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The biological fate of gadolinium-based MRI contrast agents: a call to action for bioinorganic chemists

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Abstract

Gadolinium-based contrast agents (GBCAs) are widely used with clinical magnetic resonance imaging (MRI), and 10s of million doses of GBCAs are administered annually worldwide. GBCAs are hydrophilic, thermodynamically stable and kinetically inert gadolinium chelates. In clinical MRI, 5 – 10 millimoles of Gd ion is administered intravenously and the GBCA is rapidly eliminated intact primarily through the kidneys into the urine. It is now well-established that the Gd^{3+} ion, in some form(s), is partially retained in vivo. In patients with advanced kidney disease, there is an association of Gd retention with nephrogenic systemic fibrosis (NSF) disease in patients. However Gd is also retained in the brain, bone, skin, and other tissues in patients with normal renal function, and the presence of Gd can persist months to years after the last administration of a GBCA. Regulatory agencies are restricting the use of specific GBCAs and inviting health care professionals to evaluate the risk/benefit ratio prior to using GBCAs. Despite the growing number of studies investigating this issue both in animals and humans, the biological distribution and the chemical speciation of the residual gadolinium are not fully understood. Is the GBCA retained in its intact form? Is the Gd^{3+} ion dissociated from its chelator, and if so, what is its chemical form? Here we discuss the current state of knowledge regarding the issue of Gd retention and describe the analytical and spectroscopic methods that can be used to investigate the Gd speciation. Many of the physical methods that could be brought to bear on this problem are in the domain of bioinorganic chemistry and we hope that this review will serve to inspire this community to take up this important problem.

1. Introduction

Gadolinium-based contrast agents (GBCAs) are a form of chelated Gd^{3+} ion that are used in combination with magnetic resonance (MR) imaging. The paramagnetic Gd^{3+} complex interacts magnetically with nearby water molecules and induces signal changes, i.e. contrast, in the MR image. GBCAs are used to diagnose tissue and vascular abnormalities that may be otherwise undetected in unenhanced scans,^{1–9} and are used in about 40% of all MR imaging

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Conflicts of interest

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procedures. GBCAs are highly water-soluble complexes that are designed to be completely eliminated, intact, from the body after intravenous administration. However, it is now well established that not all of the injected dose is eliminated, and in some instances the presence of gadolinium can cause a delayed onset toxic effect. Yet our understanding of the fate of GBCAs in the human body remains limited. What extent of the injected dose of the gadolinium ion remains in the body after a week, month, or year? In what tissues does the gadolinium localize? In what biological compartments is it localized? Is the gadolinium present as the intact GBCA or is it transformed, and if so, what is the result of that transformation? Is the transformed species more or less toxic than the parent GBCA? For the various GBCAs approved for human use, is their distribution and fate similar or markedly different? These questions of biological distribution and chemical speciation have real consequences for understanding toxicology and determining if some GBCAs should be preferred on a safety basis.¹⁰

The last decade has seen a rise in safety concerns regarding the use of GBCAs. In 2006, an association between GBCA enhanced MRI examinations and a rare but devastating disease called nephrogenic systemic fibrosis (NSF) was observed in patients with impaired renal function.^{11,12} This disease was recognized in 1997 and initially termed nephrogenic fibrosing dermopathy because of its skin manifestation.¹³ Subsequently, its systemic nature was reported with fibrosis affecting other organs, such as heart, diaphragm, lungs or liver, leading to its renaming as NSF.¹⁴ Extremities are first affected with the thickening and hardening of the skin and the formation of hard plaques with a peau d'orange appearance. Patients can also develop deep contractures and joint stiffness restricting movement.¹⁵ The morbidity associated with NSF can be severe with pain and immobility. Death can occur in some cases. The relationship between GBCAs and NSF has been confirmed with the presence of gadolinium in skin biopsy of NSF patients.^{16,17} GBCAs are excreted primarily through the kidneys and into the urine, and it is known that when kidney function declines the blood elimination half-life increases. It is generally accepted that this longer residency time in renally impaired patients leads to greater probability of a toxic effect. There is now a United States Food and Drug Administration (FDA) contraindication against 3 of the 8 commercially available GBCAs in patients with impaired kidney function as indicated by a glomerular filtration rate (GFR) < 30 mL/min/1.73 m².^{18,19}

The last five years have seen the emergence of GBCA safety concerns in patients with normal renal function. A series of reports beginning in late 2013 confirm long-term Gd retention in the central nervous system (CNS) and in skin and bones of patients receiving contrast-enhanced examinations.^{20–23} Moreover, in 2016, four cases of NSF were reported in patients with normal renal function who had received GBCAs.²⁴ The implications of Gd retention in the CNS remain unclear. The European Medicines Agency (EMA) recently recommended suspending the marketing authorizations for three GBCAs in response to the discovery of Gd CNS accumulation.²⁵ Other regulatory bodies in the USA, Japan, Canada, etc have issued revised precautions for GBCAs.²⁶

It is believed that NSF and CNS Gd accumulation are the result of gadolinium released from the GBCA, as NSF incidence and CNS accumulation are greater following exposure to the GBCAs which comprise an acyclic chelator compared to GBCAs that comprise a

macrocyclic chelator. The 3 GBCAs that are contraindicated by FDA in patients with poor renal function all contain acyclic chelators, as do the 3 GBCAs recently recommended for market withdrawal by the EMA. However it is also now established that all GBCAs deposit Gd in the bone and central nervous system (CNS).^{20–22,27,28} This Gd deposition appears to be irreversible and is cumulative with repeat dosing.²¹ Although Gd deposition within the CNS is not currently linked to any known toxicity, there is concern among physicians and regulators, which has triggered strong regulatory responses. A third emerging issue is the concept of “gadolinium deposition disease” which is controversial but posits that there is a spectrum of NSF-like symptoms even in patients with normal kidney function.^{29–31}

As numerous studies clearly show, gadolinium can be found in tissues months to years after a single or repeated administration of GBCAs, but one question remains to be fully elucidated: “What is the speciation of gadolinium in tissues?”. The term “speciation” refers to the chemical form of the gadolinium species in tissues: does the GBCA remain intact? Is the GBCA bound to a macromolecule? Is the Gd^{3+} ion dissociated from its chelator? If dissociated, the gadolinium ion might be chelated by low molecular weight ligands present in tissues (citrate, lactate...), be bound to macromolecules (proteins, carbohydrates...), form insoluble inorganic species with endogenous anions such as phosphate, carbonate or even be part of an inorganic entity like hydroxyapatite (Figure 1). The speciation also encompasses the localization of the gadolinium species within the tissues (extracellular or intracellular), and if intracellular, then in which cell types and organelles? Investigating the gadolinium speciation in tissues would help to better understand the potential toxicity that arises from the administration of GBCAs. The purpose of this review is to describe the state of our knowledge about the fate of GBCAs, as well as the gaps in knowledge and open questions that exist. The spectroscopic and analytical methods that should be used to solve this highly complex problem will also be discussed in an attempt to provide this community a toolbox of available techniques that, when used in combination, could help address these questions of speciation. The specialized expertise that exists in the bioinorganic and bioanalytical chemistry communities is needed to answer these questions and we hope that this review will stimulate this community to tackle these problems.

