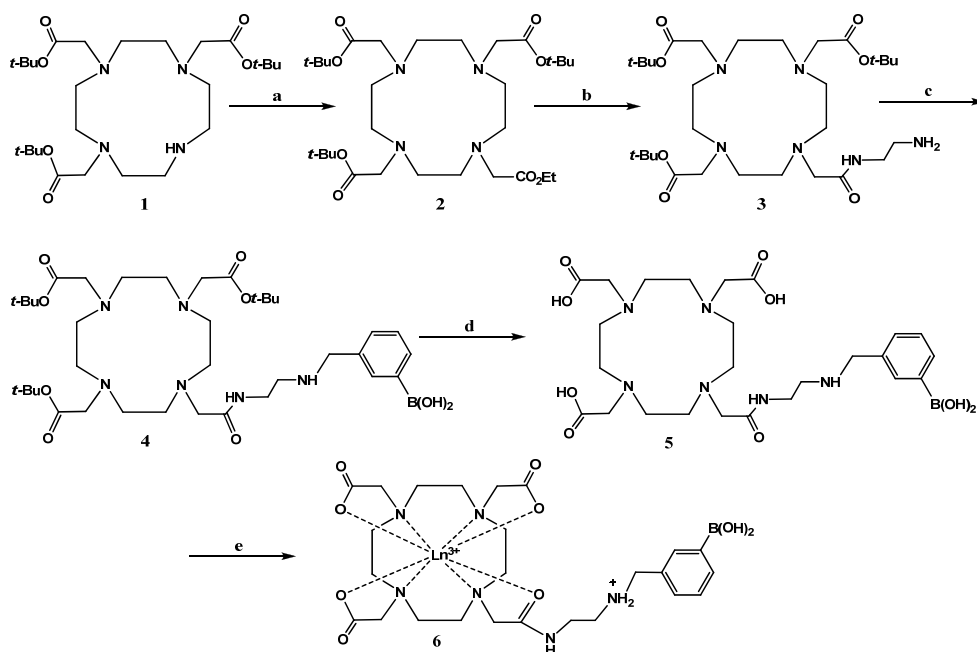


Figure 7.2. Preparation of Ln(III)-DOTA-EN-PBA: (a) $\text{BrCH}_2\text{CO}(\text{OEt})$; (b) neat $\text{NH}_2(\text{CH}_2)_2\text{NH}_2$; (c) $\text{O}(\text{H})\text{CPhB}(\text{OH})_2$, NaBH_4 ; (d) $\text{TFA}/\text{CH}_2\text{Cl}_2$; (e) $\text{LnCl}_3 \cdot 6\text{H}_2\text{O}$.

Optimization of the formulation of the TSL

Unilamellar liposomes used for the encapsulation of Ln(III)-DOTA-ENPBA targeting agent were designed to fulfill the requirements for local hyperthermia, which include high stability at 37 °C (body temperature) and presence of a PEG-coating to ensure a long circulation time in the blood stream and a T_m at 41-42 °C. At this temperature, the entrapped material should be released from the assembled system as fast as possible.²¹ Another important characteristic is the size of the liposomes. Maruyama et al. have shown that the liposome diameter determines their *in vivo* behavior;²² a relation exists between the diameter of the thermosensitive liposomes and their extravasation properties into tumor tissues and their release rate. The ideal diameter for an efficient tumor uptake was shown to be 100-200 nm (also considering the inner diameters in the human circulatory system). Therefore, for the present study we decided to use liposomes with an effective diameter of

100 (± 15) nm, choosing for the benefits of prolonged circulation time, efficient tumor accumulation and higher stability rather than for a higher amount of encapsulated drug.²³

It is known that the length of the aliphatic chain and the head-group of the phosphatidylcholines used for the composition of the lipid bilayer are determining the thermal properties of a TSL, whereas the relative amount of cholesterol (CHOL) has a stabilizing effect on the fluid-phase.²⁴ A DPPC/DSPC/CHOL/DSPE-PEG formulation was selected and the final composition of the liposome was fine-tuned for optimal performance by stepwise alterations of the ratios of the various components.

Temperature-induced release of calcein from the TSL *in vitro*

To test the releasing process a 90 mM solution of the fluorescent dye calcein was encapsulated into the liposomes. The concentration of the encapsulated dye is above its self-quenching concentration (12 μ M). Consequently, the dye becomes only observable upon its release as a result of dilution.²⁵ This enabled monitoring of the release of entrapped material *in situ* by measuring the increase of the fluorescence signal intensity (see Figure 7.3, **A** and **B**). The temperature of a sample of liposomes in HEPES buffer was increased stepwise. After each increase the sample was stabilized for 15 min and the fluorescence was measured after the system was quickly cooled down in an ice-bath to stop the release. Initially, a molar ratio of DPPC/DSPC/CHOL/DSPE-PEG of 77:5:15:3 was used in the liposomal formulation. As shown in Figure 7.3 (**A**), calcein release occurs in this case already below 37 °C, which is not suitable for *in vivo* applications. Therefore, in the next trial the ratio of DPPC/DSPC lipids was changed to 67:15. The increase of DSPC in the formulation significantly improved the thermal behavior of TSL shifting the transitional temperature to 41 °C. A massive release of the contents of the liposomes in HEPES buffer occurred between 40 and 43 °C, a maximum of 92 % was reached at 44 °C. No additional release was found upon further heating to 60 °C.

Next, temperature-induced release experiments on the TLS with the optimal formulation (DPPC/DSPC/CHOL/DSPE-PEG = 67:15:15:3, mol %) were performed in the presence of fetal bovine serum (FBS, 50 %, v/v) to mimic the *in vivo*

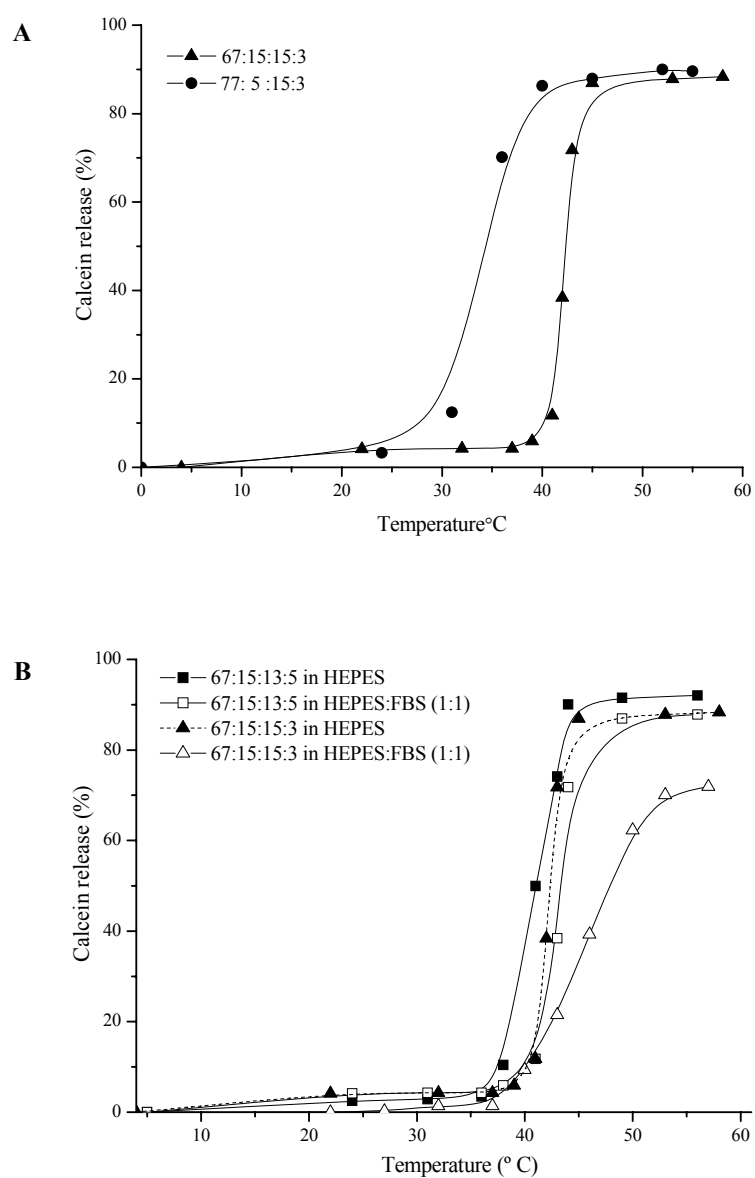


Figure 7.3. **A.** The calcein release from TSL versus temperature after incubating for 15 minutes in HEPES. **B.** Effect of an increased amount of DSPE-PEG in the TLS formulation on the calcein release in HEPES and in the presence of FBS. DPPC/DSCP/CHOL/DSPE-PEG = 67:15:15:3 (▲); 77:5:15:3 (●); 67:15:13:5 (■).

