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Metal-based redox-responsive MRI contrast agents

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Dedicated to Professor Armando J.L. Pombeiro for his outstanding achievements in Coordination Chemistry and Catalysis

Highlights

- Use of metal-based complexes and nanoparticles as relaxation and PARACEST redox responsive contrast agents for MRI is reviewed
- Ligand-based and metal-based T₁ and PARACEST redox responsiveness was reported, in some cases in small animals *in vivo*.
- Mn-based nanoparticles provide strong redox-active relaxation responses in *in vivo* animal studies.
- Ratiometric methods provide in some cases quantitative redox evaluation of cells *in vitro*.

Keywords: MRI contrast agents; Metal chelates; Inorganic nanoparticles; Redox responsive probes; Hypoxia probes; Relaxation agents; PARACEST agents

List of abbreviations

ASTM: Diacetyl-bis(N⁴-methylthiosemicarbazone)

BOLD: Blood Oxygenation Level Dependent

BPG: 2,3-D- bisphosphoglycerate

CA= contrast agent

CBT: 2-cyanobenzothiazole

CEST: Chemical Exchange Saturation Transfer

CYCLAM: 1,4,8,11-tetraazacyclotetradecane

CYCLEN: 1,4,7,10-tetraazacyclododecane

CyHBET N-(2-hydroxybenzyl-N,N',N'-trans-1,2-cyclohexylenediaminetriacetic acid

CySH/CySSCy: Cysteine/Cystine

CT: Computed Tomography

DO3A: 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic Acid

DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic Acid

DOTAM: 1,4,7,10-tetrakis(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane

DOTAM-Gly₄: N,N',N'',N'''-[1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrayltetrakis(1-oxo-2,1-ethanediyl)]tetrakis[glycine]

DOTMA = 1R, 4R, 7R, 10R)- α , α' , α'' , α''' -tetramethyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic Acid

DOTP: 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetrakis- (methylenephosphonate)

DO3A: 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic Acid

DO3ABA: DO3A-monobutylamide

DO3NI: DO3A-nitroimidazole

DTPA: 1,1,4,7,7-pentakis-(carboxymethyl)-1,4,7-triazaheptane

DTT: Dithiotreitol

EPR: Electron Paramagnetic Resonance

EPT: Exofacial Protein Thiol

GSH/GSSG: Glutathione/Glutathione Disulfide

HbA: Adult Hemoglobin

HBED: Dihydroxybenzylethylenediamine diacetic acid

HBET: Hydroxybenzylethylenediamine triacetic acid

HbF: Fetal Hemoglobin

hepPDMS: Heparin-poly(dimethylsiloxane)

HP-DO3A: 1,4,7-tris(carboxymethyl)- 10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane

HSA: Human Serum Albumin

HSAB: Hard-soft acid-base

HIF: Hypoxia Inducible Transcription Factor
5HT: 5-hydroxytryptamin
ICP-Ms: Inductively Coupled Plasma Mass Spectrometry
MT: Magnetization Transfer
MR: Magnetic Resonance
MRI: Magnetic Resonance Imaging
NADH/NAD⁺: Nicotinamide Adenine Dinucleotide
NADPH/NADP⁺: Nicotinamide Adenine Dinucleotide Phosphate
NHE: Normal Hydrogen Electrode
NMR: Nuclear Magnetic Resonance
NOX1: NADPH Oxidase 1
NSF: Nephrogenic Systemic Fibrosis
ODDA=1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diacetate
ODDM = 1,4,10,13-tetraoxa-7,16-diaza-cyclooctadecane-7,16-dimalonate
PAI: Photoacoustic Imaging
PAMAM: Polyaminoamide
PARACEST: Paramagnetic Chemical Exchange Saturation Transfer
PEG: Polyethylene Glycol
PET: Positron Emission Tomography
PLGA: Poly lactic-co-glycolic Acid
pO₂: Partial Oxygen Pressure
Poly-β-CD: Poly-β-cyclodextrin
RBC: Red Blood Cells
RE: Redox Environment
ROI: Region of Interest
ROS: Reactive Oxygen Species
RNS: Reactive Nitrogen Species
SOD: Superoxide Dismutase
SPECT: Single-Photon Emission Computed Tomography
SPION: Superparamagnetic Iron Oxide Nanoparticle
TACN: 1,4,7-triazacyclononane
TCEP: Tris(2-carboxyethyl)phosphine

TPPS₄: 5,10,15,20-tetrakis-(p-sulfonato phenyl) porphinate

Trx(SH)₂/TrxSS: Thioredoxin/Thioredoxin Disulfide

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Abstract

Given their potential in a better characterization and diagnosis of major pathologies like cancer or chronic inflammation, redox-activated Magnetic Resonance Imaging (MRI) probes have recently attracted much interest from chemists. Such redox responsive probes are capable of reporting on specific biomarkers that are related to tissue redox potential disruption or hypoxia. Lately, this research area has experienced remarkable development, including redox-responsive metal complexes and nanoparticles. Here we critically review the progress with a specific focus on metal-based probes and some nanoparticle examples. We demonstrate, via representative cases, the different molecular mechanisms that can generate a redox-modulated MRI response. They can be based on the redox activity of either the ligand or the metal center, provided the different oxidation states of the metal ion are endowed with different magnetic properties. A particular emphasis is given to recent advances and to the imaging probes that have attained *in vivo* validation. In overall, we aim to provide the reader with a comprehensive view of how intracellular or extracellular redox buffer systems can be assessed by using MRI contrast

agents based on lanthanide or transition metal ions using T₁-weighted, T₂-weighted, PARACEST ¹H or ¹⁹F MRI.

1. Introduction

Thanks to its excellent resolution, non-invasiveness and the lack of ionizing radiation, Magnetic Resonance Imaging (MRI) has become an essential tool in current clinical radiology. For three decades, Gd^{III} complexes used to enhance image contrast has largely contributed to the diagnostic potential of MRI. Once injected, commercial contrast agents distribute non-specifically in the extracellular space and their role in delineating different pathologies is solely related to a better visualization of an altered morphology or physiology.^{1,2,3}

Bringing MRI from anatomical to molecular imaging opens opportunities for characterizing tissues not only by their morphology but also by their molecular features.⁴ One can identify a multitude of molecules or tissue parameters as biologically relevant biomarkers that carry important information about the physiological or pathological *status* of a tissue. The unique capability of MRI agents to modulate the generated MRI signal in response to a specific biomarker or stimulus represents a great advantage, in contrast to other clinical modalities such as X-ray Computed Tomography (CT) or nuclear imaging. Consequently, chemists have dedicated important efforts to design smart or responsive molecular imaging probes that could enable non-invasive, repeatable and real-time *in vivo* mapping of such biomarkers.

Among those, the detection of the tissue redox state has received remarkable interest.⁵ This is first of all due to the great biological importance of this biomarker in many physiological and pathological processes, but also because of the very diverse chemical approaches that chemists can exploit for the design of redox-responsive MRI probes. First, redox-dependent MRI response can be generated by inducing redox reactions centered either on the metal center or on the ligand. While Gd^{III} itself is not redox active, many other metal ions can be potentially used and have been explored in this context, such as Mn, Eu, Fe, Cu, Co, etc. Metal-based redox switches can be further extended to nanoparticle systems, leading to signal amplification. Also, in addition to the classical T₁/T₂ weighted imaging, other contrast mechanisms can be adapted to redox mapping, including PARACEST (Paramagnetic Chemical Exchange Saturation Transfer) or ¹⁹F MRI. For all these reasons, the field of redox MRI probes is very rich from the

coordination chemistry point of view. The versatility of the approaches also holds promise of potentially successful *in vivo* translation of the probes in the future.

In addition to MRI agents, redox sensitive probes have been also extensively studied for optical imaging, positron emission tomography (PET) or single-photon emission computed tomography (SPECT).^{2,5} For optical detection, the probe can undergo redox reactions depending on the environment and this will trigger changes in the fluorescence properties. In contrast, the principle of redox-active PET and SPECT probes is fundamentally different. Indeed, once injected, a radioactive isotope will decay in the same way independently of its chemical environment. Here the principle of detection is based on specific trapping of the probes in hypoxic cells leading to accumulation of the radioactive isotope in hypoxic tumors. One should also mention photoacoustic imaging (PAI) where the oxygenation level can be quantified based on the different absorption coefficients of oxy- and deoxyhemoglobin. Each of these imaging modalities presents advantages and disadvantages. While optical imaging is excellent to produce high resolution images at the cellular level, it is less adapted to macroscopic imaging. PAI is so far limited to small animals. PET and SPECT provide low resolution images and represent an ionization burden, but have great sensitivity and hypoxia PET probes are already in clinical use. In the future, MRI probes could nicely complement the radiologists' and biologists' toolbox for redox mapping.

The scope of this review is to introduce the different molecular mechanisms that can provide redox-dependent MRI response by focusing exclusively on metal-based probes. Each mechanism will be illustrated by representative literature examples, without the pretention of being exhaustive. If available, we present examples that have been validated *in vivo*. Previously, several reviews have been dedicated to smart MRI agents in general,^{6,7,8,9} or to redox-probes in particular.^{10,11} In this quickly growing field, our intention is to survey the recent developments and to provide the reader with a comprehensive view of how intracellular or extracellular redox buffer systems can be assessed by using MRI contrast agents based on lanthanide or transition metal ions with a read-out in T₁-weighted, T₂-weighted, PARACEST ¹H or ¹⁹F MRI.

2. Intra- and extracellular redox environment

The biological aspects of redox-potential homeostasis have been described in several reviews.^{12,13,14} Here, we will summarize the most important aspects in the context of molecular imaging.

In any living organism, both extracellular and intracellular redox environments are tightly regulated^{15,16,17} and thus are important health biomarkers. In fact, their dysregulation can be associated with a wide range of pathophysiological conditions, including chronic inflammation,^{18,19} neoplastic growth¹² and ischemia,²⁰ since several triggered biochemical cascade events can damage cellular or tissue components, enabling disease progression.^{13,14,21} In healthy tissue, intracellular environments tend to be reducing whereas extracellular environments tend to be more oxidizing and subject to much larger fluctuations.¹⁷ For example, in necrotic tissue, dead cells leach components of the cytosol into the extracellular space to create a relatively reducing environment.²² Hence, the development of imaging techniques capable of monitoring changes in redox activity is of utmost interest in biomedical or clinical research as well as in medical practice.^{23,24,25}

The redox environment in a biological fluid, organelle, cell or tissue is maintained by a number of redox couples present at relatively high concentrations, which are linked to each other by biochemical redox homeostasis processes (Table 1). Any single redox couple contributes to the redox state, with a half-cell redox potential (E'_{hc}) described by a Nernst equation (1):

$$E'_{hc} = E'^{\circ}_{pH7} - \frac{59.1}{n} \log Q \quad (1)$$

where E'°_{pH7} is the standard biochemical redox potential for the couple against the normal hydrogen electrode (pH 7, 298.15 K), n is the number of electrons involved in the chemical process and Q is the respective mass action ratio, eg. $[\text{NADH}]/[\text{NAD}^+]$ and $[\text{NADPH}]/[\text{NADP}^+]$ for nicotinamide adenine dinucleotide and its phosphorylated derivative, respectively, $[\text{Trx}(\text{SH})_2]/[\text{TrxSS}]$ for thioredoxin, $[\text{GSH}]^2/[\text{GSSG}]$ for glutathione (GSH=(Glu-Cys-Gly)) and $[\text{CySH}]^2/[\text{CySSCy}]$ for cysteine (CySH). There are also contributions from other couples, such as the exofacial free thiols (EPTs) of proteins with surface cysteinyl residues. The redox environment (RE) is defined by the summation of the products of the reduction potentials weighted with the concentration of the respective reduced species for all the redox couples:²²

$$RE = \sum_i E'^{\circ}_i [X_{i \text{ red}}] \quad (2)$$

Among these, glutathione (GSH) is the major thiol-disulfide redox buffer of the intracellular space with an average concentration of 1–11 mM, much higher than the other redox active compounds.¹⁷ Therefore, the redox state of the glutathione disulfide-glutathione couple (GSSG/2GSH) can serve as an indicator of the intracellular redox

environment. Changes of the half-cell reduction potential (E_{hc}) of the GSSG/2GSH couple appear to correlate with the biological status of the cell: proliferation $E_{hc} \sim -240$ mV; differentiation $E_{hc} \sim -200$ mV; apoptosis $E_{hc} \sim -170$ mV, with even less negative potentials for necrotic cells.²² The extracellular space is typically more oxidized than the cytosol and its redox environment is dominated by the cystine-cysteine couple (CySSCy/2CySH).

Table 1. Some properties and compartmentation of biological redox buffer pairs.²²

Redox pair	E°_{pH7} (mV vs NHE)	cytosol		extracellular/plasma	
		conc. (mM)	E'_{hc} (mV)	conc. (mM)	E'_{hc} (mV)
GSSG/GSH	-240	1-11	-220 to -260	0.002-0.02	-120 to -70
CySSCy/ CySH	-250	0.1-1	-110	0.05-0.3	-80 to -20
$T_{rx}(SH)_2 / T_{rx}SS/$	-270 to -190	0.001-0.015	-270 to -190	-	-
NADH/NAD ⁺	-316	0.1 (1:10 to 1:1000)	-280	-	-
NADPH/ NADP ⁺	-315	0.1 (100:1)	-374	-	-

Besides the biological redox buffers, the local tissue concentration and reactivity of molecular oxygen (O_2), expressed by its partial pressure (pO_2), and its reactivity products (reactive oxygen species -ROS – such as O_2^- and H_2O_2 , or reactive nitrogen species – RNS- such as NO or peroxynitrite ONO^{2-}) are important targets for the design of MRI redox probes.²⁶ Oxygen has a relatively large concentration dissolved in arterial blood ($pO_2 \sim 100$ mmHg) or bound to hemoglobin and less in venous blood ($pO_2 \sim 40$ mmHg). pO_2 values vary substantially in different tissues depending on their vascular architecture and tissue demand, with typical average $pO_2 \sim 40$ mmHg for normal muscle. When local oxygen pressures are low ($pO_2 < 10-15$ mmHg), the condition known as hypoxia substantially affects cell metabolism. Hypoxia is important in tumors because rapid proliferating tumor cells require high levels of oxygen, which drives the cancer cells to promote angiogenesis. The abnormal signaling pathways in the tumor lead to formation of underdeveloped blood vessels which are chaotic in nature, leading to regions of hypoxia in non-necrotic parts and especially in necrotic cores that are severely lacking oxygen ($pO_2 < 2-3$ mmHg). Hypoxia has a significant influence on the progression of cancer and is considered as a marker of poor prognosis. This is because hypoxia has several biochemical consequences, including high expression levels of the transcription

factor HIF-1 (hypoxia inducible factor 1) and increased reductive stress, promoting tumor proliferation and resistance to therapy.

Several important enzymes contribute to the redox status of the extracellular matrix, which are of interest for MRI redox probes.^{12,13,14} ROS and NOS, important as signaling molecules, can be produced in hyperoxia or hypoxia, in the second case due to accumulation of reduced species such as NADH. The activity of the membrane enzyme NADPH oxidase 1 (NOX1) produces the superoxide radical $O_2^{\cdot-}$. In the extracellular space, superoxide dismutase (SOD) catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2 and O_2 , while glutathione peroxidases catalyze the reduction of peroxide and lipid hydroperoxides. Most ROS and RNS are formed inside the cell, but may be exported into the extracellular space, where they may oxidize the exofacial protein thiols (EPTs), which are highly sensitive reporters of the extracellular environment.

3. Background on relaxation-based and PARACEST-based MRI CAs

3.1. Relaxation-based MRI CAs

Magnetic resonance images are based on the density and the relaxation properties of water proton nuclei in tissues. Paramagnetic metal complexes used as contrast agents will reduce the longitudinal T_1 and transverse T_2 relaxation times in the various tissues leading to an increased contrast on the images. The efficiency of a contrast agent is commonly expressed by its proton relaxivity, r_1 , which is defined as the paramagnetic relaxation enhancement of the longitudinal water proton relaxation rate in the presence of 1 mM concentration of the agent (eq. 3):

$$\frac{1}{T_{1,obs}} = \frac{1}{T_{1,dia}} + r_1 c \quad (3)$$

where $T_{1,obs}$ and $T_{1,dia}$ are the longitudinal relaxation times observed in the presence and in the absence of the contrast agent, respectively, c is the concentration of the paramagnetic species and r_1 is the longitudinal water proton relaxivity.^{27,28,29}

Nuclear relaxation phenomena in the proximity of a paramagnetic metal are governed by dipole-dipole interactions between the electron spin of the metal and the proton nuclear spin, and are directly linked to the microscopic parameters of the metal chelate as described by the Solomon-Bloembergen-Morgan theory. The overall relaxivity is the sum of two major contributions, classified as outer sphere and inner sphere

relaxivity, originating from interactions of the electron spin with protons on water molecules diffusing in the surroundings of the complex, or with protons on the inner sphere water molecule, respectively. In some cases, second sphere water molecules can represent an additional contribution to relaxivity (eq. 4). Although for commercial agents, inner- and outer-sphere mechanisms have comparable contributions to relaxivity, chemists are more interested in the inner sphere term which can be modulated by ligand design, whereas the outer sphere contribution is dependent only on diffusion effects and is not amenable to improvement. All three relaxivity terms (inner sphere, second sphere and outer sphere) in eq. 4 increase with increasing electron spin, S , of the metal.

$$r_1 = r_{1IS} + r_{1,2ndS} + r_{1OS} \quad (4)$$

$$r_{1IS} = f(S, q, k_{ex}, \tau_R, T_{1,2e}) \quad (5)$$

The inner sphere relaxivity term is linearly proportional to the number of coordinated water molecules, q , and depends on the rate of water exchange between the metal-bound state and the bulk, k_{ex} , as well as on the rotational dynamics of the chelate (rotational correlation time, τ_R) (eq. 5). All these three factors are dependent on the ligand structure and therefore they are the most accessible to modification *via* rational ligand design. In addition, electron spin relaxation times, $T_{1,2e}$, also influence relaxivity, but their dependence on chelate structure is much less understood for Gd^{3+} or Mn^{2+} and their effect vanishes at clinically important magnetic fields.

The chemical design of redox responsive probes can be based on redox reactions occurring on the metal centre or on the ligand.¹¹

The first approach takes benefit of the redox capabilities of the metal ion. Several metal ions (Eu, Mn, Fe, Co, ...) have at least one redox state which is a strong relaxation agent. A change in the redox state of the metal ion might impact the relaxivity via essentially all parameters, namely the electron spin, the electronic relaxation, the water exchange rate and potentially the hydration number.

When the redox reaction occurs on the ligand, this can lead to the modulation of (i) the hydration number, (ii) the rotational dynamics, or (iii) the water exchange rate; the first two strategies being by far easier to realize and therefore more representative.

Concerning changes in the hydration number, variations between 0 and maximum $q=2$ can be reasonably exploited for a Gd^{3+} -, and $q=0$ and 1 for a Mn^{2+} -complex; higher hydration numbers would lead to substantial decrease in complex stability and to a risk of ternary complex formation with endogenously available coordinating functions (carboxylates, hydrogenocarbonate, etc). As the effect of the hydration number is independent of the magnetic field, responsive probes based on q changes can operate equally well at any field and will produce the same relative relaxivity changes. In contrast, the effect of the rotational dynamics on relaxivity is strongly field-dependent, and it might even depend on the water exchange rate (even if for typical Gd^{3+} or Mn^{2+} complexes water exchange has no limiting effect on relaxivity, thus changes in the rotational correlation time will lead to relaxivity variation). The Solomon-Bloembergen-Morgan theory predicts that the influence of rotational dynamics on relaxivity is maximum in the 20-60 MHz proton Larmor frequency range (the slower the rotation, the higher the relaxivity). Then this effect vanishes with increasing frequencies and above ~ 200 MHz, the $r_1 - \tau_R$ relationship is even inversed (the slower the rotation, the lower the relaxivity). As a consequence, responsive probes based on variation of the rotational dynamics typically work well at 20-60 MHz (several-fold relaxivity increase), but can produce limited relaxivity changes at higher frequencies. These considerations should not be neglected when designing responsive relaxation agents. Modifications in the rotational correlation times can be achieved *via* dimerization, aggregation, binding to biomacromolecules (proteins, typically serum albumin) by either non-covalent or covalent linkage.

3.2. PARACEST-based MRI probes

Chemical Exchange Saturation Transfer (CEST) agents possess protons in slow to intermediate exchange with bulk water protons, typically -NH of amides, amines or -OH of water or alcohols ($k_{ex} \leq \Delta\omega$, where $\Delta\omega$ is the chemical shift difference between the two proton pools). In a CEST experiment, the slowly exchanging protons of the agent are saturated by selective radiofrequency pulses, which results in the disappearance of their proton NMR signal, and, given the chemical exchange of these and bulk water protons, the water proton signal intensity will be also decreased. This water proton signal intensity decrease can be then visualized on an MR image. Complexes of paramagnetic metal ions that induce an important paramagnetic shift of the surrounding protons but do not have a

strong relaxation effect have specific advantages for CEST. The large chemical shifts allow for exploiting higher proton exchange rates yet remaining in the slow exchange region, and the selective saturation of the protons on the probe is also easier if its signal is far from the water proton signal. In the lanthanide series, all ions except for the highly relaxing Gd^{III} and Eu^{II} and the diamagnetic La^{III} and Lu^{III}, while among transition metals, Co^{II}, Ni^{II}, Fe^{II} can typically be exploited to create PARACEST probes. The parameters that determine the CEST effect involve the proton exchange rate, k_{ex} , the power of the pre-saturation pulse applied, B_2 , and the proton relaxation times in the two exchanging pools. Provided that the saturation of the exchangeable protons on the CEST probe is complete, the net magnetization of the bulk water protons (M_z/M_0) at steady state can be estimated as:

$$\frac{M_z}{M_0} = \left(1 + \frac{cnT_1}{111 \tau_M}\right)^{-1} \quad (6)$$

where $\tau_M (= k_{\text{ex}}^{-1})$ is the proton residence lifetime, c is the concentration of probe, n is the number of chemically equivalent exchanging protons *per* molecule that are saturated and T_1 is the spin-lattice relaxation time of bulk water protons. The optimal exchange rate providing a maximum CEST effect is related to B_2 according to $k_{\text{ex}} = 2\pi B_2$.^{27,28}

PARACEST (and CEST) imaging has advantages over classical relaxation-based MRI. The contrast is turned on by the application of the selective saturation of the exchanging protons, and thanks to the frequency-encoding of CEST, several protons in the same agents or several probes can be simultaneously visualized on a single image, if their saturation frequencies are different. CEST is well adapted to the design of responsive agents, for which the modulation of any of the parameters in eq. 6 can be used, even if the concentration of the exchangeable protons and their exchange rate are the most accessible. CEST agents are also easily amenable to ratiometric approaches, given the possibility to incorporate two exchangeable proton sites into the same molecule.³⁰ Finally, we should note that for PARACEST, higher magnetic fields are advantageous as they lead to better signal separation between the exchanging sites.

