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MRI Contrast Agents From Molecules to Particles

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Sophie Laurent
General, Organic and Biomedical Chemistry
NMR and Molecular Imaging Laboratory
University of Mons
Mons
Belgium

and

Center for Microscopy and Molecular Imaging (CMMI)
Gosselies
Belgium

Céline Henoumont
General, Organic and Biomedical Chemistry
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Chapter 1

Interest of Nanomaterials in Medicine

Abstract This introduction highlights the importance of nanomaterials in biomedical applications. The use of different nanosystems in the medical field has conducted researchers to develop numerous synthetic ways.

Since few decades, nanotechnology is a growing interesting area of research. Fabrications of nanomaterials such as nanoparticles, clusters, nanotubes, nanowires or nanorods are the main domains investigated in nanotechnology. In October 2011, the European Commission officially defined the term “nanomaterial” as followed: “A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm” [1].

As the size decreases to nanoscale, nanomaterials exhibit extraordinary physical and chemical properties different from the bulk species, which can be attributed to their dimensional anisotropy and size. An important aspect is the increased ratio of surface area to volume, making possible new quantum mechanical effects where the electronic properties of solids are altered. In addition, their optical properties, such as fluorescence, depend on the particle diameter. For example, ZnO nanowires show nonlinear optical properties while the bulk ZnO shows linear optical properties [2] or Au nanorods can be used for optothermal therapy [3] while Au nanocages can be used for controlled release of drugs [4]. These excellent properties provide potential advantages in a wide range of applications including magnetic nanoparticles or multicolor optical coding based lab-on-chip technology [5, 6], visualization of cellular structures or tracking movements [7], controlled drug delivery, medical imaging technology.

Nanotechnology plays an important role in both medical research and clinical diagnostics/therapy, as nanomaterials are commonly within the same size scale than biological entities like cells, DNA and proteins. Properties of nanomaterials, including size, shape, chemical composition, surface structure and potential, aggregation and agglomeration, and solubility, can greatly influence their interactions with biomolecules and cells. For example, quantum dots (QDs) with

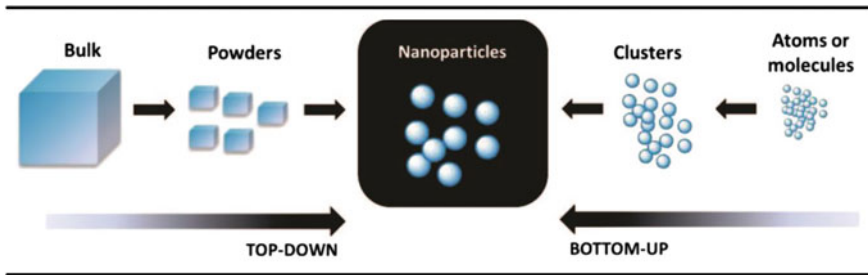


Fig. 1.1 Scheme of top-down and bottom-up approaches of the nanoparticle fabrication

size-tunable fluorescence emission have been employed to produce predominant images of tumor sites [8], or single-walled carbon nanotubes (SWCNTs) with diameters comparable to that of DNA show an impressive potential as high-efficiency delivery transporters for biomolecules into cells [9].

The discovery of the novel properties at the nanoscale has led to a great fascination for the nanoparticle fabrication. The manufacturing of the nanoparticles can be roughly categorized as either “top-down” or “bottom-up” approaches as depicted in Fig. 1.1. Top-down strategy refers to nanofabrication techniques consisting in the reduction of the size from bulk materials towards a nanometer size structure. The traditional methods of nanofabrication are principally gathered in the different methods of lithography [10–12]. This approach creates nanostructures with the expected shape and size with a high reproducibility. However, such processing is expensive and the lithography of nanostructures below 10 nm is often limited. In nanoparticle synthesis domain, researchers prefer the second approach “bottom-up” that provides a simple and powerful way to form colloidal suspensions. Contrarily to the top-down method, this approach is able to produce nanoparticles with dimensions below 10 nm. The synthesis process is not expensive. This process begins from the assembling of atoms or molecules to produce bigger entities. Many properties of nanomaterials can be controlled as the size, the surface or the shape which confer them a great interest in the organic/inorganic synthesis field [13].

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Chapter 2

Magnetic Properties

Abstract The magnetic properties of a material are the basis of their applications. Specifically, the contrast agents that will be developed in Chaps. 4 and 5 use their magnetic properties to play their role in magnetic resonance imaging. It is thus important to describe briefly the different magnetisms.

The magnetic properties of a material are characterized by its magnetic susceptibility χ , which describes the ability of the material or substance to be magnetized by an external magnetic field H .

$$M = \chi H$$

The magnetization, M , represents the net magnetic moment per unit volume that is aligned parallel to the external magnetic field. The magnetism of the material comes from either electrons (electronic magnetism) or atomic nuclei (nuclear magnetism). However, the magnetization created by one electron being 657 higher than that created by one proton, electronic magnetism masks the nuclear one. According to the behavior exhibited in the presence of a magnetic field, several classes of substances can be distinguished.

2.1 Diamagnetic Substances

The majority of atoms constituting tissues of the body are diamagnetic because they do not have unpaired electrons. The global electronic magnetic moment of each atom is zero. However, an external magnetic field B_0 may slightly alter the orbital paths of the electrons in motion and low magnetization can then be detected in a direction opposite to the external field direction B_0 . Diamagnetic agents are not used as contrast agents for their effects on T_1 and T_2 relaxation time. The consequence of diamagnetism is a very weak and negative susceptibility.

2.2 Paramagnetic Substances

Ions or molecules possessing unpaired electrons tend to move in the direction of the magnetic field and increase its effect, these compounds are called paramagnetic substances. The more unpaired electrons, the greater is the electron magnetic moment and the more important will be the paramagnetic properties. All transition metals (Cr^{2+} , Cr^{3+} , Mn^{2+} , Mn^{3+} , Fe^{2+} , Fe^{3+}) and lanthanides (Gd^{3+} , Dy^{3+}) have a significant number of unpaired electrons. The gadolinium ion, with seven free electrons, has a large magnetic moment. It was first chosen as the base of paramagnetic MRI contrast agents.

2.3 Ferromagnetic Substances

While paramagnetism is a property from the isolated ion, ferromagnetism is a property based on the cooperation of thousands of atoms in a paramagnetic crystal structure. Ferromagnetic compounds have regions or domains where there is an overall magnetization. Before being subjected to an external field, a ferromagnetic compound has no spontaneous magnetization: the different magnetic domains are oriented randomly. After application of an external field, the magnetic fields are oriented along the axis of the field and have a bulk magnetization describing a hysteresis curve (Fig. 2.1a). When the magnetic field B reaches a certain value, the saturation magnetization M_s is obtained and the magnetization stays constant. When B is stopped, the magnetization is maintained at M_s and then slowly decreases to the isothermal remanent magnetization. The coercive force (or coercivity) is the intensity of the applied magnetic field required to reduce the magnetization to zero after reaching the M_s .

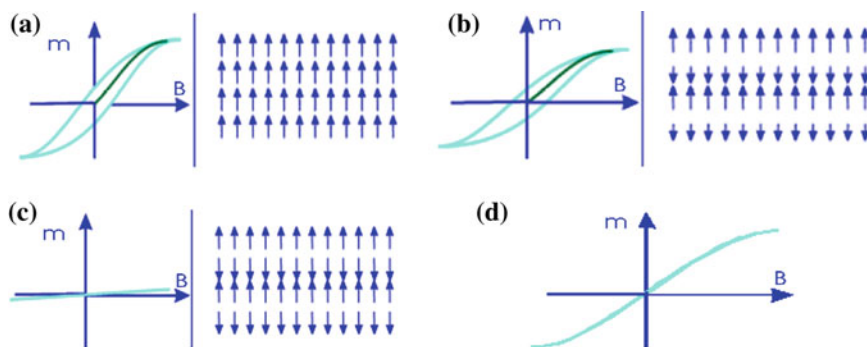


Fig. 2.1 Different kinds of magnetisms: magnetisation curves of **a** ferromagnetic **b** ferrimagnetic **c** antiferromagnetic **d** superparamagnetic material

2.4 Ferrimagnetic Substances

Ferrimagnetism is characterized by two sub-networks in which the spins are antiparallel. However, their magnetic moments are not equal and do not compensate completely (Fig. 2.1b).

2.5 Antiferromagnetic Substances

Antiferromagnetic materials are composed of ferromagnetic sub-networks whose magnetic moments are identical and of opposite directions (Fig. 2.1c). Therefore, in the absence of an external field, the resulting magnetization is zero. By heating above a characteristic temperature of the material (Neel temperature), the material becomes paramagnetic.

2.6 Superparamagnetic Substances

The concept of superparamagnetism is very close to that of ferromagnetism, the difference being the coherent strength of the magnetic domains. When the external magnetic field is stopped, different magnetic domains found a random orientation and their resultant is zero (Fig. 2.1d). The superparamagnetism appears when the crystal size becomes sufficiently small. The crystal becomes a single domain when the size is below a critical value. A single domain magnetic crystal has no hysteresis loop. A crystal is superparamagnetic when it demagnetizes completely when the field is removed. Suspensions of iron oxide nanoparticles smaller than 20 nm display superparamagnetic behavior at room temperature. The degree of alignment of magnetic moments depends of the temperature. The alignment decreases when the temperature increases and beyond a critical temperature, the magnetization becomes zero.

For magnetite and maghemite, this critical temperature is called Curie temperature T_c . For example, magnetite has a Curie temperature of 850 K [1]. At room temperature, magnetite particles smaller than 6 nm are superparamagnetic [2]. Their magnetic properties depend on their synthesis method and crystal morphology [3–6].

The bulk magnetite is a natural material in which the magnetic domains are divided in **Weiss domains** separated thanks to walls called **Bloch walls** [7]. Inside each domain, the magnetic moments possess uniform directions whereas the surrounding magnetic domains exhibit a different direction of magnetic moments (Fig. 2.2a). The disposition in magnetic domains optimizes the system energy [8].

If an external magnetic field (H) is applied, the Bloch walls move and induce an increase of the magnetic domains oriented in the same direction as the external magnetic field. Depending on the strength of the external magnetic field, the

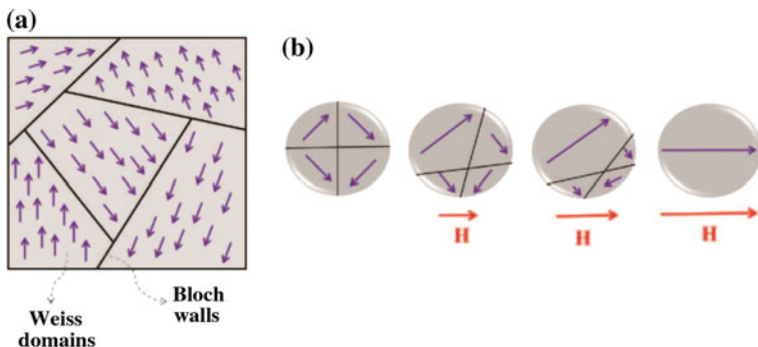


Fig. 2.2 **a** Organization of magnetic domains for ferromagnetic materials and **b** arrangement of Weiss domains as a function of applied magnetic field (H) with different intensities

predominant magnetic domains expanded much more compared to the other domains. A high magnetic field is required to completely remove the Bloch walls as shown in Fig. 2.2b. This phenomenon is at the origin of the remanent magnetization (M_r) and of the hysteresis observed [9, 10] during the measurement of magnetic moments as a function of applied magnetic field (Fig. 2.3).

Initially, the material is not magnetized (**a**) and the magnetic moment increases while an external magnetic field (H) is applied. The red curve (**b**) represents the first magnetization curve in which the Bloch walls move to promote the dominant direction of the magnetic domains present in ferromagnetic compounds as previously shown in Fig. 2.2b. The magnetic moment increases until reaching the

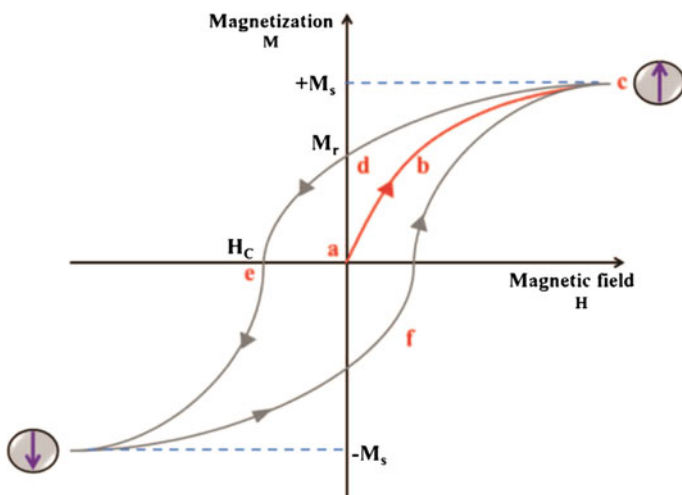


Fig. 2.3 Hysteresis curve of a ferromagnetic compound with the representation of the saturation magnetization (M_s), the coercive field (H_c) and the remanent magnetization (M_r)

saturation magnetization (M_s), corresponding to the state where all the magnetic moments are aligned with the external magnetic field (**c**). If the intensity of magnetic field is then reduced, the magnetic moment will decrease (**d**). At zero magnetic field, the magnetic moment is equal to a value corresponding to the remanent magnetization (M_r). This resulting magnetization is a fundamental characteristic of the ferromagnetic substances and it is due to the presence of domain structure [11]. To demagnetize the materials (**e**), it is necessary to apply an opposite magnetic field called coercive field (H_c). By a continuous increase of the magnetic field in opposite direction, the magnetic moment decreases to a minimum value ($-M_s$). At this point, an increase of H induces an enhancement of magnetization to the saturation magnetization; however, a larger value of H will be needed to reach a zero magnetization. The final curve describes the hysteresis cycle that is an important characteristic of the ferromagnetic compounds. It is important to determine the remanent magnetization and the coercive field. Both parameters depend on the nature and the size of the materials. On the contrary, the saturation magnetization does not depend on the material size and it is only influenced by chemical composition.

If the material size is small enough, the coercive field will become equal to zero and the remanent magnetization will disappear. Consequently, these materials react as a paramagnetic compound. However, the size of these magnetic substances must be small enough to form a single crystal domain in which these materials acquire uniform high magnetization with all the spins aligned in the same direction (Fig. 2.4). This phenomenon is called **superparamagnetism** and differs from paramagnetism. Indeed, the magnetic moments of these compounds are much larger than those of electrons responsible of paramagnetic behavior. To have superparamagnetic materials, the size must be less than a Weiss domain of the corresponding material bulk.

The magnetization curve of superparamagnetic materials is not defined by a hysteresis curve as observed for the ferromagnetic compounds. The curve is reversible with no remanence and no coercive field (Fig. 2.5).

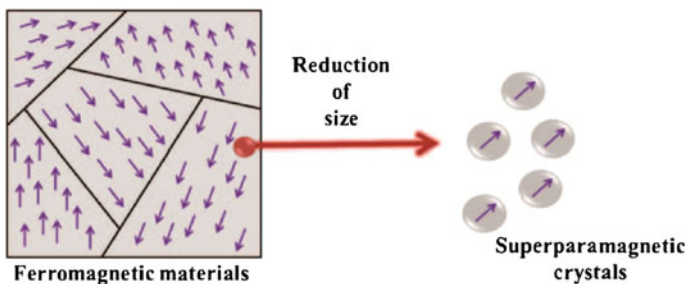


Fig. 2.4 Comparison between the multidomain structure of ferromagnetic materials and the singledomain of superparamagnetic crystals

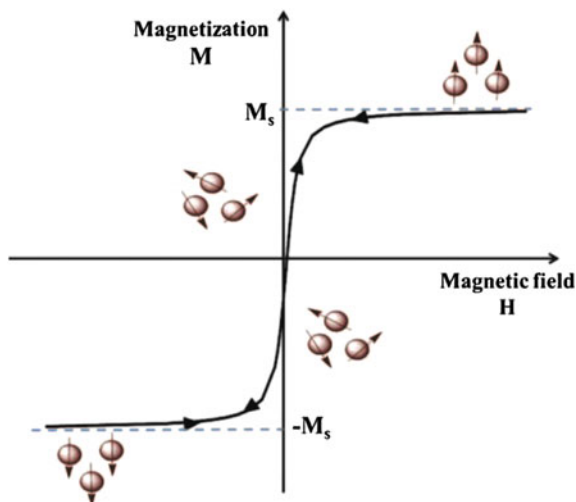


Fig. 2.5 Magnetization curve of superparamagnetic materials

The appearance of the magnetization curve of superparamagnetic materials can be defined as a Langevin function ($L(\alpha)$) as follows:

$$L(\alpha) = \coth\left(\alpha\right) - \frac{1}{\alpha} \quad \text{with } \alpha = \frac{\mu H}{k_b T}$$

Where μ is the particle magnetic moment, H is the external magnetic field, k_b is the Boltzmann constant and T is the temperature.