2. A brief overview of the structure and properties of GBCAs

The Gd^{3+} ion has seven unpaired electrons in a symmetric S ground state and this provides a large magnetic moment and a relatively long electronic relaxation time, properties which make the gadolinium ion a potent nuclear relaxation agent. GBCAs induce MR signal change by decreasing the proton relaxation times of the surrounding water molecules.^{32–34} The tripositively charged gadolinium ion has no known biological role, but is known to compete with the divalent Ca^{2+} ion as they are of similar size (ionic radius: 108 ppm for Gd^{3+} and 114 ppm for Ca^{2+}) by blocking voltage-gated Ca^{2+} channels and inhibiting the activity of calcium-dependent enzymes.^{35,36} To prevent this toxic effect while keeping its paramagnetic properties, the gadolinium ion is chelated, or encapsulated by a ligand (chelator) to form an entity called a “complex” or “chelate”. Every GBCA approved for human use is based on a polyaminocarboxylic acid ligand that derives either from the linear, acyclic compound H_5DTPA or the macrocyclic H_4DOTA (Figure 2). In these complexes, the gadolinium ion is bound to 8 coordinating atoms (oxygen and nitrogen atoms) brought by

the multidentate ligand. A coordinated water ligand occupies the ninth position available for binding to the Gd^{3+} ion, and this water ligand undergoes very fast ($10^6 s^{-1}$) exchange with bulk water in solution and this chemical exchange catalytically decreases the average relaxation time of tissue water. Chelation renders the Gd^{3+} ion soluble and greatly reduces its toxicity. For instance the LD_{50} , lethal dose at which 50% of death occurs in a group of test animals, in rats increases from 0.1 – 0.2 mmol/kg for intravenously administered unchelated gadolinium to 10 – 20 mmol/kg when chelated.³⁴

GBCAs (Figure 2) can be referred by three different names. They have a commercial name like “Dotarem®” or “Clariscan®” and a generic name, in this case gadoterate meglumine. There is also a chemical abbreviation, and in this case Gd-DOTA or $[Gd(DOTA)(H_2O)]^-$. We will use the chemical abbreviation throughout this review.

GBCAs have similar structures, but many of their properties vary. Notably the affinity of the chelator for the Gd^{3+} ion (thermodynamic stability), the affinity of the chelator for other endogenous metal ions, the rate at which the Gd^{3+} can be separated from its chelator (kinetic inertness) as well as the elimination route, all differ. Gd-DOTA, Gd-DTPA, Gd-HP-DO3A, Gd-DO3A-butrol, Gd-DTPA-BMA, and Gd-DTPA-BMEA are often referred to as extracellular fluid (ECF) agents. After intravenous injection, the ECF agents distribute in the extracellular space (intravascular and interstitial space), and are then rapidly excreted through the kidneys via glomerular filtration with an excretion half-life of 1.5 h in patients with normal renal function.³⁷ In addition to renal elimination, Gd-BOPTA and Gd-EOB-DTPA are also partially cleared through the hepatobiliary system due to their higher lipophilicity. These two GBCAs have elimination half-lives similar to the ECF agents in patients with normal renal function. MS-325 reversibly binds serum albumin and this helps to restrict this agent to the blood pool agent, but also serves to markedly increase the blood and elimination half-life compared to the ECF agents. Gd-DTPA-BMEA and MS-325 are now no longer commercially available. In patients with normal renal function, GBCAs are considered extremely safe as their excretion is nearly complete within a few days with an elimination of the injected dose close to 100%, and no metabolites reported in the blood or urine.^{38–41}

The kinetic inertness and the thermodynamic stability constants are key parameters in understanding the stability of chelates in vivo.^{42,43} An equilibrium constant describes the chemical speciation under steady state conditions, for instance how much Gd^{3+} would be displaced from a GBCA if challenged with an equal amount of Cu^{2+} ion. Thermodynamic stability constants can be used to compute different equilibrium constants and describe the fate of the Gd^{3+} ion under a given set of conditions (pH, competing ligands like phosphate or bicarbonate, competing metal ions like Zn^{2+}). Given enough time for reactions to occur, thermodynamics will predict the speciation. The kinetic inertness reflects the chelate's rate of Gd release under some set of conditions. GBCAs are designed to be inert to Gd release under most physiological conditions, however the rate at which GBCAs react and the mechanism of reaction differs widely among the GBCAs. Thus, thermodynamics may predict that iron from transferrin will ultimately displace Gd^{3+} from its chelator but the kinetics of this reaction are extremely slow under physiological conditions. Before the reaction could occur, the GBCA would be completely eliminated from the blood stream.

The macrocyclic based GBCAs Gd-DOTA, Gd-HP-DO3A, and Gd-DO3A-butrol are much more kinetically inert than the acyclic based GBCAs. The incidence of NSF was much higher in patients given the acyclic GBCAs Gd-DTPA-BMA, Gd-DTPA-BMEA, and Gd-DTPA and this has been ascribed in part to the relatively lower kinetic inertness of these compounds.

2.1. The hierarchy of data

It is important to keep in mind that the data observed in humans is paramount. Physicochemical measures of thermodynamic stability and reaction rates (kinetics) may help to explain what is seen in humans and can be very valuable in formulating hypotheses. Similarly, studies in animal models can be useful in understanding observations in humans. As described by Tweedle,⁴⁴ this collection of data should be categorized within a hierarchy where human in vivo studies are at the top, Figure 3. Animal model studies and physicochemical measurements are valuable to the extent that they can predict and explain the human in vivo result. Animal models are models and there are many differences between rodents and humans in terms of physiology and anatomy. Physicochemical measurements can be made very accurately but extrapolation to the human in vivo condition is challenging due to the complexity of the biological milieu both in terms of the number of (bio)chemical species which exist as well as tissue and cellular compartments.