As for relaxation probes, the redox process that initiates the detectable PARACEST changes can also occur either on the metal centre or on the ligand. In the first case, the magnetic properties of the metal ion change, leading to a modification of

the resonance frequency and of the relaxation rate of the ligand protons. Redox reactions on the ligand might also lead to chemical shift changes.

The various mechanisms exploitable to generate a redox-dependent MRI signal for relaxation and PARACEST agents are schematically summarized in Figure 1.

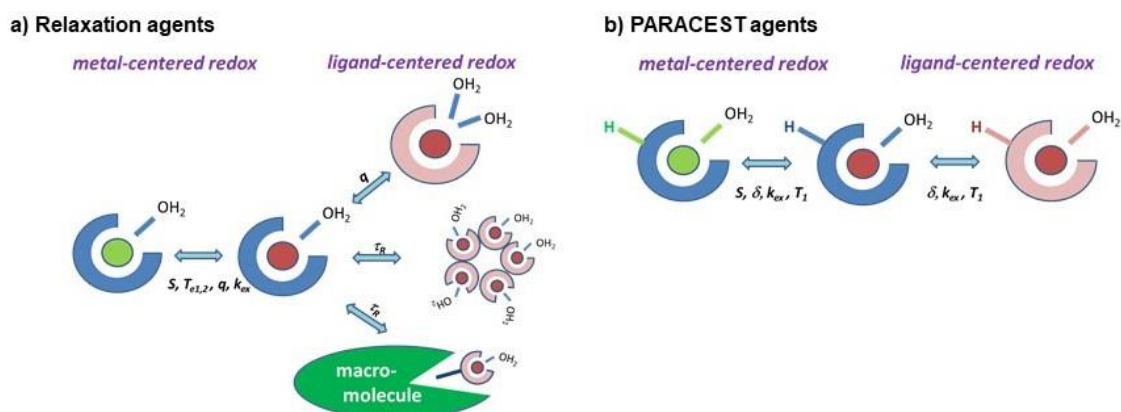


Figure 1. The main strategies to modulate a) r_1 relaxivity and b) PARACEST signal of redox probes through ligand-centred and metal-centred redox activity.

3.3. General considerations on redox-responsive, metal-based MRI agents

Any metal-based imaging probe designed for *in vivo* applications has to be biocompatible, non-toxic and water soluble. For typical polyamino-carboxylate complexes used as imaging agents, the non-toxicity is directly linked to the thermodynamic stability and kinetic inertness of the chelate which both need to be high enough to prevent release of free metal ions in the body. These issues have become particularly important following the recent concerns about Gd-toxicity in relation to Nephrogenic Systemic Fibrosis (NSF)^{31,32} and Gd-deposition in the brain.³³ In general terms, one can consider that macrocyclic complexes are more stable and inert than the open-chain analogues and that increased ligand rigidity results in higher kinetic inertness. As for any responsive probe, a strong MRI signal change, if possible a signal increase, is expected upon activation (“turn on” probe). For redox responsive probes, the redox half-cell potential has to be adapted to the potential of biological redox buffers and the redox reaction should be reversible and its kinetics has to be fast enough as compared to the time scale of the imaging experiment and of the probe elimination.

4. Redox responsive MRI contrast agents

Here, we will describe MRI contrast agents based on redox active ligands and on changes of metal redox states that can potentially respond to an alteration in the redox environment in the organism.

4.1 T₁ contrast agents

4.1.1 T₁ probes based on redox active ligands

One of the most frequently used strategies to obtain redox active MRI probes is the modulation of Gd^{III} chelates that, under a reductive/oxidative environment, undergo a change in their structure that affects their r_1 relaxivity, creating a “pseudo switch” on/off or off/on signal (better described as “ON/on” – relaxivity decrease, or “on/ON” relaxivity increase, as the loss of MRI signal is never complete).⁵ Here we will discuss characteristic examples from the literature, highlighting the structure modulation and the corresponding changes in the molecular parameters determining the relaxivity changes. The most frequently used methods involve changes a) in the number (q) and/or exchange kinetics (k_{ex}) of the water molecule(s) coordinated to the Gd^{III} center, or b) changing the rotational correlation time (τ_R) of the complex by reversible or irreversible attachment to macromolecular structures.

a) Changes of hydration state

The first mechanism for changing q based on redox active ligands was reported by Louie and collaborators³⁴ who used the known capacity of the photochromic dyes spiropyrans and spirooxazines³⁵ to isomerize under UV irradiation to the merocyanine forms. They linked Gd^{III}DO3A (DO3A= 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid) to the dye moiety to obtain potential multimodal magnetic-resonance/optical-imaging contrast agents (**Figure 2**).^{33,36,37,38}

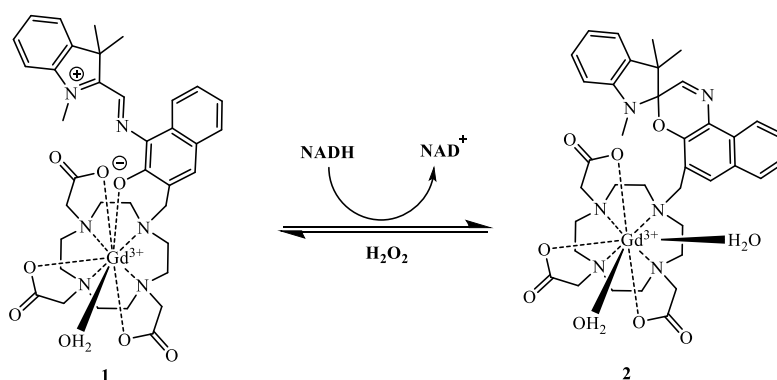


Figure 2. The conversion of the merocyanine derivative **1** into the spirooxazine form **2** by NADH increases q , and hence r_1 .³³

In the dark or under UV light, the probes are present in the colored merocyanine **1** form which can be converted into the spirooxazine derivative **2** by irradiation with visible light, leading to decreased fluorescence intensity and increased r_1 relaxivity.^{36,37} The spirooxazine derivatives have higher r_1 values than the corresponding merocyanine forms (e.g. $r_1 = 8.60$ versus $5.56 \text{ mM}^{-1} \text{ s}^{-1}$, respectively, for the spironaphthoxazine probe) due to the cleavage of the coordination bond of the phenoxide oxygen with the Gd^{III} ion, allowing the coordination number q to increase from one to two, as determined by ^{17}O NMR.^{33,38} The spirooxazine and dinitrospyrans-bound complexes also isomerize from the form **1** to the form **2**, upon reduction with NADH, although the mechanism is unclear, as it appears that no electron transfer occurs. While the reduction of the spirooxazine derivative is irreversible, the spirooxazine isomer could be partially converted back into the merocyanine derivative by H_2O_2 (**Figure 2**). Interestingly, the probes were not responsive to other biological reducing agents, like L-cysteine and L-ascorbic acid, showing their potentiality as NADH responsive probes.^{33,38}

Nagano and collaborators³⁹ proposed a family of redox active ligand probes obtained by linking a nitrobenzenesulfonamide moiety to a $\text{Gd}^{\text{III}}\text{DO3A}$ complex (see e.g. **3** in **Figure 3**). Under hypoxia conditions ($\text{pH} = 5.8\text{-}7.6$), the nitro group was reduced to an amino group with a concomitant 1.8-fold increase of the r_1 value. This is a consequence of the shift in acidity of the sulfonamide nitrogen atom upon reduction, allowing its protonation and the dissociation of the $\text{Gd}^{\text{III}}\text{-N}$ bond, thus leaving two sites for water binding (q changes from 0 to 2). Reduction of **3** was observed in the presence of rat liver microsomes, under hypoxic conditions, to give an increased contrast on T_1 -weighted MR images.

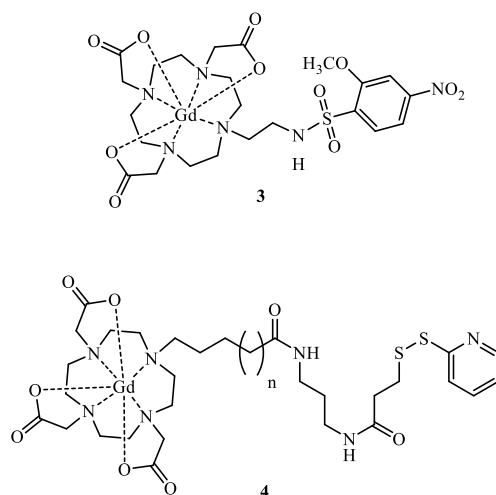


Figure 3. Redox-activated MRI probes **3**³⁹ and **4**.⁴⁰

Another strategy towards detection of reducing thiols in the extracellular microenvironment used redox probe **4**,⁴⁰ based on Gd^{III}-DO3A bearing a flexible linker ending in a 2-pyridyl-dithio moiety (**Figure 3**) that can promptly react with free thiol groups to form mixed disulfides in a reversible reaction. In the case of biological reductants also containing a carboxyl group like glutathione (GSH) or cysteine, the resulting product had about half of the relaxivity of the starting Gd^{III} complex (r_1 drop from $8.1 \text{ mM}^{-1} \text{ s}^{-1}$ to $4.1 \text{ mM}^{-1} \text{ s}^{-1}$ at 20 MHz, 25 °C and pH 7.4). This has been explained by the coordination of the carboxylate group, hence a decrease of the Gd^{III} hydration number from $q=2$ to $q=1$, on the basis of ¹H NMRD and ¹⁷O relaxation studies. Larger relaxivity differences could be achieved by forming an inclusion complex between the Gd^{III} complex and β -cyclodextrin (β -CD) or poly- β -CD.⁴⁰

b) Changes of rotational dynamics

Reversible or irreversible binding of low molecular weight Gd^{III} complexes to macromolecules has been often used to increase τ_R and to obtain higher r_1 values. This has been exploited by Raghunand and collaborators,^{41,42,43,44,45} who synthesized a series of Gd^{III}-DO3A **5**, **6** and **7** and Gd^{III}-DOTA-monoamide complexes **8** and **9** (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) bearing alkyl linkers with terminal thiol groups (**Figure 4**). These probes interacted with the Cys-34 residue of human serum albumin (HSA) via formation of an S-S bond, enhancing r_1 by slowing down the rotational dynamics of the Gd^{III} complex.^{40,41,44} In reducing environments, the Gd^{III} chelates were released and r_1 decreased. The authors have demonstrated high *in vivo*

MRI contrast enhancement in mice bearing Mia-PaCa-2 or NCI-N87 tumor xenografts when using the Gd^{III} complex with a thiol moiety on a hexyl arm (**8**). This was correlated with tumor thiol content, after the animals were treated with a glutathione synthesis inhibitor or the thiol-oxidizing anticancer drug Imexon, confirming the potentiality of this probe as an MRI reporter of the tumor redox status.^{41,43,44}

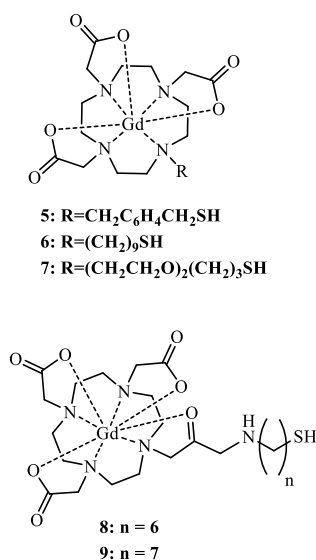


Figure 4. Redox-activated MRI probes based on thiol oxidation of the ligand followed by protein conjugation.^{41,42,43,44,45}

Exofacial protein thiols (EPTs) are a pool of thiol groups attached to membrane proteins on the external surface of various types of cells. They are present as free -SH or in the oxidized -S-S- form depending on the extracellular redox state. As the extracellular space is more oxidizing, most of them are present in the form of disulfide bridges. Aime *et al.*^{46, 47, 48} studied the relaxometric properties of derivatives of Gd^{III}-DO3A **10**, **11** and **12** or Gd^{III}-DTPA **13** and **14**, bearing a flexible linker of varying length ending in a 2-pyridyl-dithio moiety (**Figure 5**).

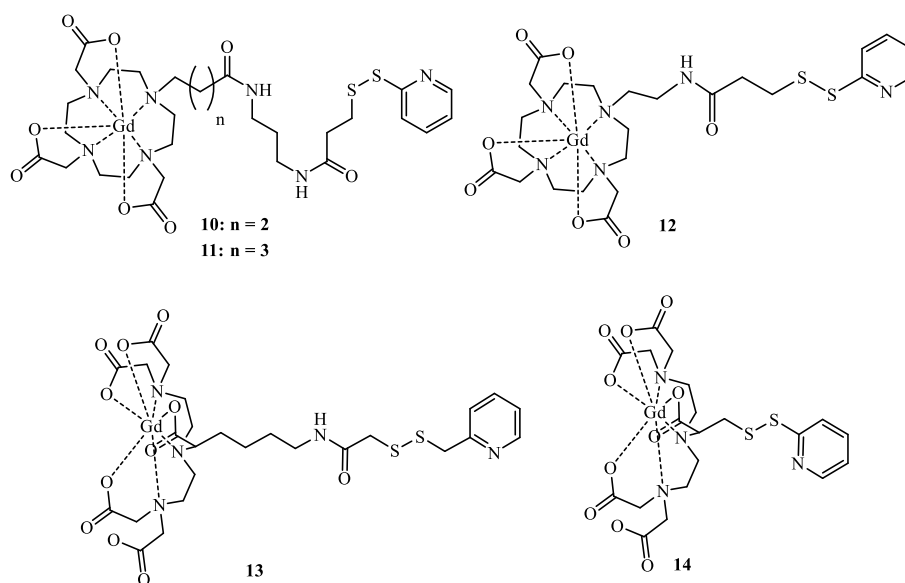


Figure 5. Redox-activated MRI probes based on S-S rearrangement of the ligand.^{46,47,48}

At 20 MHz and 25 °C, the bishydrated complex **11** had $r_1 = 9.0 \text{ mM}^{-1}\text{s}^{-1}$, while the other, monohydrated complexes had lower r_1 values ($6.3\text{-}6.9 \text{ mM}^{-1}\text{s}^{-1}$).^{46,48} It was shown that it is possible to bind these complexes to EPTs for potential application as markers for tissue hypoxia. Upon formation of a disulfide bridge, the Gd^{III}-DO3A derivatives **11** and **12** were internalized efficiently and accumulated in endosomes of different types of cells (as many as $1.2\text{-}10^{10}$ Gd atoms per single cell, a number similar to the number of EPTs at the cell surface) as demonstrated by *in vitro* MR imaging.^{46,47,48} It was also possible to label B₁₆ melanoma tumor cells *in vivo* with this type of chelates (chelate **11**) (**Figure 6**) and an increased uptake was correlated with increasing levels of EPTs, which was observed in the case of more reductive extracellular microenvironment.⁴⁷

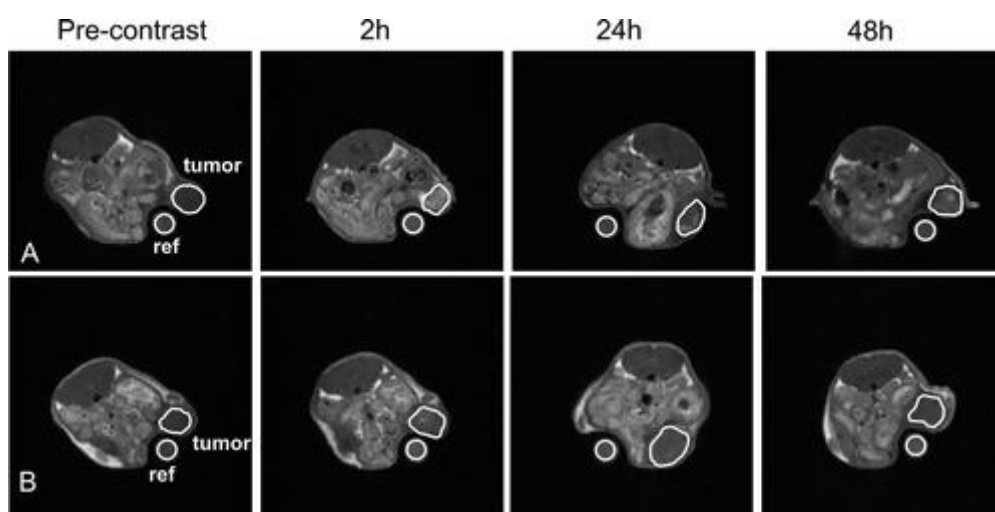


Figure 6. T_{1w} -SE axial images at 1 T (TR/TE/NEX 250/8/10) of mice subcutaneously grafted with a B₁₆ tumor. The ROIs used to measure the signal enhancement in tumor are shown. Time course of the signal enhancement from precontrast to 48 h post intratumor injection (at 0.12 μ mol) of (A) **11**; (B) Gd^{III}-DO3A. Reproduced with permission from Ref. 47, Copyright (2011) by American Chemical Society.

On the other hand, Gd^{III}-DTPA derivatives **13** and **14**, despite their capacity to react with the thiol groups of EPTs, showed a very low or non-detectable uptake. This was attributed to their negative charge, while the neutral Gd^{III}-DO3A-based ligands had efficient uptake.⁴⁸

Meade and collaborators⁴⁹ developed Gd^{III}-DOTA **15** and Gd^{III}-DTPA **16** derivative chelates linked to an octamer of arginine residues through a disulfide bond (**Figure 7**). The addition of an arginine polypeptide improved the transport of the probe across cell membranes. Upon cell internalization, the disulfide bond was efficiently cleaved by GSH, the biological reducing agent which attains higher concentration inside the cells. The separation from the cell-penetrating peptide transduction domain increased the cell-associated lifetime of the Gd^{III} probes, leading to enhanced MRI signal in labeled NIH/3T3 cells.

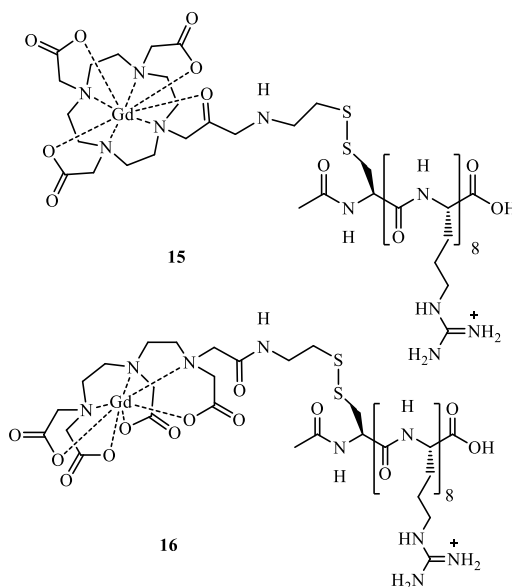


Figure 7. Redox-activated MRI probes based on S-S rearrangement of the ligands **15** and **16**.⁴⁹

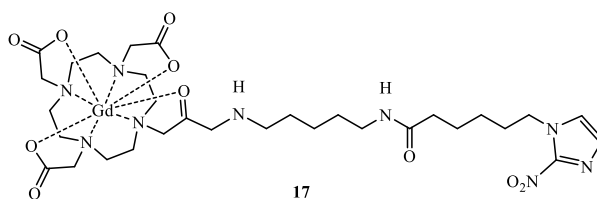


Figure 8. Redox-activated MRI probes based on reductive isomerization of the ligand **17**.⁵⁰

Probes containing nitro groups have been commonly used for imaging hypoxia. For instance, 2-nitroimidazoles are known to accumulate in hypoxic tissues due to reduction of the nitro to an amine group in reducing environments.^{51,52} Sherry and collaborators⁵⁰ developed a Gd^{III}-DO3A type **17** chelate bearing a nitroimidazole moiety for MRI imaging of hypoxia (**Figure 8**). They observed that chelate **17**, although of lower stability than Gd^{III}-DO3A-monobutylamide, showed a similar r_1 value corresponding to $q = 1$. Importantly, it was selectively trapped inside hypoxic 9L rat glioma cells, as evidenced by an increase of contrast enhancement in the *in vitro* MRI images as compared to normoxic cells. In an *in vivo* study using rats bearing prostate adenocarcinoma AT1 tumor, the same authors⁵³ clearly evidenced that **17** accumulated in the central, poorly perfused and hypoxic regions of the tumor while it showed complete clearance from the surrounding muscle tissue (**Figure 9**). These results demonstrated the utility of this nitroimidazole derivative complex to image tumor hypoxia *in vivo*.

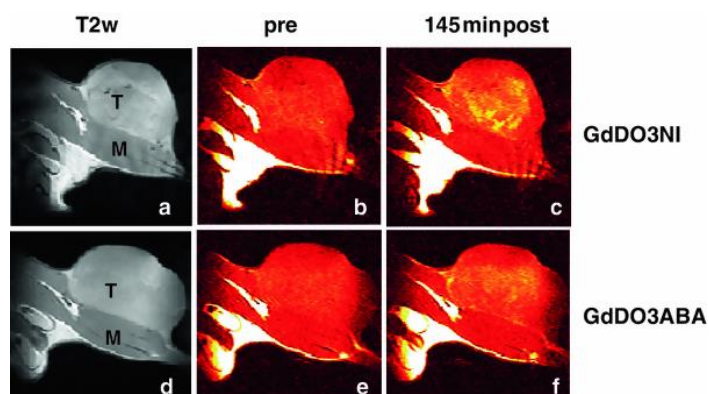
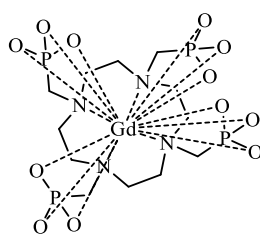


Figure 9. Grayscale MR T₂-weighted (a,d) and color T₁-weighted (b, c, e, f) images of Copenhagen rat thighs bearing syngeneic Dunning prostate R3327-AT1 tumors following injection of 0.1 mmol GdDO3NI per kg body weight (a–c) or 0.1 mmol GdDO3ABA (**17**) per kg body weight (d–f) before injection (b, e) and 145 min after injection (c, f). The

tumor and thigh muscle are labeled T and M, respectively. Reproduced with permission from Ref. 51, Copyright (2013) by Springer Nature.