The net magnetization (M) is described as the saturation magnetization (M_s) multiplied by Langevin function ($L(\alpha)$):

$$M = M_s L(\alpha)$$

The size and the saturation magnetization of superparamagnetic particles can be determined from the measurements of magnetization curve thanks to their fitting by Langevin function [12, 13]. For this, the assumptions are that all particles exhibit the same volume and that the saturation magnetization is considered as an average value. The superparamagnetism is a particular magnetic property widely exploited in the field of relaxometry and relaxing agents.

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Chapter 3

Imaging Probes

Abstract This chapter briefly describes the principle of nuclear magnetic resonance (NMR) and more particularly of magnetic resonance imaging (MRI). This is necessary to fully understand the importance of developing new MRI contrast agents.

Imaging technologies are the “spies of surgeons”, as they allow to collect and process information of patients without surgical operation. Over the past decades, medical imaging technology has been developed at amazing speed, including radiography, ultrasonography, echocardiography, magnetic resonance imaging (MRI). X-ray is mostly used in radiography for its prominent penetration, while the cancer risk induced by X-ray irradiations increases anxiety of clinicians and patients [1, 2]. Therefore, MRI and optical imaging (OI) become growing interesting challenges. However, each imaging technology has its particular advantages and disadvantages, which prompts researchers to combine different technologies to form bi-functional or multi-functional imaging technologies. The need for multifunctional imaging probes has increased the interest for nanoparticles as platform where several fluorophores, biological vectors, radioisotopic molecules, MRI contrast agents, ... can be grafted.

3.1 Magnetic Resonance Imaging (MRI)

MRI uses nonionizing radiation and has become an indispensable tool in clinical radiology, and more recently in experimental research. Compared to computer tomography (CT), MRI can also produce noninvasive and painless diagnosis of disease, but without cancer risk caused by X-ray radiation. After Felix Bloch and Edward Purcell (the Nobel Prize winners in 1952) discovered the magnetic resonance phenomenon independently in 1946, nuclear magnetic resonance (NMR) was developed and used for chemical and physical molecular analysis. In 1971 Raymond Damadian reported that the nuclear magnetic relaxation times of tumors

were different from those of tissues [3], which motivated scientists to consider magnetic resonance for the detection of disease. The 2003 Nobel Prize winners in physiology or medicine Paul Lauterbur and Sir Peter Mansfield used magnetic field gradients to determine spatial localization [4], which allowed rapid acquisition of 2D images and made MRI developing rapidly in clinical diagnosis.

There are two discrete energy states in a system of protons. In such a system the protons occupy the high or low state according to a defined probability, which is called Boltzmann distribution or Gibbs distribution. The ratio between numbers of particles N_i and N_j occupying the states possessing energy E_i and E_j , respectively, can be calculated as:

$$N_i/N_j = \exp(\Delta E/kT)$$

where $\Delta E = (E_j - E_i)$ is the energy difference between the two states, k and T are the Boltzmann constant and the absolute temperature, respectively. With the existence of an applied magnetic field B_0 and a radio frequency electromagnetic field B_1 , ΔE will increase proportionally to B_0 , following the equation:

$$\Delta E = h\nu = \frac{h(B_0 - B_e)\gamma}{2\pi}$$

where h is the Planck's constant, ν is the frequency of the B_0 field, B_e is a small magnetic field generated by the circulation of electrons of the molecule, and γ is the gyromagnetic ratio (a constant which is a property of the particular nucleus). Therefore, the difference of the protons in the low and high energy states will increase, and the NMR signal intensity increases with it, as the NMR intensity is directly dependent on the population difference. So strong magnetic field is commonly applied to enhance the NMR signal and to obtain larger signal-to noise ratio.

MRI uses the property of NMR to image nuclei of atoms inside the body. The intensity of the signal in each voxel depends on several intrinsic parameters, including the proton density, longitudinal (R_1) and transverse (R_2) NMR relaxation rates, and some extrinsic parameters, including magnetic field strength, pulse sequence, and the presence of contrast agents (CAs). The magnetic field is used to align the magnetization of atomic nuclei of ^1H in the body, and radio frequency magnetic fields are used to tune the alignment of this magnetization, which causes the nuclei to produce a rotating magnetic field detectable by the scanner. This process where the system discharges the excess energy and returns to the equilibrium state is called relaxation. Magnetic field gradients make nuclei at different locations to spin at different rates, which allow spatial information to be recovered with the help of Fourier analysis of the measured signal. By using magnetic field gradients in different directions, 2D images or 3D volumes can be obtained in any arbitrary orientation.

3.1.1 Classical Description of NMR—Description on the Macroscopic Scale

If we consider a spin packet experiencing the same applied magnetic field B_0 aligned on the z-axis, their motion may be described by a single precessing vector M , called the bulk magnetization (Fig. 3.1a). M is defined as the sum of the individual nuclear magnetic moments of the spin packet.

$$M = \sum_{i=1}^N \mu_i$$

$$|M| = \frac{\gamma \hbar}{4\pi} (n_+ - n_-) \approx \frac{\gamma^2 \hbar^2 |B_0| N_S}{16\pi^2 kT} \quad [Am^{-1}]$$

M is precessing around B_0 at the Larmor frequency. In order to simplify the analysis of its behavior, it is more convenient to introduce a new rotating reference system $[x', y', z]$ called rotating frame. The new reference system is defined as rotating around the z axis of the coordinate system $[x, y, z]$ at the Larmor frequency so that M appears as static (Fig. 3.1b).

In the rotating frame, M is defined by its longitudinal component (M_z) which is its projection on the z axis and by its transversal component (M_t) which is its projection in the xy plane (Fig. 3.1b).

The application of a radiofrequency pulse at the Larmor frequency induces a temporary transient magnetic field B_1 in the xy plane. The net magnetization is flipped from its equilibrium and spins are precessing around B_1 . The flip angle (also called pulse angle) depends on the duration of the RF pulse and on the magnitude of B_1 . A 90° pulse tips the M_0 magnetization by 90° , a two times longer radiofrequency results in a 180° pulse.

When the system is submitted to a radiofrequency wave at the Larmor frequency, spins are promoted to the higher energy state and the magnetization flips

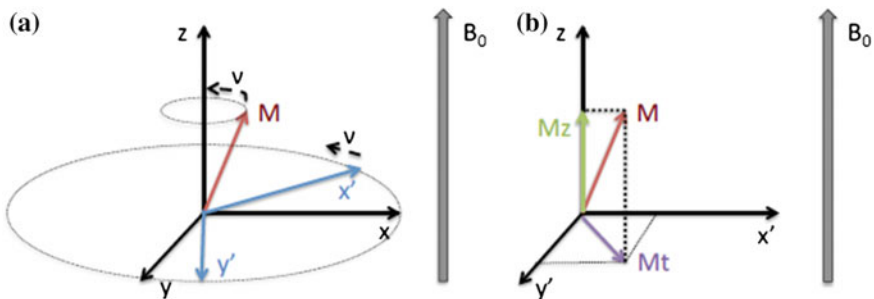


Fig. 3.1 Representation of the bulk magnetization (M) in a coordinate reference system. **a** Precession of M and of the new coordinate system $[x', y', z]$ around z at the Larmor frequency (v), **b** Representation of M in the rotating frame

away from its equilibrium position so that the longitudinal component of the net magnetization, M_z , is lower than M_0 (M at the equilibrium).

When stopping the excitation, the system progressively returns back to equilibrium, M_z grows up until it reaches its initial M_0 value (Fig. 3.2).

This phenomenon is called spin-lattice relaxation and depends on the T_1 relaxation time. T_1 is defined as the time needed to recover 63 % of the net magnetization value at the equilibrium after a 90° pulse.

The longitudinal component of the net magnetization at a time “ t ” after a 90° pulse can be expressed as an increasing exponential equation:

$$M_z(t) = M_0 \left(1 - e^{-t/T_1} \right) \quad [Am^{-1}]$$

The transversal component $M_{x',y'}$ is defined as the projection of the M vector in the xy plane and is 0 at the equilibrium.

$$M_{x',y'} = \left| \vec{M}_{x'} + \vec{M}_{y'} \right| \quad [Am^{-1}]$$

After excitation, the transverse magnetization decays because of the loss of phase coherence of the individual magnetic moments. The transverse relaxation is

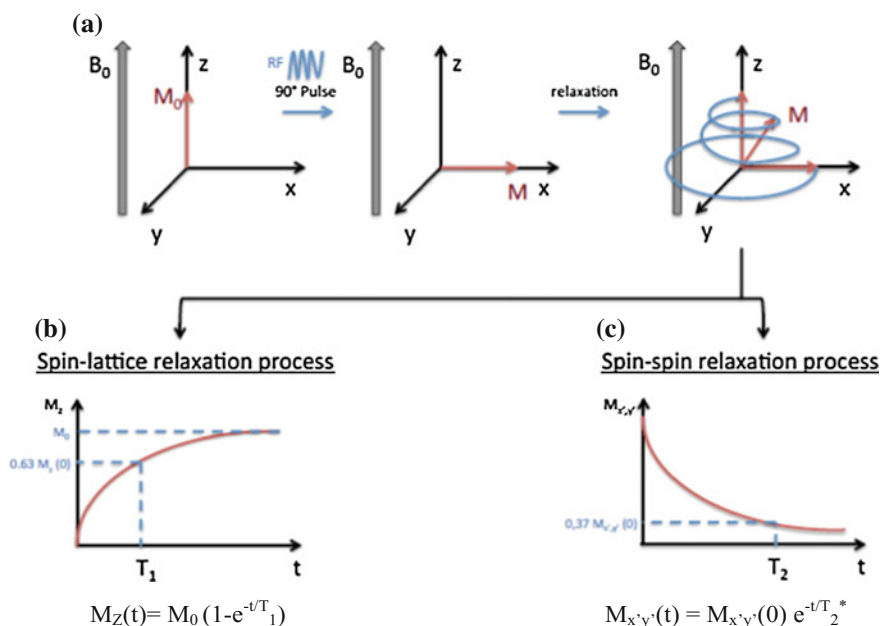


Fig. 3.2 Behavior of the bulk magnetization (M) after a 90° pulse (a). Description of the relaxation processes: the spin-lattice relaxation (b) and the spin-spin relaxation process (c)

depending on the time constant T_2 that is defined as the time needed to reduce the transversal magnetization component by 63 %. It is described by the following decreasing exponential equation:

$$M_{x'y'}(t) = M_{x'y'}(0)e^{-t/T_2} \quad [Am^{-1}]$$

The transverse relaxation is mostly due to spin-spin interactions but is also depending on the magnetic field inhomogeneities that make spins precessing at different frequencies and on the surrounding environment (temperature, interferences with other molecules, microviscosity, ...). Consequently, the time constant of the decay T_2^* is given by

$$\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_2^{inh}}$$

where T_2^{inh} is related to the inhomogeneities.

T_2 is always smaller or equal to T_1 .

3.1.2 NMR Signal Acquisition

After a 90° pulse, the magnetization starts precessing in the xy plane and generates an electromagnetic signal that can be detected by a receiver coil placed in the xy plane (Fig. 3.3).

The recorded signal is the FID that is a signal decaying with an exponential time constant T_2^* .

In Fourier Transform NMR spectroscopy broad frequency pulses are used to excite all the spins present in the sample. The application of a mathematical treatment on the FID signal (Fourier transformation) leads to the creation of a frequency spectrum. This spectrum yields information on the quantity and Larmor frequencies of the nuclei present in the sample and consequently information concerning the chemical structure of the sample is provided.

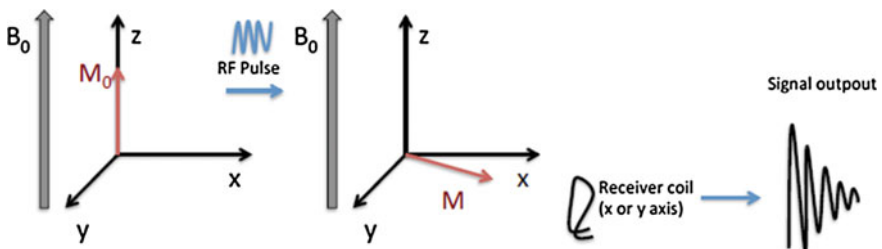


Fig. 3.3 NMR signal acquisition, registration of the FID signal thanks to a receiver coil placed on x or y axis

3.1.3 Principle of MRI

Magnetic resonance imaging is a non-invasive imaging technique based on the nuclear magnetic resonance phenomenon. It provides a cartography of the ^1H density in an object. As hydrogen constitutes approximately 63 % of the atoms of the human body, it is the most studied nucleus.

3.1.3.1 Spatial Coding

MRI requires the recording of the MR signal from each individual volume element (voxel). The spatial coding of the signal is achieved by the use of gradient fields which make the magnetic field intensity and consequently the Larmor frequency spatially dependent.

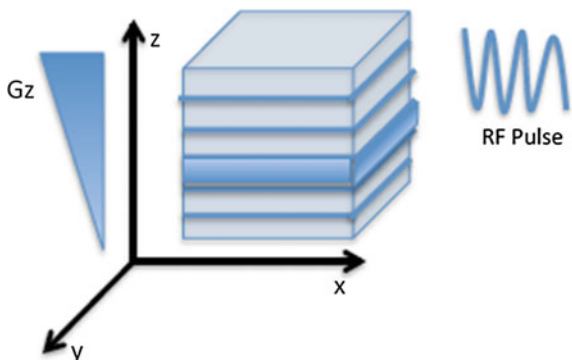
The first step of the acquisition is the excitation of a specific slice of the image object. This is made by imposing a gradient G_z to the main magnetic field B_0 (Fig. 3.4). The intensity of the resulting magnetic field increases along z and the resonant frequency (ν) at the time t and at the position z is,

$$\nu(z, t) = \frac{\gamma}{2\pi} (|B_0| + G_z(t)z) \quad [\text{Hz}]$$

Then, the slice selection gradient is turned off and a gradient along the y -axis (called the phase encoding gradient) and a gradient along the x -axis (frequency encoding gradient) allow to record the signal from each voxel.

During examinations, the typical thickness of the excited slices is several millimeters. Gradient fields are in the order of μT and can be rapidly switched on and off to encode the spatial position of the NMR signal.

Fig. 3.4 Slice selection through the application of a magnetic field gradient along z (G_z)



3.1.3.2 Excitation Sequence

The spin echo sequence is one of the most commonly used imaging sequence that aims to improve the quality of the recorded signal while making it less susceptible to inhomogeneities.

The sequence consists of a 90° pulse that flips the magnetization out of its equilibrium. Because of inhomogeneities, spins begin to spread out leading to a weaker signal. To compensate this T_2^{inh} effect, the 90° pulse is followed after a defined time by a 180° refocusing excitation pulse that consists in flipping the dephasing spins in the transversal plane so that spins begin to rephase until a time called echo time (TE) at which the signal is maximum along x' and is recorded (Fig. 3.5). The intensity of this signal depends on the TE and on the T_2 of the nuclei.

3.1.3.3 Contrast in MRI

Tissues are characterized by different proton densities, T_1 and T_2 relaxation times. These values are exploited to generate natural contrast on images.

Nevertheless, it is possible to improve the contrast by varying parameters such as the echo time (TE) and the repetition time (RT) leading to proton density, T_1 or T_2 -weighting of the spin echo sequence.