situation. As demonstrated in Figure 7.3 (B), a substantially smaller amount of calcein was released under these conditions. This is consistent with the reported hampering of the release of encapsulated material from liposomes due to interaction with the serum proteins caused by insufficient PEGylation of the surface.²⁶ Therefore, in a final modification of the liposomal formulation, the amounts of DSPE-PEG and cholesterol were increased to 5 and decreased to 15 % mol, respectively, whereas the molar ratio of the lipids DPPC/DSPC was kept the same (67:15). These alterations of the formulation resulted in a dramatic improvement of the performance of the liposomes in the presence of serum. All further experiments were performed using this optimized liposomal formulation DPPC/DSPC/CHOL/DSPE-PEG = 67:15:13:5 (mol %).

Differential scanning calorimetry (DSC) measurements confirmed that the thermotropic transition of the composed liposomes takes place at 42 °C (Figure 7.4).

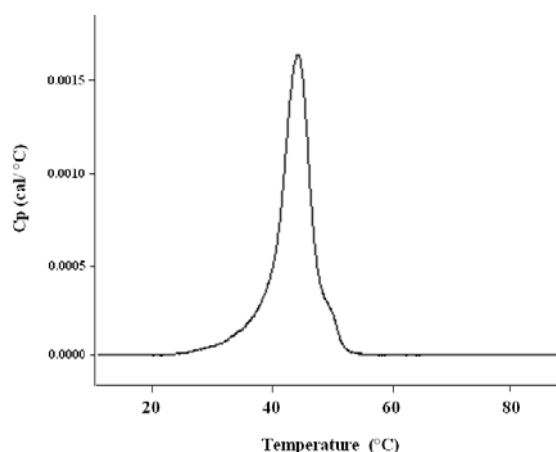


Figure 7.4. DSC analysis of the TSL with lipid composition DPPC/DSPC/CHOL/DSPE-PEG (67:15:13:5, m/m %) in FBS (50%, v/v); heat flow 1°C/min.

Stability of the TSL *in vitro*

For the applicability of the liposomes it is not only important that they have optimal releasing properties at elevated temperatures, but they have to be sufficiently stable in the blood circulation and, not less essential, under storage conditions. Therefore, prolonged incubations were performed, in which the liposomal suspension of HEPES/FBS medium (1:1) was incubated at 37, 39, 41, and 45 °C up to 5 hours. The amount of released calcein was monitored by measuring the intensity of the fluorescence signal. The data (see Figure 7.5) clearly demonstrate that, at 37 and 39 °C, only minor quantities of calcein are released during the first 5-10 min, after which the release stops. At 41 and 45 °C, the maximum release is also reached within 5-10 min, but then the amounts are dramatically higher (67 and 83 %, respectively). In all cases, almost no further increase of the intensity of the fluorescence intensity was observed during a period of 5 hours.

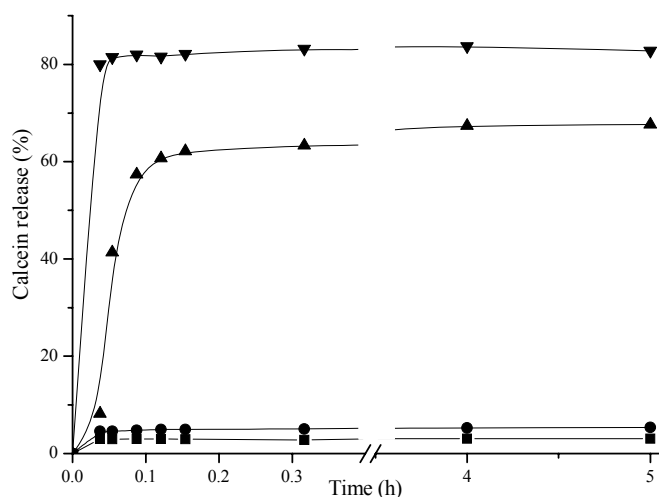


Figure 7.5. Calcein release from DPPC/DSPC/CHOL/DSPE-PEG liposomes (67:15:13:5, mol %) in HEPES/FBS (1:1) at different temperatures monitored at different time intervals in the range from 2 to 300 min: 37 °C(■), 39 °C(●), 41 °C(▲), 45 °C(▼). The break in the graph is introduced for a better visualization of the start of the release process.

The long term stability of the liposome system under storage conditions was evaluated by measuring of the calcein release and the particle size by DLS during a period of 30 days. Table 7.1 compiles the data, from which it is clear that the liposomes are completely stable under these conditions for at least one month.

Table 7.1. Long term stability study of calcein containing liposomes.

Time (days)		0	1	5	8	14	30
4 °C	Fluorescence intensity	0.107	0.119	0.115	0.105	0.117	0.111
	Mean diameter (nm)	108	114	112	114	114	114
20 °C	Fluorescence intensity	0.094	0.083	0.060	0.049	0.048	0.058
	Mean diameter (nm)	96	90	100	114	110	104

All values are means of measurements of two TSL batches with a SD of typically 5%.

Release of encapsulated La(III)-DOTA-ENPBA targeting agent from TSL

Liposomes containing the La(III)-complex of DOTA-ENPBA were prepared using a procedure similar to that used for the encapsulation of calcein. A 250 mM aqueous solution (1 ml) of the targeting agent was used for the hydration of the lipid film. ICP measurements of the crude liposomal suspension after destruction by a concentrated Triton-X solution showed that the system contained 2.47×10^{-4} mol La(III)-complex of DOTA-ENPBA, while the phosphate assay indicated 4×10^{-7} mol of lipids. After extrusion and centrifugation, the liposomal pellet obtained was re-suspended in 1 ml of HEPES buffer. The amounts of lipid and complex in the liposomal pellet were measured to be 3.25×10^{-7} and 1.72×10^{-5} moles, respectively. It may be concluded that the encapsulation efficiency of DOTA-ENPBA in the liposomes was 8.5%.

The release of La(III)-DOTA-ENPBA from the liposomes was tested in HEPES/FBS (1:1) medium. The release was evaluated by determining the amount of the complex in the liposomal pellet and in the supernatant after centrifugation of the sample. The results of these experiments, depicted in Figure 7.6, are in agreement with the thermal behavior observed during the experiments with the TSL with encapsulated calcein described above. The maximum amount of released compound at temperatures above 40 °C is somewhat lower than that of calcein, but it is still sufficiently high reaching maximal values of 70 % released compound. The release at 37 °C (5 %) is once again negligible.