The potential of a Gd^{III} complex containing phosphonate groups (**18**) (**Figure 10**) was evaluated as a hypoxia responsive agent by Aime and collaborators^{54,55}. This complex has different affinities for the two human adult (HbA) and fetal hemoglobin (HbF) conformations (R and T), acting as an allosteric effector by favoring an heterotropic interaction with the 2,3-D- bisphosphoglycerate (BPG) binding site, allowing a change in the thermodynamic equilibrium stabilizing the T conformation. The r_1 relaxivities obtained for the probe linked to R-Hb (oxy-Hb) and to T-Hb (deoxy-Hb) were quite different from the unbound chelate, suggesting that this type of probe could potentially be used as a detector of hypoxia environments. Later, the same authors⁵⁶ proposed to map tumour hypoxia via the combined use of the pO₂-responsive Gd^{III}-DOTP- and the non-responsive Gd^{III}-HPDO3A-labeled red blood cells (RBC). In fact, with this strategy, it was possible both to detect the vascular oxygen *status* and to assess the local labeled RBC concentration in a transplanted breast tumor mouse model. This circumvents an important barrier in the development of responsive agents, which is the difficulty to separate the effect arising from local concentration changes from that associated with the relaxivity response of the probe to hypoxia.



Gd-DOTP (18)

Figure 10. Redox-activated MRI probes based on Red blood Cells binding: Gd-DOTP (**18**).^{54,55,56}

Several Gd^{III} complexes containing ligands with peripheral redox-active hydroxyl groups have been studied as H₂O₂ sensors of myeloperoxidase activity (**Figure 11**). Gd^{III}-DOTA-5HT (**19**), a conjugate of serotonin (or 5-hydroxytryptamin = 5HT), containing a peripheral catechol pendant group⁵⁷ undergoes a myeloperoxidase-mediated reduction of the catechol in the presence of peroxide, yielding oligomers with a 70% increase in r_1 at

1.5 T. A similar study was undertaken with Gd^{III}- DTPA-bis-5HT (**20**), leading also to a 70% relaxivity increase.⁵⁸

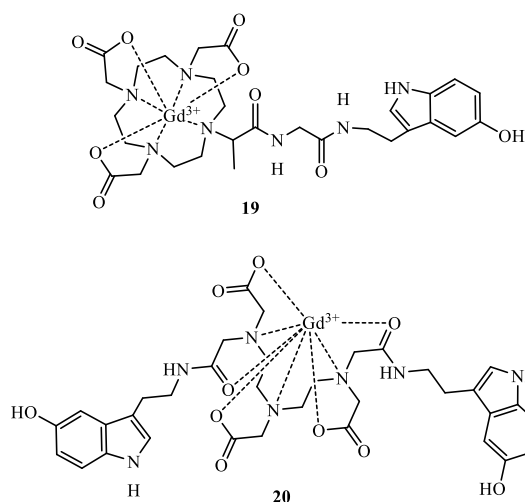


Figure 11. Gd^{III}-based MRI probes to detect myeloperoxidase activity. a) Gd^{III}-DOTA-5HT (**19**),⁵⁷ b) Gd^{III}-DTPA-bis 5HT (**20**).⁵⁸

Although Gd^{III} is the most widely used paramagnetic metal to develop MRI probes, other metal ions with similarly favorable magnetic properties, like Mn^{II} have also been investigated^{59, 60, 61}. Most literature examples of redox responsive contrast agents involving manganese take advantage of the different oxidation states of this metal, as can be seen in the next section. Albeit, Goldsmith and collaborators^{62, 63, 64} reported a series of non-cyclic Mn^{II} complexes that contain ligands with an increasing number of peripheral redox-active hydroxyl groups as H₂O₂ sensors (**Figure 12**). While the monohydrated and heptacoordinate Mn^{II} complex **21** is stable in the presence of oxygen, addition of H₂O₂ leads to an intermolecular oxidative cross-coupling reaction between two carbon atoms of the 2-hydroxy-5-methylbenzyl rings, yielding a binuclear complex.⁶² The slightly lower relaxivity of this binuclear as compared to the monomer complex ($r_1 = 3.59 \text{ mM}^{-1} \text{ s}^{-1}$ vs $r_1 = 4.39 \text{ mM}^{-1} \text{ s}^{-1}$) results from the hydration water molecule shared by the two Mn^{II} that counterbalance the effect of the increased τ_R .

A drawback of this probe was the non-reversibility of the oxidation. Later the same authors synthesized ligand **22**, containing a hydroquinone moiety that, in an oxidizing environment, is transformed into a *p*-benzoquinone with lower coordinating capacity. This allows to more easily displace the quinone part of the ligand by hydration water molecules increasing q ; however, it also leads to decreased complex stability.⁶³ The

r_1 increase observed upon addition of H_2O_2 excess was modest ($\Delta r_1 = 0.8 \text{ mM}^{-1} \text{ s}^{-1}$), due to incomplete oxidation of the ligand. However, the reaction was reversed in the presence of a reductant. Upon addition of a second hydroquinone moiety (**23**), a Mn^{II} complex was obtained with a two-fold increase of $\Delta r_1 = 1.71 \text{ mM}^{-1} \text{ s}^{-1}$.⁶⁴

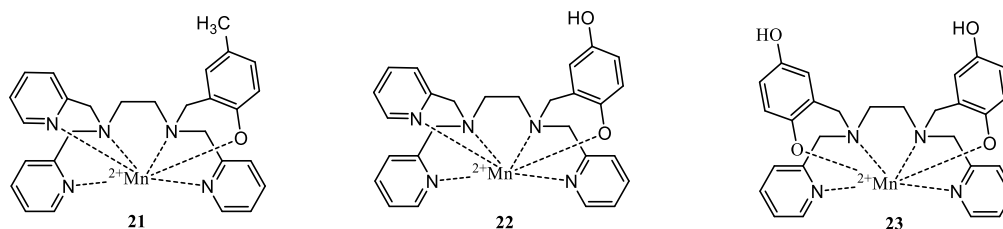


Figure 12. Redox-activated Mn^{II} based MRI probes based on hydroxyl group oxidation of the ligand: **21**,⁶² **22**⁶³ and **23**.⁶⁴

There are relatively few examples of nanoparticle-based T_1 MRI redox probes. To date, examples are primarily based on changes in the rotational correlation time (τ_R), resulting either from dissociation or association of a small Gd^{III} chelate from/with a larger molecule.

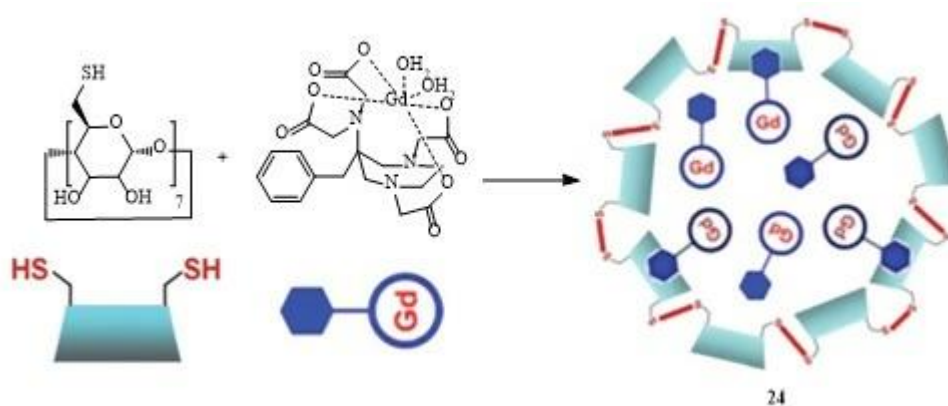


Figure 13. Nanoscale redox-activated MRI probe **24**, based on S-S rearrangement. Reproduced with permission from Ref. 65, Copyright (2011) by Royal Society of Chemistry.

Several case studies are based on a thiol/disulfide redox couple. When dissociation occurs through the S-S bonds, r_1 decreases (“turn-off” probes). The first example was a redox sensor made by encapsulating a functionalized Gd^{III} -AAZTA probe (**24**) (AAZTA=6-amino-6-methylperhydro-1,4-diazepinetetraacetic acid) in crosslinked thiolated β -CD nanocapsules via hydrophobic pendant aromatic residues (**Figure 13**).⁶⁵

This incorporation resulted in a high r_1 value of the Gd^{III} chelate ($15.2 \text{ mM}^{-1} \text{ s}^{-1}$ at 70 MHz) due to restricted mobility and good water permeability through the β -CD shell. The addition of tris(2-carboxyethyl)phosphine (TCEP) reducing agent led to S-S bond cleavage and degradation of the nanocapsules, releasing the β -CD monomers and the Gd^{III} -complexes. As a result, τ_R was shortened and relaxivity decreased from 20.7 to $9.0 \text{ mM}^{-1} \text{ s}^{-1}$ (0.47 T, 25°C).⁵⁰ Similar work was published using PAMAM dendrimers conjugated to β -CD incorporating Gd^{III} chelates, where the redox sensitive S-S bonds have been introduced in the dendrimer arms or in the hydrophobic arm of the Gd^{III} ligand responsible for its encapsulation in the CD structure.^{66,67}

A similar disassembly-based redox example was a fluorescence/ ^{19}F -MRS/ ^1H -MRI triple-functional probe consisting of Gd nanoparticles based on a precursor (**25**) Gd^{III} -DOTA monoamide linked with a quenched amino oxyluciferin fluorophore and a ^{19}F -bearing moiety attached to the amino oxyluciferin through a disulfide linker (**Figure 14**) which self-assembled through hydrophobic interactions.⁶⁸ Biothiols, like GSH, that are abundant in reducing biological environments were able to cleave the disulfide bond in probe **25** to induce disassembly of the nanoparticles and lead to fluorescence activation (~70-fold), ^{19}F -MRS signal amplification (~30-fold) and significant r_1 relaxivity reduction from $23.6 \text{ mM}^{-1} \text{ s}^{-1}$ to $7.6 \text{ mM}^{-1} \text{ s}^{-1}$ (~68% at 0.5 T, 37 °C). Molecular imaging of reducing environment in HeLa cells and *in vivo* mice bearing subcutaneous HeLa tumors was achieved using this probe.

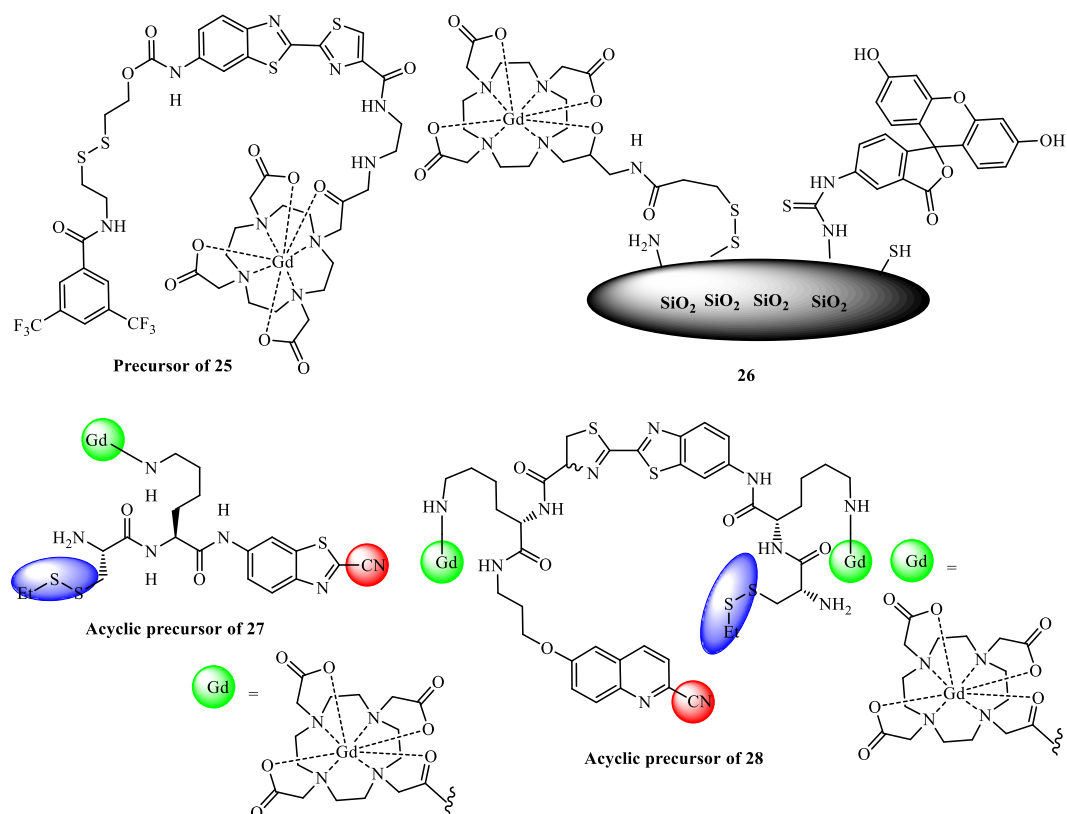


Figure 14. Nanoscale redox-activated MRI probes based on S-S rearrangement.^{68,69,70}
71 72

In another example, porous silica microparticles were surface-decorated with a Gd^{III}-HP-DO3A-like chelate (**26**) through a disulfide bond providing MRI responsivity to redox potential (HP-DO3A: 1,4,7-tris(carboxymethyl)-10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane) and rhodamine B allowing for optical imaging (**Figure 14**). Addition of TCEP to a suspension of Gd^{III}-SiMSS and reductive cleavage of the disulfide bond led to a 30% decrease of r_1 (20 MHz) from the initial value of $5.7 \text{ mM}^{-1} \text{ s}^{-1}$ due to the loss of the macromolecule effect upon release of the Gd^{III}-SH chelate. Such microparticles were interspersed with therapeutic cells (human Mesenchymal Stem Cells - hMSCs) within a biocompatible hydrogel. The onset of reducing conditions within the hydrogel was paralleled by an increased clearance of the monomer Gd^{III} complex that can be detected by MRI.⁷²

Liang et. al.⁶⁹ developed a “turn-on” probe from a precursor (**27**), Acetyl-Arg-Val-Arg-Arg-Cys(StBu)-Lys(Gd-DOTA)-CBT, for self-assembling gadolinium nanoparticles (Gd^{III}-NPs) under the action of furin in living tumor cells. (**Figure 14**). It contains a RVRR peptide sequence for cleavage and cell membrane translocation by

furin, a trans-Golgi protease overexpressed in tumors, a Gd^{III}-DOTA monoamide for MRI, disulfided Cys for supplying the 1,2-aminothiol group for condensation, and 2-cyanobenzothiazole (CBT). After entering MDA-MB-468 cells, the disulfide bond of the Cys motif of the probe is reduced by the intracellular GSH and subsequently its RVR motif is cleaved by furin on the site of this enzyme (i.e., Golgi body), resulting in an active intermediate. Two of these condense quickly, activated by disulfide reduction of the cyano group of CBT to free thiol, followed by intramolecular cyclization to yield an amphiphilic dimer which has a hydrophobic macrocyclic core for self-assembling the Gd^{III}-NPs via π - π stacking. At 25.6 MHz (*ca.* 0.6 T), the proton relaxation effect of the probe increased by 160% after self-assembly, due to the elongated τ_R , while at 64 MHz (1.5 T), the relaxation rate increased by 110%. T₁-weighted MRI of MDA-MB-468 tumor xenografts showed that the probe enhanced contrast within the tumors.

A potential limitation of the previous system is the competition from the reaction between the 2-cyanobenzothiazole (CBT) moiety in the monomer and the endogenous free cysteine that stops polymerization and self-assembly. In order to avoid the competition from free cysteine and generate a larger Δr_1 value, the self-assembly strategy was optimized by switching the concentration-dependent intermolecular polymerization to concentration-independent intramolecular macrocyclization and self-assembly, using an acyclic precursor B containing two Gd-DOTA amide chelates and a biothiol sensitive disulfide bond (**28**, **Figure 14**).⁷⁰ This precursor is reduced to form a reactive intermediate, which subsequently undergoes intramolecular cyclization to generate a more rigid and hydrophobic macrocyclic product. The following self-assembly into **28** generated a ~60% increase in r_1 from 25 to 34.2 mM⁻¹ s⁻¹ per molecule at 0.5 T and brighter MRI contrast.

These examples demonstrate that effective r_1 responses can be observed using a redox stimulus, even if reduction in r_1 and in subsequent MRI signal intensity resulting from dissolution of aggregates are less useful than relaxivity (and signal) increase due to longer τ_R .

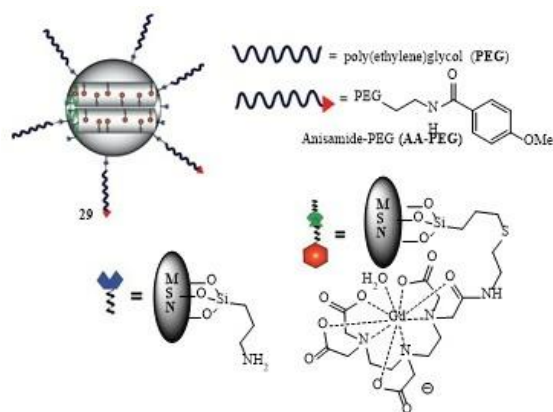


Figure 15. Nanoscale redox-activated MRI probe **29** based on S-S rearrangement. Reproduced with permission from Ref. 71, Copyright (2011) by John Wiley and Sons.

Mesoporous silica nanospheres (MSNs) surface-functionalized with cleavable Gd^{III} chelates have also been studied as redox “turn-off” MRI agents. Gd^{III}-DTPA monoamide complexes were conjugated to MSNs through a cleavable S-S linker.⁷¹ The particles were further decorated with polyethylene glycol (PEG) and an anisamide (AA) ligand **29**, **Figure 15**, to improve their biocompatibility and target specificity. The AA-PEG-Gd^{III}-DTPA-MSN particles had an r_1 of 25.7 mM⁻¹ s⁻¹ (at 3 T). Reductive S-S bond cleavage by cysteine and subsequent release of the Gd chelate, with a half-life $t_{1/2} \approx 35$ h, caused a large decrease in r_1 . The probe efficiency and its targeting ability were demonstrated *in vitro* using human HT-29 colon adenocarcinoma and pancreatic cancer cells, as well as *in vivo* in nude mice xenografts using a 3T scanner. The Gd^{III} chelate was quickly cleaved by the blood pool thiols and eliminated through the renal excretion pathway.⁷¹

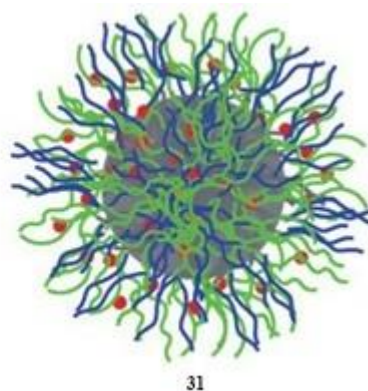
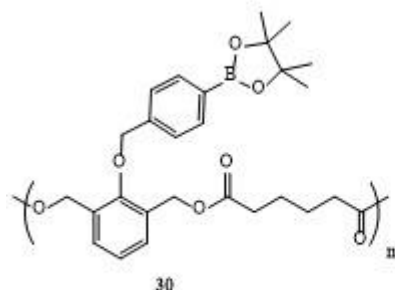


Figure 16. Other nanoscale redox-activated MRI probes. Probe **31** representation was reproduced with permission from Ref. 73,74, Copyright (2016) by Royal Society of Chemistry.

Other redox triggering mechanisms have been also explored.^{73,74} An activation mechanism was based on ultrasmall (< 5 nm) gadolinium oxide NPs encapsulated in capsules made up of a bioresponsive poly lactic-co-glycolic acid (PLGA) polymer bearing boronic ester groups (**30**, **Figure 16**) that degrades by quinone methide rearrangement upon exposure to H₂O₂, triggering the release of the NPs in response to this chemical marker of disease. Inside the hydrophobic polymeric matrix, the Gd-NPs are shielded from the aqueous environment, silencing their ability to enhance water proton relaxation ($r_1 = 0.7 \text{ mM}^{-1} \text{ s}^{-1}$). Upon disassembly of the polymeric particles, the released NPs have a r_1 of $6.7 \text{ s}^{-1} \cdot \text{mM}^{-1}$, yielding a strong positive contrast enhancement of one order of magnitude. Thus, this system could be a useful H₂O₂ MRI biosensor.⁷³

Palivan and collaborators reported an interesting redox responsive nanoplatform of 45 nm size.⁷⁴ (**31**, **Figure 16**) It results from self-assembling of an amphiphilic heparin–poly(dimethylsiloxane) (hepPDMS) block copolymer with complexation sites for Gd^{III}. A disulfide-linked amphiphilic peptide interacts with hepPDMS-Gd *via* its polyhistidine sequence and co-assembles to peptide-hepPDMS-Gd NPs **31** (p-hepPDMS-Gd-NPs), with a high $r_1 = 44.2 \text{ mM}^{-1} \text{ s}^{-1}$ (at 3 T). In a reductive environment, such as exposure to GSH or dithiotreitol (DTT), the peptides are released, increasing the accessibility of Gd^{III} for water molecules, with a 20% increase of r_1 to $54.4 \text{ mM}^{-1} \text{ s}^{-1}$. *In vitro* cellular assays demonstrated low cellular uptake and the absence of cell toxicity of the assembled NPs. The relaxivity increase observed in a reductive environment can be used to obtain increased MRI contrast for cancerous tissue based on the elevated GSH levels in the extracellular space of tumors.⁷⁴

In conclusion, the strategy of introducing a redox sensitive moiety into the ligand of Gd^{III} or Mn^{II}- based chelates or nanosystems provides relaxivity changes which are solely modulated through alteration of the ligand structure or molecular rotation. However, neither the reduced nor the oxidized forms are completely silent in terms of T₁ relaxation, thereby limiting the change in MR signal intensity obtainable upon the redox process.