2.2. Thermodynamics and Kinetics of GBCAs

The thermodynamic stability constant refers to the affinity between the ligand and the metal. For GBCAs these are all very high and expressed as the logarithm of the equilibrium constant. If the stability constant for a GBCA is much higher than for the same ligand with another metal ion, then that metal ion will not displace the Gd^{3+} from the GBCA under any circumstance. For example, Ca^{2+} and Mg^{2+} cannot displace Gd^{3+} from any of the GBCAs. The ligands used to make GBCAs are very basic and as a result there is also a competition with protons (H^+) which will increase as pH is decreased. This can be computed as a “conditional” stability constant which are conditional on the pH specified. Table 1 lists the thermodynamic stability constants as well as conditional constants at pH 7.4. and pH 4, which may be encountered in some cellular compartments. The two linear derivatives Gd-DTPA-BMA and Gd-DTPA-BMEA have the lowest conditional stability constants calculated at pH 7.4 with 14.9 and 15.0, respectively. Gd-DTPA, Gd-EOB-DTPA, Gd-BOPTA and MS-325 exhibit similar conditional stability constants at pH 7.4 (18.4 – 18.9) due to the same coordination environment and thus the same basicity. Despite their higher thermodynamic constants $\log K_{GdL}$, the two macrocyclic derivatives Gd-DOTA and Gd-HP-DO3A are characterized by lower conditional constants at pH 7.4 in comparison to the DTPA analogues with $\log K_{cond} = 17.2$ and 17.1, respectively. This is explained by the higher basicity of the ligands resulting in a stronger competition with H^+ . At pH 4, the conditional stability constants are much lower due to a higher H^+ concentration and are in the range of 9.0 – 11.6 (Table 1). Endogenous ions Fe^{3+} , Zn^{2+} , and Cu^{2+} are present in the biological milieu and are capable of displacing the gadolinium ion from its chelator. This is especially true for Zn^{2+} as its concentration in serum reaches 50 μM . Fe^{3+} and Cu^{2+} are more tightly regulated but given the very long residency times of residual GBCAs in humans, these ions

may also compete over time and they form more stable complexes with the GBCA chelator than Zn^{2+} , Table 2.

The acid-catalyzed dissociation of the GBCAs, where an excess of protons is used as a competitor, is one way to rank GBCAs with respect to lability and appears to be the most representative in vitro assay of in vivo dissociation of Gd^{3+} ,⁴⁵ Table 1. Thanks to their rigid and preorganized structure, macrocyclic derivatives show a slower dissociation rate in acidic conditions (k_1 constant), resulting in a higher kinetic inertness. The mechanism of reaction is also important. The acyclic GBCAs will react directly with Zn^{2+} or Cu^{2+} and undergo transmetallation. This reaction with acyclic GBCAs increases with decreasing pH and increasing competitor ion. The macrocyclic GBCAs do not react directly with Zn^{2+} or Cu^{2+} . Rather the Gd^{3+} must first undergo an acid assisted dissociation and then the Zn^{2+} can bind to the free ligand. As a result, this reaction does not depend on the concentration of competing metal ion.

A combination of a high thermodynamic stability and a high kinetic inertness (low dissociation rate) is ideal to minimize the release of gadolinium ion in vivo.⁴³ Frenzel et al. evaluated the stability of the GBCAs in human serum over 15 days and confirmed the trend measured in aqueous solution: chelates that exhibits a low thermodynamic stability and low kinetic inertness dissociate and release the largest amount of gadolinium in comparison to the most stable and inert compounds.⁴⁶

3. The issue of gadolinium retention

3.1. Terminology

The terms employed here are defined according to a recent review by Robert et al.⁵⁸ Thus, “residual gadolinium” and “presence of gadolinium” are used in a general way when no precise mechanism is known or suggested. “Retention” means that a gadolinium species is retained but slowly eliminated over time in opposition to “deposition” for which no excretion occurs.

3.2. Anthropogenic sources of gadolinium

Gadolinium traces, albeit very low, have been detected in human tissues from control patients without known history of GBCA administration. Murata et al. reported ng of Gd per g of wet tissue.⁵⁹ In other studies, the gadolinium background in control patients was below the LOQ.^{22,60} Gadolinium and other rare earth elements are geogenic and can be naturally detected in river and lake waters.^{61,62} Where GBCAs are extensively used, especially in megacities where health care systems are highly developed, positive anthropogenic gadolinium anomalies have been reported in rivers around the world.⁶³ These anomalies are caused by the presence of GBCAs in effluents from hospitals and waste water treatment plants due to the lack of specific recycling processes. Analysis of tap water in London (UK) and Berlin (Germany) also revealed higher gadolinium concentration⁶¹ and GBCAs have been detected intact.⁶⁴ Therefore, the low signal detected in control patients that were not exposed to a GBCA for imaging purpose might be linked to anthropogenic gadolinium, although no articles testing this hypothesis were found while preparing this review.

3.3. Residual Gadolinium detected in humans and in animal models

Even though they were designed to be fully excreted, a small amount of GBCA tends to dissociate *in vivo* and/or is retained for an extended period. After administration of GBCAs in patients suffering from renal insufficiency, the clearance is considerably slower with a half-life that may exceed 30 h depending on the patient's renal function. Such a long *in vivo* residency time of a gadolinium chelate facilitates its dissociation by transmetallation or ligand exchange with e.g. phosphate. Gadolinium is strongly associated with NSF disease. Gadolinium deposits have been found in the skin, liver, vessel wall, kidney, and heart tissue of patients.^{65,66} Most of the NSF cases normalized to the total number of doses (market share) have been observed after Gd-DTPA-BMA or Gd-DTPA-BMEA injections, and these are the least stable and least inert GBCAs.⁶⁷ Since the link between GBCA administration and NSF was established, much fewer patients with renal impairment receive GBCAs and those that do are given a macrocyclic inert GBCA. As a result, very few cases of NSF are now reported.³⁵

However, gadolinium has also been detected *in vivo* in patients with normal glomerular filtration rates after the administration of a GBCA. In 2014, Kanda et al. demonstrated a correlation between the cumulative dose of GBCA (Gd-DTPA or Gd-DTPA-BMA) and an abnormal increase in signal intensity in some brain regions (Globus Pallidus (GP) and Dentate Nucleus (DN)) prior to GBCA injection.²¹ Such an increase can be observed between brain areas (pons vs DN) or between the first and last gadolinium enhanced MRI as shown in Figure 4.²² In another study, the same group measured a mean gadolinium concentration in autopsied brain tissues of 0.25 µg/g, using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), with a higher concentration in the DN and GP (0.44 µg/g) than in other brain regions (0.12 µg/g). These patients had received between 2 and 4 injections of either Gd-DTPA-BMA, Gd-DTPA, or Gd-HP-DO3A at a dose of 0.1 mmol/kg of bodyweight. Through this study, they confirmed that the increase of the T1 weighted signal intensity detected in those brain areas was indeed correlated to the presence of gadolinium.²⁰ These results are consistent with the McDonald et al. study which showed a concentration in brain tissue in the range of 0.1 – 58 µg/g with no gadolinium detected in a control group.²² In the McDonald study, Gd-DTPA-BMA was the only GBCA used at a cumulative dose that ranged between 52 – 500 mL (287 mg Gd/mL). Such an enhancement in human brain regions can only be the result of a highly concentrated and soluble species with an access to surrounding water molecules or a species with a high relaxivity at low concentration.