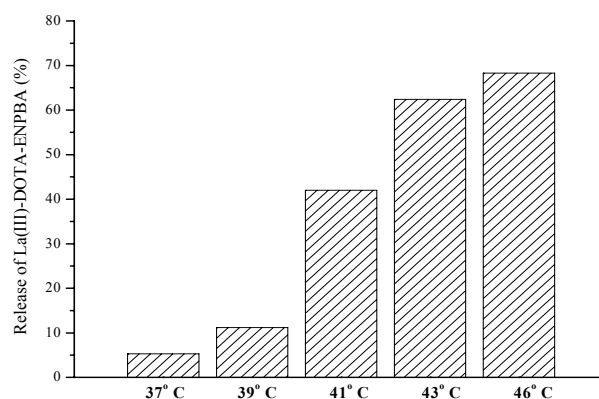


Figure 7.6. Release of encapsulated La(III)-DOTA-ENPBA targeting agent (1.75×10^{-5} moles per 3.23×10^{-7} moles lipids) in HEPES/FBS medium (1:1) from TSL as determined by ICP; DPPC/DSPC/CHOL/DSPE-PEG (67:15:13:5, mol %).

***In vivo* testing of the calcein loaded TSL by means of fluorescence microscopy**

To test the performance of the designed system, liposomes containing 90 mM buffer-solution of calcein in the inner-core and 1 mol % of rhodamine-PE in the lipid bilayer were injected into a nude mouse with a window chamber in which a human BLM melanoma tumor was implanted. Green (calcein) and red (rhodamine) fluorescence were monitored by confocal laser scanning microscopy in the living mouse upon local heating, representing the encapsulated calcein and the liposomal bilayer, respectively. The window chamber bearing mouse was anesthetized, injected with liposomes and installed under a confocal laser scanning microscope. The tumor tissue in the window chamber was heated using an in house developed heating device mounted on top of the window chamber. Online tissue temperature measurements were performed to monitor the local tissue temperature. The dose of injected liposomes used for the intravital microscopy experiments was $4.2 \mu\text{mol}$ lipids. After the injection, the accumulation of liposomes in the tumor was clearly observed

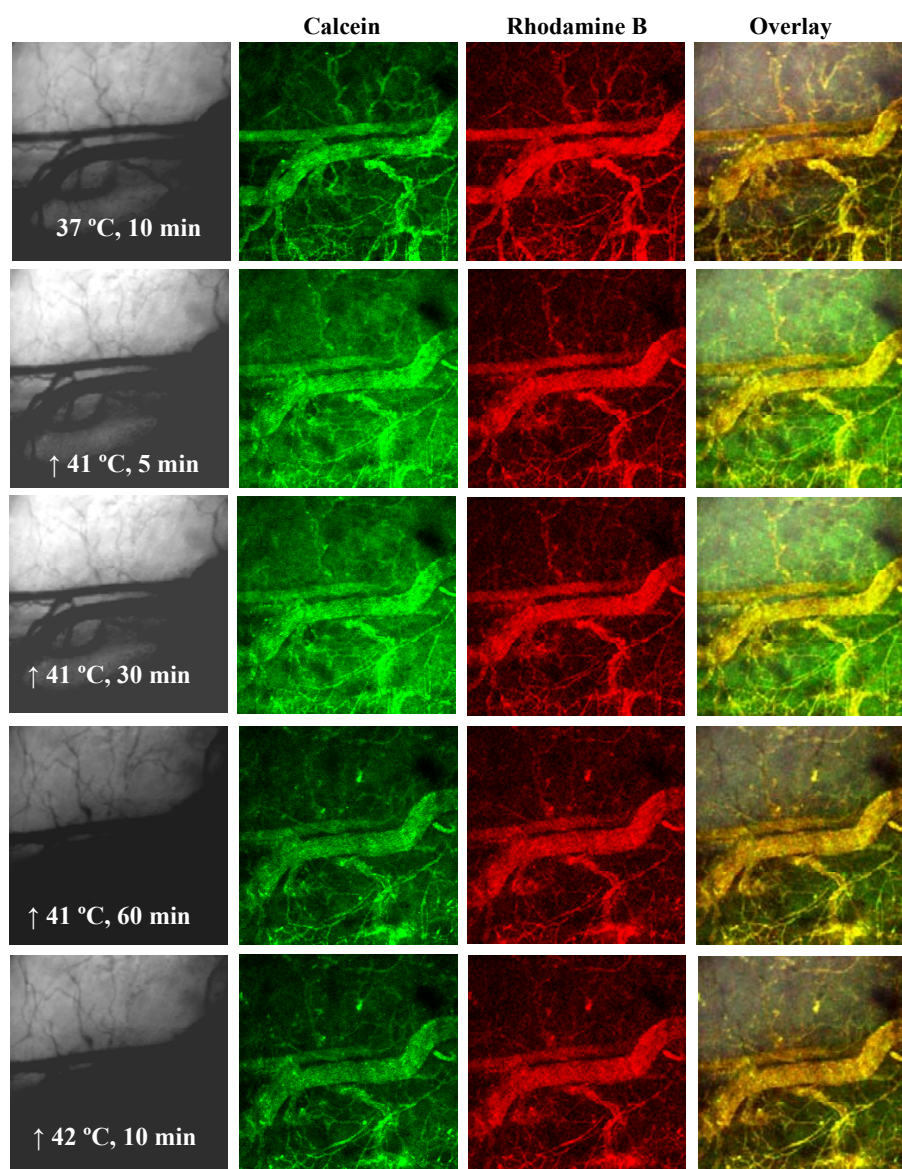


Figure 7.7. *In vivo* visualization of calcein release from rhodamine labeled TSL by means of invital fluorescence microscopy. The images (from top to bottom rows) were obtained 10 min after injection, after subsequent application of hyperthermia (41 °C, 5, 30 and 60 min) and additional hyperthermia (42 °C, 10 min). The column left represents a bright field image of the area under study.

as red fluorescence originating from the rhodamine merely present in the tumor vasculature. After 10 minutes a mild hyperthermia of 41 °C was applied. As can be seen in Figure 7.7, the green fluorescence increased strongly upon heating, demonstrating that a significant amount of calcein was released. The intensity of the green fluorescence became even more pronounced 30 min after the start of the heating. The heating was continued for one hour, but no further increase in calcein release was observed, despite the fact that liposomes were still detectable in the blood vessels. Further increase of the temperature to 42 °C did not result in an increased release of liposomal calcein.

CONCLUSIONS

The formulation of a TSL system consisting of DPPC, DSPC, CHOL and DSPE-PEG, was optimized for efficient performance of temperature-triggered drug release. *In vitro* tests of this system after encapsulation of calcein as well as of the tumor targeting agent Ln(III)-DOTA-ENPBA demonstrated a sharp transition between the gel and the liquid phase at 41-42 °C, with release of the encapsulated material (about 90% for calcein and 70% for Ln(III)-DOTA-ENPBA). Below the transition temperature, the system is essentially stable and the release of encapsulated material is negligible.

Preliminary *in vivo* studies of liposomal delivery with the TSL system to a tumor site in a nude mouse with implanted human BLM melanoma in a temperature-controlled dorsal skin-fold window chamber were performed. They show that the designed liposomes possess a remarkable potential for rapid triggered release of their tumor cell targeting content during hyperthermia, since a significant release of calcein was found already a few min after local increase of the temperature. It can be expected that the targeting agent Ln(III)-DOTA-ENPBA will behave similarly and can be delivered to the tumor sites efficiently and selectively by the liposomes. Temperature-triggered release of the targeting agent in the tumor will lead to further diffusion of the compound and subsequent tumor cell specific binding due to the interaction with the sialic acid moieties present on the tumor cells. Using this approach we will be able to specifically deliver DOTA-ENPBA-chelated MR and nuclear contrast agents as well as therapeutic radionuclides. Further research along these lines is in progress.