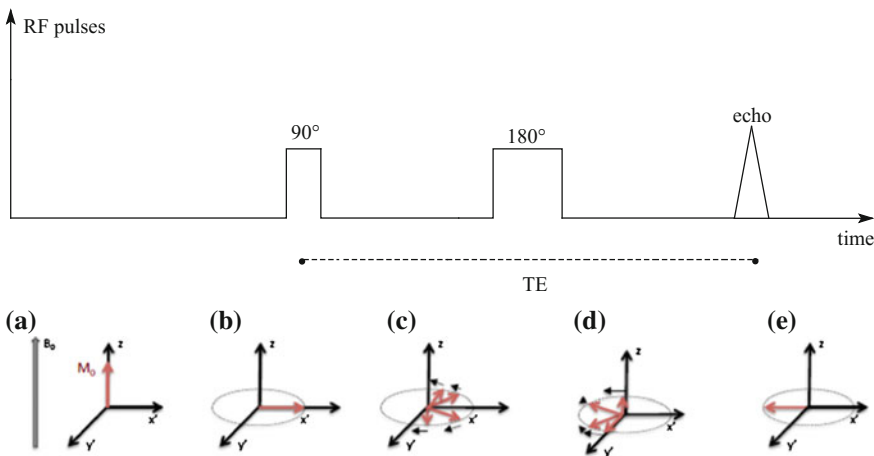


Fig. 3.5 Representation of a spin-echo sequence. **a** Bulk magnetization in the presence of a static magnetic field (B_0), after the application of a 90° pulse **(b)**, spins start to loose phase coherence due to field inhomogeneities **(c)**. The application of a 180° pulse makes them to flip in the x - y plane **(d)** and spins rephase until the TE (echo time) when the signal reaches its maximum and is measured **(e)**

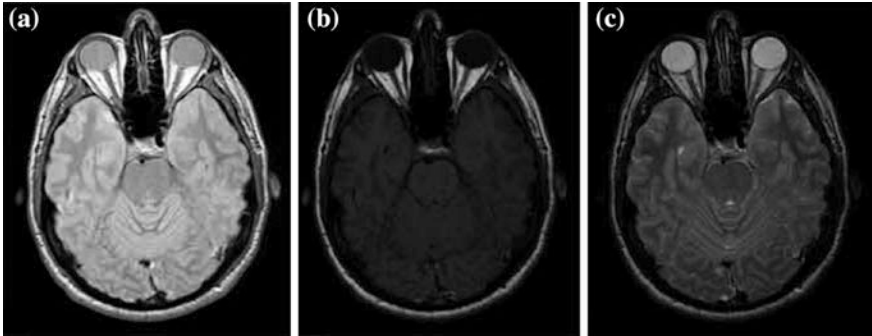


Fig. 3.6 Illustration of the TE and RT effect on the contrast of a transversal section of a human brain MR image. **a** Proton density weighted images (RT = 4 s, TE = 20 ms), **b** T₁-weighted image (RT = 500 ms, TE = 20 ms) and **c** T₂-weighted image (RT = 4 s, TE = 80 ms). *Source* http://www.thebarrow.org/Education_And_Resources/Barrow_Quarterly/205116

By setting $TE \ll T_2$ and $T_1 \ll RT$, the received signal is not affected by the relaxation phenomenon and the generated MRI image is mostly related to the proton density (Fig. 3.6a).

The differentiation between the T₁ relaxation times of tissues is emphasized if $RT < T_1$ (and $TE < T_2$) because the magnitude of the signal becomes T₁ dependent and images are T₁-weighted (Fig. 3.6b).

If $T_2 < TE$ (and $RT \gg T_1$), the sequence is mainly sensitive to T₂ relaxation times and images are T₂-weighted (Fig. 3.6c).

However, sometimes the contrast generated by the relaxation times and the differences in water content is not sufficient to discern diseased from healthy tissues. Then clinicians use compounds called contrast agents able to enhance the contrast in the area of interest where they accumulate.

3.2 MRI Contrast Agents

Multiple classifications are possible [5, 6]. Contrast agents can be classified according to their route of administration, their magnetic properties and thus their effect on the contrast, their size or their distribution in the body.

The mode of action of all MRI contrast agents is based on the reduction of T₁ and T₂ relaxation times of water protons. Two categories can be distinguished:

- T₁ agents, the main effect is a decrease of the longitudinal proton relaxation time. This relaxation time depends on the rate at which the proton transfers its excited energy to the surrounding medium.
- T₂ agents, the main effect is a decrease of the transverse proton relaxation time. T₂ depends on the rate at which protons are out of phase with each other.

T_1 and T_2 nuclear relaxation times are expressed in seconds. The relaxation rates (R_1 and R_2) are the reciprocal of relaxation time ($1/T_1$ and $1/T_2$) and are expressed in s^{-1} . The effect of a contrast agent on the relaxation rates is linearly proportional to its concentration. The essential property of contrast agents is their “relaxivity” (r_1 and r_2) defined as the contribution to the relaxation rate of water protons at a concentration of 1 mM ($s^{-1}mmol\ l^{-1}$). Relaxivity thus defines the efficiency of the contrast agent to increase the relaxation rate of surrounding protons. The r_2/r_1 ratio is sometimes used to indicate the magnitude of the T_2 effect compared to T_1 effect.

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Chapter 4

Paramagnetic Gadolinium Complexes

Abstract The mechanism of action of the paramagnetic gadolinium complexes is briefly described, with an emphasis on the different parameters that can be tuned to increase their efficiency. An overview of the different classes of paramagnetic gadolinium chelates is then presented.

Contrast agents (CAs) for various kinds of imaging methods have been used for decades in diagnostic radiology and developmental biology [1]. The synthesis and applications of paramagnetic CAs were reported with a rapidly growing rate by scientists, and more than 30 % of all clinical MRI examinations use CAs [2]. Paramagnetic metal ion with unpaired electrons can act as the relaxation enhancement agents for MRI, by increasing the relaxation rates R_1 and R_2 of close-by spins. Such enhancements on R_1 and R_2 can be used to assess the efficiency of an MRI CA, following the equation:

$$R_i^{\text{obs}} = R_i^d + R_i^p \quad i = 1, 2$$

where R_i^{obs} is the observed solvent relaxation rate in the presence of a CA, and the subscripts d and p refer to diamagnetic and paramagnetic, respectively. The paramagnetic contribution is dependent on the concentration of CAs, in the form of r_i [M] where [M] is the concentration of CAs and r_i is the relaxivity, defined as the relaxation rate R_i increased per unit concentration of CAs. The origin of the paramagnetic contribution is usually divided into two components, inner-sphere (IS) and outer-sphere (OS) mechanisms:

$$R_i^p = R_i^{\text{IS}} + R_i^{\text{OS}} \quad i = 1, 2$$

where R_i^{IS} and R_i^{OS} refer to relaxation enhancement of the bulk solvent related to solvent molecules directly coordinated to the paramagnetic ion in the innersphere and relaxation enhancement of solvent molecules in the outer sphere, respectively (Fig. 4.1a, b) [3].

Lanthanide Gd(III) ion has been employed as the metal ion for MRI T_1 agents, for its high magnetic moment ($\mu^2 = 63\mu_B^2$) and symmetric electronic ground state

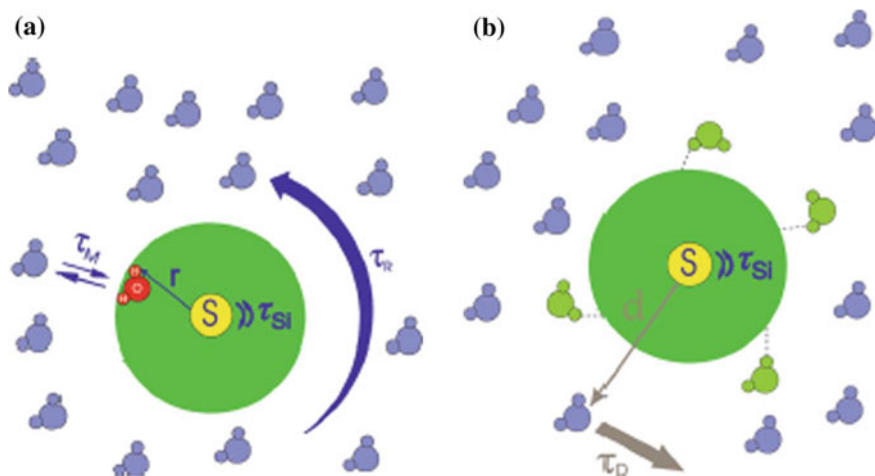


Fig. 4.1 Representation of the inner-sphere (a) and outer-sphere (b) contribution. Reproduced with permission of Ref. [3]

(S^8) [4]. Gd ions have a relatively long electronic relaxation (10^{-8} to 10^{-9} s) compared with other lanthanides, allowing efficient transfer of the magnetic information to the water molecules. These complexes have relaxivities r_1 and r_2 of the order of 3 to $10 \text{ s}^{-1} \text{ mM}^{-1}$ at 1.5 T. However, as the T_1 of the tissue is much higher than their T_2 (≈ 10 fold), the effect is much more marked on T_1 leading to an increase of the MRI signal (bright signal). These complexes are called positive contrast agents. The larger the T_1 weighting of the sequence, the greater the contrast will be. The T_1 -weighted images may be obtained with short TR and TE compared to T_1 and T_2 .

Gadolinium ion is, however, extremely toxic because its radius is close to that of the calcium ion and therefore Gd ion can replace it. Furthermore Gd ions are not stable at physiological pH and produce gadolinium hydroxide crystals that can form deposits and block vessels. To overcome these problems, the Gd ion is complexed by a linear polycarboxylate-type organic ligand, diethylenetriaminepentaacetic acid (DTPA) or macrocyclic tetra-azacyclododecanetetraacetic acid (DOTA) [34] (Fig. 4.2). Since Gd-DTPA (Magnevist®) was approved in 1988, around 10 kinds of Gd chelates have been approved or are in clinical trials as MRI CAs, and over 600 references on Gd(III) chelates appear every year.

The first six contrast agents (Gd-DTPA, Gd-DTPA-BMA, Gd-DTPA-BMEA, Gd-DOTA, Gd-HP-DO3A and Gd-BT-DO3A), called first generation products are administered at a dose 0.1 mmol/kg body weight in the form of an aqueous solution of 0.5 M in Gd. These complexes are distributed in the extracellular space, do not exhibit tissue specificity and are rapidly excreted renally.

The three last Gd complexes are part of the second generation of contrast agents. Gd-EOB-DTPA [5] and Gd-BOPTA [6] are hepatobiliary agents because they are specifically taken up by healthy hepatocytes and allow to highlight lesions in the

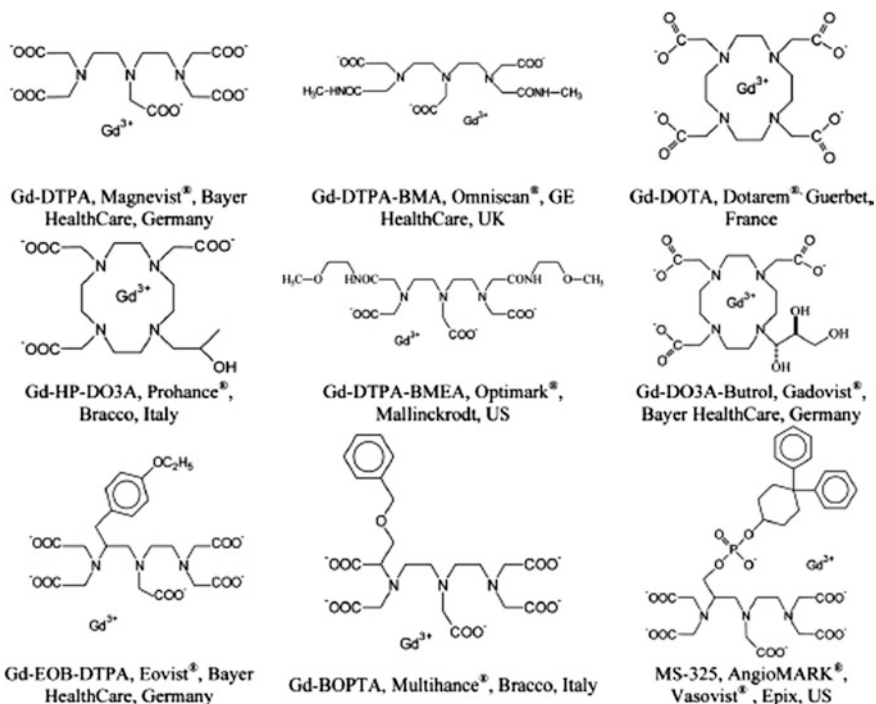


Fig. 4.2 Structure of approved Gd complexes as MRI contrast agents

liver. MS-325 [7] is an angiographic agent with a high affinity for human serum albumin (HSA) which gives it a high residence time in vessels.

4.1 Relaxation Mechanisms of Gadolinium Complexes

For small gadolinium chelates, two main contributions can be distinguished in the relaxation mechanisms: the innersphere mechanism (IS) dealing with the water molecules bound to the first coordination sphere of the gadolinium ion and the outer-sphere mechanism (OS) concerning water molecules diffusing near the paramagnetic center.

For some chelates, an additional contribution defined as the second-sphere (SS) mechanism is necessary but this contribution is generally neglected.

Therefore, the effect of Gd chelate on the longitudinal water relaxation rate (R_{1p}) is given by

$$R_1^p = R_1^{IS} + R_1^{OS} (+ R_1^{SS})$$

4.1.1 *Innersphere Mechanism*

The innersphere mechanism allows the propagation of the paramagnetic effect from the paramagnetic center to the bulk solution (Fig. 4.3). It is based on the chemical exchange of water molecule(s) in the first coordination sphere of the gadolinium ion and the water molecules constituting the bulk.

The innersphere contribution was described by Salomon-Bloembergen theory [8, 9] as an exchange of water molecules between two sites (Fig. 4.3) and is expressed by the following equation for solutions containing low concentrations of paramagnetic chelates.

$$R_1^{is} = fq \frac{1}{T_{1M} + \tau_M}$$

where f is the molar fraction of the paramagnetic agent, q is the number of water molecules coordinated to the first coordination sphere, T_{1M} is the longitudinal

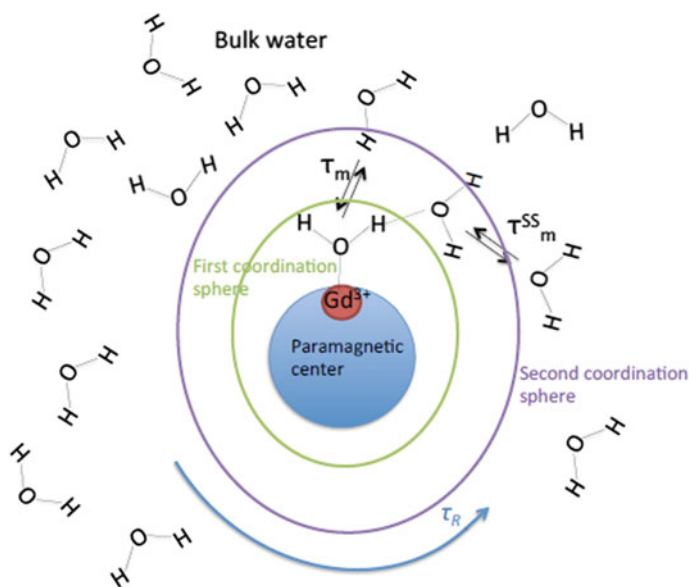


Fig. 4.3 Schematization of a paramagnetic complex with one innersphere water molecule, one second-sphere water molecule and several outersphere water molecules (bulk water molecules)

relaxation time of the coordinated water molecules and τ_M is the residence time of water in the first coordination sphere.

A dipolar mechanism governs the longitudinal relaxation rate of the water molecules coordinated to gadolinium chelates following the equation:

$$\frac{1}{T_{1M}} = \frac{1}{T_{1M}^{DD}} = \frac{2}{15} \left(\frac{\mu_0}{4\pi} \right)^2 \gamma_I^2 \gamma_S^2 \hbar^2 S(S+1) \frac{1}{r^6} \left[\frac{7\tau_{c2}}{1 + (\omega_S \tau_{c2})^2} + \frac{3\tau_{c1}}{1 + (\omega_I \tau_{c1})^2} \right]$$

where γ_I and γ_S are the nuclear (I) and the electron (S) gyromagnetic ratios, respectively ($\gamma_I = 2.675 \times 10^8 \text{ rad s}^{-1} \text{ T}^{-1}$, $\gamma_S = 1.76 \times 10^{11} \text{ rad s}^{-1} \text{ T}^{-1}$), ω_S and ω_I are the Larmor pulsations of the electron and of the proton, respectively, r is the effective distance between the electron charge and the ^1H nucleus, τ_{ci} are the correlation times that modulates the interaction, with $i = 1, 2$.

These can be expressed by the equation:

$$\frac{1}{\tau_{ci}} = \frac{1}{\tau_R} + \frac{1}{\tau_M} + \frac{1}{\tau_{si}}$$

where τ_R is the rotational correlation time and τ_{S1} and τ_{S2} are the longitudinal and transverse relaxation times, respectively, of the impaired electrons. They can be expressed as,

$$\begin{aligned} \frac{1}{\tau_{S1}} &= \frac{1}{5\tau_{SO}} \left[\frac{1}{1 + \omega_S^2 \tau_V^2} + \frac{4}{1 + 4\omega_S^2 \tau_V^2} \right] \\ \frac{1}{\tau_{S2}} &= \frac{1}{10\tau_{SO}} \left[3 + \frac{5}{1 + \omega_S^2 \tau_V^2} + \frac{2}{1 + 4\omega_S^2 \tau_V^2} \right] \end{aligned}$$

where τ_V is the relaxation time at zero field and depends on the tensor of the ZFS (zero field splitting).