In 2004, Gibby et al. reported residual gadolinium *in vivo* in patients with normal renal function.⁶⁸ Using inductively coupled plasma atomic emission spectroscopy (ICP-AES), the authors measured the concentration of gadolinium in human bones from patients who had undergone a GBCA-enhanced MRI, three days before hip replacement surgery. The patients were administered either Gd-HP-DO3A or Gd-DTPA-BMA. In both cases, gadolinium was detected in tissue samples, however the concentration was 2.5 times higher for Gd-DTPA-BMA (1.18 ± 0.787 µg Gd/g bone) than for Gd-HP-DO3A (0.466 ± 0.387 µg Gd/g bone) reflecting the lower stability of the linear GBCA. Using a more sensitive technique (ICP-MS), a higher concentration was measured for Gd-DTPA-BMA (1.77 ± 0.704 µg Gd/g

bone).⁶⁰ Since the clearance rate of both of these GBCAs is the same, the difference in measured gadolinium concentration in the bone shows greater retention of Gd-DTPA-BMA compared to Gd-HP-DO3A. The authors did not investigate the speciation of gadolinium in the bone samples. However, the speciation in brain tissues was investigated in an animal study where Gd-DTPA-BMA or Gd-HP-DO3A were injected in healthy rats at a cumulative dose of 13.2 mmol per kilogram of bodyweight over a period of 8 weeks.⁶⁹ When Gd-HP-DO3A was administered, the total gadolinium concentration measured in brain tissues corresponded only to the intact GBCA in opposition to Gd-DTPA-BMA for which insoluble species were mainly detected. However, the rats were sacrificed only three days after the last injection of GBCA leading to the question of whether the macrocyclic derivative is excreted over time or if dissociation occurs.

It is not yet clear whether or not the macrocyclic GBCAs are involved in gadolinium deposition in humans, but they are certainly retained. Retrospective analyses of repeat dose human MRI studies looking for hyperintense signal in the DN on T1-weighted scans has been controversial. Several studies have reported no enhancement in the DN after 7.06 ± 1.20 doses of macrocyclic GBCAs (cumulative dose = 162.41 ± 45.20 mL),^{27,70,71} but others have reported an increase in signal intensity in the brain after the administration of a macrocyclic derivative (Gd-DOTA or Gd-DO3A-butrol).^{72–75} These latter reports have been criticized due to several methodological limitations.^{76–78} It is important to keep in perspective that the hyperintense T1-weighted signal in the DN requires more Gd retained there than in surrounding brain tissue. If the distribution of Gd is more uniform throughout the brain, then there would be less or no contrast between the DN and surrounding tissue. Moreover, unenhanced T1 weighted MRI is limited in its sensitivity. Typically an increase in T1-weighted signal is only observed after at least 5 GBCAs injections, but no change in intensity does not mean no gadolinium deposition or retention.⁷⁹ Ex vivo analysis of brain and bone tissues using ICP-MS revealed that gadolinium was detected even in patients that were administered Gd-DO3A-butrol or Gd-HP-DO3A.⁵⁹ That study did not reveal if a hyperintensity was previously observed in brain regions and there was no examination of Gd speciation.

In animal studies the brain can be analyzed in detail ex vivo at the end of the study providing a better understanding, albeit in a model organism. Robert et al.⁸⁰ injected healthy rats with either Gd-DOTA, Gd-DTPA-BMA, Gd-BOPTA or Gd-DTPA at a cumulative dose of 12.0 mmol per kg of bodyweight over five weeks and sacrificed 4 weeks after the last injection. No change in T1 weighted SI was observed with Gd-DOTA in opposition to the three linear derivatives. Using ICP-MS, they reported the following concentration in the cerebellum: 3.75 ± 0.18 nmol/g (Gd-DTPA-BMA), 1.67 ± 0.17 nmol/g (Gd-DTPA), 1.21 ± 0.48 nmol/g (Gd-BOPTA) and 0.27 ± 0.16 nmol/g (Gd-DOTA). The authors drew the conclusion that no significant gadolinium amount was retained for the macrocyclic derivative compared to the control (saline, 0.09 ± 0.12 nmol/g). Using the same protocol, Bussi et al. measured the concentration of gadolinium in healthy rats after injection of one of the three macrocyclic GBCAs (Gd-DOTA, Gd-DO3A-butrol and Gd-HP-DO3A) by means of ICP-MS.⁸¹ Interestingly, gadolinium was detected for the three GBCAs, but lower gadolinium concentrations were observed with Gd-HP-DO3A compared to Gd-DOTA and Gd-DO3A-butrol in the cerebellum, cerebrum and kidneys. The gadolinium concentration measured in

the cerebellum with the three macrocyclic GBCAs was in the range of 0.150 – 0.292 nmol/g of tissue, similar to the concentration reported by Robert et al. for Gd-DOTA (0.27 ± 0.16 nmol/g).⁸⁰ The faster clearance of Gd-HP-DO3A has been ascribed to its lower viscosity in conjugation to its lower molecular weight and osmolality.⁸¹ The authors hypothesized that the GBCAs remain intact as demonstrated in another animal study,⁶⁹ but speciation was not investigated in this study.

Frenzel et al. compared the presence of gadolinium in healthy rats following the injections of a GBCA (Gd-DTPA-BMA, Gd-DTPA, Gd-BOPTA, Gd-DOTA and Gd-DO3A-butrol).⁸² They received 10 injections of 2.5 mmol/kg bodyweight (5 per week). Only the intact form was found for the two macrocyclic derivatives whereas a mix of insoluble species and macromolecules was detected for the linear derivatives. The presence of high molecular species might explain the increase in T1 weighted signal intensity observed for several GBCAs in some brain areas. This is also supported by the increase of the longitudinal relaxivity observed over time when the Gd-DTPA-BMA complex (Gd-DTPA-BMA) is placed in an artificial cerebrospinal fluid in presence of polysialic acid.⁶⁹ This carbohydrate polymer is present in high concentration in brain tissue and is able to coordinate the released gadolinium ions. Of course, those results must be confirmed by human studies, the highest level on the hierarchy of the relevance of data.

3.4. Knowledge gaps

There is no doubt that a residual amount of gadolinium is present in vivo, both in humans and in animals, following GBCA administration. Since the first article in 2014 raised concern about the presence of gadolinium in brain regions, progress has been made to better understand this issue. However, serious knowledge gaps exist, and further studies are required.