4.1.2 T₁ probes based on metal redox states

For an effective clinical application, it is preferable that redox probes exhibit a completely silent signal either in the oxidized or in the reduced form (“OFF/ON” or “ON/OFF”), thus maximizing the r_1 relaxivity change (Δr_1) and consequently the change of contrast in T_1 -weighted images. This is not the case in the ligand-based redox Gd-probes described above (they are “on/ON” or “ON/on”). Alternatively, complexes of redox-active metal ions that present different magnetic properties in their reduced and oxidized forms can be used to design MRI agents that directly sense redox changes of their environments. For some metal ion redox systems, the relaxivity of either the reduced or oxidized form is zero or negligible, and, in these cases, on/off sensors can be designed with maximum sensitivity.^{5,10}

The $\text{Eu}^{\text{III}}/\text{Eu}^{\text{II}}$ redox couple could be a good candidate for such systems since Eu^{II} , with seven unpaired f electrons in an $^8\text{S}_{7/2}$ ground state, is isoelectronic with Gd^{III} . Thus, the Gd^{III} and Eu^{II} complexes can be expected to have similar proton relaxivities. Eu^{III} is a very poor relaxing agent due to its small magnetic moment, resulting from contributions from the thermally accessible $^7\text{F}_1$ and $^7\text{F}_2$ states above the diamagnetic $^7\text{F}_0$ ground state, and also to its very fast electronic relaxation ($\tau_s \sim 0.01 - 0.05$ ps).⁷⁵ Therefore, the paramagnetic relaxation effect of Eu^{III} is negligible relative to Eu^{II} .

In some early studies towards developing Eu^{II} -based MRI agents,^{76,77,78} Eu^{II} complexes have been found to have faster (more favorable) water exchange kinetics than the corresponding Gd^{III} analogues, due to the larger ionic radius (1.25 Å for Eu^{II} vs. 0.938 Å for Gd^{III}) and lower charge (lower charge density) of Eu^{II} . On the other hand, Eu^{II} has a shorter electronic relaxation time than Gd^{III} in most of the complexes investigated, leading to r_1 relaxivities often somewhat lower than that those of typical Gd^{III} chelates.

However, the use of Eu^{II} complexes as *in vivo* redox probes requires high thermodynamic stability and redox stability. The thermodynamic stability constants of the Eu^{II} polyaminopolycarboxylate (eg. $[\text{Eu}^{\text{II}}(\text{DTPA})(\text{H}_2\text{O})]^{3-}$) (**32**) and macrocyclic azacrown ether carboxylate derivatives ($[\text{Eu}^{\text{II}}(\text{ODDA})(\text{H}_2\text{O})]$ (**33**) and $[\text{Eu}^{\text{II}}(\text{ODDM})]^{2-}$ (**34**) ODDA^{2-} =1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diacetate, ODDM^{4-} = 1,4,10,13-tetraoxa-7,16-diaza-cyclooctadecane-7,16-dimalonate, see **Figure 17**) initially studied are very close to those of the Sr^{II} analogues and far below those of the corresponding Gd^{III} chelates.⁷⁸ Additionally, most Eu^{II} polyaminopolycarboxylate complexes have redox potentials significantly lower than that of the $\text{Eu}^{\text{II}}(\text{H}_2\text{O})_8^{2+}$ aqua ion ($E_{\text{III/II}} = -0.39$ V vs. NHE), reflecting the instability of the Eu^{II} oxidation state. A small improvement has been found for the azacrown derivatives, with $E_{\text{III/II}}$ values of -0.58 V

and -0.68 V for the $[\text{Eu}^{\text{II}}(\text{ODDA})(\text{H}_2\text{O})]$ (**33**) and $[\text{Eu}^{\text{II}}(\text{ODDM})]^{2-}$ (**34**) complexes, respectively, but their negative values severely limit the potential for their use *in vivo*.^{77,78}

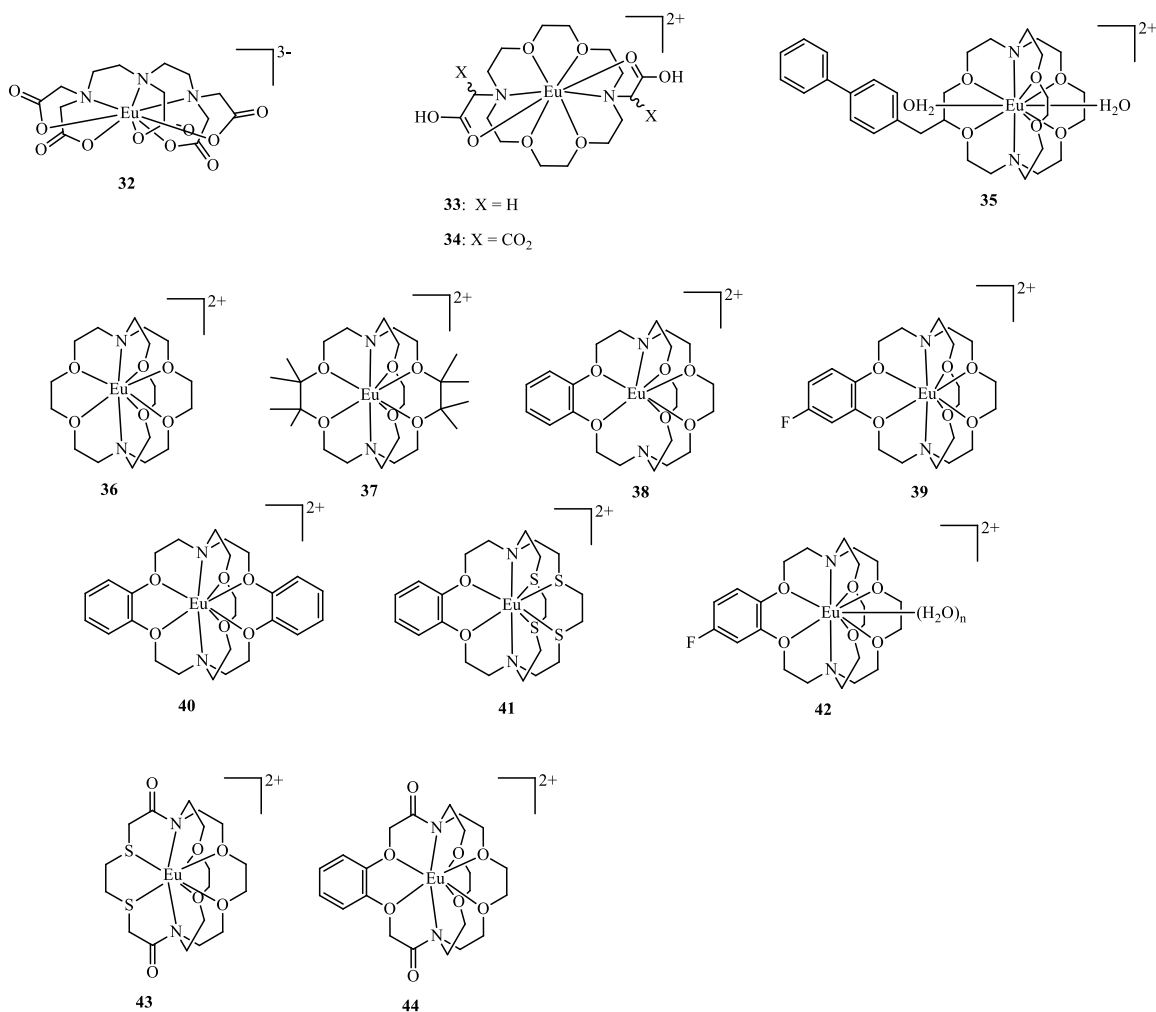


Figure 17. Chemical structures of Eu^{II} complexes discussed.

Eu^{II} cryptates (**Figure 17**) proved to be more stable toward oxidation and offered further advantages. For example, $[\text{Eu}^{\text{II}}(2.2.2)(\text{H}_2\text{O})_2]^{2+}$ (**35**) (2.2.2 = 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane), with a better size match of its cryptand 2.2.2 cavity (1.4 Å) to the Eu^{II} ion (1.25 Å) relative to the Eu^{III} ion (1.07 Å), showed a thermodynamic stability seven orders of magnitude higher than for the corresponding Eu^{III} cryptate and a better redox stability ($E_{\text{III/II}} = -0.39$ V vs. NHE) than the $\text{Eu}^{\text{II}}(\text{H}_2\text{O})_8^{2+}$ aqua ion. It was also shown to have a fast water-exchange rate (10^8 s⁻¹), a 10-fold slower electron-spin relaxation than **32** that does not limit the relaxivity and two inner-sphere water molecules in a ten-coordinate complex affording a $r_1 = 8$ mm⁻¹ s⁻¹ at 0.47 T and 298 K.^{79,80}

While this Eu^{II} cryptate has some promising properties, its propensity to be oxidized in water is still too high, preventing *in vivo* MRI applications. Further improvements on the redox stability of the Eu^{II} cryptates have been achieved by the group of Allen. They modified the structure of the cryptand **35** using coordination chemistry principles to stabilize the electron-rich Eu^{II} ion,^{81,82} to obtain cryptands **36–41** (**Figure 17**).⁸³ The first goal was to increase the steric bulk surrounding cryptand **36** to minimize interactions between Eu^{II} and its environment, by adding methyl groups to the ethylene carbon atoms between the oxygen atoms, resulting in ligand **37**. This substitution pattern was chosen to block the metal–environment interactions occurring between the unmodified ethylene groups. The second goal was to reduce the Lewis basicity of cryptand **36** to favor the electron rich Eu^{II} over Eu^{III}. Phenyl rings were introduced to decrease the electron donating ability of the adjacent oxygen atoms of ligands **38–40** by a resonance withdrawing effect, which was modulated by varying the electron density of the phenyl ring through the addition of a fluorine atom (**39**) or by increasing the number of rings (**40**). The third goal was to decrease cavity size of the cryptand to better match the size of the Eu^{II} ion, due to the effect of each phenyl ring in cryptands **38–40**. The final goal was to modify the hard–soft acid–base (HSAB) properties of cryptand **36**, which was done by introducing relatively soft sulfur-atom donors in cryptand **41** in place of oxygen-atom donors to explore the HSAB preferences for the softer Eu^{II} ion relative to the harder Eu^{III} ion. The redox potential of these Eu^{II} cryptates could be tuned over a range of 300 mV. Cryptates **39** and **41** had the highest redox potential increases, with E_{III/II} values that were 0.622 V (E_{III/II} = +0.329 V vs NHE) and 0.666 mV (E_{III/II} = +0.283 V vs NHE), respectively, more positive than the aqueous Eu^{II}(H₂O)₈²⁺ ion.

The relaxivity of several Eu^{II}-containing cryptates (**35**, **36**, **38**, **Figure 17**) was studied at different field strengths (1.4, 3, 7, 9.4, and 11.7 T), which showed higher r₁ values at higher fields (7 and 9.4 T) than at lower fields (1.4 and 3 T), unlike common Gd^{III}-containing contrast agents, *eg.* [Gd(DOTA)(H₂O)]⁻.^{84,85} Their higher relaxivities relative to [Gd(DOTA)(H₂O)]⁻ at ultra-high fields are due to the ability to accommodate two water molecules in the inner sphere, the increase in water-exchange rates and changes in rotational correlation rates compared to [Gd(DOTA)(H₂O)]⁻. The differences in relaxivities among the different cryptates arise mainly from the changes in the rotational correlation rates that are proportional to molecular weight differences.

The biphenyl derivative Eu^{II}-cryptate (**35**) is capable of interacting with human serum albumin (HSA). The influence of HSA binding on the r₁ relaxivity was investigated

at different field strengths (1.4, 3, 7, 9.4, and 11.7 T). Relaxometric measurements showed that HSA binding led to a r_1 enhancement of **35** of 170 % at the lower field of 1.4 T, as expected from the increased rotational correlation time (τ_R) upon protein binding, while had no effect at high fields ($\geq 3T$).⁸⁶ It was also shown that the number of metal-bound water molecules (q) decreases from two in the free complex to one in its bound form, while the water exchange of the remaining bound water molecule was not hindered by the protein side chains.

Besides high relaxivity, other requirements for effective MRI CAs, including kinetic stability, need to be considered for *in vivo* use. Eu^{II}-containing cryptates **36** and **38** were found to be stable in the pH 3-10 region and also to transmetallation in the presence of endogenous ions such as Ca²⁺, Mg²⁺ and Zn²⁺.⁸⁴ That was not the case for cryptates **43** and **44**, where weak metal binding can be attributed to the presence of the amide groups in the cryptand structure, which, by lowering the basicity of the neighboring nitrogen atoms, affect their metal coordination capacity.

The increased redox stability in water and air-sensitivity of these Eu^{II} cryptates led to their proposal as pO₂ sensitive MRI redox probes. This was recently tested *in vivo* for the first time by performing T₁-weighted MRI before and after intra-tumoral injection of the Eu^{II} cryptate (**39**) into a 4T1 mammary carcinoma xenograft mouse model (**Figure 18, a–d**).⁸⁷ Positive contrast enhancement was observed during the whole experiment, but the contrast location changed with time. While heterogeneous contrast was observed in the whole tumor immediately post injection, after 120 min it was only observed in a localized core of the tumor, indicating a lack of oxygen and the persistence of the reduced (Eu^{II}) probe in that part for at least 120 min.

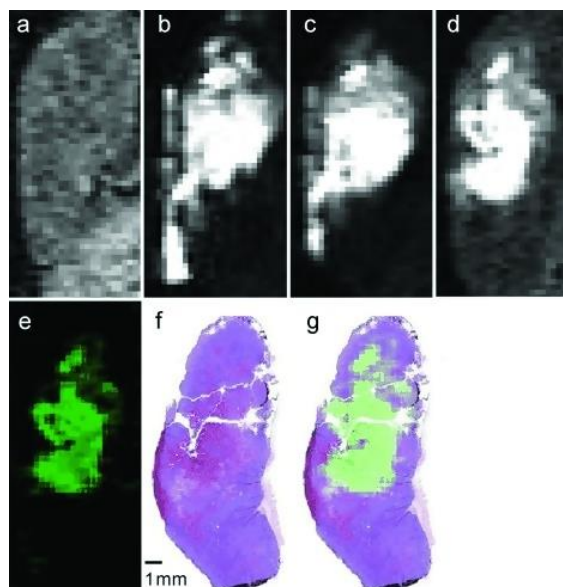


Figure 18. T₁-weighted *in vivo* sagittal plane images of a 4T1 tumor in a xenograph mouse carcinoma model injected with **39**. Images are recorded a) pre-injection and b) 3 min, c) 20 min, and d) 120 min post-intratumoral injection. e) The difference between the 120 min and pre-injection images (image (d) minus image (a)) colored using the ImageJ green lookup table. f) Hematoxylin- and eosin-stained slice of the tumor imaged in (a–e) and g) the sum of images (e) and (f). All images are on the same scale. Selected imaging parameters: echo time = 1.5 ms, repetition time = 11 ms, flip angle = 40°, field of view = 30 mm x 90 mm, in-plane resolution = 0.352 mm x 0.352 mm. Reproduced with permission from Ref. 87, Copyright (2015) by John Wiley and Sons.

The presence of necrotic tissue in the tumor was confirmed by histological staining, excised after the MRI experiment, with hematoxylin and eosin dyes (**Figure 18, f**).⁸⁷ Hematoxylin stains nuclei (blue) and eosin stains elements of the cytoplasm (pink) to differentiate nuclei abundant nuclei-deficient areas. Areas associated with necrosis (in the mid-to-upper half of the tumor) stained pink to a greater extent than non-necrotic areas, because of the lack of cells and their corresponding nuclei in necrotic regions. Consistent with the staining results, most of the positive contrast enhancement was observed in the mid-to-upper half of the tumor, suggesting that Eu^{II}-**39** provided positive contrast enhancement in the necrotic core of the tumor (**Figure 18, g**).⁸⁷ This probe retention was confirmed by analysis of the tumor Eu content as a function of time by inductively coupled plasma mass spectrometry (ICP-MS). The parallel decrease of MRI tumor contrast (85%) and of Eu content (80%) at 3h post-injection showed the slow clearance of the Eu^{II} probe. The clearance process could not be observed by MRI because

of the fast probe oxidation in tissues or fluids of relatively higher oxygen content to the Eu^{III} form, which is MRI contrast silent.

The dependence of the *in vivo* contrast enhancement on the type of probe injection was studied by performing intravenous, intraperitoneal and subcutaneous injections of the Eu^{II} cryptate **36** in mice.⁸⁸ The observed contrast was very sensitive to the route of administration. An inverse correlation was observed between tissue oxygen content and expected rates of diffusion with the persistence of Eu^{II}-based contrast enhancement. Biodistribution studies showed Eu clearance through the liver and kidneys for intravenous and intraperitoneal injections, but no contrast enhancement was observed in organs associated with clearance. This study helped understand the behavior of Eu^{II}-based complexes *in vivo* and might be helpful in their preclinical application.

Given the *in vivo* correlation between tissue pO₂ and the persistence of Eu^{II}-based positive contrast enhancement,^{87,88} oxidation reactions of a set of Eu^{II} complexes with O₂, bromate (BrO₃⁻) and glutathione disulfide (GSSG) have been studied *in vitro*.⁸⁹ These helped rationalize the observed Eu^{II} oxidation responses and understand the possible products of probe oxidation *in vivo*. The reaction between Eu^{II}_(aq) and O₂ readily led to superoxide formation, which was detected using electron paramagnetic resonance (EPR) spectroscopy and spin traps. The oxidation kinetics of three Eu^{II}-containing complexes, including Eu^{II}_(aq) and the cryptate **39**, with BrO₃⁻ and GSSG was also studied. The reactions with BrO₃⁻, with negative Gibbs free energies of electron transfer were all thermodynamically allowed. However, the oxidation rate of cryptate **39** was relatively low (one order of magnitude lower than with Eu^{II}_(aq)), indicating that the ligand responsible for the oxidative stability of the cryptate also inhibits the access of water to the metal center by decreasing the available coordination sites from 9-10 in Eu^{II}_(aq) to 1 in **39**.

In contrast, oxidation was not observed for any Eu^{II}-containing complex with GSSG. The lack of reactivity between **39** and GSSG can be explained by a positive Gibbs free energy, indicating that spontaneous reactions should not be observed. However, in the case of Eu^{II}_(aq), spontaneous reaction is expected from the negative free energy of the reaction.^{87,88} This lack of reactivity points to a kinetic activation barrier, possibly related to the inability of the disulfide bond to access the Eu^{II} metal center, preventing easy disulfide reduction. Altogether, the GSSG data and *in vivo* imaging experiments^{87,88} indicate that disulfide bonds or other bulky oxidizing agents are likely to have a minor contribution to Eu^{II} redox probe oxidation in *in vivo* MRI experiments of short duration.

Manganese is a biogenic element that plays an important role in the organism as a cofactor for enzymes and receptors, possessing several stable oxidation states (Mn^{II} , Mn^{III} , Mn^{IV} , Mn^{VI} and Mn^{VII}). In its divalent state, Mn^{II} , it is a very efficient paramagnetic relaxation agent, ($3d^5$ configuration, high spin $S = 5/2$), with long (in the 0.1 – 1 ns range) longitudinal electronic relaxation times and labile water exchange.⁷ Such characteristics also provide an efficient, although smaller, positive contrast enhancement, making it an attractive alternative to Gd^{III} in MRI probes^{59,60,61} Mn^{III} ($3d^4$ configuration) is also paramagnetic, usually giving rise to high spin $S = 2$ compounds. While water exchange is also fast,^{90,91} the longitudinal electronic relaxation times are shorter (≤ 10 ps),⁷⁵ making them less efficient relaxation agents. Both Mn^{II} and Mn^{III} oxidation states are accessible under biologically relevant conditions.

The first Mn-based redox probe was reported by Aime and collaborators⁹² (**Figure 19**), using a $\text{Mn}^{\text{II}}/\text{Mn}^{\text{III}}$ water-soluble porphyrin (5,10,15,20-tetrakis-(p-sulfonato phenyl) porphinate, TPPS₄) as an oxygen sensor. As shown in **Figure 20**, the nuclear magnetic relaxation dispersion (NMRD) profiles of the $\text{Mn}^{\text{II}}\text{-TPPS}_4$ (**45**) and $\text{Mn}^{\text{III}}\text{-TPPS}_4$ (**46**) complexes are significantly different: while at ¹H frequencies below 60 MHz the r_1 relaxivity of the Mn^{II} complex is higher than that of the Mn^{III} complex (over 3-fold higher at very low fields, below 0.1 MHz), their differences (Δr_1) are quite small within clinically relevant fields (¹H frequencies of 60-120 MHz at 25°C). An efficient solution to circumvent this consisted in the incorporation of the complexes into poly- β -cyclodextrin (poly- β -CD), which led to a significant (three-fold) amplification of Δr_1 at 20 MHz between the reduced and oxidized forms (**Figure 20**). This was due to the fact that only the r_1 of **45** was affected by the incorporation in the rigid supramolecular adduct since it is controlled by the rotational motion (τ_R), while r_1 of **46** was not, as it is dominated by the electronic relaxation rate.⁹³ Oxygen was found to react with the poly- β -CD adduct of **45** (**47**) to give the adduct of **46** (**48**) (with a corresponding 50% drop in r_1) in an extent which was roughly proportional to $p\text{O}_2$ values less than 40 mmHg. Because the $p\text{O}_2$ of venous blood vessels is ~ 40 mmHg, this system is not suitable for *in vivo* studies, but its modification may lead to probes useful for the MR differentiation of arterial and venous blood vessels. This might involve tuning of the redox potential, controlling the kinetics of the redox processes and optimizing Δr_1 values at clinically relevant fields by derivatization of this type of porphyrin ligand.