EXPERIMENTAL

General methods

The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphacholine (DPPC, $T_m = 42\text{ }^{\circ}\text{C}$), 1,2-distearoyl-*sn*-glycero-3-phosphacholine (DSPC, $T_m = 56.0\text{ }^{\circ}\text{C}$), distearoylphosphatidyl ethanolamine-poly(ethylene glycol)-2000 (DSPE-PEG) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl)-ammonium salt were obtained from Avanti Polar Lipids. The solvents used, were of analytical grade. *tert*-Bu₃DO3A·HBr (**1**) was synthesized according to a published procedure.²⁷ All other chemicals were purchased from Sigma-Aldrich and were used without further purification. All reactions were monitored by TLC on silica plates detecting the components by UV light or by applying the Dragendorff reagent. Ion-exchange chromatography was carried out on Dowex 50-WX8-200 (H⁺).

NMR spectroscopy was performed on a Varian Inova-300 spectrometer at 25 °C using 5mm NMR tubes and operating at 300, 75, and 96 MHz for ¹H, ¹³C, and ¹¹B respectively. Fluorescence was measured on a Jasco-J-815 CD spectrometer in Hellma quartz cuvettes, with a sensitivity of 630 V, using emission and excitation wavelengths of 520 and 495 nm respectively. The extrusions were carried out on a LipexTM extruder using nucleopore track-etch membranes with pore sizes of 100 and 200 nm (Whatman Schleicher & Schnell). ICP measurements were performed with a Perkin Elmer Optical Emission Optima 4300 DV Spectrometer. The measurements of the lanthanum and boron contents in the liposomes were calibrated using standard solutions of La(NO₃)₃ and H₃BO₃, respectively. Differential scanning calorimetry (DSC) was performed on a Capillary Cell MicroCalorimeter (MicroCal VP-DSC) instrument. The dynamic light scattering (DLS) was studied with a Perkin-Elmer photon counter, equipped with a JDS Uniphase 633 nm 35mW laser, an ALV sp 125 s/w 93 goniometer, and a fiber detector; 3 ml cylindrical scattering cells were used with an internal diameter of 12 mm at 20 °C. Particle size distributions were calculated using the Contin method.²⁸

Synthesis of the targeting agent

Tris-1,4,7-*tert*-butoxycarbonylmethyl-10-ethoxycarbonyl-1,4,7,10-tetraazacyclododecane (2). To a solution of the HBr salt of tris-1,4,7-*tert*-butoxycarbonylmethyl-1,4,7,10-tetraazacyclododecane (**1**) (3.05 g, 5.04 mmol) in 28 ml of acetonitrile, K₂CO₃ (4.72, 34.15 mmol) was added and stirred for 1 h at room temperature. After the suspension was cooled down to 0 °C in an ice-water bath, a solution of ethyl bromoacetate (0.95 g, 5.67 mmol) in 7 ml of acetonitrile was added dropwise over 15 min. The reaction mixture was stirred overnight at ambient temperature. The suspended salts were removed by filtration and the solvent was evaporated under reduced pressure. After purification of the residue by column chromatography (silica, CH₂Cl₂/MeOH gradient from 95:5 to 80:20) 2.63 g (88%) of **2** were obtained. ¹H NMR (CDCl₃, 25 °C, TMS): δ = 1.16 (t, J = 7.2 Hz, 3H), 1.45 (s, 18H), 1.47 (s, 9H), 1.80–3.80 (broad multiplets, 24H), 4.17 (q, J = 7.2 Hz, 2H). ¹³C NMR (CDCl₃, 25 °C, TMS): δ = 14.14, 30.95, 27.91, 28.02, 51.96, 53.50, 54.71, 54.91, 55.67, 55.73, 62.26, 82.08, 82.16, 172.92, 173.02, 173.60.

Monoamide of ethylenediamine and tris-1,4,7-*tert*-butoxycarbonylmethyl-10-carboxymethyl-1,4,7,10-tetraazacyclododecane (3). This compound was prepared and characterized according to a published procedure²⁹ in 63% yield (2.01 g).

Conjugate of 3 and 3-formylphenylboronic acid (4). To a solution of **3** (2.01 g, 3.27 mmol) in 15 ml of methanol, 3-formylphenylboronic acid (0.53 g, 3.58 mmol) was added. After addition of 8 ml of freshly distilled triethylamine, the reaction mixture was stirred for 2 h at room temperature. The reaction was followed by NMR. After the conversion of the amine to the imine, NaBH₄ (1.50 g, 39.61 mmol) was carefully added to the reaction mixture. The resulting solution was stirred at room temperature for 12 h. The solvent was removed under reduced pressure to give a light-yellow foam. The residue was taken up in water and the resulting mixture was extracted three times with dichloromethane. After evaporation of the combined organic phases, 2.34 g of compound **4** (97%) were obtained.

Removal of the *tert*-butyl protective groups from 4. A solution containing **4** (2.34 g, 3.13 mmol) in 5 ml CH₂Cl₂/TFA (3:1) was stirred overnight. The solvent was removed under reduced pressure; the residue was washed several times with methanol, which was removed each time by evaporation, until a light yellow foam was formed. The residue was purified by ion-exchange chromatography. Therefore, the crude product was dissolved in water and the pH was adjusted to 12. This basic solution was brought on the column and rinsed with water until the pH of the eluent became neutral. The final product was eluted with a mixture of pyridine (10% in water)/ethanol (1:1). The fractions obtained were analyzed by TLC. Lyophilization of the purified material gave 615 mg (1.06 mmol, 33%) of **5**. ¹H NMR (D₂O, 25 °C, pH 12, internal reference *t*-BuOH at 1.20 ppm): δ = 2.78–3.48 (m, 20H), 3.68–3.71 (m, 8H), 4.70 (s, 2H), 7.49–7.73 (m, 4H). ¹³C NMR (75 MHz, D₂O, 25 °C, pH 12, internal reference *t*-BuOH at 31.20 ppm): δ = 35.98, 46.59, 48.66, 48.94, 50.64, 51.04, 51.23, 52.23, 56.70, 57.12, 128.96, 130.60, 132.66, 134.91, 135.42, 137.85, 169.81, 173.06, 178.46. ¹¹B NMR (D₂O, 25 °C, pH 12, external reference 0.1M H₃BO₃ at 0 ppm): δ=−16.79.

Complexations were carried out by mixing LnCl₃·6H₂O and compound **5** (5 mol % excess) in water, adjusting the pH to 7.4 followed by stirring for several hours. The absence of free lanthanide(III) ion was confirmed by a xylenol orange test.³⁰

Preparation of liposomes

Liposomes were prepared from DPPC/DSPC/CHOL/DSPE-PEG (67:15:13:5, mol %) by the thin film hydration technique followed by extrusion and ultracentrifugation to remove non-encapsulated compounds. The calculated amounts of lipids with a total weight of 90 mg were dissolved in 10 ml of chloroform. The solvent was evaporated under reduced pressure to form a lipid film, which was subsequently dried with nitrogen. Unilamellar liposomes were formed by hydration of the lipid film with a solution of either calcein (90 mM) or of targeting agent (250 mM) in 10 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), containing 135 mM NaCl, pH adjusted to 7.2. The resulting mixture was stirred vigorously followed by extrusion above the transition temperature (50 °C). The crude liposome suspension was extruded under nitrogen pressure (500 psi): three times over a membrane with a cut-off of 200 nm and then three times over a membrane

with a cut-off of 100 nm. For the preparation of liposomes for *in vivo* experiments a filter with 0.2 μm cut-off was used to sterilize the calcein solution and the final suspension of liposomes. In order to remove low molecular weight material, the suspension of the liposomes was then centrifuged twice for 1 h at 4 °C and 45,000 rpm in a Beckman ultra-centrifuge. The pellet obtained was re-suspended in 500 μl of HEPES buffer and stored under nitrogen atmosphere at 4 °C for the following studies. The effective diameter of the liposomes was 100 nm (SD $\pm$ 5%) as determined by DLS. The phosphate concentration was determined using the colorimetric Rouser assay.³¹ To test the transition temperature 500 μl of the liposome suspension was dissolved in 1 ml 10 mM HEPES and, after degassing for 7 min, analyzed by DSC-scanning three times in the temperature range from 10 to 90 °C, 60 °C/h as compared to the reference capillary containing only HEPES. For the *in vivo* experiments, 1 mol % of the lipid-grafted rhodamine dye was included in the lipid composition.