First, all the studies discussed above clearly illustrate that each GBCA has its own properties and its own behavior regarding its in vivo retention or deposition. The results obtained for one ionic linear GBCA cannot be extrapolated for all the ionic linear GBCAs, as a different amount of gadolinium is retained within this class of GBCAs. It is the same for the macrocyclic class. Therefore, each GBCA should be studied as one pharmaceutical and the categorization of the GBCAs should be avoided. There is a need for studies involving all the GBCAs, evaluating the retention of the FDA-approved GBCAs under identical conditions, which would greatly contribute to better understand this issue.

The difficulty in retrieving information from retrospective studies in humans comes from the heterogeneity between the patients, the cumulative dose, the type of GBCA used and the time between the last injection and the analysis. When several GBCAs have been used, no conclusion can be drawn about neither which GBCA is responsible for the presence of gadolinium nor to what extent. Moreover, the medical records of patients are often incomplete or inaccurate. In vivo studies are often limited by a small number of cases and selected patients are suffering from multiple sclerosis, brain tumors and other diseases that are a potential source of bias.

Most of the human ex vivo studies investigated the gadolinium deposition in brain or bone. Samples from more organs should be harvested to better understand the biodistribution of gadolinium as its concentration might also be high in kidneys, liver or heart as it has been demonstrated in the case of NSF patients.⁸³

It is also important to investigate the behavior of GBCAs in more acidic conditions. It has been hypothesized that GBCAs may encounter intracellular vesicles where the pH is lower, but this remains to be studied. Moreover, interactions between the intracellular and extracellular milieu are still unknown and should be understood.

Little is known about the chemical form of the gadolinium species present in vivo. Hence, the speciation is one of the major knowledge gaps that has to be filled in to understand the metabolism, the biodistribution and the biological effects in humans. If the GBCA remains intact, what percentage of the injected dose is retained and in which tissues? Does the retained intact GBCA slowly clear from the body and at what rate? Does the retained intact GBCA interfere with any kind of metabolic process? If the Gd is released from the GBCA, then what is the chemical form of the Gd containing species? Does it exist in the form of insoluble species (GdPO_4) or bound to macromolecules? Are the Gd containing species excreted over time? Do they interfere with specific metabolic processes and do they elicit toxic responses? What are the long-term effects? So far, besides patients suffering from NSF, no established link has been made with symptoms or side effects in patients with normal renal function and an hyperintensity in brain regions.⁸⁴ But knowing the chemical form may help understanding the long-term consequences, finding a treatment and adapting medical practice.

As detailed above, gadolinium speciation is a complex problem to solve. Further studies are necessary to shed light on the issue of speciation and these will likely require the expertise of researchers from many disciplines to fill the many knowledge gaps that remains. The following section is dedicated to the analytical and spectroscopic methods that have been used to investigate the gadolinium speciation.

4. Approaches to gadolinium speciation

Gadolinium is not an essential element in living systems and its presence in humans is mainly related to the use of GBCAs (section 3.2). Since the background signal arising from the biological milieu is very low or even undetectable, the detection of gadolinium is straightforward. Several techniques can provide information on the concentration, the distribution or on the chemical form of gadolinium species in tissues, Table 3. A flow chart of a general procedure summarizing the main methods used when investigating the presence and speciation is shown in Figure 5. The challenge is to determine the chemical form of gadolinium entities without interfering with the species or disturbing the equilibria established in human body. A sample treatment can remove soluble species or also modify the entity in a way that the one detected might be different from the one in tissue. Controls have to be prepared to validate the method employed and to ensure the full recovery of Gd. For instance, the extraction of soluble species from tissue has to be done in mild conditions but the extraction efficiency has to be proved by means of blank tissue samples that are

spiked with a known amount of GBCA. Moreover, because the concentration of gadolinium in tissue is often very low (sub-ppm), care should be taken to avoid cross-contamination between samples.

4.1. In vivo detection

Determining the speciation in vivo appears to be an ideal solution. But as previously discussed, T1 weighted MRI lacks sensitivity. Moreover, no relaxivity value can be obtained as the species responsible for the signal hyperintensity in brain regions, as well as its concentration, remain unknown. Recently, two radiation-based techniques have been developed to detect and quantify gadolinium non-invasively in the living human: prompt gamma neutron activation analysis (PNAA)⁸⁵ and X-ray fluorescence (XRF).^{86–88} PNAA consists in irradiating the sample with a beam of neutrons. The neutron capture reactions $^{155}\text{Gd}(n, \gamma)$ and $^{157}\text{Gd}(n, \gamma)$ product $^{156}\text{Gd}^*$ and $^{158}\text{Gd}^*$ which emit γ rays when decaying to the stable ^{156}Gd and ^{158}Gd isotopes. A pilot study with 10 human participants proved the ability of this technique to detect the presence of gadolinium in the lower leg using a low radiation dose (0.6 μSv). However, the limit of detection (LOD) for this method is relatively high at 0.58 ppm. In vivo XRF involves excitation of an electron of a well-defined energy level of the gadolinium followed by the measurement of the resulting X-ray fluorescence. XRF was used to quantify the amount of gadolinium in the tibia of patients that had received a GBCA and the authors were able to detect the presence of gadolinium five years after the injection.⁸⁷

4.2. Spatial distribution of gadolinium in tissues

The spatial distribution of gadolinium within tissue sections has been studied by means of various techniques, most of them allowing the detection of other elements in the process. ICP-MS is the most sensitive technique to quantify gadolinium. Usually, the tissue samples are digested in acid (HNO_3 or HClO_4) and analyzed. ICP-MS is a destructive technique and informs only on the amount of elemental gadolinium but gives no information about its chemical form. Coupled to a Laser Ablation system (LA-ICP-MS), the distribution of gadolinium species within a tissue section can be assessed. To do so, a laser beam volatilizes a small amount of tissue and this is aspirated into the ICP-MS. The laser is then rastered across the sample and elemental maps can be attained at low micron resolution. Other elements can be simultaneously detected. For instance phosphorus and calcium have shown the same distribution pattern as gadolinium within brain⁸⁹ and skin⁹⁰ samples which is a suggestive of a transmetallation process and the formation of inorganic gadolinium species, even if a correlation cannot always be found.²³ In their recent article, Fingerhut and coworkers analyzed a deep frozen brain section using LA-ICP-MS.⁸⁹ The patient received one injection of Gd-DTPA-BMA (16 mL, 0.5 mmol/L) two years prior to death. The gadolinium concentration measured in the dentate nucleus (DN) (0.80 $\mu\text{g/g}$) was much higher than in other brain regions (basal ganglia, frontal lobe and pons) where gadolinium concentrations ranged between 0.01 to 0.30 $\mu\text{g/g}$ (Figure 6). Interestingly, the concentration found in a parallel tissue section was 16-fold lower when stained with hematoxylin and eosin and this was attributed to repeated washes in the staining process which removed soluble gadolinium species. This finding points out that fresh or frozen samples are preferred over formalin-fixed and paraffin-embedded tissues when investigating both concentration

and speciation of gadolinium. That means that gadolinium concentration might be even higher than those actually measured when such treatment was performed. Using LA-ICP-MS, Roberts et al. found a gadolinium concentration of 1.01 $\mu\text{g/g}$ in the DN whereas no T1-weighted hyperintensity was detected, reflecting the higher sensitivity of ICP-MS.⁷⁹ That patient was administered at least four GBCA injections with Gd-DTPA and/or Gd-DTPA-BMA, the medical record being incomplete. The authors did not specify the sample's treatment procedure.