4.1.2 Outer-Sphere Mechanism

The outer-sphere relaxation was described by Freed [10, 11] and comes from the dipolar interaction between the diffusing water molecules close to the gadolinium ion and the gadolinium ion itself. It is only governed by the relative motion of two entities and their distance of closest approach. The outer-sphere relaxation rate is given by the following equation:

$$R_1^{\text{os}} = \frac{32\pi}{405} \gamma_I^2 \gamma_S^2 \hbar^2 S(S+1) \frac{\text{NA}}{1000} \frac{[\text{C}]}{\text{dD}} [7j(\omega_S \tau_D) + 3j(\omega_I \tau_D)]$$

where N_A is the Avogadro number, $[C]$ is the concentration of paramagnetic entities and τ_D is the time modulation characterizing the outersphere mechanism named the diffusion correlation time and expressed by equation:

$$\tau_D = \frac{d^2}{(D_I + D_S)} = d^2/D$$

where d is the distance of closest approach between the gadolinium ion and the ^1H nucleus of the diffusing water molecules and D_I and D_S are the molecular diffusion coefficients of water and of the paramagnetic entity, respectively.

$$j(\omega\tau_D) = \text{Re} \left[\frac{1 + \frac{1}{4}(i\omega\tau_D + \tau_D/\tau_{S1})^{1/2}}{1 + (i\omega\tau_D + \tau_D/\tau_{S1})^{1/2} + \frac{4}{9}(i\omega\tau_D + \tau_D/\tau_{S1}) + \frac{1}{9}(i\omega\tau_D + \tau_D/\tau_{S1})^{3/2}} \right]$$

4.1.3 Second-Sphere Mechanism

Second-sphere mechanism concerns water molecule protons that are not directly coordinated to the paramagnetic centers but which interact with coordinated protons through hydrogen bonds for example. They constitute the second coordination sphere (Fig. 4.3).

$$R_1^{SS} = f q^{SS} \frac{1}{T_{1M}^{SS} + \tau_M^{SS}}$$

where q^{SS} is the number of water molecules in the second coordination sphere and τ_M^{SS} , their residence time.

If we define $\tau_{C1,2}^{SS} = \tau_{Ci}^{SS}$, the correlation times modulating the interaction are given by

$$\frac{1}{\tau_{Ci}^{SS}} = \frac{1}{\tau_R^{SS}} + \frac{1}{\tau_M^{SS}} + \frac{1}{\tau_{si}}$$

For gadolinium chelates, $1/T_{1M}^{SS}$ can be expressed as:

$$\frac{1}{T_{1M}^{SS}} = \frac{2}{15} \left(\frac{\mu_0}{4\pi} \right)^2 \gamma_I^2 \gamma_S^2 \hbar^2 S(S+1) \frac{1}{r_{SS}^6} \left[\frac{7\tau_{c2}^{SS}}{1 + (\omega_S \tau_{c2}^{SS})^2} + \frac{3\tau_{c1}^{SS}}{1 + (\omega_I \tau_{c1}^{SS})^2} \right]$$

where r^{SS} is the distance between the unpaired electron spins and the water protons constituting the second-sphere coordination and τ_R^{SS} is the reorientational time of the water molecule in the second sphere. The second-sphere mechanism can often be neglected.

4.1.4 Influence of the Different Parameters on the Innersphere and Outersphere Contributions

4.1.4.1 Innersphere Contribution

According to the theories developed by Solomon-Bloembergen-Morgan [8, 9, 12], the paramagnetic relaxivity of water protons due to the innersphere contribution depends on several parameters including those listed below.

Number of water coordinated molecules (q) The relaxation rate is proportional to the number of water molecules in the first coordination sphere of the metal ion. A higher q leads to a high relaxivity but is often accompanied by a low thermodynamic stability. The main contrast agents have a single coordinated water molecule.

Residence time of the coordinated water molecule (τ_M) This parameter plays an important role in the proton relaxivity because it modulates the efficiency of the exchange of the water molecules in the first coordination sphere with the bulk solvent water molecules. This factor can be extremely limiting for the observed relaxivity. Rapid exchange of the water coordinated molecule is therefore necessary to have a high relaxivity.

Rotational correlation time (τ_R) This parameter strongly influences the observed relaxivity between 0.5 and 3 T corresponding to the magnetic fields used in clinical MRI. An increase in the value of τ_R causes a marked improvement in the relaxivity values at these magnetic fields. Many studies have been focused on optimizing τ_R and have led to the production of contrast agents with higher molecular weight.

Electronic relaxation time (τ_{Si}) The choice of the Gd^{3+} ion is due to a high unpaired electron number but also to its larger electronic relaxation times, which allow a favorable interaction with the protons of the water coordinated molecule.

4.1.4.2 Outersphere Contribution

This mechanism comes from the freely diffusing water molecules in the vicinity of the paramagnetic center, which can therefore interact with the electron spin. This phenomenon is governed by the relative movement of two molecules and by their distance of smaller approach. The correlation time modulating this magnetic interaction is called the translational correlation time τ_D .

4.1.4.3 NMRD Profiles

Nuclear magnetic relaxation dispersion (NMRD) is a powerful tool that provides the evolution of the relaxivity as a function of the magnetic field [13, 14]. As the magnetic field has no influence on the chemical state of the sample, it is a valuable tool for the study of the interaction mechanisms and dynamic processes influencing the relaxation behavior.

NMRD profiles are recorded using the fast field cycling technique. The theoretical adjustment of these curves provides the efficiency of the contrast agent at a specific magnetic field and a good estimation of several parameters described above such as τ_R , τ_M , τ_V , q , Nevertheless, NMRD profiles are influenced by many parameters increasing the complexity of their analyses. Two additional techniques are generally used to probe some parameters: the EPR (Electron Paramagnetic Resonance) for τ_{Si} and ^{17}O NMR for τ_M and q .

The shape of the NMRD profiles of low molecular weight T_1 contrast agents is typical (Fig. 4.4).

4.2 Different Classes of Gadolinium Based MRI Contrast Agents

4.2.1 Vascular Contrast Agents

They are characterized by a long residence time in the vascular system, making them suitable for use in angiography. Two types of products are reported: the covalent complexes, obtained by grafting a low molecular weight contrast agent (<1000 Da) to a macromolecule (>10,000 Da) and the non-covalent complexes in which the small contrast agent binds non-covalently to endogenous

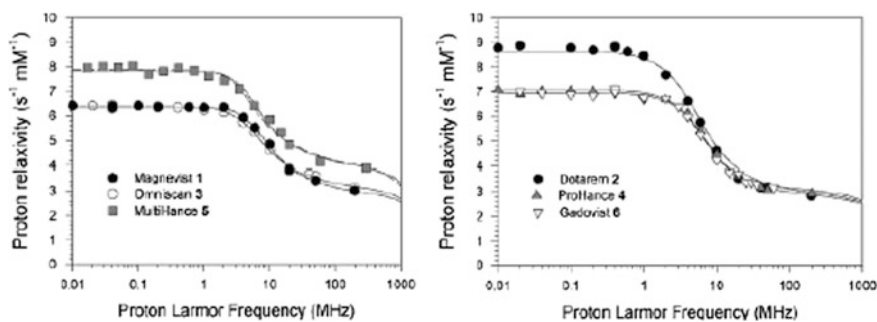


Fig. 4.4 NMRD profiles of several small commercial paramagnetic gadolinium chelates recorded at 37 °C. Reproduced with permission of Ref. [15]

macromolecules (such as HSA). Complexes of intermediate molecular weight (5–10 kDa), for example P792 [16] and P760 [17], are also developed for angiography.

Covalent complexes involve the grafting of a contrast agent, a derivative of Gd-DTPA or Gd-DOTA, on a synthetic or natural polymer, a protein, a dendrimer ... [18]. The residence time of some of these macromolecular contrast agents in the body is long and their elimination is sometimes incomplete, increasing the risk of cellular uptake and dissociation of the complex.

Many studies on non-covalent complexes have allowed the development of Gd chelates with high affinity for HSA. Several researches have demonstrated the need for the presence of hydrophobic residues and negative charges to have high affinity. Among these molecules, Gd-EOB-DTPA [5], Gd-BOPTA [6] and MS-325 [7] have been involved in numerous works. In these non-covalent complexes, there is an equilibrium between the free form of the small Gd chelates and the complex bound to the macromolecule. This allows a rapid elimination through the kidneys and a low toxicity.

4.2.2 Smart Contrast Agents

The efficiency of these contrast agents depends on biochemical parameters such as pH [19], temperature [20], oxygen pressure [21], presence of an enzyme [22] or a metal ion (Ca^{2+} , Zn^{2+} , ...) [23]. These activatable agents possess two distinct states. One state is *off* and corresponds to low contrast enhancement, while the other state, the *on* state, corresponds to high contrast enhancement. An activatable agent can be *switched* from one state to the other by the occurrence of a metabolic or physiological event. The design of this new class of agents exploits the fundamental means by which a paramagnetic species affects the intensity of an image acquired by MRI.

Some *switches* used to activate (or inactivate) Gd complexes are parameters of the Solomon Bloembergen equations and include q , τ_R , and τ_M , which were described earlier. Increasing q or τ_R or decreasing τ_M to an optimal value leads to a decrease in T_1 resulting in higher contrast enhancement.

4.2.2.1 PH Sensitive

As reported by Aime et al. [24], Gd complexes with ionisable ligands which are derivated from 1, 4, 7, 10- tetraazacyclododecane with three acetate oxygens and one phosphonate group on the fourth nitrogen can form ions pairs with poly-aminoacids like polyornithine or polyarginine which makes these entities sensitive to pH. Because pH is an important physiological indicator (as markers for abnormal tissue), many research groups have designed pH sensitive contrast agents. Mikawa et al. [25] developed a MRI contrast agent based on a micro environmental responsive polyion complex in the form of a mixture of two polymers. The complex

exhibits a fifty percent increase in relaxivity upon decreasing pH from 7 to 5. Aime and coworkers [26] have prepared a pH sensitive contrast agent with 30 Gd chelates and 114 ornithine residues. The chelates are conjugated to the amino acid chain via squaric esters. At low pH, the amines are protonated and do not interact with the squaric ester linkers. When the pH rises, the amine side chains become deprotonated and interact with the squaric ester residues. This interaction rigidifies the polymer creating an increase in τ_R , so an increase in relaxivity. Lowe et al. [27] synthesized a DO3A-derivative with sulfonamide nitrogen which at low pH is protonated and unable to chelate the paramagnetic ion. As a result, water access to this ion is restored creating a detectable signal. At a high pH the deprotonated amine chelates prevent water access and the MR signal is low. Hovland et al. [28] have prepared a DO3A compound with a tertiary amine-containing 2 long alkyl chains. When the amine is protonated (pH = 3–6) the relaxivity is low. Upon deprotonation (pH = 8–10), the agents form colloidal aggregates due to the higher lipophilicity. The aggregation causes an increase in τ_R and a subsequent increase in relaxivity. A Gd-DOTA-tetramide derivative has been prepared by Zhang et al. [29]. Its relaxivity increases when pH increases from 4 to 6 but decreases between 6 and 8.5 and is constant between 8.5 and 10.5 and then increases again. This usual pH dependence of the relaxivity could be accounted for by the presence of the uncoordinated phosphonate groups. The proton of the ionisable groups between 9 and 6 can catalyse the exchange of protons between the bound water molecule and the bulk by providing an efficient hydrogen bond network which could be disrupted at lower pH by protonation. Gd-DOTA-tetraamide bearing hydroxypyridyl substituent has exhibited 2 regions of enhanced relaxivity: a small enhancement at lower pH = 2–4 attributed to an increase in the prototropic exchange of the coordinated water molecule and a slightly larger enhancement at higher pH = 6–9 due to deprotonation of the ligand amide protons [30].

Aime et al. [31] have also synthesized a ternary complex between a Gd chelate and carbonate ions. The relaxivity of this complex can be affected by the saturation of its coordination sphere by 2 water molecules or a bidentate ligand such as hydrogenocarbonate. The relaxivity of the complex changes from $7.5 \text{ mM}^{-1} \text{ s}^{-1}$ at low pH to $1.9 \text{ mM}^{-1} \text{ s}^{-1}$ at high pH, reflecting the replacement of 2 water molecules in the first coordination sphere by a carbonate ion.

4.2.2.2 Metal Sensitives

Intracellular calcium plays an important role in muscular contraction, neuronal transduction, hormonal secretion. Li et al. [32, 33] have developed a contrast agent that can specifically detect Ca ions. In the absence of Ca^{2+} , the aromatic aminoacetates interact with the 2 Gd ions. In the presence of Ca^{2+} , the aromatic aminoacetates rearrange to bind Ca allowing water to bind directly to Gd. This increase in q number yields an increase in relaxivity. This mechanism is reversible (Fig. 4.5).



Fig. 4.5 Example for calcium sensitive contrast agent

Nagano et al. [23, 34] have used a similar scheme for detection of zinc ions, with a DTPA ligand coupled with *N,N,N',N'*-tetrakis (2-pyridylmethyl)ethylenediamine (TREN) as a zinc specific chelator. Without zinc ion, a water molecule is bound to the gadolinium ion. In the presence of zinc ion, the carboxylic acid and pyridine moieties are coordinated to zinc ion thus restricting the access of the water molecule to the Gd ion. This decrease in q yields a decrease in relaxivity in the presence of zinc.

An iron-sensitive contrast agent was synthesized by Aime et al. [35] by functionalizing DTPA with salicylate moieties. Upon addition of iron (III), the Gd-DTPA-salicylate complexes bind to the iron ions via the salicylate functional groups. This binding yields an increase in τ_R and in relaxivity.

Another iron-sensitive contrast agent was prepared by Comblin and Jacques [36, 37]. DOTA was conjugated to a phenanthroline derivative. Self assembly around iron (II) ions leads to an increase in τ_R and the relaxivity increases from 5.1 to 12.5 $\text{mM}^{-1} \text{s}^{-1}$. PhenHDO3A is a ditopic ligand featuring a tetraazacyclododecane unit substituted by three acetate arms and one 6-hydroxy-5,6-dihydro-1,10-phenanthroline group. This ligand was specially designed so as to obtain highly stable heteropolymetallic assemblies [38]. Costa et al. [39] have developed the same kind of rigid chelates obtained by self-assembly of Fe(II) ions and two terpyridine based Gd-DTTA. $\text{Fe}[\text{Gd}_2\text{bpy}(\text{DTTA})_2(\text{H}_2\text{O})_4]_3^{4-}$ is a self-assembled, metallostar-structured contrast agent, with six Gd ions confined into a small molecular space. The relaxivity is particularly remarkable at very high magnetic fields ($r_1 = 15.8 \text{ mM}^{-1} \text{ s}^{-1}$ at 200 MHz, 37 °C) [40]. Another high-molecular weight

tetrametallic supramolecular complex $[(Ln-DTPA-phen)_3Fe]^-$ ($Ln = Gd, Eu, La$) has been prepared upon self-assembly around one iron (II) ion of three 1,10-phenanthroline-based molecules substituted in 5'-position with the DTPA ligand, $DTPA-phen^{4-}$ ([41], Fig. 4.6).

4.2.2.3 Enzyme-Activated Contrast Agents

The first enzymatic sensitive contrast agent was reported by Louie et al. [42]; it was developed in response to the need to correlate biological events with gene expression during an imaging experiment. The mechanism is based on 2 distinct relaxation states, a weak and a strong ones. By blocking the one remaining open coordination site, water protons are excluded from the innersphere and the effect of the Gd ions on the relaxivity is diminished. The agent «Egad» was activated by the enzyme β -galactosidase. The enzyme substrate (sugar) occupies all nine coordination sites, inhibiting water access to the Gd ion. The contrast agent is irreversibly turned «on» when β -galactosidase cleaves the sugar and water becomes accessible to the paramagnetic ion, thus modulating q number (Fig. 4.7). These agents have been successfully used in vivo.

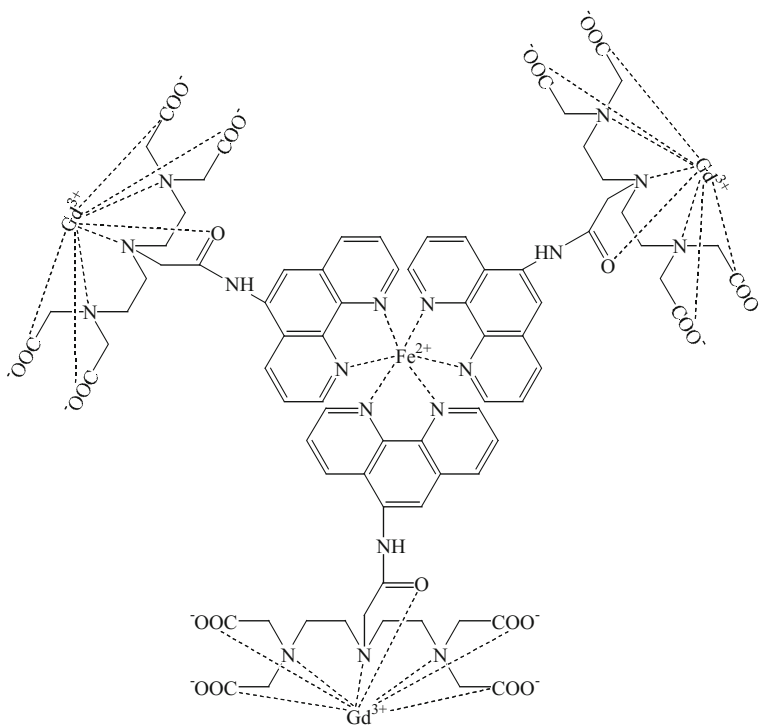


Fig. 4.6 Structure example of iron sensitive contrast agents

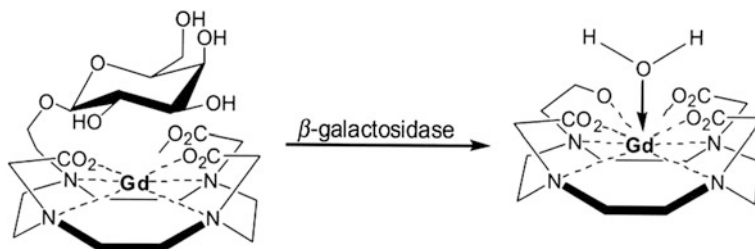


Fig. 4.7 Enzymatic cleavage of the Gd-DOTA derivative with β -galactosidase

Anelli et al. [43] have synthesized a DTPA compound which can detect carbonic anhydrase. The Gd complex contains a sulfonamide group helping to selectively target the enzyme carbonic anhydrase. Upon binding to the enzyme, the relaxivity increases (five fold at 40 MHz).