***In vitro* temperature-induced calcein release measurements**

The release of calcein at elevated temperature was determined fluorometrically by measuring the increase of fluorescence signal as a result of self-quenching. The sensitivity of the photomultiplier was optimized on the solution of the liposomes in buffer after their destruction with Triton-X (10%) and dilution to a non-quenching concentration (< 12 μM). After the dilution degree was determined (see Figure 7.8), the liposomes were loaded into a 10 mm cuvette and incubated for 15 min at the desired temperature. After rapidly cooling down in an ice-bath, the fluorescence of the sample was measured at 4 °C. The percentage calcein release was calculated using Equation 7.1, where F is the experimental fluorescence value (at $\lambda = 520$ nm) at a given temperature, F_i is the initial fluorescence and F_T is the fluorescence measured after solubilization of the liposomes by addition of 2 μl of Triton-X (100%). For the experiments where the calcein release was studied in the presence of serum, the pellet of the liposomes obtained after centrifugation was suspended in a medium containing 50% heat-inactivated Fetal Bovine Serum (FBS) in HEPES buffer.

$$\text{Calcein}(\%) = \frac{F - F_i}{F_T - F_i} \times 100 \quad 7.1$$

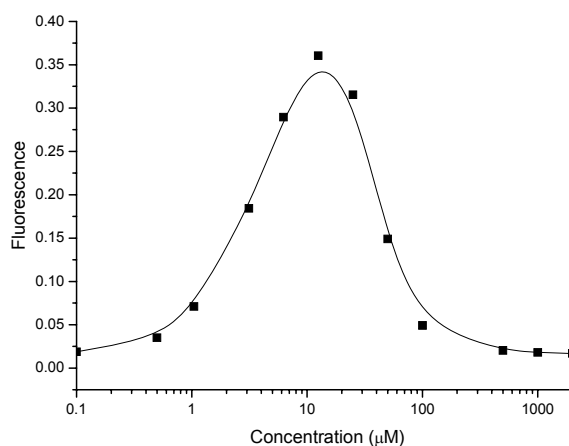


Figure 7.8. Intensity of the fluorescence signal of sample of calcein as a function of its concentration. The total concentration of calcein in TSL samples for temperature-triggered release was below the maximum (12 μM), determined from this calibration curve.

Encapsulation and release of La(III)-DOTA-ENPBA

For the preparation of the TSL containing the targeting agent, the procedure described above was applied but now using 1 ml of a 250 mM solution of La(III)-DOTA-ENPBA for the hydration of the lipid film. The common characterization procedures were applied after extrusion and centrifugation steps. The amount of encapsulated material was determined by ICP by measuring both the lanthanum and the boron contents per amount of lipids of the Triton-X treated liposomal suspensions: i) after the hydration step, and ii) after harvesting of the liposomal pellet after centrifugation. The values are expressed as the molar percentages of La/B per normalized molar amount of lipids.

For the temperature-triggered release measurements, the pellet of liposomes with the determined amount of targeting agent was re-dissolved in 1 ml of HEPES/FBS medium (1:1), and divided in five vials, each containing 200 ml of suspension. These samples were incubated at 37, 39, 41, 43 and 46 °C, respectively. After 15 min of incubation, the vials

were placed into an ice-water bath to stop the release process and then centrifuged. The amount of released compound was determined by ICP measuring samples of supernatant and Triton-X treated suspension. Xylenol orange tests were negative, showing that no free lanthanide was present in the samples.

Animal model

Specific pathogen-free nude mice (weighing 20 g) were purchased from Harlan-CPB, and fed a standard laboratory diet ad libitum (Hope Farms). A human BLM melanoma xenograft tumor model was used for this study. All animal studies were done in accordance with protocols approved by the committee on Animal Research of the Erasmus MC (Rotterdam, the Netherlands).

Preparation of the dorsal skin fold window chamber

Preparation of the dorsal skin-fold chamber was performed as described previously.³² Briefly, mice were anesthetized [100 μ L of a 2:1:1 (v/v/v) mixture of saline, ketamine, and xylazine] and hair was removed from the back of the animal. After dissecting the skin, leaving the fascia, and opposing the skin, the skin-fold of the mouse was sandwiched between two frames, fixed with two light metal bolts and sutures. A small piece of the BLM tumor (0.1 mm³) was transplanted in the fascia, and on both sides, the window was closed with a 12-mm diameter microscopic cover glass of 0.13 to 0.16 mm thickness. The mice were housed in an incubation room with an ambient temperature of 32 °C and a humidity of 70%. Experiments started 10 to 14 days after implantation of tumor in the dorsal skin-fold chamber.

Intravital fluorescence microscopy during hyperthermia

At given time points, mice were anesthetized by isoflurane-based inhalation anaesthesia and fixed to the heated microscope stage of a Zeiss LSM 510 META confocal laser

scanning microscope. Before examination a temperature sensor was placed in the tissue in the window chamber for online temperature measurements during hyperthermia. An in house-developed conductive heating device was placed on top of the window for applying hyperthermia. Liposomes were injected i.v. via the tail vein at a dose of 4.2 μmol of lipids. Randomly selected tumor regions were examined with a 10 $\times$ objective with long working distance. Scans were made with a 488 nm argon laser for detection of calcein (505-550 nm band pass filter) and a 543 nm helium-neon laser for detection of rhodamine (560-615 nm band pass filter). During hyperthermia tumor accumulation and triggered drug release were monitored in the living animal.

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Molecular imaging has become a highly important tool in medicine. Recent technological developments allow visualization of molecular events *in vivo* on a level that has never been achievable before. Each of currently available imaging techniques possesses unique strengths and weaknesses concerning spatial resolution and detection limits. However, the complementary information generated from different imaging modalities is highly valuable and encourages the search for combined technologies and, consequently the development of novel reporter probes. The importance of chemistry in medical science is strongly growing due to the demand of specific agents for biological targets. Chapter 1 gives a short overview of the currently available imaging techniques and evaluates the perspectives in the development of contrast media.

Ln(III) complexes of polyaminocarboxylates are widely used in MRI as contrast agents. The paramagnetic properties of the metal ion contribute to the increase of ^1H relaxation rates, while the chelate offers a stable binding with the metal. The number of water molecules, coordinated directly to the Ln(III) ion, is very important for the relaxivity and, thus, the efficacy of these contrast agents. Chapter 2 describes a convenient method to determine this parameter by measuring the Ln(III) induced shifts of the water ^{17}O NMR resonance.

Chapter 3 deals with the application of the method reported in the previous chapter. The ^{17}O NMR shifts of aqueous samples of lanthanide triflates were measured and analyzed. In these systems the triflate anion does not enter the first coordination sphere. The contact contribution to the shifts showed a break at Eu(III), which reflects a change of the number of water molecules in the first coordination sphere of the Ln(III) ion from 9 for La-Sm to 8 for the heavier lanthanides. This change in hydration number is accompanied by a change in the parameters governing the pseudocontact shifts. Fitting of the data with tricapped trigonal prism and square antiprismatic geometries obtained by DFT (density function theory) calculations showed that the crystal field parameters for these geometries differ by an order of magnitude. The hyperfine coupling constant for both geometries was determined to be $A/\hbar = -4.2 \times 10^6 \text{ rad s}^{-1}$.