Synchrotron X-Ray Fluorescence (SXRF) microscopy is a powerful imaging technique that can inform on the distribution of gadolinium within a tissue section with a high resolution and sensitivity. George et al. used SXRF to investigate the gadolinium distribution in autopsy skin tissues from a patient with NFS who had received Gd-DTPA-BMEA.⁹¹ P, Ca and Zn were simultaneously detected and shown a similar distribution, again implying a transmetallation process.

Scanning electron microscopy combined with energy dispersive X-ray (SEM-EDX) was also used to determine the elemental composition of electron dense deposits on SEM.^{16,17,66,91–93} However, this method is sensitive to concentrated mineralized deposits but is much less sensitive to the presence of soluble or protein bound gadolinium species that might be dispersed throughout the sample.

Similar to LA-ICP-MS, mass spectrometry (MS) imaging gives access to the spatial distribution of gadolinium but also informs on its speciation. Species present in the tissue section are ionized by means of a laser beam and the ratio of mass to charge, m/z , is then detected by MS. As in LA-ICP-MS, the laser can be rastered across the sample to create images. Hence, the identification of gadolinium species and the deduction of their chemical form might be achieved. MS is particularly interesting to detect gadolinium species as gadolinium shows a specific pattern relative to its isotopic distribution. MALDI-imaging has been used to detect gadolinium contrast agents in mouse liver⁹⁴ and in myocardial infarcts.⁹⁵ However, the use of a matrix might be considered as an extraction, so the detected gadolinium species might not be representative of those present in the sample. Additionally, the quantification might be inaccurate as the desorption and ionization might differ between different Gd-containing species. Controls must be prepared to validate the results.

Finally, NMR imaging has been used to investigate the gadolinium distribution and speciation in heart and kidney autopsy samples from patients with NSF.⁸³ Combining relaxation time measurements T1, T2, and T2* with ICP-MS measurements can provide estimates of tissue relaxivities. Gd is a potent T1 relaxation agent of water but for this to occur, water must have access to the Gd. If the Gd is present in tissue as an insoluble deposit, e.g. GdPO_4 , or if it is sequestered inside an endosome then the T1 relaxivity will be low. On the other hand, if the T1 relaxivity is high then the Gd is likely in a form that has water access, e.g. it is intact or protein bound. Gadolinium also shortens T2 and T2*. In tissue, T2* is especially shortened if large magnetic susceptibility gradients are created. This can occur if the Gd is localized spatially in tissue at high concentrations, e.g. in solid deposits. These relaxation time measurements are bulk effects and would provide the information on the dominant type of speciation.

4.3. Probing the coordination environment of the Gd³⁺ ion

Knowing the chemical form of the gadolinium species in tissue might be achieved by probing its coordination environment and detecting, for instance, the Gd-N and Gd-O bonds of the GBCA. To do so, X-ray absorption fine structure spectroscopy (EXAFS) can be used.⁹⁶ After X-ray absorption, the emitted photoelectron wave is scattered by the nearest neighbor atoms. This scattering is measured and provides information on the local environment of the gadolinium ion such as the number, the type of neighboring atoms and the length of the bonds. This technique has been used in coordination chemistry to explain the mechanism of formation of lanthanide complexes in solution.⁹⁷ George et al. used EXAFS to investigate the gadolinium speciation in skin tissues from an autopsy of a patient with NSF.⁹¹ The difference between the EXAFS spectrum from the skin tissue sample and the spectra of pure Gd-DTPA and Gd-DTPA-BMA (Figure 7) indicates that most or all of the Gd in the skin sample is dissociated because the tissue spectrum has a completely different pattern. Moreover, the detection of features attributable to Gd-O-P bonds suggests the presence of GdPO₄.

Electron nuclear double resonance spectroscopy (ENDOR) can also inform on the environment of the Gd ion and should also be considered when investigating the gadolinium speciation: no separation is required and it can be used in complex matrices like bones.⁹⁸ It can measure the interaction between the gadolinium and close atoms, especially when it is coordinated by phosphate oxygens. The structure of the species can be elucidated by means of model complexes.

4.4. Biodistribution investigations in animal models

The isotope ¹⁵³Gd has been used to evaluate the biodistribution and measure the residual amount of gadolinium in mice and rats of four ¹⁵³Gd-labeled GBCAs.⁹⁹ While this technique is very sensitive, it does not discriminate gadolinium species. However, the dissociation of a chelate can be proven with the simultaneous use of ¹⁵³Gd and ¹⁴C-labeled chelator if these isotopes are no longer associated.¹⁰⁰ The use of radioactive material implies the preparation of a solution of GBCAs that will differ from the commercial formulation and most likely can only be used in an animal.

Another elegant strategy consists in using lanthanide surrogates. Europium and terbium ions flank gadolinium in the periodic table and form complexes of similar properties. By injecting EuL1, GdL2 and TbL3 in one animal, the biodistribution of the three complexes can be studied and compared by detecting the lanthanides by ICP-MS. The role and influence of the chelate stability on the presence of gadolinium in vivo can also be understood. Di Gregorio et al. injected either Gd-DTPA or La-DTPA in healthy mice at various doses and measured the amount of retained lanthanum and gadolinium in several organs at different time points after administration (Figure 8).¹⁰¹ They attributed the higher retention of La to the lower thermodynamic stability constant of La-DTPA (log $K_{\text{cond}} = 14.69$) compared to Gd-DTPA (log $K_{\text{cond}} = 18.49$). Bone, cerebellum, cerebrum and liver shows the largest difference in accumulation. Even though this method can only be used in animals, it might help understanding the full biodistribution and identify organs where dissociation occurs more readily.