Nivorozhkin et al. [44] prepared an agent that is sensitive to the presence of human carboxypeptidase B which has been implicated in thrombotic disease. TAFI cleaves a trilycine masking group attached to the agent exposing an aromatic functional group which has a high affinity for human serum albumin. The contrast agent binds HSA leading to an increase in τ_R . This event is known as a receptor induced magnetization enhancement (RIME). The trilycine chain makes this agent a pro-RIME agent because the trilycine chain inhibits interaction with HSA.

Bogdanov et al. [45] prepared a peroxidase activatable agent. This agent consists of a Gd chelate linked to benzene-1,2-diol that acts as a monomer. In the presence of peroxide, the monomers are oligomerized yielding a threefold increase in relaxivity due to an increase of τ_R . This MRI signal amplification can detect peroxidase concentration in vitro and has been used to detect E-selectin expression on human endothelial cells in cell culture by the high local enzymatic activity of antibody bound peroxidase associated with the plasma membrane of these cells.

Duimstra et al. [46] have synthesized a new class of enzyme activated contrast agents using a self immolative mechanism for detection of β -glucuronidase. Querol et al. [47] have prepared new DTPA bisamides. These derivatives bear thyraindo or 5-hydroxytryptamido groups that could be oligomerized in situ in the presence of peroxidase/ H_2O_2 pair resulting in a net increase in r_1 relaxivity. Chen et al. [48] showed that activatable paramagnetic imaging agents can be used to directly image myeloperoxidase (MPO). Plaque rupture in atherosclerotic disease is the major cause of morbidity and correlates well with MPO secretion by activated macrophages and neutrophils in human. Gd-DOTA-serotonin (3-(2-aminoethyl)-5-hydroxy indole) was efficiently polymerized in the presence of human neutrophil MPO resulting in a 70–100 % increase in proton relaxivity.

4.2.2.4 pO₂

The use of deoxyhemoglobin as MRI contrast agent was discovered by Thulborn et al. [49]. Ogawa et al. [50] have shown that the signal was dependent on the oxygenated state of blood and that the blood oxygen level-dependent (BOLD) signal could be used for noninvasive mapping of human brain function. Activable contrast agents sensitive to the partial pressure of O₂ (pO₂) have been synthesized [51]. The oxidation state of a europium ion is varied to trigger the signal on and off by the environmental pO₂. Eu³⁺ is reduced in Eu²⁺ (isoelectronic with Gd³⁺) and enhances the observed MR signal upon reduction. Aime et al. [52] have developed a redox switch to increase τ_R as a pO₂ sensitive compound. These complexes use manganese (Mn²⁺) as the redox ion. By coupling the product to polycyclodextran, the Mn²⁺ porphyrin aggregates increasing the Mn²⁺ concentration and τ_R . The water relaxation of Mn²⁺ is much greater than the Mn³⁺ one, creating a redox switch dependent on pO₂. This technique can quantify the oxygen concentration in the surrounding environment.

4.2.3 *Specific Contrast Agents for Molecular Imaging*

To develop specific contrast agents for molecular imaging, it is necessary to graft molecules targeting receptors overexpressed in pathology.

Contrast agents can be accumulated in a pathological site by passive or active targeting mechanisms. Thanks to the passive targeting, most nanoparticles can be accumulated in tumors due to the pathophysiological characteristics of the tumor blood vessels [53]. Tumors have higher retention time than normal tissues because tumors lack a well-defined lymphatic system [54, 55]. This can explain an enhanced permeability and retention (EPR) effect.

Active targeting is based on the use of vectorized ligands. A lot of vectors as antibodies, peptides or proteins, aptamers, polysaccharides, ... can be grafted to target cellular biomarkers.

MRI contrast agents are used to help in the diagnosis of many diseases (various cancers like breast [56], liver [57], ovarian [58], lung [59], ...), however, the agents described so far have the limitation of being non-site specific. The inability to localize selectively in a desired area lessens the diagnostic potential of these agents. The first step toward improving the diagnostic capability of contrast agents is to make them target specific and accumulate in desired biological locations. Many attempts have been made to deliver gadolinium chelates to specific biological targets. An aminoxy-functionalized DTPA derivative that is tumor specific has been synthesized by taking advantage of the fact that tumor cells overexpress sialic acid residues [60]. A DOTA derivative conjugated to the HIV-Tat peptide has the ability to be internalized into many different cell lines unlike unmodified chelates that are not internalized into cells [61]. Liposomes containing gadolinium were conjugated to mouse antibodies and successfully targeted to ICAM-1, an endothelial leukocyte

receptor upregulated on cerebral microvasculature during experimental autoimmune encephalitis [62]. Polylysine labeled with Gd-DTPA and transferrin can image cells expressing the transferrin receptor [63]. Further, this polymer has been modified with an anti-CEA F(ab')₂ immunoconjugate to image colorectal carcinoma [64]. Dendrimers labeled with Gd-DTPA have been conjugated to folate and used to successfully image tumor cells which are expressive of the high-affinity folate receptor in mice [65, 66]. Specific, non invasive imaging of angiogenesis in rabbits was accomplished using an antibody to the angiogenesis marker endothelial integrin $\alpha_v\beta_3$ conjugated to gadolinium containing liposomes [67] and perfluorocarbon-nanoparticles [68].

The blood brain barrier (BBB) is one of the most challenging physical hurdles when designing contrast agents intended for the brain. The BBB contains various mechanisms for minimizing the flow of material into and out of the brain [69]. This barrier prevents most passive diffusion except by extremely small molecules such as water and ethanol. Electric charge, lipid solubility, and molecular weight can be used to help predict whether or not a molecule will cross the BBB [70]. Brain tumors have a fenestrated BBB and diseases such as multiple sclerosis involve temporary weakening of the BBB. Gd-DTPA [71, 72], Gd-BOPTA [73], have all been used to image these BBB breakdowns. One recent development in the delivery of contrast agents into the brain involves the conjugation of putrescine (an endogenously occurring polyamine that is known to increase BBB permeability) to a peptide linked to Gd-DTPA. This agent successfully crossed the BBB as predicted [74].

Targeted contrast agents may greatly improve the accuracy and scope of diagnostic imaging.

4.2.4 *How to Obtain High Relaxivity with Paramagnetic Gd-Derivatives by Increasing τ_R*

Several dinuclear complexes have been envisaged to obtain higher relaxivity [75–77]. Gd-DTPA or Gd-DOTA with large substituents have been studied [78–81]. Port et al. have developed several Gd macrocyclic compounds based on a Gd-DOTA substituted by hydrophilic arms. The structure of P760 [78, 79] and P792 [80–82] has been optimized to obtain a high relaxivity in the field for MRI (P760: 24.7 s⁻¹ mM⁻¹ at 20 MHz and P792: 29 s⁻¹ mM⁻¹ at 60 MHz), a high biocompatibility profile and a high molecular volume (Fig. 4.8).

Macromolecules To obtain a better relaxivity, intense research has been carried out in the covalent coupling of contrast agents with macromolecules like polymers, dendrimers, proteins, ... First of all, a compromise must be found between the size of the macromolecules (to be eliminated by glomerular filtration) and tolerability in the body. They must be biocompatible and the grafted complexes kinetically stable. Albumin was the first molecule to which Gd-DTPA units were attached [83].

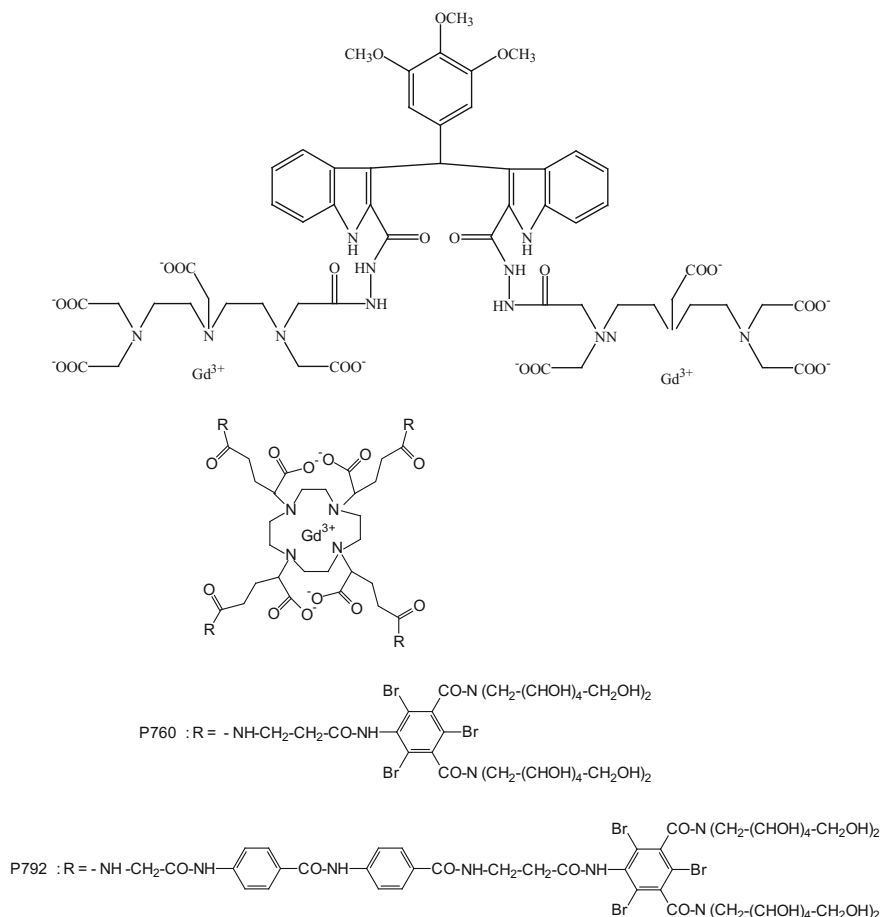


Fig. 4.8 Some examples of Gd-DTPA or Gd-DOTA derivatives with large substituents

Gd-DTPA complexes have also been grafted onto a lysine residue without [84] or in presence of PEG [85] which increases the relaxivity. Gd complexes have been attached to linear polymer chains as side chains [86–89] or incorporated in the linear polymer itself [90]. The relaxivities of these molecules have been found to be lower than expected. This was explained by the highly flexible nature of the macromolecule. Higher efficiency has been obtained for Gd-DTPA-bisamide copolymers, $[(\text{Gd-DTPA-BA})-(\text{CH}_2)_n]_x$ ($n = 6, 10$ or 12) which contain hydrophobic parts in the polymer [91].

Dextran containing a spacer arm was also used [92]. The advantage of dextran is its high capacity for functionalization (carbamate, epichlorhydrine, periodate, carboxymethyl) [93–100].

Several Gd chelates have also been grafted on polysaccharide like inulin [101].

For all these macromolecules, the increase in relaxivity is not as large as predicted by the theory. The macromolecular contrast agent must lead to a slowing of the rotational speed and to an increase in the relaxivity via τ_R . The polymer allows a certain rotational freedom and probably, the movements of the Gd complexes are more rapid than the polymer molecule itself.

Gd-DTPA-polylysine, Gd-DOTA-polylysine, Gd-SCN-Bz-DOTA-polylysine were also investigated [102].

Relaxivity studies indicated that polyaspartamide Gd complexes containing sulfadiazine groups possess higher relaxivities than that of Gd-DTPA. MRI showed that the macromolecular PAEA-Gd-DTPA-SD greatly enhanced the contrast of MR images of hepatoma in the lower limb of mice and prolonged intravascular duration [103]. Most currently macromolecular contrast agents are not biodegradable. Wen et al. [104] have synthesized and characterized poly(glutamic acid) Gd chelates as biodegradable blood pool compounds. Biodegradable macromolecular chelates, Gd-DTPA cystine copolymers were grafted with PEG of different size to modify the physicochemical properties and in vivo MRI contrast enhancement of the agents and to study the effect of PEG chain length (MW = 550, 1000 and 2000) on these properties [105]. PEG 2000 showed more prominent enhancement in the blood pool for a longer period of time. Polydisulfide-based biodegradable polymer Gd complexes have been modified to improve the in vivo retention time and MRI contrast [106].

The Gd complexes have been grafted on PAMAM dendrimers [107–110]. The increase in relaxivity can be explained by the steric hindrance which predominates at the periphery and which produces an increase in τ_R . Relaxivity studies showed that the dendritic Gd complexes possess high efficiency. Dendritic Gd-chelates containing pyridoxamine groups enhanced the contrast of the MR images of the liver, provided prolonged intravascular duration and produced highly contrasted visualization of the blood vessels [111].

Two things have guided the development of contrast agents interacting with HSA: the blood pool compound for angiography and the increase of τ_R for higher relaxivity and so better efficiency. In the last case, two techniques can be used, the covalent (see above) or non-covalent interaction with a macromolecule.

Several ways have been investigated for conjugation of contrast agents to a protein: DTPA bisanhydride as the acylation agent [112–114], DTPA mixed anhydride [86, 115], N-hydroxysuccinic ester [116], squaric acid esters linker [117], isothiocyanatobenzyl groups [89]. The most studied system is represented by albumin labeled with Gd-DTPA. The frequency and temperature dependence of proton and oxygen-17 relaxivities of (Gd-DTPA)₄₅-HSA have been studied [118]. The observed behaviour is typical of systems whose relaxivity is limited by a long exchange lifetime of the coordinated water molecule [119]. Sieving et al. [120] have synthesized a poly-(L-lysine) containing 60–90 chelating groups (DTPA or DOTA). The relative small relaxation enhancement shown by Gd complexes when bound to polylysine is accounted for by the high internal mobility of the paramagnetic moiety. An analogous result was observed by using the squaric acid unit as linker between the macromolecule and the Gd-chelates. Labeling of monoclonal antibodies with Gd-DTPA has also been considered for targeting tumors [64, 121].

The clinical application of Gd chelates grafted with polymers is limited by the slow excretion of the complexes and so the accumulation of toxic Gd. To avoid this problem, biodegradable polydisulfide macromolecular complexes have been prepared based on the disulfide—thiol exchange to allow degradation of the macromolecules by endogenous thiol and facilitate excretion of Gd chelate [122].

Zeolite and tungstosilicates Gd doped zeolite NaY nanoparticles have been envisaged as a contrast agent for MRI [123–125]. The relaxivity of GdNaY increases when the Gd content in the zeolite is decreasing [126]. The relaxivity increases drastically (from 11.4 to 37.7 s⁻¹ mM⁻¹ at 60 MHz and 37 °C) as Gd loading decreases [127]. Gd sandwiched complexes with tungstosilicates have also been tested in vivo and in vitro [128]. They are favorable candidates for hepatic contrast agents but they exhibit high toxicity and need to be modified before clinical use.

Nanosystems To enhance the relaxivity of Gd complexes, Aime et al. have trapped Gd-HP-DO3A in apoferritin [129]. The relaxivity shown by each Gd complex included in the protein at 20 MHz and 25 °C is very high (about 80 s⁻¹ mM⁻¹), value about 20 times higher than the value of the free complex in water.

Novel materials having stronger proton relaxivity and higher signal enhancement have been tested for a new generation of contrast agents. Recently, polyhydroxylated Gd metallofullerenes have been proposed by several groups. The team of Wilson [130, 131] have found $r_1 = 20 \text{ s}^{-1} \text{ mM}^{-1}$ (20 MHz, 40 °C), Zhang et al. [132] have reported a relaxivity of $r_1 = 47 \text{ mM}^{-1} \text{ s}^{-1}$ (400 MHz) and Shinohara et al. [133] measured a particularly high proton relaxivity for Gd@C₈₂(OH)₄₀ (81 mM⁻¹ s⁻¹ at 25 °C, 40 MHz). Kato et al. have [134] reported endohedral metallofullerenols M@C₈₂(OH)_n with M = La, Gd, Ce, Dy, Er. The origin of the high relaxivities and the mechanism involved have not been fully elucidated. Recently, Bolskar et al. [135] have obtained the first water soluble Gd@C₆₀ derivatives. Toth et al. [136, 137] have reported the physicochemical characterization of Gd@C₆₀[C(COOH)₂]₁₀ and Gd@C₆₀(OH)_x.