In Chapter 4, ^{17}O NMR is used to evaluate the water exchange rates on Gd(III) complexes of pyridine-containing ligands: **L**¹ (=N,N'-Bis[(6-carboxy-2-pyridyl)methyl]ethane-1,2-diamine-*N,N'*-diacetic acid), and its phosphonate analogues with ethane-1,2-diamine, **L**² (=N,N'-Bis[(6-carboxy-2-pyridyl)methyl]ethane-1,2-diamine-*N,N'*-methylene phosphonic acid) or cyclohexane-1,2-diamine backbones, **L**³ (=N,N'-Bis[(6-carboxy-2-pyridyl)methyl]cyclohexane-(1*R*,2*R*)-diamine-*N,N'*-methylenephosphonic acid). The exchange rate of Gd**L**¹(H₂O)_q (k_{ex}^{298}) is found to be $5.0 \times 10^6 \text{ s}^{-1}$, which is slightly faster than in [Gd(DOTA)(H₂O)]¹⁻ (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate). Modification of the **L**¹ by substituting the carboxy groups by the more bulky phosphonate groups dramatically increases the water exchange rate on Gd**L**²(H₂O)_q to $k_{\text{ex}}^{298} = (7.0 \pm 0.8) \times 10^8 \text{ s}^{-1}$, which is comparable to that of the Gd(III) aquo ion. This fast water exchange could be rationalized in terms of a very flexible inner coordination sphere. The amount of water molecules directly coordinated to the lanthanide ion (*q*) was found to vary between 0.5 and 0.8 suggesting the presence of an equilibrium between complex species with *q* = 0 and 1. Further modification on the amine backbone structure, in case of Gd**L**₃(H₂O)_q, by introduction of the cyclohexyl group resulted in a decrease of water exchange rate by a factor of 5 ($k_{\text{ex}}^{298} = (1.5 \pm 0.8) \times 10^8 \text{ s}^{-1}$) due to increased rigidity.

Chapter 5 reports a multinuclear NMR study of the interaction between phenylboronic acid (PBA) and sialic acid (Neu5Ac). The latter compound is known to be overexpressed on the cell surface of tumor cells. The results of this investigation suggest that the binding of PBA to sialic acid is pH dependent. ^{17}O NMR experiments with glycolic acid as model compound prove that an interaction at the α -hydroxycarboxylate occurs at pH < 9, while a study on threonic and erythronic acids shows that the PBA group interacts selectively with the vicinal diol functions at higher pH. Similarly, Neu5Ac binds PBA through its α -hydroxycarboxylate at low pH (< 9) and through its glycerol side chain at higher pH values. The conditional stability constant of the phenylboronate ester at pH 7.4 is 11.4. On cell surfaces, sialic acid is connected to the neighboring sugar unit through the 2-hydroxy group. To mimic this, the 2- α -O-methyl derivative of Neu5Ac was included in this study. The *erythro* configuration of the hydroxy substituents prevents a stable-complex formation at positions C7 and C8 and, consequently, the strongest interaction is observed at positions C8 and C9, leading to a five-membered 2-boron-1,3-dioxalate. In addition, a relatively

small amount of the C7-C9 six-membered complex was observed. Molecular modeling studies confirm that the C8-C9 boronate complex has the lowest energy.

Chapter 6 describes a study on the molecular recognition of sialic acid end groups on the surface of human glioma cells by ^{160}Tb -DTPA-EN, $^{160}\text{Tb(III)}$ -DTPA-(ENPBA)₂ and $^{160}\text{Tb(III)}$ -DTPA-(PBA)₂ complexes. The over-expression of sialic acid on the surface of cancer cells compared to normal ones makes this 9-carbon sugar an attractive biomarker for molecular diagnosis and therapy. The results show Tb(III) -DTPA-(ENPBA)₂ to be the most efficient targeting agent, due to the electrostatic interaction between its two positively charged ammonium groups and the negatively charged cell surface, which provides an additional stabilization of the covalent binding through the PBA moieties and the sialic acid diol functions. Up to 5.5 nmol Tb/mg protein is taken up by the cells. ICP analysis after incubation experiments with non-radioactive Tb(III) -DTPA-(ENPBA)₂ suggests that dissociation of Tb from this complex occurs after its binding to the cell surface. Most likely, most of the free Tb remains adsorbed on the surface of the cells, although internalization of a small amount cannot be excluded.

In Chapter 7, the idea of recognition of sialic acid is further developed. Thermo-sensitive liposomes are exploited as carriers of phenylboronated targeting agents. The stability aspects of the chelating part of the developed targeting agent that emerged from the previous study were tackled by substitution of the DTPA back-bone for a DOTA unit, since the latter chelate is known to form thermodynamically and kinetically more stable Ln(III)-complexes. The lipid formulation of the liposomes was carefully tuned to obtain a gel-to-liquid transitional temperature of 41–42 °C, which is suitable for mild hyperthermia. Intravital fluorescence microscopy was applied to prove the principle of the application of the designed liposomes for drug delivery *in vivo*. Therefore, calcein/rhodamine containing liposomes were prepared and injected into a nude mouse with a human BLM tumor implanted under a dorsal skin chamber. A dual wavelength detector was used to monitor simultaneously the arrival of the liposomes and the release of the contents after the temperature in the chamber was increased. Preliminary results demonstrated not only a rapid accumulation of the designed liposomes at the tumor site, but also an efficient release of calcein. In further steps, the new tumor-targeting agent will be loaded into thermosensitive liposomes and its release and binding to the tumor site will be studied.

Moleculaire imaging is een zeer belangrijk onderdeel van de moderne geneeskunde geworden. De recente technologische vooruitgang maakt het mogelijk om moleculaire gebeurtenissen *in vivo* zichtbaar te maken op een manier, die tot nu toe ondenkbaar was. Elk van de nu beschikbare imaging technieken heeft zijn eigen sterke en zwakke punten met betrekking tot ruimtelijke resolutie en detectiegrens. De complementaire informatie, die verkregen kan worden met de verschillende imaging modaliteiten, is uitermate waardevol gebleken. Het bevordert onderzoek naar nieuwe gecombineerde technieken en, als gevolg daarvan, de ontwikkeling van nieuwe reporter-probes. Het belang van chemie in de medische wetenschap is sterk groeiende dankzij de vraag naar specifieke doelzoekende reagentia voor biologische targets. Hoofdstuk 1 geeft een beknopt overzicht van de beschikbare imaging technieken en evalueert de vooruitzichten in de ontwikkeling van contrastmedia.

Ln(III) complexen van polyaminocarboxylaten worden op grote schaal als MRI contraststof toegepast. De paramagnetische eigenschappen van het metaalion daarin zijn verantwoordelijk voor de versnelling van de ^1H relaxatiesnelheid, terwijl het chelaat voor een stabiele binding van het metaalion zorgt. Het aantal watermoleculen dat rechtstreeks gecoördineerd is aan het Ln(III) ion is uitermate belangrijk voor het relaxatie versnellend vermogen (de relaxivity), en zodoende voor de werkzaamheid van deze contrast reagentia. Hoofdstuk 2 beschrijft praktische methoden om deze parameter te bepalen door het meten van Ln(III) geïnduceerde chemische verschuivingen van de ^{17}O NMR resonantie van het water.