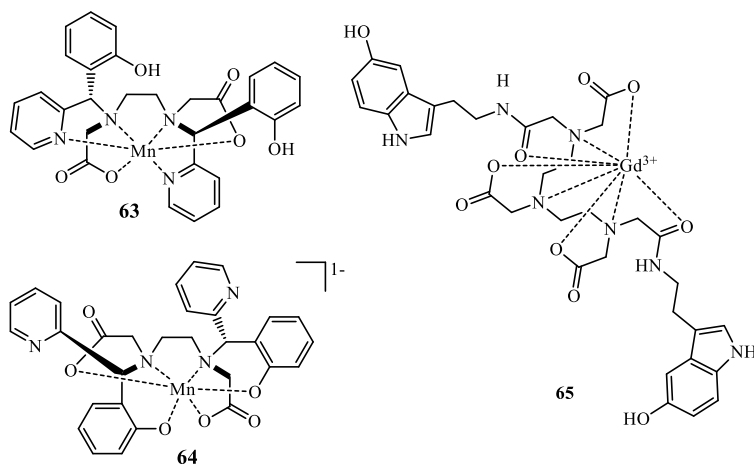
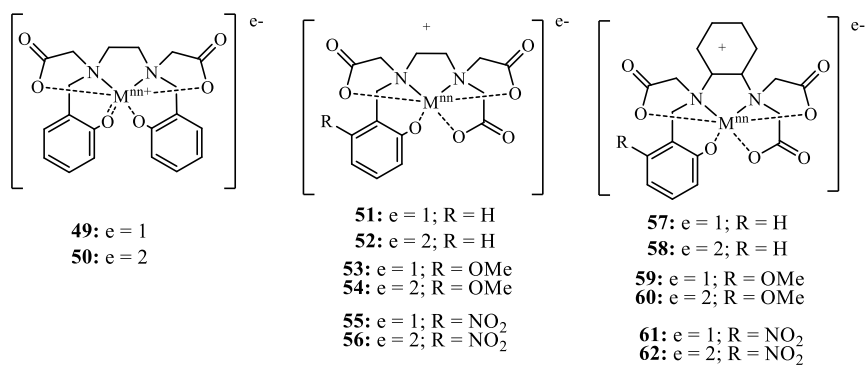
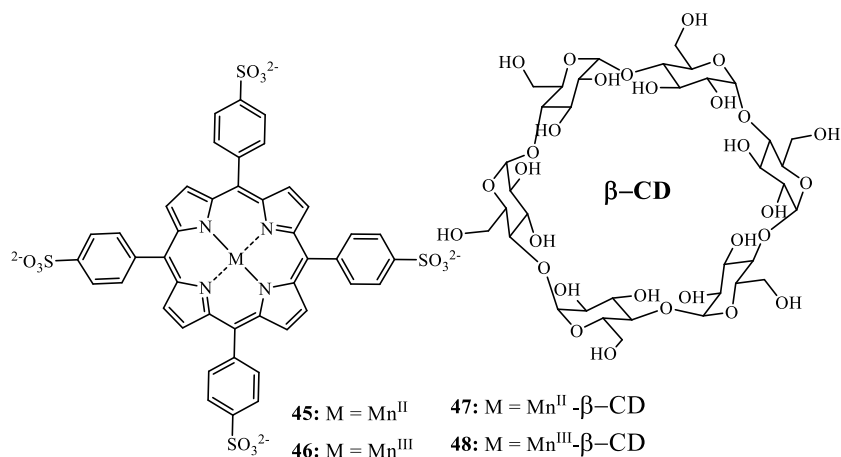


Figure 19. Manganese redox probes.

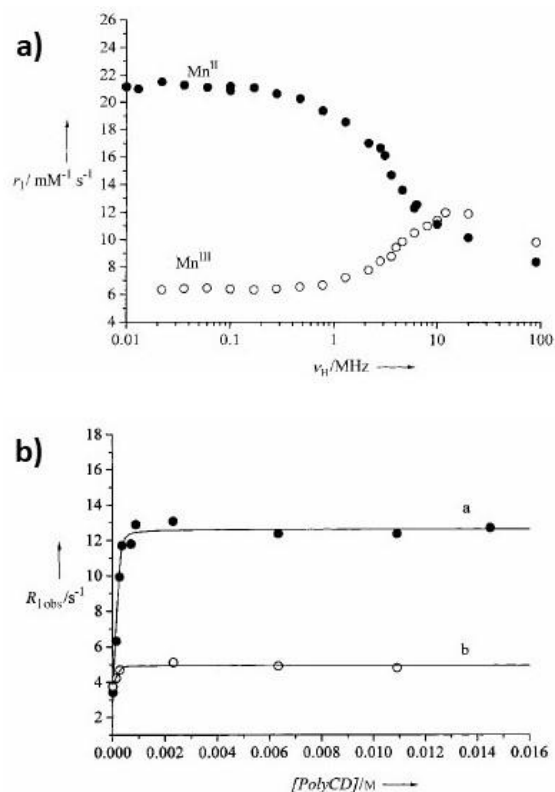


Figure 20. a) r_1 NMRD profile of aqueous Mn^{II} -TPPS₄ (●) and Mn^{III} -TPPS₄ (○) at pH 7 and 25 °C; b) Effect of supramolecular adduct formation with poly- β -CD on the relaxation rate of 0.2 mM aqueous Mn^{II} -TPPS₄ (●) and Mn^{III} -TPPS₄ (○) (20 MHz, pH 7 and 25 °C). Reproduced with permission from Ref. 92, Copyright (2000) by John Wiley and Sons.

Another family of redox-responsive $\text{Mn}^{\text{II}}/\text{Mn}^{\text{III}}$ probes was reported recently by Caravan and collaborators (**Figure 19**). They are based on complexes of EDTA derivative ligands containing one (hydroxybenzylethylenediamine triacetic acid, **HBET**, **51/52**) or two (dihydroxybenzylethylenediamine diacetic acid, **HBED**, **49/50**) phenolato donor groups, capable of stabilizing both Mn^{II} and Mn^{III} oxidation states.⁹⁴ While **Mn-EDTA** favors the Mn^{II} oxidation state ($E_{\text{III/II}} = 0.633$ V vs. NHE), the two phenolato donors of **Mn-HBED** strongly favor the Mn^{III} state (**50**) ($E_{\text{III/II}} = 0.016$ V vs. NHE). When a single phenolato donor is present in **Mn-HBET** ($E_{\text{III/II}} = 0.356$ V vs. NHE), both oxidation states (**51** and **52**) are stabilized making it a suitable probe for biological redox buffer systems. At 4.7 T (200 MHz), **Mn^{II}-HBET (51)** showed a 3.3-fold higher relaxivity ($\sim 4 \text{ mM}^{-1} \text{ s}^{-1}$) than its Mn^{III} counterpart (**52**) ($\sim 1.2 \text{ mM}^{-1} \text{ s}^{-1}$) due to a difference in the electron relaxation properties, as well as to a decrease in the number of inner-sphere molecules (q) from one in **51** (heptacoordinate) to zero in **52**. The higher r_1 value of **51** was clearly demonstrated by phantom MR images. Reduction of **52** to **51** was achieved using low-

millimolar concentrations of GSH, as monitored by relaxometry and UV–vis spectroscopy. The reaction was direct, without the formation of any by-product. Based on the intracellular levels of 1–10 mM GSH, **52** might be used as an intracellular GSH sensor if delivered inside the cells, which switches on in the presence of GSH with a half-life of about 3–30 min. However, it is expected to be much longer-lived in extracellular compartments, *e.g.* over a week in blood plasma, which typically contains only 2–20 μM glutathione.²² The authors also observed reversibility between the two oxidation states by cyclic voltammetry and testing the probe oxidation using H_2O_2 . However, the probe does not have satisfactory kinetic inertness for *in vivo* applications, as it was not kinetically stable in the presence of EDTA.

The same group performed a systematic study of ten Mn-containing complexes obtained through modification of the **HBET** and **CyHBET** (N-(2-hydroxybenzyl-N,N',N'-trans-1,2-cyclohexylenediaminetriacetic acid) structures by introduction of different substituents (-H, -OMe, -NO₂) at the 5-position of the phenolate ring (**Figure 20**, compounds **51–62**), with the objective of establishing structure-redox-relaxivity relationships that allowed optimizing this kind of redox probes.⁹⁵ In all cases, the Mn^{II} complexes had higher relaxivities than the respective oxidized forms, with a turn-on signal of Mn^{II} *versus* Mn^{III} which is influenced by the surrounding ligand environment, with increases from 2.5 to 7.5-fold in r_1 (maximum for Mn^{II}-**CyHBET**, **57** to **58**). The stability of the Mn^{II} oxidation state decreased as the electron-releasing nature of the phenolate substituent increased. The redox potential, and therefore the Mn^{II/III} reversibility, could be tuned over a range of 0.30 V ($E_{\text{III/II}} = 0.27 - 0.57$ V). The Mn^{III} complexes could be reduced by cysteine, the main extracellular reducing agent,²² with faster kinetics promoted by electron-withdrawing phenolate substituents (*eg.* -NO₂).

In a further improvement, Caravan and collaborators⁹⁶ developed a “Janus” ligand, bearing a **HBED**-type binding set to Mn^{III} and a **BPED**-type binding set to Mn^{II} in its structure (**Figure 19**). Upon oxidation or reduction, the **Mn-JED** complex **63/64** (JED = “Janus HBED/BPED”), isomerizes between these two binding modes that stabilize Mn^{III} or Mn^{II}, supporting both states in biological media such as plasma, as opposed to what was observed with the previously developed **HBET-ligand**.⁹⁴ Cyclic voltammetry measurements revealed irreversible oxidation and reduction events, with potentials at 0.91 and 0.01 V vs NHE, respectively. Peroxidase-mediated oxidation of **Mn^{II}-JED** (**63**) to **64** is rapid ($k_{\text{obs}} = 19.1 \text{ s}^{-1}$) in human blood plasma supplied with a steady state of H_2O_2 . However, this Mn^{II/III} interconversion in blood plasma alone or in

the presence of H₂O₂ alone, is very slow or negligible. **Mn^{III}-JED (63)** is also readily reduced by cysteine present in blood plasma. Such redox switching processes provided a 9-fold decrease in relaxivity upon reduction, leading to notable MRI signal changes in T₁-weighted images (**Figure 21**). This system provides much better and faster relaxivity changes than that observed with the best Gd-based peroxidase sensor available, Gd^{III}-DTPA-bis 5HT (**65, Figure 19**).⁵⁸

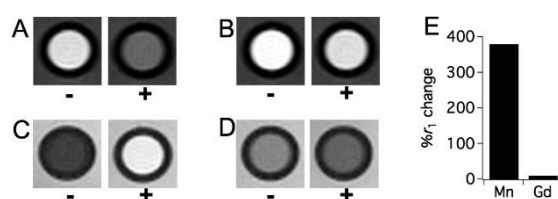


Figure 21. MR phantom images at 4.7 T and RT of water containing 0.5 mM (A, C) **Mn^{II}-JED (63)** or (B, D) **Gd-bis-5HT-DTPA 65** before (–) and after (+) incubation with H₂O₂/peroxidase (45 units/mL). Images acquired (A, C) using a T₁-weighted FLASH sequence or (B, D) with a 325 ms inversion prepulse to generate large positive contrast in the oxidized sample. The high contrast between the samples containing **Mn^{II}-JED (63)** and **Mn^{III}-JED (64)** and the low contrast for the Gd-based system are notable. (E) Percentage r₁ changes after peroxidase-mediated oxidation of the Mn- and Gd-based agents. Reproduced with permission from Ref. 58, Copyright (2016) by American Chemical Society.

Manganese-based nanostructured T₁ MRI CAs have been designed to respond to redox potential variations.⁹⁷ The redox-induced contrast changes are amplified by the large number of redox active ions present in the nanoparticles. The contrast mechanism may result directly from intrinsic changes in the magnetic properties of the Mn cations depending on their oxidation state, or indirectly through changes in integrity and hydration state in the nanomaterial as a whole. A number of Mn-based nanomaterials have been studied, such as manganese dioxide (MnO₂) and other oxides, Mn silicates, Mn phosphates, Mn-doped nanostructures and Mn-containing core-shell nanoparticles. In relation to the Δr₁ performance upon redox activation, two groups can be distinguished. Some show an “on/ON” behavior with a relaxivity increase Δr₁ of 2 to 25-fold upon activation. This group involves manganese silicates, phosphates, hydroxides, Mn^{II}-doped materials, and some of the Mn_xO_y nano structures. In some cases, like those based on

Mn₃O₄ nanostructures,^{98,99} high final r_1 values of 25.1 and 16.1 mM⁻¹ s⁻¹, respectively, have been reported. In the second group, all the structures are based on MnO₂ and display larger Δr_1 upon activation (>50-fold) and, in most cases, they show a real “OFF/ON” behavior, with r_1 values before activation lower than 0.1 mM⁻¹ s⁻¹.

Some examples of *in vivo* application of redox responsive Mn-based CAs using complex nanoplatforms are illustrated in **Figure 22**. A nanoplatform able to combine multimodal imaging and therapy was designed by incorporating MnO₂ NPs stabilized by bovine serum albumin into a nanoscale coordination polymer also containing high-Z element hafnium (Hf) ions and a cisplatin anticancer prodrug. The system is capable of enhancing radiotherapy, to work as a chemotherapeutic agent resulting from the reduction-induced release of cisplatin and enable the non-invasive responsive MRI imaging of tumors *in vivo*¹⁰⁰. **Figure 22 A** shows that 12 h post-administration of these nanostructures, the T₁-weighted image intensity of a region of interest (ROI) localized in the tumor showed a two-fold increase with respect to the baseline. The release of Mn^{II} ions allowed to follow the clearance route of the nanostructures. A significant signal increase observed in the kidneys suggested the renal clearance of Mn. In another example, PEG modified hollow MnO₂ nanoshells co-loaded with a photodynamic agent and the chemotherapy drug doxorubicin were designed to dissociate at lower pH, leading to a remarkable *in vivo* synergistic therapeutic effect and were used to combine diagnosis and therapy.¹⁰¹ The reduction of MnO₂ to Mn^{II} ions led to a strong MRI signal increase in the tumor (up to 4-fold 2 h post-injection), providing a clear difference between tumor and muscle (**Figure 22 B**). In a third example, Mn^{II}-calcium phosphate pH-sensitive NPs with a PEG -shell were used. At low pH, such as in solid tumours, the NPs disintegrates and releases Mn^{II} ions. Binding to proteins increases the relaxivity of Mn^{II} and enhances the contrast.¹⁰² **Figure 22 C** shows 3D T₁-weighted images of a colon tumor after the injection of these Mn-Ca phosphate NPs. In this work, the performance of these NPs is compared to that of clinical (Gd-DTPA) and non-responsive NPs (Mn₂O₃). The results unequivocally show the potential of such responsive systems, with a 70% higher MRI signal *versus* commercial and non-responsive NPs.

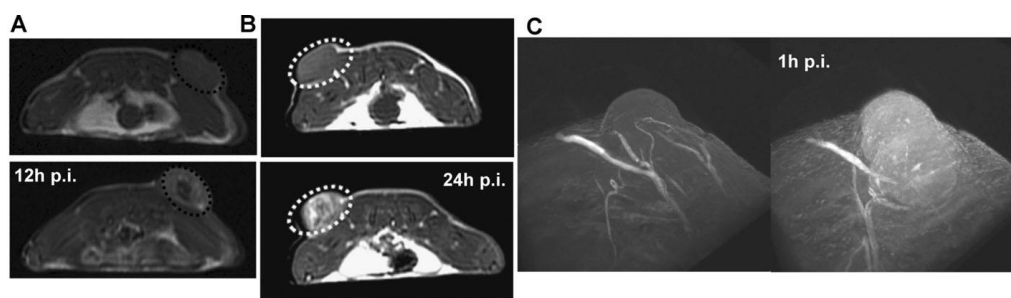


Figure 22. *In vivo* MRI of responsive Mn-based NP CAs: A) Use of MnO₂ NPs stabilized by bovine serum albumin and encapsulated in nanoscale coordination polymers for T₁ imaging of breast tumor in Balb/c mice;¹⁰⁰ B) Use of hollow MnO₂ NPs as T₁-weighted CAs for imaging of breast tumor model in Balb/c mice,¹⁰¹ C) Effect of Mn-calcium phosphate NPs as responsive CAs in 3D T₁-weighted imaging of a colon tumor model in mice. Figure 22A was reproduced with permission from Ref. 100, Copyright (2017) by John Wiley & Sons. Figure 22B was reproduced with permission from Ref. 101, Copyright (2017) by Nature Publishing Group. Figure 22C was reproduced with permission from Ref. 102, Copyright (2016) by Nature Publishing Group.

Other examples involve the use of core-shell based nanoplateforms. A Fe₃O₄@Mn₃O₄/polysorbate behaves as an “on/ON” T₁/T₂ bimodal redox-pH activated agent after reduction with GSH, which simultaneously generates large amounts of magnetically decoupled Mn^{II} ions and allows Fe₃O₄ to interact with the water protons.⁹⁹ Another example is the use of SiO₂@Mn silicate/doxorubicin as an *in vivo* theranostic redox activated “on/ON” T₁ agent with pH responsive delivery of doxorubicin in tumors of animal models.¹⁰³

Other transition metal redox pairs have been also explored in the development of redox-active probes. In the case of copper, while Cu^I (3d¹⁰ configuration) is diamagnetic, Cu^{II} (3d⁹ configuration, S = 1/2) with a small magnetic moment, long (1 – 5 ns range) longitudinal electronic relaxation times and labile water exchange ($k_{\text{ex}} \sim 10^9 \text{ s}^{-1}$), is not a very efficient paramagnetic relaxation agent (*e.g.* Cu^{II}(H₂O)₆ has a $r_1 = 1.4 \text{ mM}^{-1} \text{ s}^{-1}$ at 10 MHz, 298 K).⁷⁵ However, with the advent of better imaging software and higher field strengths, limited relaxivity changes are less prohibitive. Spickett and collaborators¹⁰⁴ (**Figure 23**) studied three series of Cu^{II} macrocyclic frameworks (**66-68**), obtaining the reduced forms by using ascorbic acid as reductant. They observed considerably low Δr_1 changes (0.11-0.44 mM⁻¹.s⁻¹), the highest was reported for the member of the **67** family with X = -(CH₂)₂OH. The ability of the compounds to act as redox sensing CAs requires

that they have redox reversibility between the Cu^{II} and Cu^I forms and redox potentials amenable for biological applications (< 0.80 V). Cyclic voltammetry studies gave E_{II/I} values in the 0.65 – 1.44 V vs. NHE range, with the lowest values for the **67** family. The addition of the pendant arms at the ring nitrogen atoms raised the potential above 1.00 V and made the redox reaction irreversible.

An interesting strategy to overcome the low r₁ associated to paramagnetic Cu^{II} emerged with the use of ¹⁹F MRI, which has favorable NMR properties and no detectable background signal in biological systems, as a tool for detection of the turn-on/turn-off signal in hypoxia environments. Actually, it is known that the presence of paramagnetic metal ions nearby fluorine atoms in a probe will decrease the transverse relaxation time (T₂) and attenuate the MR signal (paramagnetic relaxation enhancement mechanism; PRE).^{105, 106} Bearing that in mind, Que and collaborators¹⁰⁷ designed two fluorinated probes based on Cu^{II}-ATSM (**69**) (ASTM = diacetyl-bis(N⁴-methylthiosemicarbazone), whose ⁶⁴Cu^{II} analogue is widely used for PET imaging of hypoxia cells. Introducing one or two CF₃ groups near the copper ion (**Cu-ATSM-F₃** (**70**) and **CuATSM-F₆** (**71**)) (**Figure 23**), they found that, as theoretically predicted, the ¹⁹F signal was turned off. The Cu^I/Cu^{II} redox properties of complexes 53-55 were studied by cyclic voltammetry in DMF solution, showing a reversible redox process with E_{II/I} values of -0.39 V (**69**), -0.32 V (**70**) and -0.25 V (**71**) vs. NHE. As a value of E_{II/I} below – 0.26 V is required for differentiation of hypoxic and normoxic cells by ATSM type scaffolds,¹⁰⁸ **71**, had more potential than **72** to exhibit selectivity towards hypoxic cells, and was chosen for further studies. As a proof of concept, the reduction of **Cu^{II}-ATSM-CF₃** (**72**) to **Cu^I-ATSM-CF₃** was achieved with sodium dithionite, and the ¹⁹F signal was turned on in the reduced form. An *in vitro* ¹⁹F MRI study with MCF-7 (human breast cancer cells) was also performed in normoxic and hypoxic conditions, showing the selective switch on of the MRI signal in the hypoxic cells, thus confirming the potentiality of this probe to act as reporter to track hypoxic cells using ¹⁹F MRI. Following the previous work, the Que group¹⁰⁹ introduced a polyethylene glycol linker with variable length between the ATSM backbone and the fluorinated group (**Cu-ASTM-PEG_n-F₉**, n = 1-4, **73**, **Figure 23**) allowing to modulate the probe lipophilicity and making it more biocompatible, the Cu^{II}/Cu^I redox properties and the ¹⁹F relaxivity. Reduction potentials of -0.39 V obtained for the complexes with n = 3, 4 indicate sufficient separation between Cu^{II} and the fluorinated moieties to provide redox properties for hypoxia selectivity. This allowed, for the first time, the quantitative evaluation of the ¹⁹F PRE effect as a function of the distance

between Cu^{II} and F atoms in the Cu-ATSM derivatives, showing a 129-fold T₂-decrease between the reduced and oxidized forms of **73**. The potential utility of these complexes for sensing reductive environments was demonstrated using both ¹⁹F MR phantom imaging and ¹⁹F NMR, including experiments in intact live normoxic and hypoxic MCF-7 cells.

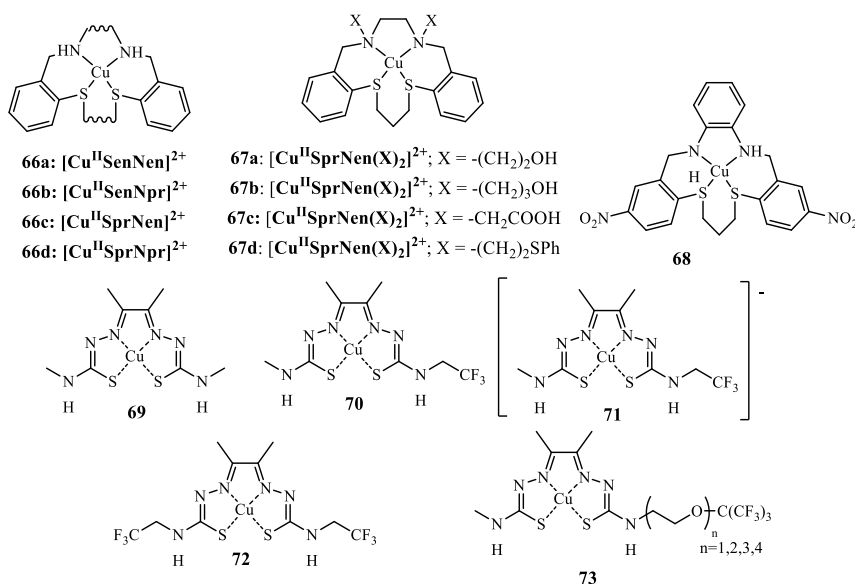


Figure 23. Copper redox probes: a) Cu-N₂S₂ aromatic substituted macrocyclic derivatives (**66-68**);¹⁰⁴ b) CuATSM derivatives (**69-73**).^{107,109}

Cobalt is a transition metal with two main biologically useful redox states, Co^{II} and Co^{III}, which have quite different magnetic properties. Co^{II} (3d⁷) complexes can have low spin configuration (S=1/2), predominantly in planar or square pyramidal complexes, with relatively long electronic relaxation times (T_{1e} in the 0.1-1 ns range), leading to broad and strongly shifted NMR lines. In the more common high spin configuration (S=3/2), paramagnetically shifted NMR lines appear with linewidths that depend on the symmetry of the complex: signals are broader for octahedral (shorter T_{1e} of 1-5 ps) than for tetrahedral complexes (longer T_{1e} around 1 ps). High spin Co^{III} (3d⁶) complexes (S = 3) have quite short T_{1e} around 1 ps, leading to relatively broad and shifted resonances. On the other hand, low spin Co^{III} complexes (S=0) are diamagnetic, leading to unshifted and sharp NMR signals.⁷⁵ Thus, the Co^{II/III} redox couple provides opportunities for designing redox responsive probes, due to the paramagnetic nature of Co^{II} and the tendency for Co^{III} complexes to be low spin and diamagnetic. These probes can be designed to switch between these states in a large tunable electrochemical window.