More recently, another way to reach high relaxivity has been opened by use of carbon-nanotubes. Nanowires of magnetic metals (Fe, Gd, Co) have been prepared inside the hollow interiors of single wall nanotubes (SWNTs) by filling SWNTs with precursor metal chlorides and subsequent reduction [138]. A metallofullerene, Gd@C₈₂, encapsulated in the inside space of single walled carbon nanotube has been identified as an STM image of a carbon cage and a Gd atom by a measurement of the tunnel currents onto the SWNT surface. This fact indicates that there is a substantial local interaction between the wall of SWNT and the Gd ions close to the wall [139]. In a preliminary study [140], surprisingly high relaxivities (500 s⁻¹ mM⁻¹ at low frequency and about 180 s⁻¹ mM⁻¹ at high frequency) have been found. At this time, no theory can explain this observation.

Numerous organic or inorganic nanoparticles for encapsulation of Gd based contrast agents, such as lipid nanoparticles (liposomes, solid lipid nanoparticles, ...), silica nanoparticles, dendrimers or polymer nanoparticles have been described.

Liposomes

Liposomes, thanks to their biocompatibility, low toxicity and high structural flexibility are widely used as vehicles for therapeutic and/or diagnostic purposes. Due to their structure, liposomes can encapsulated hydrophilic contrast agents in the hydrophilic compartment or internalize hydrophobic complexes into the membrane.

Encapsulation within the cavity generally led to a decrease in relaxivity, due to the limited interaction between the contrast agent and the surrounding water molecules [141, 142]. For example, encapsulation of Gd-HPDO3A in liposomes causes a decrease in its longitudinal relaxivity of three to nineteen times according to the nature of the lipid used [$r_1 = 3.75 \text{ mM}^{-1} \text{ s}^{-1}$ for free Gd-HPDO3A and between 0.2 and $1.1 \text{ mM}^{-1} \text{ s}^{-1}$ for encapsulated (at $25 \text{ }^\circ\text{C}$ and 20 MHz)] [143]. The incorporation of the functionalized lipid gadolinium chelates in the membrane causes an enhancement of relaxivity, due to a facilitated interaction between the water protons and the gadolinium, and to a lesser free rotation of the gadolinium chelate [141]. For example, the Gd-DOTA functionalized with a phosphoethanolamine (DSPE-GdDOTA) is an integral part of the liposome membrane and has a longitudinal relaxivity of approximately $15 \text{ mM}^{-1} \text{ s}^{-1}$ at $37 \text{ }^\circ\text{C}$ and 60 MHz [142]. In this way, it is possible to include large amounts of gadolinium chelates. For example, 34,000 gadolinium centers could be incorporated into the bilayer of liposomes of 100 nm diameter [142]. To gain relaxivity, it is also possible to optimize the size, permeability and organization of the liposome membrane [144]. Thus, liposomes prepared from Gd-DTPA-BSA and Gd-DOTA-DSPE did not give the same results in terms of efficiency (respectively $r_1 = 7.5$ and $12.8 \text{ mM}^{-1} \text{ s}^{-1}$ at $37 \text{ }^\circ\text{C}$ and 60 MHz) and proton exchange rate (respectively $\tau_M = 1200$ and 250 ns) while Gd-DTPA and Gd-DOTA exhibit similar values. This difference in behavior is explained by a difference in orientation of the polar heads at the liposome/water interface. Indeed, in Gd-DTPA-BSA, Gd-DTPA is at the interface and BSA is the amphiphilic tail. In contrast, in the case of Gd-DOTA-DSPE, the phosphate moiety is placed at the interface and Gd-DOTA is removed from the rest of the nanoparticle to the aqueous medium [145] (Fig. 4.9).

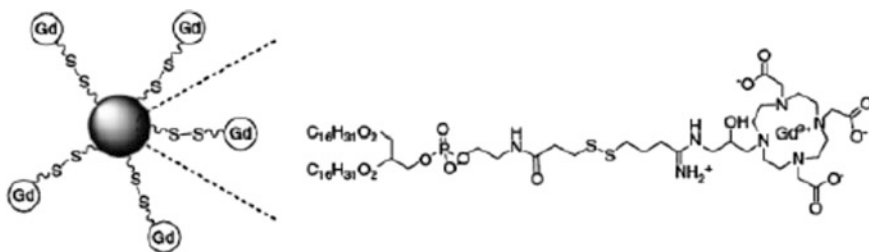


Fig. 4.9 Example of Gd complexes conjugated to phospholipids in liposomes [146]

Polymers

Nanocapsules or nanospheres can encapsulate or adsorb contrast agents. PLGA nanoparticles were tested for encapsulating commercial contrast agents. If the encapsulation of T_2 agents was successfully observed, the results obtained for T_1 agents were worse than what was hoped [147, 148]. The incompatibility between the hydrophobic matrix and contrast agent induced premature release and a restriction of exchange between the water molecules and the Gd ion. For these reasons, when the polymer matrix is hydrophobic, the exaltation of the relaxivity of the paramagnetic agent proceeds rather by its grafting to the surface of the nanoparticle than by its containment. Surface functionalization of the PLGA nanoparticles with gadolinium complexes allows to observe a relaxivity gain of approximately three to four times that of the free agent (483). It is possible to optimize the gain by varying the length or the architecture of the spacer group linking the chelate to the nanoparticle. Thus, when the Gd-DTPA and Gd-DOTA are grafted on the surface of PLGA nanoparticles via a spacer of polyethyleneimine type (PEI), their relaxivity is increased up to approximately $17 \text{ mM}^{-1} \text{ s}^{-1}$ (as compared to 4 for the free complex) at 25°C and 60 MHz.

An improvement in relaxivity per Gd center can be achieved by containment of contrast agents in hydrogels formed from hydrophilic polymers as chitosan. The longitudinal and transverse relaxivities of Gd-DOTA may be increased to achieve respective values of $r_1 = 72.3 \text{ mM}^{-1} \text{ s}^{-1}$ and $r_2 = 177.5 \text{ mM}^{-1} \text{ s}^{-1}$ (37°C , 60 MHz) [149]. It is the same for the Gd-DOTP ($r_1 = 98 \text{ mM}^{-1} \text{ s}^{-1}$ and $r_2 = 109.5 \text{ mM}^{-1} \text{ s}^{-1}$ at 37°C , 20 MHz) [150]. The results confirm a relaxivity exalted by Gd center via second sphere mechanisms.

Other types of polymeric nanoparticles are able of increasing the relaxivity of Gd complexes. For example, dendrimers can incorporate several contrast agents, increasing the longitudinal relaxivity r_1 of the Gd-DTPA from $5 \text{ mM}^{-1} \text{ s}^{-1}$ to values between 20 and $30 \text{ mM}^{-1} \text{ s}^{-1}$ for Gd-DTPA associated with dendrimers (between 0.5 and 1.5 T at 37°C) [151].

Inorganic nanoparticles

Wartenberg et al. have significantly increased the relaxivity of Gd-(ebpatcn) simply by incorporation into silica nanoparticles via the sol-gel process. Thus the relaxivity of encapsulated Gd chelates (encapsulated $r_1 = 84 \text{ mM}^{-1} \text{ s}^{-1}$ at 25°C and 45 MHz) is 18 times greater than that of the free Gd complexes ($r_1 = 4.7 \text{ mM}^{-1} \text{ s}^{-1}$ at 25°C and 45 MHz) [152]. The decrease in relaxivity observed when the nanoparticles increase in size is explained by a larger charging rate of contrast agent hindering movement of water molecules and thus reducing exchanges.

Other systems based on the confinement of Gd complexes have allowed a significant increase of the relaxivity values. This is the case, for example, with Gd-fullerenes, Gd-zeolites or complexes of Gd (III) included in the cavity of the apoferritin [153–155].

The gadofullerenes consist of a carbon cage (produced by electric arc from composite materials Gd/graphite) enclosing one or more Gd ions. In this cage, the Gd^{3+} ions have their movement slowed and the presence of hydroxyl groups on the surface of fullerene promotes exchanges with the surrounding water molecules. Relaxivity can then be increased by approximately 10–40 times compared to commercial Gd complexes [153, 155].

Zeolites are microporous aluminosilicate crystals in which the ion Gd^{3+} can be fixed. Relaxivity can reach between 20 and 40 $\text{mM}^{-1} \text{s}^{-1}$ which is better than the commercial contrast agents but remains far from the theoretical maximum. This moderate increase can be explained by the low diffusion of water molecules through the pores [155].

Finally improved relaxivity is greater when the contrast agent is confined rather than bound to the nanoparticle, provided that the structure thereof is permeable to water molecules. When both conditions are met, relaxivity values close to the theoretical maximum can be reached ($100 \text{mM}^{-1} \text{s}^{-1}$ at 25 °C and 20 MHz). This is explained by the fact that the confinement of the Gd chelate in the particle limits the free rotation but also that of water molecules therein, moderating indeed anarchic reorientation of Gd– H_2O vectors. When the Gd complex is bound to the particle by a covalent bond, the nanoparticle minimizes the overall rotation of the building but local rotational movement of the chelates, at their covalent bond with the nanoparticle structure, remains. This results in an enhancement of relaxivity but this increase is limited.

4.2.5 PARACEST Agents

Another class of activatable lanthanide based agents makes use of a chemical exchange saturation transfer (CEST) event as the “switch” [156] (Fig. 4.10).

Conventional low-molecular weight, extracellular, Gd^{3+} -based contrast agents enter the extracellular space and highlight only those tissue regions that temporally accumulate the paramagnetic complex. This accumulation of paramagnetic complex results in a shortening of the bulk water spin-lattice relaxation time (T_1) and hence brightening of the image in that region. This mechanism of altering bulk water relaxation cannot be controlled externally and hence one cannot modulate (turn on/off) the relaxation effects of a T_1 shortening agent; one can only compare image intensities pre- and post injection of the agent.

There has been considerable effort to engineer Gd^{3+} -based complexes with substantially higher relaxivities, but it is not always easy. As shown before, one of the ways to gain a substantial increase in relaxivity is to slow rotation of Gd^{3+} complexes by attachment to a larger structure such as biopolymer, a nanoparticle, or a naturally occurring polymer like albumin.

As an alternative to T_1 shortening agents for MR contrast enhancement, one could also introduce contrast by altering the proton density or total water signal detected. This can be accomplished using a NMR technique called magnetization

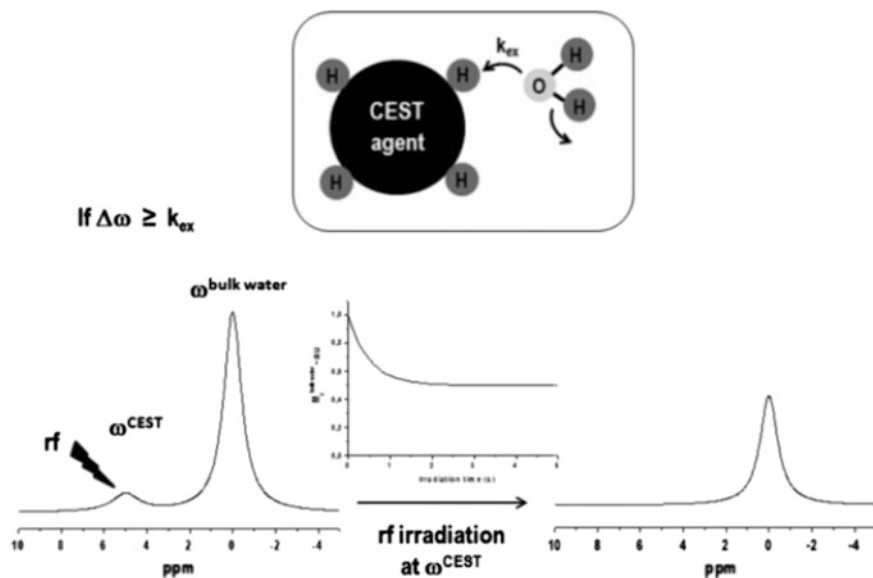


Fig. 4.10 Schematic view of the saturation transfer mediated by chemical exchange. The plot reported on the *arrow* indicates the dependence of the bulk water magnetization on the irradiation time [172]

transfer (MT). Ward et al. [157, 158] introduced the idea of using the MT effect to induce contrast into an image and proposed a new class of MRI contrast agent based on chemical exchange saturation transfer (CEST) between intrinsic metabolites like amino acids, sugars, nucleotides or other heterocycles having exchangeable OH or NH groups that exchange protons with bulk water. They demonstrated using simple diamagnetic molecules that MRI contrast can be switched on/off by applying a saturating irradiation pulse at an exchangeable site a few ppm away from the bulk water resonance. CEST agents take advantage of the difference in proton chemical shift between the amide proton and the innersphere water protons of the contrast agent. Aime et al. [159–161] and Zhang et al. [162–166] have used this kind of CEST agents. The concentration dependence is avoided by using two different exchange sites within a contrast agent, as in the OH and NH bonds. When external RF is directed at the frequency of one of the proton pool resonances, this causes saturation transfer to the water resonances and decreases the bulk water intensity, allowing for external manipulation of the signal intensity. Aimé et al. [167] have shown that Yb complex can be sensitive to biological substrates such as lactate. The free Yb complex and the lactate bound complex have two different sets of amide frequencies to irradiate. The concentration in lactate can be determined by irradiating the amide protons of the unbound complex. Aime et al. have [167] also developed a pH sensitive CEST agent, a Yb-DOTA-tetraglycineamide complex. Saturation of the amide protons that are in close proximity to the paramagnetic center results in an excellent CEST agent. The

observed effect is pH-dependent since the exchange rate for amide protons is pH modulated. By irradiating the amide N–H resonances, saturation transfer to the water resonance occurs, leading to a decrease in the water signal intensity which determines the contrast in MRI. Several strategies have been developed for improving the activity of CEST agents: to increase the number of mobile proton per molecule [168, 169] or to increase the chemical shift difference between the exchangeable protons and the bulk water with paramagnetic shift reagents interacting with the diamagnetic system bearing the mobile protons [170]. This CEST method can be use with liposomes that intrap a paramagnetic shift complex [171]. This system called Lipocest, allows a very low detection limit and opens the possibility of use in the field of magnetic molecular imaging.

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Chapter 5

Superparamagnetic Iron Oxide Nanoparticles

Abstract Thanks to their unique properties, inorganic nanostructures have become the center of modern material science. Among existing nanomaterials, magnetic iron oxide nanoparticles (IONP) have attracted a lot of interest. These nanoparticles are suitable for many technological applications such as contrast agent for magnetic resonance imaging. When comparing to molecular MRI probes (Gd-based organic complexes), IONP present many advantages such as a better sensitivity, a poor toxicity, and the possibility to easily modify their surface to develop some properties as multimodality, modulable half-life or specificity. However, one must stress that these properties are related to IONP's composition, shape and stability (physical and chemical). In that paper, we will introduce some concepts related to IONP's physico-chemical properties, the synthetic ways to obtain such structures and we will finish with some concepts governing their stability and surface modification.

Superparamagnetic iron oxide nanoparticles have a hydrodynamic diameter ranging from 5 to 300 nm (Tables 5.1 and 5.2). These nanomaterials are coated with a natural or synthetic polymer or a small ionic molecule for ensuring a stable suspension [1, 2]. They have a very large magnetic moment and higher transverse relaxivity values than paramagnetic Gd complexes. This magnetic moment creates a local heterogeneity of the magnetic field in the region where water molecules are diffusing. This process leads to an acceleration of proton nuclear spin dephasing and a decrease of transverse relaxation time T_2 . With a greater relaxivity r_2 than r_1 , these contrast agents have a so-called negative T_2 predominant effect, leading to a decrease of the signal (dark MRI signal). Contrast induced by superparamagnetic agents is optimum using a T_2 -weighted (increasing of TR and extending the TE). It is also possible, at low magnetic field to obtain a positive contrast using T_1 -weighted sequences.