Een van de toepassingen van deze methode wordt behandeld in hoofdstuk 3. De ^{17}O NMR chemische verschuivingen van waterige oplossingen van lanthanide triflaten werden gemeten en geanalyseerd. In deze systemen is het triflaat anion niet in staat om de eerste coördinatie sfeer binnen te gaan. De contactbijdrage aan de chemische verschuiving vertoont een breuk bij Eu(III) als gevolg van de vermindering van het aantal water moleculen in de eerste coördinatiesfeer van het Ln(III) ion van 9 voor Ln-Sm naar 8 voor de zwaardere lanthaniden. Deze verlaging van het hydratatiegetal gaat gepaard met veranderingen in de parameters die de pseudocontactbijdrage aan de chemische

verschuivingen bepalen. Fitten van de data met "tricapped trigonal prism" en antiprismatische conformaties, verkregen met DFT (density function theory) berekeningen, tonen aan dat de kristalveldparameters voor deze conformaties een order van grootte verschillen. De hyperfijne koppelingsconstante voor beide conformaties werd bepaald op $A/\hbar = -4.2 \times 10^6 \text{ rad s}^{-1}$.

Hoofdstuk 4 beschrijft de bepaling van wateruitwisselingssnelheden in Gd(III) complexen van pyridine-houdende liganden met behulp van ^{17}O NMR: $\mathbf{L}^1$ (= *N,N'*-Bis[(6-carboxy-2-pyridyl)methyl]ethaan-1,2-diamine-*N,N'*-diazijnzuur), en zijn fosfonaat variant met ethaan-1,2-diamine-, $\mathbf{L}^2$ (= *N,N'*-Bis[(6-carboxy-2-pyridyl)methyl]ethaan-1,2-diamine-*N,N'*-methyleenfosfonzuur) of cyclohexaan-1,2-diamine basisskelet $\mathbf{L}^3$ (= *N,N'*-Bis[(6-carboxy-2-pyridyl)methyl]cyclohexaan-(1*R*,2*R*)-diamine-*N,N'*-methyleenfosfonzuur). De uitwisselingssnelheid van $\text{GdL}^1(\text{H}_2\text{O})_q$ (k_{ex}^{298}) was $5.0 \times 10^6 \text{ s}^{-1}$, hetgeen enigszins sneller is dan die van $[\text{Gd}(\text{DOTA})(\text{H}_2\text{O})]^{-1}$ (DOTA=1,4,7,10-tetraazacyclododecaan-1,4,7,10-tetraacetaat). Modificatie van $\mathbf{L}^1$ door vervanging van de carboxyl groepen door de grotere fosfonaatgroepen leidt tot aanzienlijke verhoging van de wateruitwisselingssnelheid van $\text{GdL}^2(\text{H}_2\text{O})_q$ tot $k_{\text{ex}}^{298} = (7.0 \pm 0.8) \times 10^8 \text{ s}^{-1}$, een waarde die vergelijkbaar is met die voor het Gd(III) aquo ion. Deze snelle uitwisselingsnelheid kan verklaard worden door de zeer flexibele eerste coördinatiefeer. Het aantal watermoleculen dat rechtstreeks aan lanthanide ion gecoördineerd is (q) bleek te variëren tussen 0.5 en 0.8. Dit kan verklaard worden door een evenwicht tussen complexen met $q = 0$ en 1. Verdere veranderingen in de structuur van de amine-backbone, door introductie van een cyclohexyl groep, zoals in het geval van $\text{GdL}^3(\text{H}_2\text{O})_q$, leidde tot een afname van de wateruitwisselingssnelheid met een factor 5 ten gevolge van de verminderde flexibiliteit ($k_{\text{ex}}^{298} = (1.5 \pm 0.8) \times 10^8 \text{ s}^{-1}$).

Hoofdstuk 5 beschrijft een NMR studie van de interactie tussen fenylboorzuur (PBA) en siaalzuur (Neu5Ac). Resultaten van dit onderzoek suggereren dat PBA siaalzuur op twee verschillende manieren kan binden, namelijk via de α -hydroxycarboxylaate functie en via de glycerol zijketen. Deze interacties blijken pH afhankelijk te zijn. ^{17}O NMR experimenten met glycolzuur, als modelverbinding, laten zien dat een interactie met α -hydroxycarboxylaate plaats vindt bij $\text{pH} < 9$, terwijl onderzoek aan aan threon- en erythronzuur aantoont dat de fenylboronaatgroep bij hogere pH's naar de vicinale diol functies verschuift. Analooq vindt de binding aan PBA van Neu5Ac via de

α -hydroxycarboxylaat functie plaats bij pH < 9 en via glycerol zijketen bij hogere pH's. De conditionele stabiliteitsconstante van de fenylboronaatester bij pH 7.4 is 11.4. Siaalzuur is gebonden aan de naburige suiker op het celoppervlak via zijn 2-hydroxy groep. Om de *in vivo* situatie na te bootsen en de diol-interactie te stimuleren, werd Neu5Ac omgezet naar zijn 2- α -O-methyl derivaat. De *erythro* configuratie van de hydroxyl substituenten is niet gunstig voor de vorming van een stabiel complex op posities C7 en C8, en als gevolg daarvan, is er een sterkere interactie op posities C8 en C9 onder de vorming van een 2-boor-1-3-dioxalaat vijfkring, en een geringe hoeveelheid C7-C9 zesring.

Hoofdstuk 6 beschrijft een *in vitro* studie aan de moleculaire herkenning van siaalzuur op het oppervlak van menselijke glioma cellen door $^{160}\text{Tb(III)-DTPA-EN}$, $^{160}\text{Tb(III)-DTPA-(ENPBA)}_2$ and $^{160}\text{Tb(III)-DTPA-(PBA)}_2$ complexen. De over-expressie van siaalzuur in het membraan van tumorcellen in vergelijking tot gezonde cellen maakt deze suiker een aantrekkelijke biomarker voor moleculaire diagnose en therapie. De experimentele data wijzen erop dat $\text{Tb(III)-DTPA-(ENPBA)}_2$ de meest efficiënte doelzoekende verbinding is dankzij een elektrostatische interactie tussen zijn twee positief geladen ammonium groepen en het negatief geladen celoppervlak. Dit verschaft een additionele stabilisatie van de covalente binding tussen PBA en diolgroepen van het siaalzuurmolecuul. Er werd tot maximaal 5.5 nmol Tb/mg proteïne door de cellen opgenomen. ICP analyse na afloop van incubatie met niet-radioactieve $\text{Tb(III)-DTPA-(ENPBA)}_2$ duidt op de dissociatie van Tb uit het complex nadat dat geadsorbeerd is aan het celoppervlak. Het is zeer waarschijnlijk dat het grootste deel van het vrije Tb aan het oppervlak geadsorbeerd blijft, alhoewel het niet uitgesloten kan worden dat een kleine hoeveelheid Tb door de cellen geïnternaliseerd wordt.

In hoofdstuk 7 wordt het concept van herkenning van sialaalzuur verder ontwikkeld. Thermo-gevoelige liposomen worden toegepast als dragers voor $\text{Ln(III)-DOTA-ENPBA}$ doelzoekende reagentia. De stabiliteitsaspecten van het DTPA-chelaat, die in de voorafgaande studie naar voren kwamen, worden aangepakt door het in de molecuul te vervangen door een DOTA-analoog. De laatste staat bekend als een thermodynamisch en kinetisch stabiel chelaat. De lipidensamenstelling van de gebruikte liposomen werd zodanig bijgesteld dat een faseovergang tussen 41 en 42 °C bereikt werd. Deze temperatuur is geschikt voor milde hyperthermia. Intravitale fluorescentie microscopie werd toegepast om het thermische gedrag van de ontwikkelde liposomen *in vivo* te testen. Daartoe werden

liposomen die calcein/rhodamine als markers bevatten geïnjecteerd in een muis met een BLM melanoma tumor geïmplanteerd onder de rughuid. Door middel van een dubbele golflengte detector werd de accumulatie van de liposomen in het tumorweefsel en het vrijkomen van de inhoud na het verhogen van de temperatuur gevolgd. De efficiëntie van de ontwikkelde liposomen werd aangetoond in een eerste serie experimenten. In een vervolgstudie zullen de Ln(III)-DOTA-ENPBA geladen liposomen gebruikt worden om de tumorherkenning te realiseren.