A $\text{Co}^{\text{II}}\text{NODA-CF}_3$ (**74**) (**Figure 24**) probe was recently developed and studied as a potential redox-responsive contrast agent.¹¹⁰ ^{19}F T_1 and T_2 relaxation times were significantly shortened in the reduced form causing NMR signal attenuation. Upon oxidation to the Co^{III} form, for instance using H_2O_2 , a chemical shift change and an enhancement of the ^{19}F NMR signal was observed (turn-on effect). This metal redox-based sensing mechanism could enable the MRI detection of multiple radical oxygen species (ROS) associated with oxidative stress.

Elevated levels of ROS and peroxidase expression are often associated with inflammation and inflammatory diseases. Que *et al* developed two novel Co^{II} complexes (**75** and **76**, **Figure 24**) that can be used to detect oxidative activity associated with inflammation using ^{19}F MRI.¹¹¹ These agents display a large change in ^{19}F shift upon oxidation from Co^{II} to Co^{III} , facilitating selective visualization of both species using chemical shift selective pulse sequences. The large shift difference results from the large magnetic anisotropy of the high spin Co^{II} complexes. The different reactivity of the two agents **75** and **76** allowed detection of either H_2O_2 production and/or the activity of peroxidase enzymes, providing two useful procedures for ^{19}F MR imaging of oxidative events associated with biological inflammation.

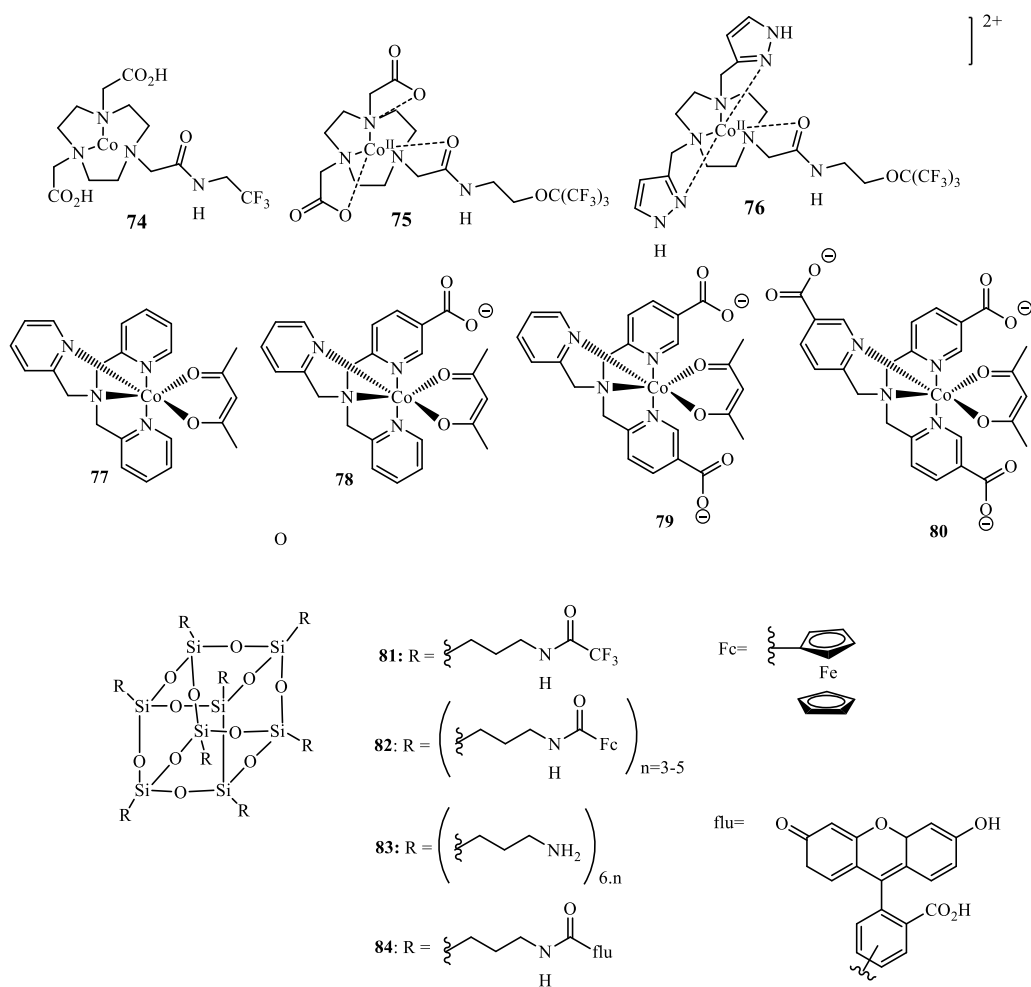


Figure 24. Cobalt and iron redox probes: a) CoNODA-CF₃ (**74**), **75** and **76**; b) TPA0 = [Co(III)TPAacac] (**77**), TPA1 = [Co(III)TPA(COO⁻)acac] (**78**), TPA2 = [Co(III)TPA(COO⁻)₂acac] (**79**), TPA3 = [Co(III)TPA-(COO⁻)₃acac] (**80**); c) Fc-POSS (**82**) and flu-POSS (**84**).

Cobalt bio-reductive pro-drugs, such as Co^{III}TPAacac, (TPA0 = cobalt(III)tris((2-pyridylmethyl)amine)acetylacetonate (**77**), **Figure 24**), which selectively release toxic payloads upon reduction to the Co^{II} form in hypoxic cells of tumours, have shown great promise as anticancer agents. The cobalt complex can work as a turn-on redox responsive MRI CA upon reduction from diamagnetic Co^{III} to paramagnetic Co^{II}, allowing monitoring the bio-reduction process. Co^{III}TPAacac was found to be an efficient *in vitro* hypoxia-responsive MRI contrast agent.¹¹² Previous studies on cobalt TPA complexes as potential pro-drugs have explored optimization by varying the number of pyridyl carboxylate groups, and hence their overall charge. The redox and relaxivity properties in solution of four of such complexes (TPAx, x = 0-3, **77-80**, **Figure 24**) were further

investigated.¹¹³ Their redox potentials, $E_{III/II}$, were found in the -0.635 V to -0.491 V range. Increasing the number of carboxylate groups led to an increase in the stabilization of the Co^{III} state, but only TPA0 was a reversible redox switch. The complexes showed, as expected, very small r_1 and r_2 relaxivities ($Co^{III}(H_2O)_6$ has $r_1 = 0.18$ $mM^{-1}s^{-1}$ at 10 MHz and 298 K⁷⁵ with $r_2/r_1 \sim 4.0$ to 8.7). The TPAX probes were taken up by tumour spheroids, as shown by LA-ICP-MS. MR images of the tumour spheroids previously incubated with TPAX showed that TPA3 was selectively activated and accumulated in the hypoxic cells. The combined study of the TPAX complexes using LA-ICP-MS and MRI enabled the evaluation of their diagnostic utility for developing cobalt pro-drugs in tumour spheroids.¹¹³

The two main biological redox states of iron, Fe^{II} and Fe^{III} , also present a variety of magnetic properties. Fe^{II} ($3d^6$) octahedral complexes in the low spin state are diamagnetic ($S=0$), while the paramagnetic high spin state ($S = 2$), with very short T_{1e} (0.1-1 ps range), leads to relatively sharp and highly shifted proton resonances. Its complexes have very small relaxivities (e.g. $Fe^{II}(H_2O)_6$ has $r_1 = 0.88$ $mM^{-1}s^{-1}$ at 10 MHz and 298 K). Fe^{III} ($3d^5$) is paramagnetic in the two spin states available. The high spin state ($S = 5/2$), with relatively long T_{1e} (0.01-1 ns), induces large paramagnetic shifts and broadenings and causes relatively high proton relaxivities (e.g. $Fe^{III}(H_2O)_6$ has $r_1 = 13$ $mM^{-1}s^{-1}$ at 10 MHz and 298 K). The low spin state ($S = 1/2$) causes large shifts but very small relaxation effects, due to the very short T_{2e} values (0.1-1 ps).⁷⁵ Thus a redox switch involving the high spin Fe^{III} /low spin Fe^{II} states provides the ideal turn-off contrast effect.

Superparamagnetic iron oxide nanoparticles (SPION) have been extensively explored as T_1 MRI contrast agents, but so far not so much as redox responsive probes due to the difficulty of modulating the contrast effect.^{114, 115, 116} Concerning other types of Fe-containing nanoparticles, a water soluble dendrimer containing a cubic octameric silesquioxane of 1.5 nm diameter with covalently attached ferrocene units as redox switching modules and trifluoroacetyl groups as ^{19}F NMR reporters (**Fc-POSS (82)**)¹¹⁷ (**Figure 24**) was explored as a reversible redox active probe. Upon oxidation of the ferrocene (Fe^{II}) moiety to the ferrocenium cation (Fe^{III}) by ammonium persulfate (APS), the relaxation of the nearby ^{19}F atoms was accelerated via PRE, and, as a result, the ^{19}F NMR signal of the probe was decreased. This process was reversible. A bimodal ^{19}F NMR/fluorescence probe (**Flu-POSS (84)**, **Figure 24**) obtained by further derivatization with fluorescein, also based on the difference of the fluorescence quenching ability between ferrocene and ferrocenium cation, was also constructed.

4.2 T₂/T₂*contrast agents

The blood oxygen level-dependent MRI (BOLD-MRI) method is based on the reversible change between the diamagnetic oxyhemoglobin under high pO₂ and paramagnetic deoxyhemoglobin under low pO₂.¹¹⁸ Therefore, the variations in oxy/deoxyhemoglobin levels in the bloodstream changes the water T₂ and particularly T₂* relaxation, allowing blood oxygenation levels to be measured by MRI. This capacity of tracing blood oxygenation allows hypoxia to be detected in humans.¹¹⁹ BOLD effects have been proven to correlate well with standard pO₂-measurement strategies.¹²⁰ Recent studies have shown the feasibility of the BOLD-MRI technique for monitoring oxygenation fluctuations and differentiate hypoxia from normoxia in tumors.^{121,122,123} Such a technique has several advantages, such as non-invasiveness and real-time detection of the variations in oxygenation levels in different organs and tissues.

The number of reports using superparamagnetic iron oxide nanoparticles (SPION) as redox or hypoxia sensitive T₂ CAs has been relatively scarce.¹²⁴ A general disadvantage of T₂/T₂* MRI agents is their negative contrast effect associated with a water signal intensity decrease.¹²⁵ Also, most of them are “always ON” systems whose negative MR contrast effect is difficult to modulate through interaction with target cells or biomarkers, resulting in poor target-to-background ratios. However, some recent examples involving core-shell systems have appeared where the modulation of the NP shell effectively exposed the SPION to the water molecules. An example of this strategy is the core-nanostar shell based Fe₃O₄@Mn₃O₄/polysorbate nanoplatfrom (RANS),⁹⁹ described in the previous section, that behaves as an “on/ON” T₂ redox-pH due to the pH induced shell peeling effect that allows the core Fe₃O₄ to interact with the water protons, leading to a large Δr₂ increase from 92.5 to 258.6 mM⁻¹ s⁻¹, with a notable dual T₁/T₂ response (**Figure 25**).

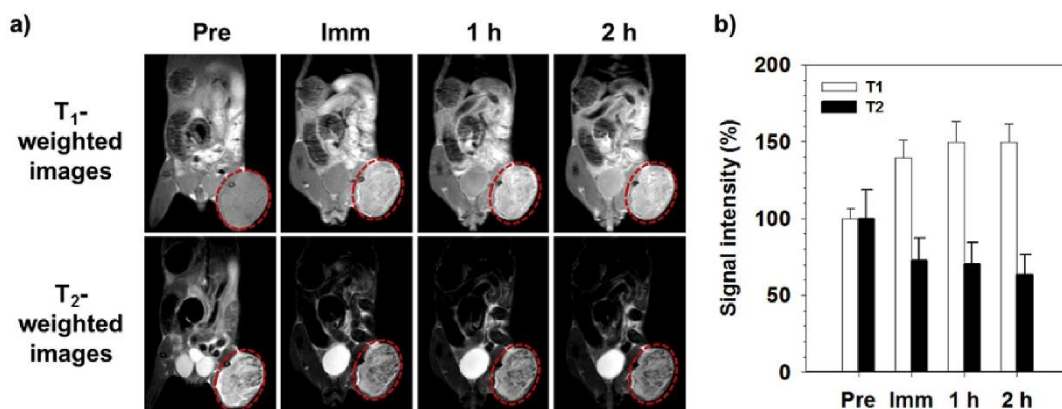


Figure 25. a) In vivo T₁- and T₂-weighted MR images of MKN-45 tumor-bearing mice obtained before (Pre) and after (immediate, 1 h, and 2 h) the intravenous injection of the RANS probe. b) relative T₁ and T₂ signal intensities (%) of tumor sites (red dotted circle) at different time points. Reproduced with permission from Ref. 99, Copyright (2016) by Elsevier.

Other relevant examples involve redox responsive multifunctional theranostic systems fabricated for efficient cancer therapy, some of which are SPION-based core-shell nanoparticles. One of such examples are DSSCe6@Fe₃O₄ NPs obtained by loading Fe₃O₄ nanoparticles in redox-responsive chlorin e6 (Ce6)-conjugated dextran nanoparticles for near-infrared (NIR)/magnetic resonance (MR) dual-modality imaging. The fluorescence signal of Ce6 in the obtained NPs could turn “ON” from a self-quenching state in a reductive intracellular environment, through cleavage of the disulfide linker between Ce6 and dextran, thus releasing Ce6 from the NPs. These NPs also showed a high transverse relaxivity $r_2 = 194.4 \text{ mM}^{-1}\text{s}^{-1}$ (at 3 T). T₂-weighted MRI of *in vitro* labeled MCF-7 cells and *in vivo* BALB/c mice bearing MCF-7 tumors showed efficient tumor uptake of the NPs upon subcutaneous injection and negative contrast, confirming their potential as an efficient redox responsive CA in T₂-weighted MRI.¹²⁶

5. Redox responsive PARACEST MRI contrast agents

PARACEST agents are paramagnetic lanthanide or transition metal complexes containing an inner-sphere water molecule and/or –NH, –OH or –SH protons on the ligand which are in slow exchange with bulk water.^{27,28,29,127} These protons are subject to large dipolar and/or contact hyperfine shifts induced by the paramagnetic ion (which should have limited relaxation effect). The paramagnetic induced shifts (and the relaxation effect) are dependent on the redox state and magnetic properties of the metal ion as well as on the geometric orientation and distance of the proton with respect to the metal ion.⁷⁵ In this section, we will review some recent examples of lanthanide and transition metal complexes explored as PARACEST probes responsive to biologically important redox active molecules.

5.1. Lanthanide^{III} PARACEST agents

Similarly to the T₁-agents described previously, the PARACEST effect can be modulated by redox active ligands or by changes of the metal oxidation states.

5.1.1 Ln-PARACEST probes based on redox active ligands

In principle, all paramagnetic Ln^{III} ions, except the highly relaxing Gd^{III}, can be suited for PARACEST probe design. Nevertheless, due to the sometimes extremely large proton induced dipolar shifts, Eu^{III} complexes formed with (DOTA)-tetraamide ligands have been the most popular. Among all Ln^{III} complexes, (DOTA)-tetraamide derivatives show the slowest exchange of the metal-bound water molecule, with an extreme sensitivity to electronic effects of the ligand pendant arms and changes in the coordination geometry.¹²⁸ The most commonly used parameters which are modulated to provide redox responsiveness to PARACEST agents are the proton (water) exchange rate k_{ex} and T_1 (eq.6).

Sherry and collaborators¹²⁹ prepared a series of Eu^{III}-DOTA tetraamide complexes through selective trialkylation of cyclen with bromoacetylglycinate, followed by alkylation of the remaining nitrogen with different *p*-substituted bromoacetylglycinate and complexation with Eu^{III}(OTf)₃. The water exchange kinetics and the corresponding CEST properties of the complexes were highly influenced by changes in the electronic properties of the ligand. In an early proof-of-principle example, it was shown that the Eu^{III} complex bearing a *p*-nitro group (*p*-NO₂) (**85**, **Figure 26**) exhibited a 30% stronger CEST signal intensity than the reduced *p*-amino form (*p*-NH₂), obtained by Pd^{II}-catalyzed hydrogenation. This was attributed to a reduction in the water residence time, τ_M resulting from the replacement of the electron-withdrawing *p*-NO₂ by the electron-donating *p*-NH₂ group. However, the main drawback of this probe is that its redox potential was outside the biologically relevant range.

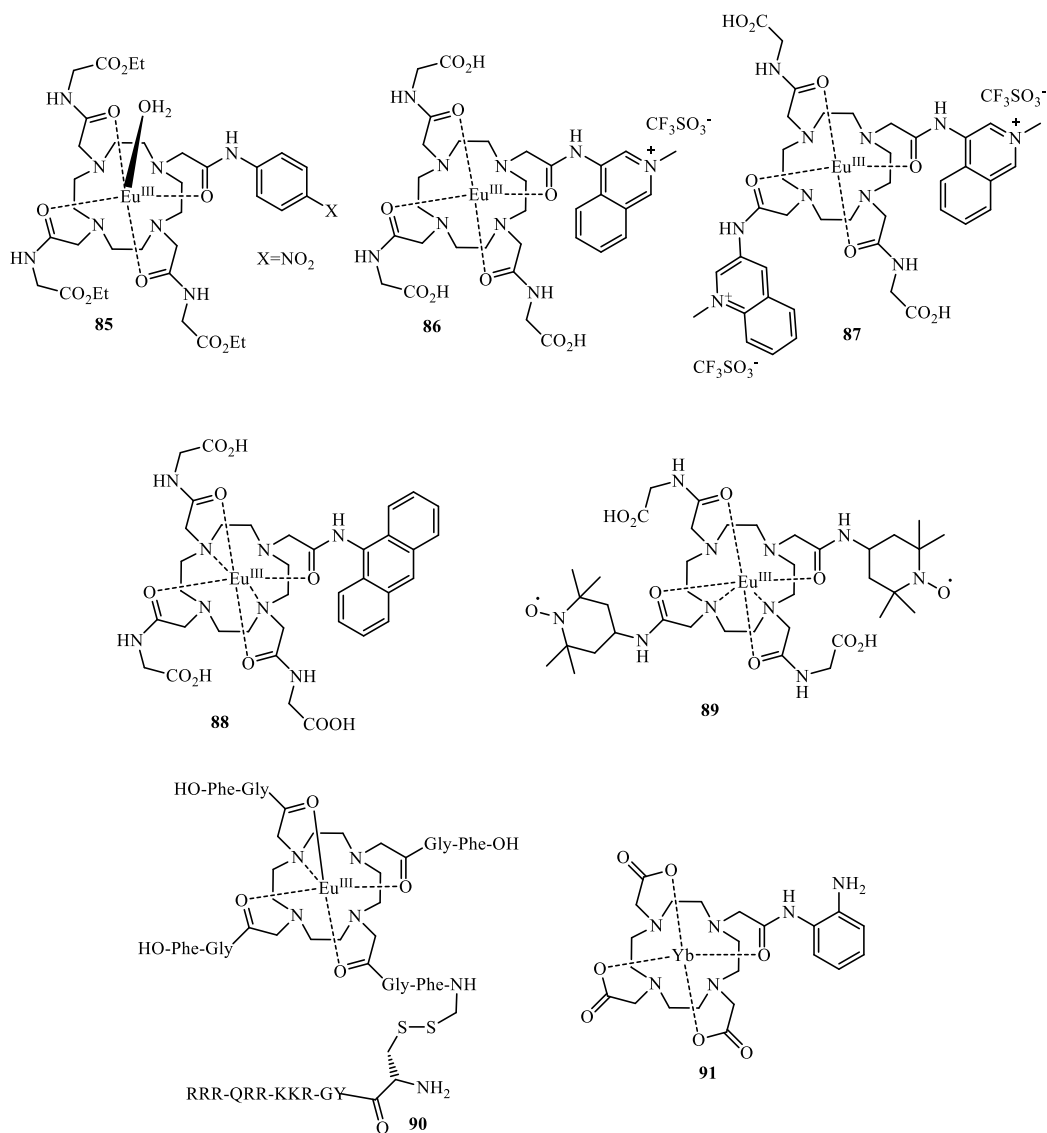


Figure 26. Redox responsive, Ln^{3+} PARACEST agents based on ligand redox reaction.

Following this initial example, biologically more relevant agents have been developed. The same authors¹³⁰ created a new probe by mimicking the NADH/NAD⁺ biological redox system. The Eu^{III} -DOTA tetraamides containing one or two N-methylquinolinium moieties as redox active functional groups (**86**, **87**, **Figure 26**) were nearly CEST-silent (turn-off) in the oxidized form, but were turned on (increased CEST signal) after reduction to the dihydroquinoline derivative by added NADH (simulating hypoxia conditions). The CEST increase observed was the largest (15%) for Eu^{III} 2Q-DOTAM-(gly)₂ (**89**) of the two complexes, with a shift of 7 ppm (**Figure 27**). The larger CEST signal upon reduction arose from the slower, more optimal water exchange ($\tau_{\text{M}} =$

90 ms) of the reduced relative to the oxidized form. However, the redox couple was not reversible, as shown by cyclic voltammetry.