4.5. Extraction, separation and identification

To date, the most important progress in gadolinium speciation has been made thanks to the use of combined techniques and extraction processes.^{23,69,82,90,102} Birka et al. investigated the presence of gadolinium and its speciation in a skin biopsy of a NSF patient who received an injection of Gd-DTPA-BMA and one of Gd-HP-DO3A, respectively eleven and eight years prior the skin biopsy.⁹⁰ Soluble species from the skin sample were extracted in a mixture of water and acetonitrile (4:1) for 72 h. After removal of high molecular weight species by filtration, they analyzed the filtrate by Hydrophilic Interaction Liquid Chromatography combined with ICP-MS (HILIC-ICP-MS). As shown in Figure 9, the intact GBCA Gd-HP-DO3A was detected in the sample as opposed to Gd-DTPA-BMA. The soluble species represented a small amount of the gadolinium species, the most important part being identified as insoluble species (GdPO₄) using LA-ICP-MS. Gd-BOPTA and Gd-DTPA have also been detected in their intact form in a skin sample using the same method.²³ However, the extraction procedure can lack efficiency as mild conditions are used to avoid any alteration of the species. An extraction combined to sonication might be more efficient as it induces cell lysis.¹⁰² Speciation information can also be obtained by using HILIC coupled to ESI-MS as the species can be characterized by its retention and its ratio m/z. Finally, size exclusion chromatography, also known as gel permeation chromatography (GPC), gives interesting information on the molecular weight of gadolinium containing species, especially to characterize Gd-containing macromolecules.⁸²

5. Conclusions

The increased concern surrounding the long-term safety of GBCAs has led to scrutiny of the retention of Gd in the human body. While it is becoming clear that some Gd is retained in the body after administration of all GBCAs, our understanding of the amount, distribution, and speciation of gadolinium throughout the body is still in its infancy. A particular challenge is the speciation which will require a collaborative research effort among clinicians, pathologists, bioanalytical and bioinorganic chemists. There is no one ideal method to address this problem and a combination of approaches will likely yield the most information. We hope that this review will spur activity in this direction.

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Fig. 1. Schematic drawing of gadolinium-containing species that may be retained in tissues.

Fig. 2.
Chemical structures and names of FDA-approved GBCAs.

Fig. 3.
Hierarchy of data, adapted from ref. 44.

Fig. 4. Axial T1-weighted MR images through, A, C, E, basal ganglia and, B, D, F, posterior fossa at level of dentate nucleus. Images are shown for a control group patient (A, B), and a patient who received contrast agent after their first (C, D) and last (E, F) examination. Regions of interest used in quantification of signal intensity are shown as dashed lines for globus pallidus (green), thalamus (blue), dentate nucleus (yellow), and pons (red). Reprinted with permission from ref. ²². Copyright 2015, Radiological Society of North America.

Fig. 5.
Flow chart summarizing the strategy and methods that can be used to investigate the speciation

Fig. 6. Analysis of thin sections of autopsy brain tissue from a patient after administration of Gd-DTPA-BMA 726 days before death. Microscopic image of the cerebellum without H&E-staining (a) and of an adjacent thin section of the cerebellum after H&E-staining (d), corresponding quantitative distribution maps of gadolinium investigated by LA-ICP-MS (b, e) showing that Gd was lost in the tissue processing procedure for H&E staining. Relevant areas are marked with dotted lines. Red circles denote blood vessels. Reprinted with permission from ref. ⁸⁹. Copyright 2018, Elsevier.

Fig. 7.

Extended X-ray absorption fine structure (EXAFS) spectra at the Gd L3-edge and analysis of the tissue sample compared with that from selected gadolinium-based contrast agents. (Left) EXAFS spectra and (right) Fourier transforms (FTs) with simulated fits of (a) skin tissue, (b) Gd-DTPA-BMA, (c) Gd-DTPA. The Fourier transforms are phase corrected assuming Gd–O interactions. Indicated are the atomic origins of the observed peaks. Reprinted with permission from ref. ⁹¹. Copyright 2010, John Wiley & sons.

Fig. 8.

Amount of retained Gd and La in the various tissues/organs as a function of time after the administration of 0.6 mmol La-DTPA/kg and 0.6 mmol Gd-DTPA/kg ($n = 5$ each time point; the error bars represent standard deviations). Reprinted with permission from ref. ¹⁰¹.

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Fig. 9. Speciation analysis using HILIC-ICP-MS chromatograms of the skin sample aqueous extract from an NSF patient. Reprinted with permission from ref. ⁹⁰. Copyright 2015, American Chemical Society.

Table 1

Thermodynamic stability constants ($\log K$ and $\log K_{\text{cond}}$) and the rate constants of the acid-assisted dissociation of the GBCAs.

	<i>Thermodynamic stability constants</i>		<i>Kinetic inertness</i>	
	$\log K_{\text{GdL}}$	$\log K_{\text{cond}}^h$	$k_1 \text{ (M}^{-1}\text{s}^{-1})^i$	
		pH 7.4	pH 4.0	
DTPA-BMA	16.85 ^a	14.9	10.8	12.7
DTPA-BMEA	16.84 ^b	15.0	10.9	8.6
DTPA	22.46 ^a	18.4	11.2	0.58
EOB-DTPA	23.46 ^c	18.7	11.5	0.16
BOPTA	22.59 ^d	18.4	11.1	0.41
MS-325	22.06 ^e	18.9	11.6	2.9.10 ⁻²
DO3A-butrol	21.8 ^f	16.1	9.0	2.8.10 ⁻⁵
HP-DO3A	23.8 ^g	17.1	9.9	6.4.10 ⁻⁴ , 2.6.10 ⁻⁴
DOTA	24.7 ^a	17.2	9.5	8.4.10 ⁻⁶ , 1.8.10 ⁻⁶

^aRef. 42

^bRef. 47

^cRef. 48

^dRef. 49

^eRef. 50

^fRef. 51

^gRef. 52

^hCalculated from corresponding protonation constants $\log K_{\text{H/L}}$ and thermodynamic stability constant $\log K_{\text{GdL}}$ at pH 7.4 and 4

ⁱRef. 53.

Table 2

Thermodynamic stability constants $\log K_{ML}$ of Gd^{3+} , Fe^{3+} , Cu^{2+} and Zn^{2+} complexes.

	$\log K_{ML}$			
	Gd^{3+g}	Fe^{3+}	Cu^{2+}	Zn^{2+}
DTPA-BMA ^a	16.85	-	12.04	13.05
DTPA-BMEA	16.84	-	-	-
DTPA ^b	22.46	28.0	21.4	18.29
EOB-DTPA ^c	23.46	-	20.2	18.78
BOPTA ^d	22.59	27.6 ^h	22.80	17.04
MS-325 ^b	22.06	26.66	21.3	18.7
DO3A-butrol ^e	21.8	25.7	20.8	19.0
HP-DO3A ^f	23.8	-	22.84	19.37
DOTA ^e	24.7	29.4	22.72	18.7

^aRef. 54

^bRef. 50

^cRef. 34

^dRef. 55

^eRef. 51

^fRef. 56

^gFrom Table 1

^hcalculated from $\log K_{Cond}$ at pH 7.4, Ref. 57.