Two types of superparamagnetic nanoparticles were developed [1, 2] for MRI of liver or lymphatic system:

- Ultrasmall superparamagnetic iron oxide nanoparticle (USPIO, Fig. 5.1) and nanocompound monocrystalline iron oxide (MION) with a hydrodynamic

Table 5.1 Some examples of “ultrasmall superparamagnetic particle of iron oxide” (USPIO) (37 °C, 1.5 T) [2]

USPIO (coating)	Hydrodynamic diameter (nm)	Relaxivity ($s^{-1} mM^{-1}$)
AMI-227, Sinerem [®] , Combidex [®] (dextran)	17–30	$r_1 = 19.5$ $r_2 = 87.6$
Ferumoxytol (carboxymethyl-dextran)	30	$r_1 = 15$ $r_2 = 89$
SHU-555C, Supravist [®] (carboxydextran)	30	$r_1 = 10.7$ $r_2 = 38$
Feruglose, Clariscan [®] (pegylated starch)	20	$r_1 = 20$ $r_2 = 35 (0.47 T)$
VSOP-C184 (citrate)	7	$r_1 = 14$ $r_2 = 33.4$
MION-46L (dextran)	8–20	$r_1 = 3.95$ $r_2 = 19.6$

Table 5.2 Some examples of “superparamagnetic particles of iron oxide” (SPIO)(37 °C, 1.5 T) [2]

SPIO (coating)	Hydrodynamic diameter (nm)	Relaxivity ($s^{-1} mM^{-1}$)
AMI-25, Endorem [®] , Feridex [®] , Ferumoxides (dextran)	80–150	$r_1 = 10.1$ $r_2 = 158$
SHU-555A, Ferucarboxydextran, Resovist [®] (carboxydextran)	60	$r_1 = 9.7$ $r_2 = 189$
AMI-121, Ferumoxil, Lumirem [®] , Gastromark [®] (silicone)	300	$r_1 = 2$ $r_2 = 47$

diameter between 20 and 30 nm, those systems are monocrystalline (single crystal per particle). AMI-227, developed by Advanced Magnetics and Guerbet, is composed of particles with a hydrodynamic diameter of 17 nm and is obtained from a separation column of AMI-25 (Table 5.1).

- Superparamagnetic iron oxide nanoparticles (SPIO, Table 5.2, Fig. 5.1) have a hydrodynamic diameter higher than 60 nm and have several magnetic grains per particle. Among them are the AMI-25, known in Europe under the name Endorem[®].

These particles are composed of a polycrystalline iron oxide core entrapped in a crown of dextran. The interactions between the magnetic core and dextran are low. Stabilization can be improved by cross-linked dextran molecules to enhance their interactions with the crystal core (CLIO) [3]. Another class of versatile particles USPIO (VUSPIO) has also been developed [4]. In this case, the dextran molecules are covalently bound via silanol on the surface of nanoparticles.

The majority of iron oxide nanoparticles is often composed of magnetite (Fe_3O_4) and its oxidized form maghemite ($\gamma-Fe_2O_4$). These iron oxides exhibit an inverse

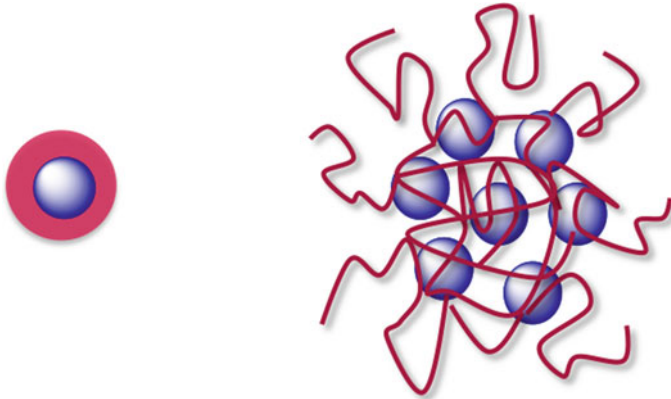


Fig. 5.1 Schematic structures of USPIO and SPIO

spinel structure with a general formula of AB_2X_4 where A and B are cations and X are anions [5]. These crystals adopt a face-centered cubic crystalline structure. Anions delimit the network of the face-centered cubic crystal system and the cations are distributed in tetrahedral and octahedral sites. Specifically, the crystalline structure of magnetite is based on 32 anions of oxygen and contains both divalent and trivalent iron as cations. For one unit cell of magnetite containing 32 oxygens, 64 tetrahedral and 32 octahedral sites are generated. For each unit cell, 8 divalent atoms and 8 trivalent atoms of iron fill the octahedral sites of magnetite (Fig. 5.2) [6]. In the tetrahedral sites, 8 trivalent iron ions are present. Overall, each unit cell of magnetite contains 56 atoms in which there are 32 ions of oxygens, 16 ions of iron (III) and 8 ions of iron (II).

Iron oxide nanoparticles have been the subject of extensive scientific researches during the last decade, and several iron oxide based nanosystems have been

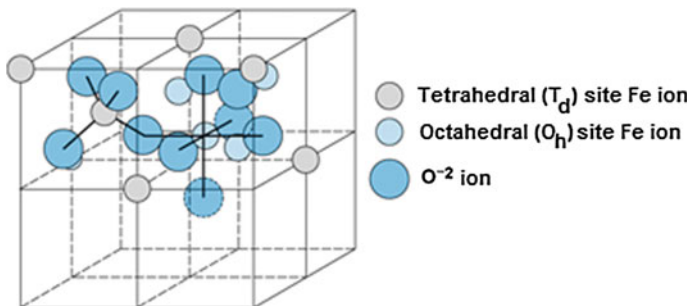


Fig. 5.2 Schematic illustration of inverse spinel of magnetite in which 8 octahedral sites are surrounded by 6 oxygens and 16 tetrahedral sites are surrounded by 4 oxygens. With permission of Ref. [7]. Available from: <http://www.intechopen.com/books/modern-surface-engineering-treatments/surface-modification-of-nanoparticles-used-in-biomedical-applications>

approved as T_2 agents. They include magnetite (Fe_3O_4), maghemite ($\gamma\text{-Fe}_2\text{O}_3$) and other ferrites which have bivalent metal ions to replace Fe^{2+} , such as MnFe_2O_4 [8], ZnFe_2O_4 [9] and CoFe_2O_4 [10].

5.1 Superparamagnetic Properties of Iron Oxide Nanoparticles

5.1.1 Relaxometric Properties

5.1.1.1 Relaxation Processes of Single Domain Iron Oxide Nanoparticles

Superparamagnetic iron oxide nanoparticles are composed of a single magnetic domain with a size of a few nanometers, the global size being much smaller than a Weiss domain of the corresponding bulk ferromagnetic materials [11, 12].

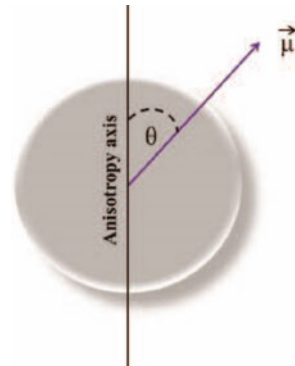
For these nanoparticles, the directions of the magnetization vector are generally aligned on preferred positions called “easy direction magnetization” or “anisotropy axes” in order to minimize the anisotropy energy of the system. The anisotropy energy can be defined as the energy required to move the magnetization between the positions of easy magnetization axis [13].

The anisotropy energy (E_a) of a single domain is proportional, in a first approximation, to its volume (V) [14]. In the simplest case of uniaxial anisotropy (Fig. 5.3), the energy barrier between two easy magnetization directions is proportional to $K_a V$ and is defined as:

$$E_a = K_a V \sin^2 \theta$$

where K_a is the anisotropy constant and θ is the angle between both anisotropy axis positions.

Fig. 5.3 Uniaxial anisotropy axis for superparamagnetic materials



K_a constant can be influenced by four contributions for isolated nanoparticles: (i) the chemical composition and the crystallographic structure of the material, (ii) the nanoparticle shape, (iii) the coating nature and (iv) the inter-distance between the nanoparticles. In the case of agglomerated nanoparticles, only the distance between the nanoparticles contributes to K_a [15].

The anisotropy energy of magnetic crystal dramatically increases with the crystal volume and thus with the particle radius [16]. Consequently, for large crystal, the energy barrier is high and the transitions from an easy axis to another one are not favored. The magnetization vector is thus blocked on an anisotropy axis. On the contrary, for small particles, the transitions of the magnetization from one easy axis to another one are thus easier [17].

The changeover of magnetic moment vector from an easy axis to another is observed when the thermal energy is sufficient to break the anisotropy energy barrier. The average time to switch from one direction to another is called “Néel relaxation time (τ_N)” and is given by

$$\tau_N = \tau_0 E_a e^{\frac{E_a}{k_b T}}$$

where τ_N is the Néel relaxation time, k_b is the Boltzmann constant, T is the temperature and $\tau_0 (E_a)$ corresponds to the preexponential factor of the Néel relaxation time expression.

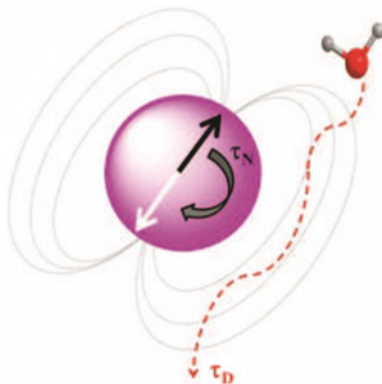
The alignment of magnetization vector is influenced by two contributions: the anisotropy energy ($K_a V$) and the thermal agitation energy ($k_b T$). The ratio between both energies induces two situations as described below:

- If $K_a V \gg k_b T$, the magnetic moment is locked in one of the easy directions. Consequently, the changeover of the magnetic vector to another easy axis involves the complete rotation of the particle.
- If $K_a V \ll k_b T$, the thermal agitation is a dominant factor, the free transition of the magnetization vector on the easy axes occurs without the particle rotation.

For superparamagnetic crystals dispersed in a liquid medium, the return of the magnetic moment to equilibrium state is defined by two different processes: the **Néel relaxation** and the **Brownian relaxation**.

The Néel relaxation is related to the permanent changeover of the magnetization from an easy direction to another one and depends on temperature and crystal volume. The values of the Néel relaxation time τ_N are generally included in the range of 10^{-9} – 10^{-11} s. When an external magnetic field is applied, the magnetic moments stay long enough on one easy magnetization axis to produce a resulting magnetization proportional to the strength of magnetic field. The behavior of these particles can be compared to the paramagnetic substances. However, the magnetic susceptibility of superparamagnetic materials is very much higher (100 or 1000 times) due to the collective interactions of electrons, and this explains why these compounds are called superparamagnetic.

Fig. 5.4 Illustration of water proton relaxation due to the presence of superparamagnetic particles



For large crystals, the Néel relaxation time becomes longer and any changeover of the magnetization is observed because the magnetic moments are locked on an easy axis. The blocking volume (V_B) is defined as a crystal critical volume. This parameter leads to a lock of the magnetization during the measuring time at a given temperature. In this case, only the total rotation of particles submitted to the thermal agitation can be observed. The characteristic time of this rotation is given by the Brownian relaxation times (τ_B):

$$\tau_B = \frac{3V\eta}{k_bT}$$

where V is the crystal volume, η is the fluid viscosity, k_b is the Boltzmann constant and T is the temperature.

In summary, the global magnetic relaxation rate of colloidal suspensions (Fig. 5.4) is modulated by Néel and Brownian relaxation times following this equation:

$$\frac{1}{\tau} = \frac{1}{\tau_N} + \frac{1}{\tau_B}$$

The fastest process dominates the relaxation of system. The Néel relaxation is thus dominant for small crystals, whereas, the relaxation of large crystals is almost exclusively governed by Brownian contribution.

5.1.1.2 Superparamagnetic Relaxation: Theoretical Model

Due to their particular magnetic properties, superparamagnetic particles are used for MRI applications. The high magnetization of these compounds influences greatly the surrounding water relaxation rate.

The understanding of the superparamagnetic relaxation phenomena is possible with the accepted theory established by Roch-Muller-Gillis (commonly called RMG model or SPM model) [13, 18, 19]. This theory is based on the classical outer-sphere relaxation theory, but extended to include the presence of materials with a strong anisotropy. The relaxation induced by superparamagnetic nanoparticles is due to the coupling between the magnetic moments of water protons and the electron magnetic moments of particles. The modulation is caused by Néel relaxation (flip of the magnetization vector of particles from one easy axis to another) and the diffusion of water protons.

RMG model of large crystals will be firstly described and the model of small crystals will be then discussed.

- **For the large crystals (particle radius > 7.5 nm)**

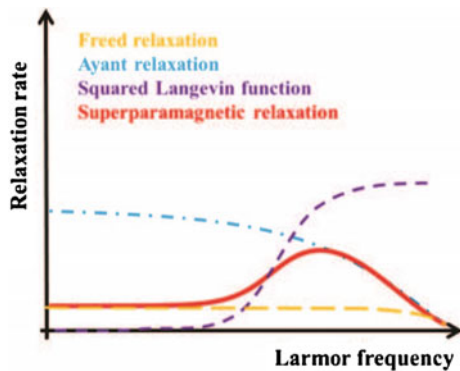
The anisotropy barrier is high, inducing the lock of magnetic moment of superparamagnetic materials on one anisotropy axis when high magnetic fields are applied. Depending on the strength of external magnetic field, several limiting cases can be described as below.

At *low magnetic fields*, the magnetic moments can easily move from one anisotropy direction to another causing drastic magnetic fluctuations on the water diffusion in the vicinity of magnetic particles. Consequently, the dipolar interactions between water protons and magnetic core are modulated by the translational correlation time of the water molecules (τ_D) and the Néel relaxation time (τ_N) as shown in Fig. 5.5. Both modulations can be gathered together in a global correlation time (τ_{CI}) as follows:

$$\frac{1}{\tau_{CI}} = \frac{1}{\tau_N} + \frac{1}{\tau_D}$$

where τ_D is equal to the square of crystal radius (r) divided by the diffusion constant (D).

Fig. 5.5 Relaxation model for large magnetic nanoparticles with each contribution



The proton longitudinal and transversal relaxation rates can be expressed from Freed spectral density (J_F) [20]:

$$R_1 = \frac{1}{T_1} = \frac{32\pi}{405,000} \gamma^2 \mu^2 \left(\frac{N_A C}{r^3} \right) [10J_F(\omega_I, \tau_D, \tau_N)]$$

$$R_2 = \frac{1}{T_2} = \frac{32\pi}{405,000} \gamma^2 \mu^2 \left(\frac{N_A C}{r^3} \right) [8J_F(\omega_I, \tau_D, \tau_N) + 2J_F(0, \tau_D, \tau_N)]$$

$$J_F(\omega_I, \tau_D, \tau_N) = R_e \left[\frac{1 + \frac{1}{4}\Omega^{\frac{1}{2}}}{1 + \Omega^{\frac{1}{2}} + \frac{4}{9}\Omega + \frac{1}{9}\Omega^{\frac{3}{2}}} \right]$$

$$\Omega = i\omega_I \tau_D + \frac{\tau_D}{\tau_N}$$

With γ is the proton gyromagnetic ratio, μ is the electron magnetic moment, N_A is the Avogadro number, C corresponds to the molar concentration of superparamagnetic compound, r is the crystal radius and ω_I is the proton angular frequency.

At **high magnetic fields**, the magnetization vector remains onto one of the easy axes. In this case, the Néel relaxation time is longer due to a high anisotropy. Therefore, the modulation of relaxation solely results from water proton diffusion. This modulation is described thanks to Ayant spectral density (J_A) [21] and the relaxation rates are described as below:

$$R_1 = \frac{1}{T_1} = \frac{32\pi}{405,000} \gamma^2 \mu^2 \left(\frac{N_A C}{r^3} \right) [9L^2(\alpha)J_A(\sqrt{2\omega_I \tau_D})]$$

$$R_2 = \frac{1}{T_2} = \frac{32\pi}{405,000} \gamma^2 \mu^2 \left(\frac{N_A C}{r^3} \right) \left[\frac{9}{2}J_A(\sqrt{2\omega_I \tau_D}) + 6J_A(0) \right]$$

with

$$J_A(\mu) = \frac{1 + \frac{5\mu}{8} + \frac{\mu^2}{8}}{1 + \mu + \frac{\mu^2}{2} + \frac{\mu^3}{6} + \frac{4\mu^4}{81} + \frac{\mu^5}{81} + \frac{\mu^6}{648}}$$

At **intermediate magnetic fields**, the water relaxation induced by superparamagnetic particles is modulated by both previous contributions. A linear combination of the equations with Langevin function [$L(\alpha)$] must be used to take into account that the magnetization is divided in two parts: the first one is locked with external magnetic field and the second one is influenced by Néel relaxation.

$$R_1 = \frac{1}{T_1} = \frac{32\pi}{405,000} \gamma^2 \mu^2 \left(\frac{N_A C}{r^3} \right) \left\{ \left(\frac{L(\alpha)}{\alpha} \right) 21 J_F(\omega_I, \tau_D, \tau_N) \right. \\ \left. + 9 \left[1 - L^2(\alpha) - 2 \left(\frac{L(\alpha)}{\alpha} \right) \right] J_F(\omega_I, \tau_D, \tau_N) + 9 L^2(\alpha) J_A(\sqrt{2\omega_I \tau_D}) \right\}$$

$$R_2 = \frac{1}{T_2} = \frac{32\pi}{405,000} \gamma^2 \mu^2 \left(\frac{N_A C}{r^3} \right) \left\{ \left(\frac{L(\alpha)}{\alpha} \right) 19.5 J_F(\omega_I, \tau_D, \tau_N) \right. \\ \left. + \left[1 - L^2(\alpha) - 2 \left(\frac{L(\alpha)}{\alpha} \right) \right] \frac{9}{2} [J_F(\omega_I, \tau_D, \tau_N) + 6 J_F(0, \tau_D, \tau_N)] \right. \\ \left. + L^2(\alpha) \left[\frac{9}{2} J_A(\sqrt{2\omega_I \tau_D}) + 6 J_A(0) \right] \right\}$$

In summary, for large crystals, the relaxation is differently modulated depending on the strength of external magnetic field. This theory predicts the evolution of water proton relaxation rate with magnetic field. Each modulation is shown in Fig. 5.5.