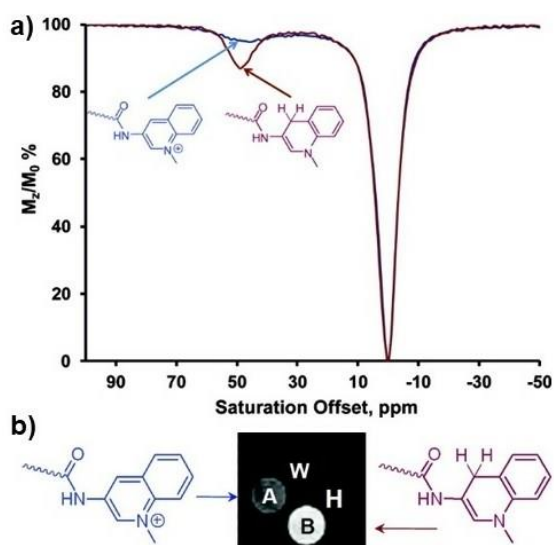


Figure 27. a) CEST spectra of the oxidized (blue) and reduced (red) forms of **89**; b) PARACEST images acquired for a phantom containing four tubes: (A) **89** (20mM) in HEPES buffer (pH 7, 0.25M); (B) **89** (20mM) plus 2 equiv of β -NADH in HEPES buffer (pH 7, 0.25mM); (W) water; (H) HEPES buffer at pH 7. Reproduced with permission from Ref. 130, Copyright (2012) by American Chemical Society.

Another example of redox modulation of k_{ex} on PARACEST probes is a Eu^{III} DOTAM-(gly)₃ derivative (**88**, **Figure 27**) bearing a 9-anthryl pendent group which can react with singlet oxygen to lead to a more electron-withdrawing anthryl endoperoxide functionality.¹³¹ This reaction leads to 12 % decrease and the shift of the CEST peak, in proportion to the concentration of $^1\text{O}_2$. The signal decrease results from the increased τ_M from 90 μs to 137 μs upon reaction. Such a PARACEST probe might be useful for imaging and quantification of photodynamic therapy agents that produce singlet oxygen.

The PARACEST signal can also be redox-modulated by changing the longitudinal relaxation time (T_1) of the bulk water pool. This strategy was proposed by Kovacs et. al¹³² based on prior knowledge that nitroxide radicals are capable of shortening T_1 of the bulk water protons due to the paramagnetic effect of the single unpaired electrons. The authors designed a Eu^{III} -DOTA tetraamide complex containing two nitroxide radicals (**89**, **Eu^{III}DOTANO**, **Figure 26**). The CEST signal of **89** was very weak due to T_1 shortening of bulk water protons by the paramagnetic nitroxides. However, reduction with ascorbic acid to a diamagnetic bis(hydroxylamine) derivative restored the T_1 value to the typical

range for Eu^{III} diamagnetic ligand complexes, significantly increasing (20%) the CEST signal, without affecting the τ_{M} value (85 *versus* 81 ms after reduction). Healthy mice were intravenously injected with **89** and *in vivo* PARACEST MRI was realized in the bladder, given the efficient renal filtration of the probe. No CEST signal was observed despite the slightly brighter T_1 -MR images obtained in the presence of the oxidized agent **89** (**Figure 28, a-f**). However, upon injection of *L*-ascorbic acid, **89** was reduced *in vivo*, and the diamagnetic hydroxylamine derivative formed generated an enhanced CEST signal in the bladder (**Figure 28, g-i**). This and similar probes could have potential applications as PARACEST reporters of hypoxia and ischemia.

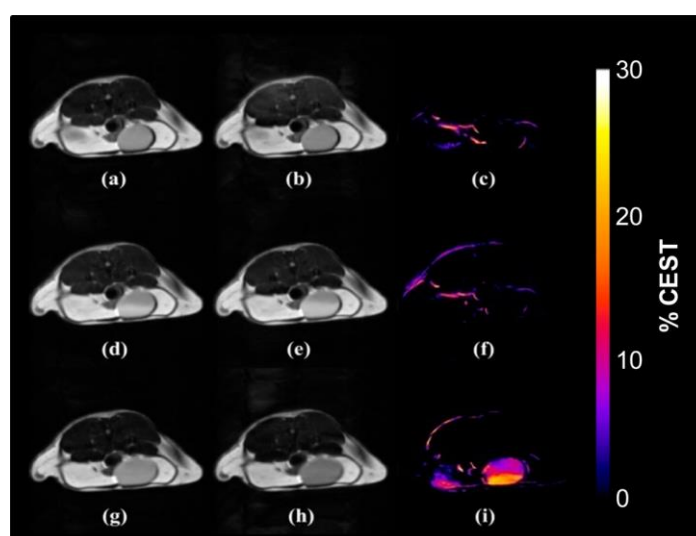


Figure 28. CEST images of the bladder of a female black-6 mouse. Top row: pre-injection spin-echo images with saturation at (a) -43 ppm and (b) $+43$ ppm. The CEST difference image (c) was obtained by subtracting on-resonance image (b) from the off-resonance image (a). Middle row: similar off-resonance (d), on-resonance (e), and difference (f) image taken 10 min after injection of Eu^{III} complex **89** into the same mouse *via* tail vein catheter. Bottom row: off-resonance (g), on-resonance (h), and difference (i) image taken 1 h after an intravenous dose of *L*-ascorbic acid. Reproduced with permission from Ref. 132, Copyright (2013) by American Chemical Society.

A Eu^{III} -DOTAM tetraamide based agent tethered with a cell-penetrating TAT peptide (transactivator of transcription peptides)¹³³ was obtained by conjugating one pendant arm of Eu^{III} -DOTAM-(Gly-Phe-OH)₄ to a TAT polypeptide containing a disulfide bond (**90, Figure 26**). Such a conjugate is expected to undergo reductive disulfide cleavage by intracellular GSH. Thus the Eu^{III} -TAT conjugate could serve as a GSH intracellular sensor, since the cleaved Eu^{III} -DOTAM agent would be trapped inside the cell. Despite the promising *in vitro* CEST data, *in vivo* studies have not been reported.

An Yb^{III}-DO3A orthoaminoanilide derivative (**Yb^{III}-DO3A-oAA, 91, Figure 26**) was investigated as an irreversible redox responsive PARACEST agent.¹³⁴ It exhibited two CEST effects at -11 ppm and +8 ppm, which were assigned to chemical exchange from the amide and amine functional groups, respectively. The probe underwent an irreversible dimerization that converted the two aromatic amino groups to a triazene bridge in the dimer in the presence of NO and O₂. This led to the irreversible disappearance of both PARACEST effects on *in vitro* MR images, as well as to a positive contrast in T₁ weighted images. Such a system could detect tissue NO and/or pO₂ changes *in vivo*.

Aime and co-workers have developed highly sensitive lipoCEST agents based on liposomes loaded with high concentrations of paramagnetic Ln-based shift reagents.^{30,135,136} These agents show two water peaks, one from extraliposomal bulk water, and another from intraliposomal water, containing a large number of exchangeable protons with a limited exchange rate across the lipid membrane. Selective saturation of the intraliposomal resonance results in a decrease in the signal of extraliposomal water due to CEST. Terreno and collaborators¹³⁷ reported a redox active dual T₁-CEST spherical liposomal agent with a shift reagent (Tm-DOTMA, DOTMA = 1R, 4R, 7R, 10R)- α , α' , α'' , α''' -tetramethyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) in the aqueous core and Gd^{III}-DO3A complexes on the external surface conjugated to 3-(2-pyridyl)dithiopropionate (PDP), which contains a redox-cleavable disulfide bond. (**LipoCESTS-SGd, 92, Figure 29**). As expected, by performing MR images at 7T and 37°C, a good T₁ contrast but no CEST effect was observed (**Figure 30**). Reduction of the S-S linkage using TCEP released the liposome bound Gd^{III} complex, decreasing the T₁ relaxation of the bulk water pool (Δr_1 increase of 20%) and consequently restoring the lipoCEST signal.

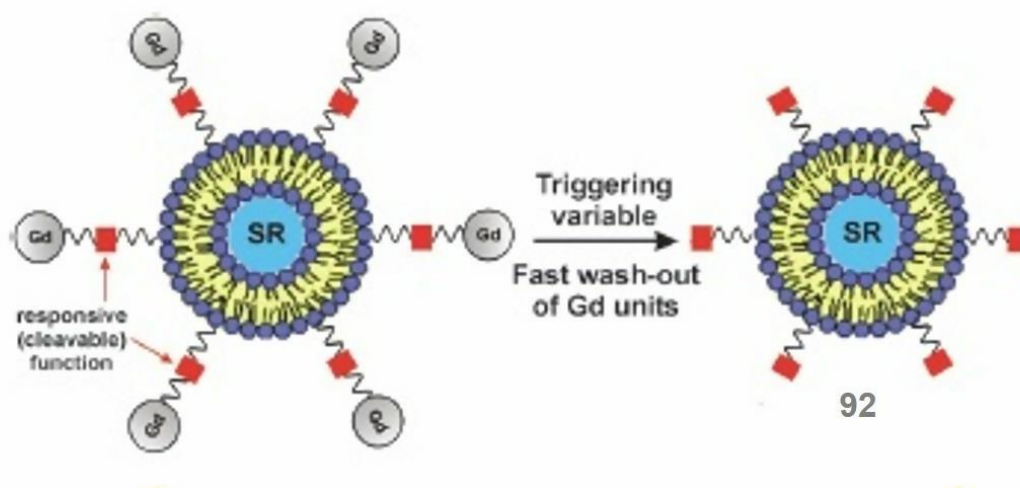


Figure 29. A redox responsive LipoCEST agent (**92**). Reproduced with permission from Ref. 137, Copyright (2011) by Royal Society of Chemistry.

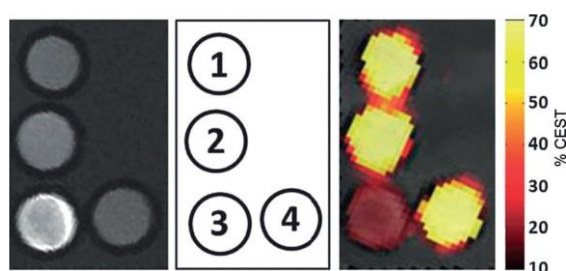


Figure 30. MR images of a phantom containing: (1) LipoCEST-PDP, (2) LipoCEST-SH, (3) **92**, and (4) sample 3 after reduction with TCEP and removal of the detached Gd^{III} -DO3A-SH complex. Left: T_1 -weighted images; right: CEST map upon irradiation at 3.5 ppm overlaid on a T_2 -weighted image of the phantom. The MR experiments were performed at 7 T and 37°C . Reproduced with permission from Ref. 137, Copyright (2011) by Royal Society of Chemistry.

5.1.2 Ln-PARACEST probes based on redox active metal centers

In the previous examples, the PARACEST change was due to redox transformation of the ligand. PARACEST redox probes may also rely on the modulation of the metal redox state. In this class, the $\text{Eu}^{\text{II/III}}$ pair is ideally suited for the design of dual T_1 /PARACEST redox probes. As described above (section 5.1.1), Eu^{II} has efficient T_1 relaxation properties, which are similar to those of Gd^{III} , and it is easily oxidized by O_2 under physiologically relevant conditions to Eu^{III} , a very poor T_1 relaxer inducing large hyperfine shifts suitable for PARACEST.

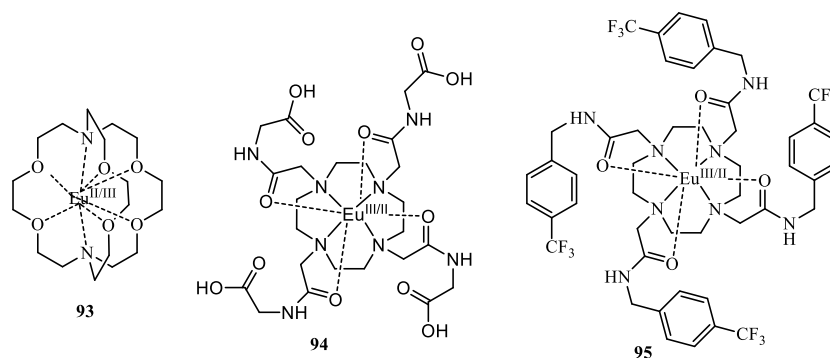


Figure 31 - PARACEST redox contrast agents based on metal redox reactions.

Allen and collaborators¹³⁸ reported an oxidative responsive T_1 /PARACEST agent based on the encapsulation of the $\text{Eu}(2.2.2)^{\text{II}}$ cryptate **36**, previously described as a T_1 redox probe,⁷⁵ into liposomes (**93**, **Figure 31**). Positive T_1 contrast enhancement was observed in the original Eu^{II} system, while PARACEST was observed after oxidation of Eu^{II} to Eu^{III} upon diffusion of air oxygen into the buffer and across the liposome membrane, which also quenched the T_1 effect. *In vitro* MR images of phantoms confirmed these observations.

Allen and collaborators¹³⁹ assessed the potentiality of **94** (also known as Eu^{II} -DOTAM(gly)₄) (**Figure 31**) as a redox responsive T_1 /PARACEST agent. The authors presented electrochemical, luminescence, electron paramagnetic resonance, UV-visible, and NMR spectroscopic data demonstrating complete redox reversibility between the 3+ and 2+ oxidation states. The Eu^{II} -analogue showed kinetic stability comparable to clinically approved Gd^{III} -containing complexes. The well-known Eu^{III} derivative **94** had a CEST signal at -55 ppm which disappeared upon reduction to the Eu^{II} analogue due to T_1 shortening of the water protons. With re-oxidation, the authors observed regeneration of the initial CEST signal, confirmed by *in vitro* CEST MRI. The time course of oxidation of **94** was studied *in vitro* and *in vivo* in healthy male mice by T_1 -weighted and CEST imaging (**Figure 32**).¹⁴⁰ The signal enhancement in T_{1w} images at the injection site decreases over ~15 min, while the CEST signal remained evident over a period of about 20 min before disappearing. This system was also studied as a bimodal oxidation-responsive contrast agent for MRI and PAI (photoacoustic imaging). PAI contrast enhancement was observed in the +2 but not in the +3 oxidation state.¹⁴¹

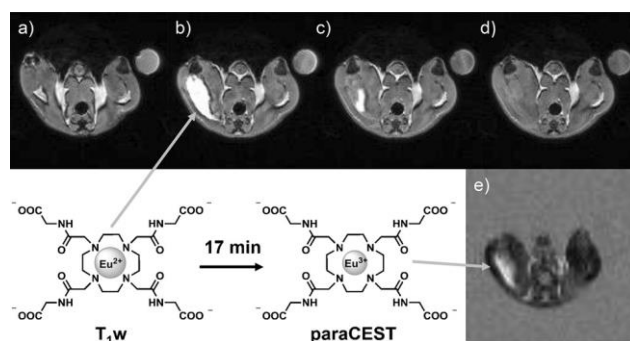


Figure 32. T_{1w} and CEST imaging of an intramuscular injection of Eu^{II} complex **94** into the thigh muscle of a healthy male C57BL/6J mouse at $B_0=9.4$ T. T_{1w} images at a) pre-injection, b) 5 min, c) 12 min, d) 16 min and e) the CEST image at 17 min. Reproduced with permission from Ref. 140, Copyright (2016) by John Wiley and Sons.

Finally, Ratnakar and collaborators^{140,142} performed an electrochemical study of the $\text{Eu}^{\text{III/II}}$ redox couple in complexes with different numbers of glycinamide and acetate pendant arms. This study revealed positive shifts in the $\text{Eu}^{\text{III/II}}$ redox potentials with each replacement of a carboxylate with a glycinamide pendant arm in the ligand scaffold. Among all the water-soluble complexes, the one containing four glycinamide coordinating pendant arms (**94**) presented the less negative redox potential and, therefore, the highest oxidative stability.

The combination of T_1 -weighted, PARACEST and ^{19}F MRI via multimodal contrast agents has been proposed and studied *in vitro* in phantoms and *in vivo* in mice.¹⁴³ For $\text{Eu}^{\text{III}}\text{DOTA-CF}_3$ (**95**, **Figure 31**),¹⁴⁴ Allen and collaborators observed ^{19}F and CEST signals in *in vitro* MR images, but no T_1 -weighted enhancement relative to the buffer. For the corresponding reduced Eu^{II} derivative, no ^{19}F and CEST signals were observed, but a 2-fold signal increase was detected in T_1 -weighted images. This redox-dependent behavior was confirmed with T_1 -weighted MRI and ^{19}F -MRS *in vivo* in mice upon peritoneal injection of the Eu^{II} complex.

5.2. Transition metal ion PARACEST agents

An efficient PARACEST probe requires a metal ion which induces large paramagnetic shifts, but decreases the T_1 relaxation time of bulk water protons as little as possible. This requires the electronic relaxation time of the metal ion, T_{1e} , to be in the picosecond range.⁶⁸ Besides Ln complexes described above, transition metal ions also

have magnetic properties capable of producing extremely large hyperfine shifts, of both dipolar and contact origin, and they often induce strong line-broadening effects. As they can exist in different oxidation (typically $M^{III/II}$) and spin states which determine their shift and relaxation properties, they offer advantages for the design of redox responsive PARACEST agents. The spin states depend on the ligand field of the complex, thus the magnetic and redox properties can be optimized by appropriate ligand design, which led to their consideration for PARACEST. Morrow and collaborators have recently developed several Fe^{II} , Co^{II} and Ni^{II} complexes with exchangeable -NH or -OH ligand protons and demonstrated their potential as PARACEST MRI contrast agents (**Figure 33**).^{36,38,145}

The choice of both the macrocyclic framework and the pendent arms is very important to tune the magnetic properties of the $M^{III/II}$ states, to ensure high thermodynamic stability and kinetic inertness of the complexes in aqueous solution, as well as to tune their reduction potential to biologically relevant values. For the divalent metal ions considered here, azamacrocycles including 1,4,7-triazacyclononane (TACN), 1,4,7,10-tetraazacyclododecane (CYCLEN), 1,4,8,11-tetraazacyclotetradecane (CYCLAM), and 1,4,10-trioxa-7,13-diazacyclopentadecane have been considered. A variety of three (or two) groups have been appended to TACN derivatives originating six (or five) donors for hexadentate (or pentadentate) coordination, creating octahedral, pseudo-octahedral or five-coordinate complexes of different geometries with those metal ions. The ligands (**Figure 33**) include pendants such as the *N*-donor pyridyl, imidazolyl, amino or benzimidazolyl or the *O*-donor amido, hydroxy or carboxy groups. The CYCLEN and CYCLAM macrocycles, with different ring sizes and four pendant groups, provide eight coordination sites and the possibility of inner-sphere water coordination. The coordination cages of different sizes might optimally suit for binding cations of various size. **Figure 33** shows that the four pendants used for these tetraaza rings provide, in all cases, *O*-donor amido (**107**, **108**, **110**) or hydroxyl (**109**, **111**) groups. The 1,4,10-trioxa-7,13-diazacyclopentadecane, with two nitrogen and three oxygen metal coordinating atoms in their 15-membered rings are complemented by two *N*-donating (amino, **113**) or *O*-donating (amido, **112**) pendants, providing a maximum heptacoordination. The sepulcrates (**114** and **115**) form a very inert cage with an octahedral coordination sphere provided by six amino groups.

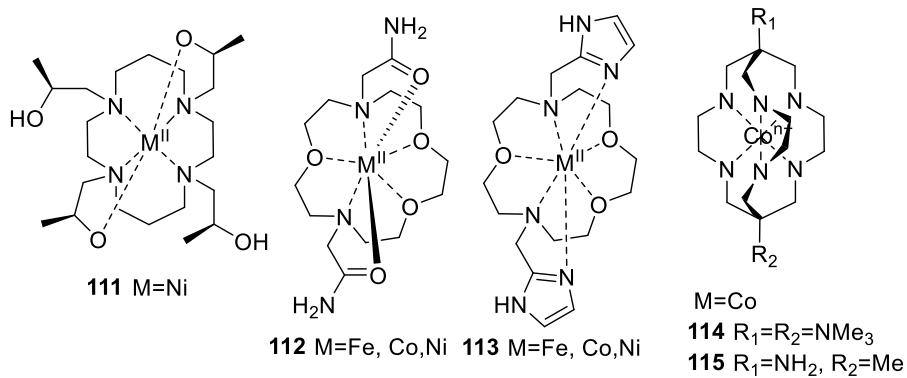
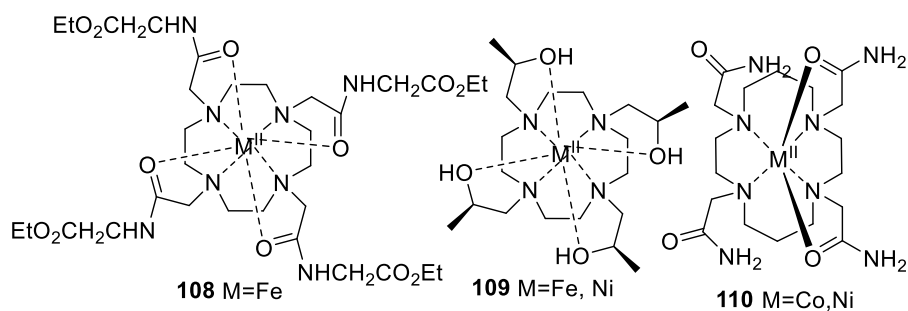
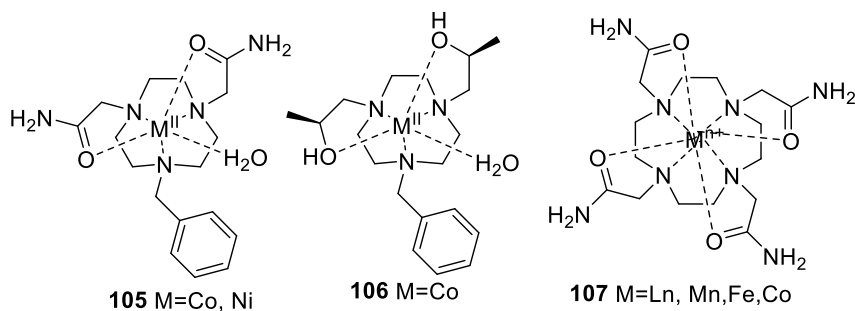
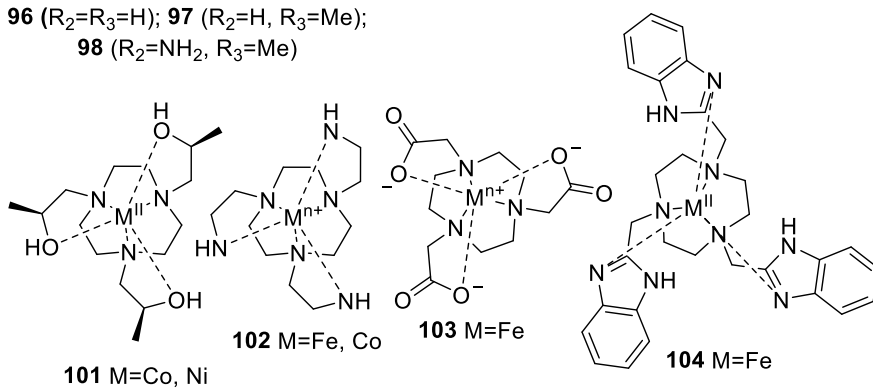
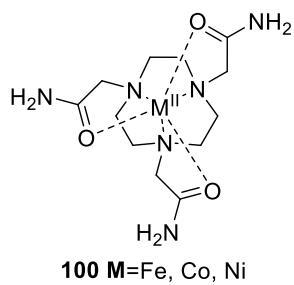
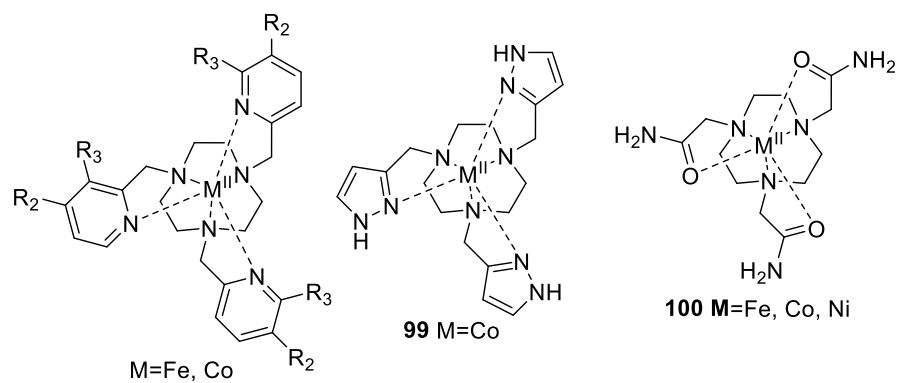


Figure 33. Macrocyclic and aza-cage transition metal complexes of interest as redox PARACEST probes.