Table 3

List of the analytical and spectroscopic methods and their characteristics. These techniques can give access to gadolinium concentration, distribution or chemical form and should be used in combination when investigating the speciation.

Methods	Sample preparation	Method principle	Information provided	Limitations of the technique
Unenhanced T1 weighted MRI ^{20-22,27,70-75}	In vivo	MRI	Increase in T1 weighted signal intensity in some brain regions	Not very sensitive: trace amounts of Gd species can't be detected. No information on the chemical form
Neutron activation ⁸⁵	In vivo	Irradiation by a beam of neutrons. The neutron capture reactions $^{155}\text{Gd}(n, \gamma)$ and $^{157}\text{Gd}(n, \gamma)$ produce $^{156}\text{Gd}^*$ and $^{158}\text{Gd}^*$ which emit γ rays when decaying to the stable ^{156}Gd and ^{158}Gd .	In vivo quantification of Gd	No information on the chemical form Low sensitivity: detects ppm concentration of Gd Limited to extremities
In vivo XRF ^{86,87}	In vivo	Gd excitation through a Cd-109 source and measurement of produced fluorescent K α 1 (42.3 keV) and K α 2 (43.0 keV) X-Rays	In vivo quantification of Gd	No information on the chemical form Low sensitivity: detects ppm concentration of Gd Limited to extremities
SXRF microscopy ⁹¹	Tissue sections	Fluorescence peaks of Gd are detected	Spatial distribution with μm resolution Quantification of elemental Gd and correlation with other elements	No speciation
SEM-EDX ^{16,17,66,91-93}	Tissue sections	Detection of Gd deposits using SEM (appears brighter than background tissue). EDX allows the detection of Gd	Elemental Gd SEM can show the presence of insoluble deposits in tissues SEM/EDX: association of Gd with other elements (Ca, P...)	Detection of Gd-containing insoluble deposits. No information on soluble species that might be present as well
EXAFS ^{91,97}	Tissue sections	Measure of the scattering of absorbed X-ray by nearest neighbor atoms around the Gd $^{3+}$ ion (L3-edge).	Detection of nearest neighbor atoms: P, O, Gd (for inorganic salts), N Unique signature of chelate.	Bulk method. If multiple species present, then this will show as weighted average
ICP-MS ^{20,22,79,90}	Tissue digestion in acid	Sample ionization through a plasma torch, detection of metals and non-metals by MS with a high sensitivity.	Elemental Gd Quantification of Gd Correlation with other elements	Destructive technique No information on the chemical form
HILIC-ICP-MS ^{23,90}	Extraction in H ₂ O/ACN (4:1), centrifugation, filtration of the supernatant with a 3kDa filter and filtrate analysis	Separation of soluble species on the column. The retention time allows a clear characterization of the presence of GBCAs in the extract. The presence of Gd is confirmed by ICP-MS.	Separation, identification and quantification of the extracted species	Only the low molecular weight and soluble species can be detected. The soluble species that differ from GBCAs can't be identified. The extraction is poorly efficient as mild conditions are employed to prevent the alteration of the species. The concentration and the ratio of the detected species might not be representative of the sample.
LA-ICP-MS ^{23,79,89,90}	Tissue sections are deparaffinized Frozen samples can be used	A laser beam allows the removal of a small amount of tissue in which Gd amount is then detected using ICP-MS.	Elemental Gd Spatial, μm distribution of Gd Quantification possible	Quantification can be challenging. Sample processing may alter speciation or distribution

Methods	Sample preparation	Method principle	Information provided	Limitations of the technique
GPC-ICP-MS ⁸²	Tissue extraction, centrifugation and analysis of the supernatant.	Separation of the species according to their molecular weight. Detection of Gd-containing species using ICP-MS	Presence of macromolecules and intact GBCAs can be detected	No direct speciation
MALDI imaging ^{94,95}	Deposition of a matrix on the tissue section	The laser allows a gentle desorption and ionization of the molecules mixed with the matrix. The m/z ratio is then detected by MS.	Molecular weight of the Gd species Spatial distribution within the tissue	The use of a matrix can be considered as an extraction and therefore the detected Gd species might not reflect the Gd species of the sample. Quantification is inaccurate as the desorption and ionization might differ between Gd species
NMR ⁸³	Tissue sections	Measurement of the relaxation times T1, T2 and T2*. - Higher T1 relaxivity than GBCA suggests Gd bound to protein - Lower T1 relaxivity suggests compartmentalized or insoluble Gd - T2* or susceptibility could indicate high local Gd concentration	Distribution, concentration and information on the type of Gd species and their environment (precipitated, endosomal, high/low molecular weight...)	No clear species identification
ENDOR ¹⁰³	Tissue samples	Measure the interaction between Gd and close atoms	The detection of Gd-N and Gd-O-P bounds informs on the chemical form of the Gd species	Bulk method. If multiple species present, then this will show as weighted average
Radioactive tracers ^{99,100}	Animal study	Detection of ¹⁵³ Gd (electron capture, $t_{1/2} = 240$ days, γ (41 keV, 102 keV)) It can be used with other radioisotopes (¹⁴ C...)	Quantification of Gd	It can only be used for animal studies. The radiolabeled GBCA has to be prepared No speciation
Lanthanide surrogates ¹⁰¹	Animal study Tissue digestion in acid	Injection of a GBCA (GdL1) and its analogous complex formed with another lanthanide ion (LaL1: a less stable complex) Detection of the metal by ICP-MS Simultaneous injections of one gadolinium complex GdL1 with TbL2 and EuL3 (Tb ³⁺ , Eu ³⁺ form complexes of similar stability as with Gd ³⁺) Detection of the metal by ICP-MS	Evaluating the difference of behavior between two complexes of similar physicochemical properties (viscosity, elimination rate...) but different stability. Investigating the biodistribution of several complexes in a same animal at the same time by detected each metal.	Only for animal studies The GBCA analogues formed with Tb ³⁺ or Eu ³⁺ must have the exact same behavior as the Gd complexes

EDX: Energy Dispersive X-ray, ENDOR: Electron Nuclear Double Resonance, EXAFS: Extended X-ray Absorption Fine Structure, GPC: Gel Permeation Chromatography, HILIC: Hydrophilic Interaction Liquid Chromatography, ICP-MS: Inductively coupled plasma Mass spectrometry, LA: Laser Ablation, NMR: Nuclear Magnetic Resonance, MALDI: Matrix Assisted Laser Desorption Ionization, MRI: Magnetic Resonance Imaging, SEM: Scanning Electron Microscopy, (S)XRF: (Synchrotron) X-ray Fluorescence