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11. D. R. Jansen, J. R. Zeevaart, Z. I. Kolar, K. Djanashvili, J. A. Peters, G. C. Krijger. ^{31}P NMR study of the valence stability of tin in its 1-hydroxyethylene-diphosphonate(HEDP) and N,N,N' -trimethylenephosphonate-polyethyleneimine (PEI-MP) complexes. *Polyhedron.* **2008**, 1779.
12. Z. Kotková, G. A. Pereira, K. Djanashvili, J. Kotek, J. Rudovský, P. Hermann, L. Vander Elst, R. N. Muller, C. F. G. C. Geraldes, I. Lukeš, J. A. Peters. Lanthanide(III) complexes of phosphorus acid analogues of H_4DOTA as model compounds for the evaluation of the second-sphere hydration. *Eur. J. Inorg. Chem.* **2008**, in print, DOI: 10.1002/ejic.200800859.
13. K. Djanashvili, J. A. Peters. Design of Targeted Contrast Agents for MRI. *Proceedings of International Seminar on Chemistry – 2008*, Indonesia.

Oral presentations

1. "Molecular Recognition of Sialic Acid End Groups by Phenylboronates", COST D18 Workshop "Evaluation of present and planning of future collaboration in research of phosphorus derivatives of dota and dtpa", Prague, Czech Republic, June 24, **2004**.
2. "Molecular Recognition of Sialic Acid End Groups by Phenylboronates", COST D18 Annual Workshop and 8th MC meeting in A Coruña, Spain, September 24, **2004**.
3. "Targeting of tumor cells by phenylboronate containing ¹⁶⁰Tb-complexes: a gamma counting study", DUT-HST-BioMedical Engineering meeting, Bilthoven, Netherlands, November 28, **2005**.
4. "In vitro targeting of tumor cells by molecular recognition of glycoproteins using phenylboronate containing ¹⁶⁰Tb-complexes", Final meeting if the COST D18 Action "Lanthanide Chemistry for Diagnosis and Therapy", Orléans, France, March 31, **2006**.
5. "Tumorjacht. Herkenning van tumoren met nieuwe contrastvloeistoffen voor MRI". Voorjaarbijeenkomst *Daden van Delft*, Delft, Netherlands, 10 mei **2006**.
6. "Phenylboronate ¹⁶⁰Tb complexes for molecular recognition of glycoproteins expressed on tumor cells". Imagination 2007, Erasmus MC, Rotterdam, Netherlands, March 14, **2007**.
7. "Liposomal Delivery of DOTA-phenylboronates for Diagnosis and Therapy of Tumors". Working groups (WG3/WG5) COST D38 Action meeting "Metal-Based Systems for Molecular Imaging Applications". Athens, Greece, October 2, **2008**.
8. "Design of Targeted Contrast Agents for MRI". International Seminar on Chemistry-2008, University of Padjadjaran, Bandung, Indonesia, October 30, **2008**. *Invited lecture*.

Posters

1. K. Djanashvili, L. Frullano, T. Maschmeyer J. A. Peters, “NMR-Study of interactions of phenylboronic acid with natural carbohydrates”, NCC4, Noordwijkerhout, the Netherlands, March 8-10, **2003**.
2. K. Djanashvili, L. Frullano, T. Maschmeyer, J. A. Peters, “An investigation on the applicability of phenylboronic moieties for the targeting of saccharides by means of MRI contrast agents”, IFE’5, Geneva, Switzerland, September 24-29, **2003**.
3. K. Djanashvili, L. Frullano, T. Maschmeyer, J. A. Peters, “Phenylboronic moieties for the targeting of saccharides.”, NMR Discussion group meeting, Gronigen, the Netherlands, October 24, **2003**.
4. K. Djanashvili, L. Frullano, J. A. Peters, “Molecular recognition of sialic acid by boronated MRI Contrast Agents”, NCC5, Noordwijkerhout, the Netherlands, March 8-10, **2004**.
5. M. Mato-Iglesias, C. Platas-Iglesias, K. Djanashvili, R. N. Muller, L. Vander Elst, J. A. Peters, A. de Blas, T. Rodríguez-Blas “Lanthanide chelates containing pyridine units and acetate pendants as potential MRI contrast agents”, Annula Workshop COST D18, La Coruña, Spain, September 23-25, **2004**.
6. K. Djanashvili, A. J. G. M. van der Meer, H. T. Wolterbeek, J. A. Peters “Targeting of tumor cells by phenylboronate containing ^{160}Tb -complexes: a gamma counting study”, Annula Workshop COST D18, Colone, Germany, September 6-7, **2005**.
7. M. M. Iglesias, C. Platas-Iglesias, K. Djanashvili, J. A. Peters, É. Tóth, E. Balogh, R. N. Muller, L. Vander Elst, A. de Blas, T. Rodríguez-Blas. “The Highest Water Exchange Rate Ever Measured for a Gd(III) Chelate”, Annula Workshop COST D18, Colone, Germany, September 6-7, **2005**.

8. K. Djanashvili, A. J. G. M. van der Meer, H. T. Wolterbeek, J. A. Peters “Targeting of tumor cells by phenylboronate containing ^{160}Tb -complexes: a gamma counting study”, Meeting of the study groups “Design & Synthesis, Structure & Reactivity, Biomolecular Chemistry, Lunteren, the Netherlands, October 17-19, **2005**.
9. K. Djanashvili, C. Platas-Iglesias, J. A. Peters. “The structure of the lanthanide aquo ions in solution as studied by ^{17}O NMR spectroscopy and DFT calculations”. 42nd Meeting of the Dutch NMR Discussion Group, Nijmegen, the Netherlands, October 12, **2007**.
10. K. Djanashvili, C. Platas-Iglesias, J. A. Peters. “ ^{17}O NMR spectroscopy and DFT calculations on the lanthanide aquo ions in solution”. Meeting of the study groups “Design & Synthesis, Structure & Reactivity, Biomolecular Chemistry, Lunteren, the Netherlands, October 29-31, **2007**.
11. K. Djanashvili, C. Platas-Iglesias, J. A. Peters. ^{17}O NMR evaluation of the number of inner-sphere water molecules in Ln(III)-complexes. COST D38 WG 2 meeting, Orléans, France, February 29 – March 1, **2008**.
12. K. Djanashvili, C. Platas-Iglesias, J. A. Peters. The number of inner – sphere water molecules in Ln(III)-complexes studied by ^{17}O NMR. Annula Workshop COST D38, Lisbon, Portugal, April 27, **2008**.

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Kristina Djanashvili was born on June 30, 1970 in Leipzig (Germany) and raised in Tbilisi (Georgia), where she lived until 1994. She obtained her secondary school diploma in 1987 at 63rd High School and in the same year she passed the entrance exams for the study of chemistry and biology at Georgian State Pedagogic University. In 1989 she graduated from the Music Academy (piano class), where she had qualified simultaneously. In 1992 she obtained her university degree with the thesis "*The Influence of Adrenaline on the Glucose Level in Blood*", which was produced during her internship at the Research Centrum Animal Physiology in Tbilisi. After graduation she spent one year with teaching activities and in 1993 she became a PhD student at the State Polytechnic University of Georgia. After moving together with her family to the Netherlands she started an additional study at Rotterdam University of Professional Educations in 1997 and obtained her engineering degree in organic chemistry in 2001 with the thesis "*Synthesis of Activated Acids for Enzymatic Resolution of Secondary Alcohols*" that was accomplished during an internship at the Delft University of Technology in the group of Applied Organic Chemistry and Catalysis. After graduation she took up a position at the same university in the group of Biocatalysis and Organic Chemistry, where she became responsible for the NMR section and performed research in the field of MRI contrast agents under supervision of Dr. Joop A. Peters.