Concerning the Fe^{III/II} pair, among the four possible redox and spin states, high spin octahedral Fe^{II} complexes are ideal for PARACEST because the paramagnetic center induces a large ligand proton shift with a relatively low relaxivity.⁶⁸ This is the case for the TACN based Fe^{II} complexes **96**, **98** and **100**, containing three pyridine pendants (**96**, **97**) that stabilize Fe^{II} relative to Fe^{III} (high E° = + 1.0 V vs NHE for **96**) and air stable complexes, while the amide pendant groups of **98** are moderately stabilizing the Fe^{II} oxidation state.¹⁴⁶ Narrow CEST peaks have been observed at + 6 ppm (**96**) and + 69 ppm (**100**) relative to water, and CEST images of phantoms could be recorded on a MRI 4.7 T scanner. These Fe^{II} complexes have been compared with another triaza complex **104** (CEST at +53 ppm, NH protons) and the tetraza complexes **107** and **108** (+ 50 and +53 ppm, respectively, amide protons) and **113** (+ 55 ppm, very broad, OH protons).^{147,148,149} The oxaza-crown-based Fe^{II} complexes **112** and **113** were also investigated. Both have a distorted pentagonal bipyramidal structure, obtained by X-ray crystallography. While **112** gave a CEST peak at + 92 ppm assigned to the amide protons, **113** had none due to oxidation in water at neutral pH.^{150,151}

Four macrocyclic Fe^{II} complexes (**96**, **98**, **100** and **107**) were evaluated towards potential *in vivo* application as PARACEST agents.¹⁵² The complexes, with moderate to high thermodynamic stability (log K values between 13.5 for **107** and 19.2 for **96**), remained intact in the presence of carbonate, phosphate and Cu^{II}. However, none of the complexes showed appreciable redox cycling as measured by consumption of ascorbate in the presence of oxygen, corresponding to their highly stabilized Fe^{II} oxidation state (E° between +0.800 and +0.970 mV vs. NHE). None of them produced hydroxyl radical in the presence of peroxide and ascorbate. Therefore, a redox response of such Fe^{II} - based agents in typical *in vivo* reducing conditions will require further structural modification so as to decrease their redox potential without compromising other physicochemical characteristics.

Similar to Fe^{II}, the clue to the successful design of macrocyclic Ni^{II}-based redox responsive PARACEST agents is the control of the spin state, the metal ion dissociation kinetics and the redox tuning. Stabilization of the divalent oxidation state under biologically relevant conditions is easier for Ni^{II} than for Fe^{II}. The Ni^{II} ion (3d⁸, S = 1) has a relatively short electronic relaxation time T_{1e} in octahedral or bipyramidal (~ 0.1 ns) and

especially tetrahedral (~ 1 ps) geometries that produce highly shifted and narrow proton resonances, due to the low T_1 relaxation effects.⁸⁴ Ni^{III} ($3d^7$, low spin $S = 1/2$) also causes large hyperfine shifts and slow T_1 relaxation due to short T_{1e} values. Therefore, PARACEST peaks at different shift values are expected for complexes in both oxidation states.

To probe different coordination geometries, two Ni^{II} complexes (**100** and **110**, **Figure 33**) were first studied by Morrow's group¹⁵³ These complexes are six-coordinate and octahedral or twisted prismatic, showing two CEST peaks at + 72/+11 ppm for **100** resulting from the two sets of non-equivalent amino protons, while **110** has only one broad peak at + 76 ppm. These results were confirmed by CEST MRI of phantoms at 4.7 T. In the two oxaaza-derived complexes **112** and **113**,^{150,151} the nickel ion is bound to only two of the three ether oxygens and three nitrogens in a distorted octahedral geometry. **112** and **113** gave a CEST peak at + 72 ppm (amide protons) and + 55 ppm (imidazole NH), respectively. Finally, a comparative study including five Ni^{II} macrocyclic complexes in water was undertaken using triaza (TACN) derivatives with three (**100**) or two (**105**) amino and three OH (**101**) groups; it also includes two CYCLEN and CYCLAM tetraaza complexes with four OH pendants (**109** and **111**). These complexes were characterized by X-ray diffraction, ^1H NMR, CEST and ^{17}O NMR relaxometry.¹⁵⁴ These macrocycles have five (**105**), six (**100**, **101**) or eight (**109**, **111**) donor groups for coordinating the Ni^{II} ion. All complexes are six-coordinate with distorted octahedral geometries, but complex **105** has one inner sphere water molecule, as shown by X-ray diffraction and ^{17}O NMR relaxometry. The strongest CEST peak appeared for complex **105** at + 72 ppm, resulting from the amide protons and not from the coordinated water molecule, whose k_{ex} value ($4 \times 10^4 \text{ s}^{-1}$, as obtained by ^{17}O NMR) was still too fast to produce a CEST signal. The low intensity of the CEST peaks for **109** (at +94 ppm) and **111** (at +104 ppm) and the absence of a CEST peak for **101** reflect too rapid exchange of OH protons to produce optimal CEST intensities of these complexes with alcohol pendent arms. No redox response studies have been reported so far for any of the described Ni^{II} systems, perhaps because they are difficult to oxidize, as shown by the redox potential of aquated Ni^{II} ($E^0 = +2.290 \text{ mV vs NHE}$). Despite the large hyperfine shifts observed, further studies are needed to take such compounds to *in vivo* evaluation.¹⁵⁴

The $\text{Co}^{\text{II/III}}$ redox couple again provides opportunities for designing redox responsive PARACEST probes. Co^{II} ($3d^7$) in the common high spin ($S=3/2$) state induces strongly shifted NMR lines, while the low spin Co^{III} ($3d^6$, $S=0$) is diamagnetic, with

unshifted resonances.⁸⁴ Therefore, “turn-off” probes can be designed to switch between those states within the biological relevant electrochemical window. All the Co^{II} complexes shown in **Figure 33** (except **104**, **108**, **109** and **111**) have been investigated by Morrow’s group.^{11,145} Those complexes have distorted octahedral geometries except for the distorted pentagonal bipyramidal oxaaza complexes **112** and **113**.

The accessibility of both oxidation states in biological systems is also very important, as it requires $E^{\circ} < +0.800$ mV vs. NHE. Although aquated Co^{II} has $E^{\circ} = 1.940$ mV vs NHE, this redox potential can be tuned to biological ranges by variation of the number and the type of donor atoms in the coordination sphere. In comparison with anionic carboxylate groups, neutral amide groups favour the reduced state. Co^{II} complexes of macrocycles with amide donor groups (**100**, **107**, **110**, **112**) are stable in the presence of oxygen, suggestive of a large positive reduction potential. However, the redox potentials can be brought down to the biological range, such as in the presence of heterocyclic pyrazole donors in **99** ($E^{\circ} = -107 \pm 11$ mV vs. NHE) or amino coordinating groups, such as in the TACN-based hexamine complex **102** ($E^{\circ} = -120$ mV vs. NHE). Also, early studies on macrocyclic hexamine Co^{III} cage complexes **114** and **115** have reported reduction potentials of +0.010 V to -0.380 mM vs. NHE, respectively.^{11,145} Other important features of Co^{III/II} cage complexes involve the kinetics of electron transfer, with self-exchange rates which vary by ten orders of magnitude (6.8×10^2 to 8.5×10^{-8} M⁻¹ s⁻¹), the electrochemical reversibility of the Co^{III/II} redox couple and the kinetic inertness in both oxidation states under biologically relevant conditions.^{11,145}

Optimal conditions have been met by **99**.¹⁵⁵ It oxidizes in air to produce the Co^{III} analogue and addition of dithionite or cysteine restores the Co^{II} complex. **99** has sufficiently low r_1 and r_2 relaxivities (0.093 and 0.50 mM⁻¹ s⁻¹, respectively) for CEST to be observed. In fact, the pyrazole -NH protons of **99** give narrow CEST peak at +135 ppm away from the bulk water resonance, while the chemical shift of the oxidized Co^{III} diamagnetic complex is close to water. (**Figure 34**). The advantage of the large shift of CEST signal is that it should allow *in vivo* CEST imaging with less interference from the tissue magnetization transfer (MT) background. The redox properties of **99** are also encouraging, because the Co^{III/II} redox couple is reversible with a moderately negative reduction potential ($E^{\circ} = -107 \pm 11$ mV vs. NHE). Oxidation of **99** has a second-order rate constant of 0.43 M⁻¹ s⁻¹, corresponding to a half-life in arterial blood (with high pO₂) $t_{1/2} = 2.6$ h, which could increase up to 26 h under hypoxic conditions.¹⁵⁵ Therefore, **99** is

a promising candidate for the development of redox-sensitive PARACEST MRI contrast agents.

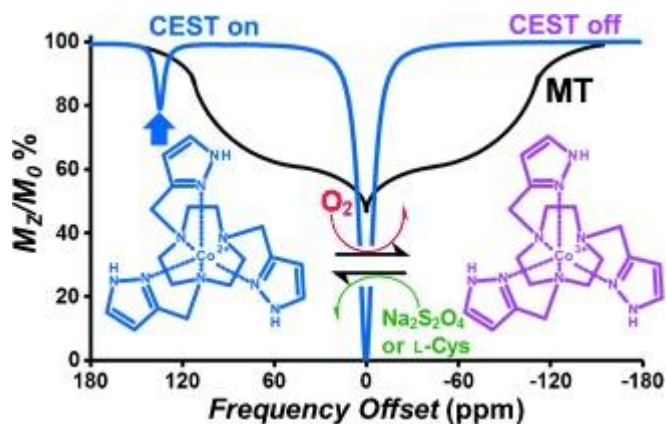


Figure 34. Structures of Co^{II}/Co^{III} complexes **99** and CEST spectra of their solutions recorded at 11.7 T. Reproduced with permission from ref. Reproduced with permission from Ref. 155, Copyright (2013) by John Wiley and Sons.

Subsequently, the same group reported five air stable amide-appended Co^{II} chelates containing triaza, tetraaza or oxaaza macrocycles, **100**, **107**, **110** and **112**.¹⁵⁶ They showed a variable number of CEST peaks with 8 to 12% intensities, characteristic of amide protons. **112** has two highly separated CEST peaks at +59 and -19 ppm from two magnetically inequivalent amide protons, due to restricted C–N bond rotation of the amide groups or to the low complex symmetry. **100** and **107** only showed CEST peak at +32 and +45 ppm, respectively, because the second amide proton resonance was under the water peak or had an unfavorable k_{ex} value. In contrast, **110** originated four CEST peaks (+112, +95, +54, and +45 ppm) of similar intensity and a smaller CEST peak at -15 ppm, which was assigned to the low symmetry of the complex. The CEST properties of Co^{II}-**112** were later compared with those of the corresponding Fe^{II} and Ni^{II} analogues.¹⁵⁰ One CEST peak was also observed at +32 ppm for the related Co^{II} oxaaza amide complex **113** which was attributed to the imidazole NH proton.¹⁵¹

Finally, a recent publication compared the CEST properties of three triaza macrocyclic complexes **101**, **105** and **106** with the complex **100** previously studied.¹⁵⁷ They contained either three (**101**) or two (**106**) pendant alcohol groups or three (**100**) or two (**105**) amides. Complexes **100** and **101** had no water ligands, while **105** and **106** had one bound water molecule occupying the coordination site left free by the five ligand donor groups. All complexes produced CEST peaks through either alcohol OH (at +140

ppm for **101** and + 37 ppm for **106**) or amide NH (+60 ppm for **105** and +32 for **100**) proton exchange. The water ligands exchanged too rapidly to produce a CEST effect as shown by variable-temperature ^{17}O NMR spectroscopy studies. The large hyperfine shift of the CEST peak observed for complex **101** is quite remarkable.

6. Concentration-independent redox-responsive MRI probes

A major limitation of the *in vivo* use of responsive contrast agents is related to the concentration dependence of the generated MRI signal. This makes it difficult to separate MRI contrast changes due to stimulus response (such as the tissue redox status) from effects due to a concentration change of the un-activated agent given by its pharmacokinetics. Several strategies have been developed to design concentration-independent responsive contrast agents,¹⁵⁸ but only few examples refer to redox probes.

One way to address this problem is through the design of ratiometric responsive MRI probes. Ratiometric probes based on CEST agents are attractive and relatively easy to design.¹⁵⁹ They possess two magnetically different sets of exchanging protons with distinct responses to the environmental variable being studied (shifting their frequencies differently, for instance). If the response of the two individual CEST signals is known, the ratio of CEST effects is independent of probe concentration and thus can be used to quantitatively monitor the variable. The two exchanging pools may belong to the same species or to different molecules with equal biodistribution. As opposed to the first case, in the second situation the knowledge of the concentration ratio of the two molecules is still required. Such ratiometric imaging has so far not been used for redox activated probes, but has been applied to MRI agents responsive to pH,^{160,161} temperature,¹⁶² endogenous cations¹⁶³ and enzymes¹⁶⁴. For example, two distinct CEST peaks might be observed in PARACEST agents which have exchangeable protons in donor groups which can be different,^{30,160,161,165,166} (one of them is often a Ln^{III} -coordinated water molecule^{160,161,161,162}) or which are identical but in different environments.^{55,163} A novel ratiometric CEST method was also recently proposed by Aime and co-workers, in which the so-called ratio of radiofrequency power mismatch was calculated by the quotient of saturation transfer at two different radiofrequency powers.¹⁶⁷

Ratiometric strategies for redox-activated MRI probes are more challenging to design. One example involves the irreversible reaction of probe **88** (**Figure 26**) with singlet oxygen in which the observed decrease and shift of the CEST peak was

proportional to the concentration of $^1\text{O}_2$.¹³¹ Other strategies might include metal ion redox couples where each redox state has distinct MRI contrast properties.

Changes in r_1 relaxivity alone are not sufficient for the design of concentration-independent Gd^{III} -based responsive contrast agents. Here, ratiometric imaging can be based on the ratio of changes in transverse and longitudinal relaxation rates (R_{2p}/R_{1p}) of solvent water protons induced. This ratiometric approach has been used for Gd^{III} pH or enzymatic probes based on τ_R changes^{168,169} and to external magnetic field strength¹⁷⁰. However, this technique has not yet been applied to redox responsive probes.

An alternative approach to ratiometric methods is to combine two different imaging modalities. In such dual-mode imaging, the probe can be detected using two or more imaging modes such as ^1H - (T_1 -weighted, T_2 -weighted, or PARACEST), ^{31}P - or ^{19}F -MRI, PET, SPECT, optical imaging, PAI, etc. In dual-mode imaging, the response to a biomarker can be detected by the ^1H -MRI mode while the probe location and concentration can be obtained by the other mode. *In vitro* examples include the use of pH or Zn^{II} sensitive Gd^{III} -probes as T_1 agents for ^1H -MRI together with a fluorinated analogue for ^{19}F -MRI,¹⁷¹ an analogue containing ^{18}F for PET detection,¹⁷² or isostructural $^{165}\text{Er}^{\text{III}}$ ¹⁷³ or $^{166}\text{Ho}^{\text{III}}$ ¹⁷⁴ complexes for SPECT imaging. All these examples rely on external standards and an imaging modality capable of quantifying the concentration of the agent.

An alternative to using external standards or ratiometric techniques is to use an internal standard to indirectly monitor the biodistribution of a responsive contrast agent. In this case, one must assume (and demonstrate) sufficiently similar pharmacokinetics of two separate complexes. An early attempt to obtain quantitative images of tissue pH was to use dynamic contrast enhancement MRI after sequential injections of a pH-responsive Gd^{III} T_1 agent and a chemically similar but non-responsive agent.^{175,176,177} Another example, relevant to redox probes, was the development by Aime et al. of an MRI probe for quantitative O_2 sensing based on the use of RBCs labeled with Gd-DOTP^{5-} (DOTP = 1,4,7,10-tetraazacyclododecane- $\text{N},\text{N}',\text{N}'',\text{N}'''$ -tetrakis- (methylenephosphonate)) or (Gd-HPDO3A) (HPDO3A = 10-(2-hydroxypropyl)-1,4,7-tetraazacyclododecane-1,4,7-triacetate). Here the first probe served as an O_2 -responsive agent and the second one reported on the local RBCs' concentration (**20**, **Figure 10**, Section 4.1.1).⁵⁶

Other redox probes that do not require the knowledge of contrast agent concentration are those combining T_1 -weighted and PARACEST ^1H -MRI capabilities to follow the response to oxidation, such as the $\text{Eu}^{\text{II}}/\text{Eu}^{\text{III}}$ probes **93**¹³⁸ and **94**¹³⁹ (**Figure 31**) described in section 5.1.2.¹⁴⁰

Finally, Parker and co-workers demonstrated a ratiometric pH response using ligand field-induced chemical shifts that might be exploited for redox probes.¹⁷⁸

7. Conclusions and perspectives

The design of metal-based probes capable of mapping tissue redox status by *in vivo* MRI has been found to be more complex than for other types of responsive probes such as those activated by pH, temperature or enzyme activity. Small molecular complexes as well as supramolecular structures, including inorganic nanoparticles, based on Ln or transition metal ions, have been reported for MRI redox sensing. The first examples relied on changes in r_1 (in some cases r_2) proton relaxivity or in PARACEST properties triggered by redox reactions either at the metal center or at reactive functionalities located on the ligand, such as thiol groups. Despite this early progress, many limitations in terms of molecular design were still to be overcome before such agents could be successfully used *in vivo*, as pointed out by two reviews published in 2014.^{10,11} Here, we will briefly summarize those challenges and highlight the most important, recent advances.

1) In relation to the image contrast itself, strong changes in relaxivity or in CEST are desirable, preferably producing signal increase (“turn-on” probe; OFF/ON). Complexes or nanoparticles based on redox-active metal ions like Mn are much more sensitive relaxation redox sensors than those based on Gd^{III} chelated to redox sensitive ligands; indeed, these latter are never completely silent either in the oxidized or in the reduced form. In this respect, Mn-based nanosystems (section 4.1.2)^{97,98,99,100,101,102,103} lead to the greatest signal changes and thus represent the most important advances.

2) High thermodynamic stability and kinetic inertness of the metal complexes, in both reduced and oxidized states, should be maintained in order to ensure *in vivo* safety. Therefore, macrocyclic agents are preferable to linear ones.

3) The redox potential of the probe must be fine-tuned and adapted for *in vivo* sensing. Several agents described in this review, such as Ni^{II} PARACEST agents, do not undergo redox chemistry over a biologically reasonable range. For many others, such as the Eu^{II/III} complex **94**,¹³⁹ the Mn^{II/III} complexes **63/64**⁵⁸ and the Co^{II/III} complex **99**,¹⁵⁵ the redox potentials are compatible with biological redox buffers.

4) The redox reaction should be reversible and fast in the timescale of an MRI experiment. As discussed, Co^{II/III} electron transfer kinetics can have fast self-exchange rates. Future probe design would benefit from more detailed understanding of the redox kinetics with

biologically relevant reductants or oxidants. This, together with information on pharmacokinetics, can help create agents suitable for uptake in tumors or other tissues of interest.

5) Although more difficult than for other types of triggers, probe design should more frequently incorporate ratiometric features in order to provide quantitative redox mapping. While progress has been registered for *in vitro* systems as described in section 6,^{63,65,131,140} *in vivo* translation has not yet been achieved for redox probes.

6) Cell permeability and intracellular delivery of the probe should be facilitated. Some *in vitro* cell work and *in vivo* small animal MRI studies using redox agents were already reviewed previously,^{47,48,49,54} and recently, the number of *in vivo* studies, mostly in mouse tumor models, have substantially increased. These include Gd^{III} and Mn^{II} nanoparticles as T₁ agents,^{65,68,69,98,100,101,102,103} a Co^{II/III} complex as T₁ agent,⁷⁶ a core-shell Fe₃O₄@Mn₃O₄ nanoplatform as T₂ agent⁹⁹ and Eu^{II/III} complexes as dual T₁/PARACEST agents.^{88,139} In all examples, tumors were well delineated, and in some cases pO₂ sensitive probes allowed differentiation between normoxic and hypoxic tumor regions.^{46,54,88} Mn^{II} nanoparticle-based theranostic platforms have also been used with success.^{100,101} However, many of these agents, except for those transported into cells via EPTs,⁴⁶ probably remain in extracellular spaces *in vivo*. Redox differences between the various tissue compartments must be taken into account and the intracellular or extracellular location of the redox sensor should be assessed. Cell penetrating peptides^{49,133} or other mechanisms of intracellular delivery of redox probes should be considered in future work.

In conclusion, the field of MRI redox sensing is slowly evolving. In the last four years, many proof-of-principle model systems have been studied *in vitro* and some, particularly nanosystems, were sensitive enough to be tested *in vivo* in tumour mouse models. The remaining challenge is to ensure that all the requirements described above are combined in a given molecular sensor in order to monitor the redox environment of tissues in different diseases.

8. Acknowledgments

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