The RMG model allows a good fitting of experimental points obtained for large crystals (Fig. 5.6a). However, this model does not fit the particles presenting a lower anisotropy energy ($r < 7.5$ nm). The experimental data show dispersion at low frequency that does not agree with the theoretical predictions. An example is shown in Fig. 5.6b. Consequently, an adaptation of RMG model has been elaborated.

- **For small crystals (particle radius < 7.5 nm):**

These materials are characterized by a small anisotropy energy in which the magnetic moments can fluctuate from one easy magnetization axis to another one.

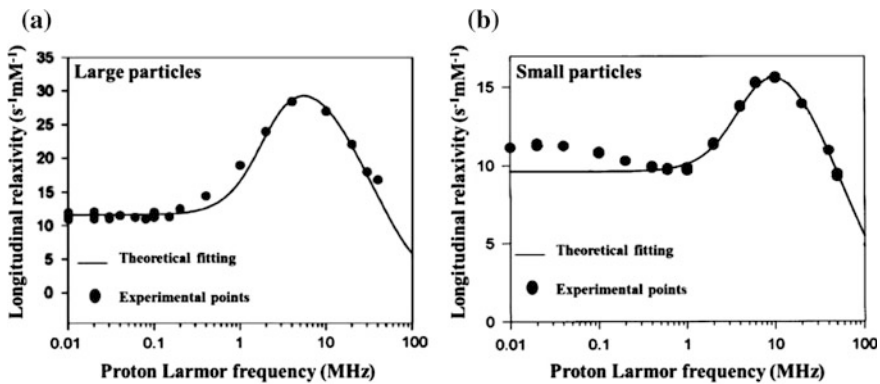


Fig. 5.6 **a** Relaxation evolution of a suspension of large particles (Endorem[®]: $r = 15$ nm) as a function of Larmor frequency and **b** relaxation evolution of a suspension of small particles ($r = 5$ nm) as a function of Larmor frequency. The *line* represents the theoretical fit developed for large magnetic crystals reproduced with permission of Ref. [2]

Therefore, a new theory is required to explain the superparamagnetic relaxation of small magnetic cores. The objective is to insert the anisotropy energy as a quantitative parameter. However, the analysis requires a long calculation time which is not easy to practice. Therefore, Roch et al. [18, 19] developed an alternative heuristic method to include this parameter. This model reproduces the gradual vanishing of the low field dispersion through a linear combination of the rate for infinite and zero anisotropy energy.

$$R_1 = \frac{1}{T_1} = \frac{32\pi}{405,000} \gamma^2 \mu^2 \left(\frac{N_A C}{r^3} \right) \left\{ \left(\frac{L(\alpha)}{\alpha} \right) 21 P J_F(\omega_I, \tau_D, \tau_N) + 21(1 - P) J_F(\omega_I, \tau_D, \tau_N) \right. \\ \left. + 9 \left[1 - L^2(\alpha) - 2 \left(\frac{L(\alpha)}{\alpha} \right) \right] J_F(\omega_I, \tau_D, \tau_N) + 9 L^2(\alpha) J_A(\sqrt{2\omega_I \tau_D}) \right\}$$

$$R_2 = \frac{1}{T_2} = \frac{32\pi}{405,000} \gamma^2 \mu^2 \left(\frac{N_A C}{r^3} \right) \left\{ \left(\frac{L(\alpha)}{\alpha} \right) 19.5 J_F(\omega_I, \tau_D, \tau_N) \right. \\ \left. + \left[1 - L^2(\alpha) - 2 \left(\frac{L(\alpha)}{\alpha} \right) \right] \frac{9}{2} \left[J_F(\omega_I, \tau_D, \tau_N) + 6 J_F(0, \tau_D, \tau_N) + L^2(\alpha) \left[\frac{9}{2} J_A(\sqrt{2\omega_I \tau_D}) + 6 J_A(0) \right] \right] \right\}$$

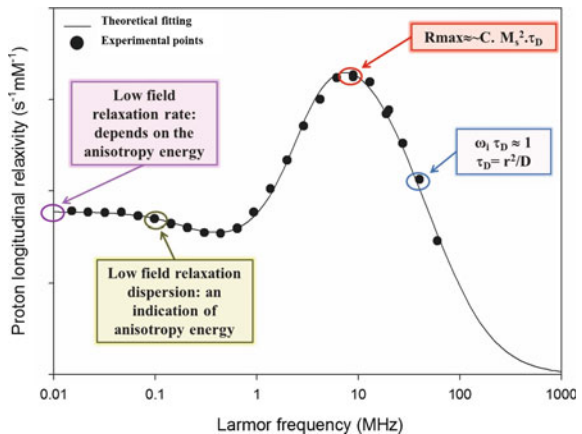
where P is a weighing factor of the linear combination and ω_s is the electron angular frequency.

The modified model allows a good interpretation of the relaxation for small particles (Fig. 5.7). These predictions have been confirmed with the experimental results for materials with low anisotropy energy [2].

5.1.1.3 Nuclear Magnetic Resonance Dispersion Profiles (NMRD)

NMRD profiles are essential tools to evaluate the relaxometric properties of MRI contrast agents as a function of magnetic field. The measurements are performed

Fig. 5.7 NMRD profiles of superparamagnetic particles. With permission of Ref. [2]



thanks to fast field cycling relaxometer. The method allows a rapid analysis of the properties for new contrast agents and can be exploited to control the reproducibility of the nanoparticle synthetic protocol. The fitting of NMRD profiles with suitable theoretical models provides information about the magnetic crystals (Fig. 5.7) such as their average radius (r), their saturation magnetization (M_s), their anisotropy energy (E_a) and their Néel relaxation times (τ_N).

The dependence of each parameter is explained as follows:

– **Average radius (r):**

At high magnetic field, the relaxation rate only depends on the diffusion correlation time (τ_D) as shown by the equation of Ayant spectral density. The inflection point corresponds to the condition of $\omega_I \tau_D \gg 1$ in which ω_I is the proton Larmor frequency. The diffusion correlation time is defined as a ratio of the square of crystal radius divided by diffusion constant ($\tau_D = r^2/D$) and thus the average radius can be estimated. An increase of crystal size modifies the position of inflection point, which moves towards lower frequency.

– **Saturation magnetization (M_s):**

At high magnetic fields, the saturation magnetization is reached and can be estimated from the relaxivity maximum by $M_s (R^{\max}/c\tau_D)^{1/2}$, where c and R^{\max} respectively correspond to a constant and the maximum relaxation rate.

– **Crystal anisotropy energy (E_a):**

The dispersion observed at low magnetic fields informs on the presence of crystals with low anisotropy energy. For materials with high anisotropy energy, no pitch dispersion is observed. The dispersion at low frequencies gives qualitative information on the magnitude of anisotropy energy of magnetic compounds in solution.

– **Néel relaxation time (τ_N):**

The Néel relaxation time obtained from the theoretical fitting is an approximate value. It can be used as qualitative information in addition to the crystal size and the specific magnetization by theoretical model.

The magnetic and relaxometric measurements give a complete description of the physico-chemical properties of iron oxide nanoparticles. It is important to notice that the RMG model is based on the assumption that nanoparticle size is uniform, and consequently, the theoretical parameters extracted from the theoretical fitting are average values. However, in the real situations, the size distribution of magnetic crystals is often inhomogeneous. If the size distribution is large, the nanoparticle suspensions contain crystals with different sizes or agglomerated nanoparticles, and consequently, the obtained theoretical parameters don't represent the effective characteristics of nanoparticle suspension.

5.2 Synthesis of Magnetic Nanoparticles

The preparation of magnetic materials in bulk form is easy. However, reducing the crystal size to the nanometer scale, brings some new complexities to synthesize these functional materials.

Due to their great potential in a wide range of domains, numerous synthetic methods were developed to produce iron oxide nanoparticles [22–28].

The synthetic way is very important and has a high impact on the characteristic of the SPIONs: size, size distribution, shape, surface chemistry, ... Preparation routes can be classified into three main categories [29]: physical (aerosol, gas phase deposition, pulsed laser ablation, electron beam lithography, laser induced pyrolysis), biological (production from fungi, bacteria or proteins) and chemical (coprecipitation, thermal decomposition, microemulsion, sonochemical, electrochemical deposition) methods. Chemical methods represent most of the published protocols because they are easy, cheap and allow the best control on size, shape and composition.

The synthesis of magnetic nanoparticles in a solution by a chemical reaction consists in the formation of nuclei, followed by particles growth. When the formed monomers achieve saturation and then the supersaturation state, the nucleation is initiated such that monomers gather to form nuclei, followed by subsequent growth to result in the formation of nanoparticles. The relationship between supersaturation and nucleation and growth is defined by the following simplified equations,

$$B = k_b \Delta C^b$$
$$G = k_g \Delta C^g$$

where B and G are the nucleation and growth rates, respectively; the subscripts b and g refer to nucleation order and growth order, respectively; k is a constant and ΔC is the supersaturation [30]. It is well acknowledged that a drastic burst of nucleation followed by slow controllable growth is critical to obtain monodisperse nanoparticles.

Synthesis of nanoparticles with uniform size and morphology is possible under conditions of rigorous control on kinetics of the nucleation and growth. The optimal control can be achieved when the two processes are separated in time, where nucleation needs to be completed before the beginning of growth of nuclei.

5.2.1 Coprecipitation Method

Coprecipitation technique is the most simple and efficient way to prepare iron oxide nanoparticles. In this method, nanoparticles are produced by heating 2/1 stoichiometric mixture of ferric and ferrous salts in alkaline medium [31, 32]. The size, shape, and composition of the particles depend on several parameters as the salts,

the pH of the solution, the temperature and the ionic strength of the media [33–35]. Chemical reaction of magnetite (Fe_3O_4) formation can be written as:



A complete precipitation of Fe_3O_4 occurs at a pH between 9 and 14, and under a non-oxidizing oxygen-free environment. Fe_3O_4 is not very stable and is rapidly oxidized into $\gamma\text{-Fe}_2\text{O}_3$ in the presence of oxygen. Nitrogen is used to produce non oxidative environment but also to reduce the particle size [36, 37].

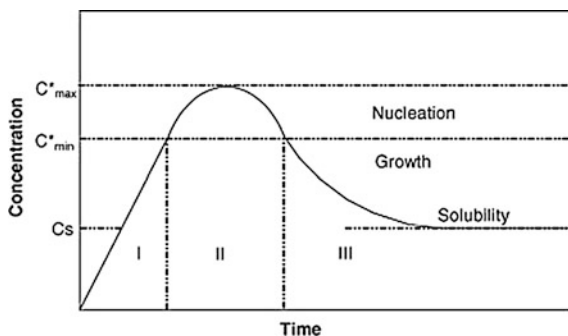
There are three phases in the coprecipitation process. In phase I, monomer concentration increases up to its saturation point. Then (phase II), small nuclei are formed when the concentration of the species reaches critical supersaturation. This second phase is followed by the growth of the crystal (phase III). The nucleation and growth steps need to be separated for producing nanoparticles [38, 39]. This phenomenon can be illustrated in the LaMer diagram (Fig. 5.8).

The presence of chelating organic molecules with negative charges (carboxylate ions, such as gluconic, citric, oleic acid, ...) or polymers (natural or synthetic as dextran, polyvinyl alcohol, starch, polyethyleneglycol, ...) during the formation of nanoparticles can help to control their size. Addition of organic ions can inhibit nucleation or crystal growth and lead to larger or smaller particles.

The most common synthesis is the Massart's method [41]. FeCl_3 and FeCl_2 are mixed in alkali to produce the nanoparticles. In the original synthesis of Massart, the magnetite particles have a mean diameter of 8 nm [42]. The influence of the base (NaOH , NH_4OH or CH_3NH_2), the pH, the cations (Na^+ , Li^+ , K^+ , NH_4^+ , $\text{N}(\text{CH}_3)_4^+$, and CH_3NH_3^+), and $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio were studied. Depending on these different parameters, particles with size between 4.2 and 16.6 nm can be obtained. The influence of pH and ionic strength were also studied, and particle size between 2 and 15 nm were produced. The shape of the particles is correlated with the electronic surface density of the nanoparticles [43, 44].

The synthetic medium can be aqueous media or organic solvents [45]. Massart [42] reported also the synthesis of $\gamma\text{-Fe}_2\text{O}_3$ by a rapid method which allows various coatings, such as α -hydroxyacids, aminoacids, hydroxamates, or dimercaptosuccinic acid.

Fig. 5.8 LaMer diagram illustrating the nucleation and growth during the coprecipitation synthesis. The nucleation process rapidly occurs with a subsequent nuclei formation. With the permission of reference [40]. Available from https://www.researchgate.net/figure/215475567_fig13_LaMer-diagram



The influence of $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio on the size, shape, composition, and magnetic properties of the nanoparticles has been studied [46]. When the ratio is too small, $\text{FeO}(\text{OH})$ are formed in the solution. When the ratio is equal to 0.3, two phases are present. The first one has a Fe^{2+} and Fe^{3+} ratio of 0.07 and produces 4 nm particles; the other one has a ratio of 0.33 and gives larger particles. When this ratio is 0.35, only one phase exists. When this ratio is 0.5, the particles are homogeneous in size and in chemical composition.

The pH and ionic strength has a strong influence on the particle size. Due to their effect on the electrostatic surface charge, the particle size and size distribution width decreases when the pH and the ionic strength are increased [47].

The mixing rate can also influence the particle size: if the mixing rate increases, the size of the particles decreases. The influence of temperature has also been studied: the number of magnetite nanoparticles decreases when the temperature increases [33].

In aqueous synthesis, coprecipitation is widely employed to obtain different ferrites (MFe_2O_4 , $\text{M} = \text{Fe}, \text{Mn}, \text{Co}, \text{Mg}, \text{Zn}, \text{Ni}$, etc.). Coprecipitation of thermodynamically favourable phases is propitious to obtain well-crystallized anhydrous oxides, even from diluted solutions.

The general reaction formula is:



Fe_3O_4 , for example, has been synthesized by a simple coprecipitation of Fe^{3+} and Fe^{2+} in 2:1 molar ratio with NaOH above 70°C [48]. Fe_3O_4 nanoparticles can also be obtained by coprecipitation using tetramethylammonium hydroxide with or without aqueous ammonia as the OH^- source [49]. ZnFe_2O_4 nanoparticles of 3–4 nm were similarly prepared from Zn^{2+} and Fe^{3+} solutions at temperatures ranging from 40 to 100°C [50]. The relaxivities have been shown to be dependent on the size of magnetic particles, which can be controlled during the synthesis process by changing the conditions of coprecipitation. CuFe_2O_4 nanoparticles were obtained by coprecipitation in alkaline medium of a ferrite stoichiometric mixture of Cu^{2+} and Fe^{3+} [51]. By controlling the pH and ionic strength, variations of size and shape can be observed, without complexing agents [52]. The nanoparticles obtained from coprecipitation did not have to be annealed at a high temperature, which can avoid their agglomeration in some degree. It is especially helpful for the medical applications, where the agglomeration of particles should be avoided.

5.2.2 *Hydrothermal Method*

In the hydrothermal way, reactions are performed in the aqueous media in reactors or autoclaves at high pressure (higher than 2000 psi) and high temperature (above 200°C) [53, 54].

Two main routes are used for the formation of ferrites: hydrolysis and oxidation or neutralization of mixed metal hydroxides. These two reactions are similar, except that ferrous salts are used in the first method [55]. The solvent, the temperature and the reaction time have a large effect on the particle characteristics [56]. The size of the particles increases when the reaction time or when the amount of water increase. At higher temperatures, the nucleation process is faster than the crystal growth and the size of the particles decreases. With longer reaction time, the crystal growth dominates and larger particles can be formed. Magnetite nanoparticles with a size up to 27 nm have been obtained by hydrothermal method [57].

5.2.3 Thermal Decomposition Method

Thermal decomposition of iron precursors in the presence of hot organic surfactants is probably the best method to control the size, the size distribution, and to obtain a good crystallinity of the synthesized nanoparticles [58].

Numerous types of thermal decomposition of iron complexes ($\text{Fe}(\text{Cup})_3$, $\text{Fe}(\text{CO})_5$, or $\text{Fe}(\text{oleate})_3$) have been developed. For example, Sun et al. described a high temperature mediated reaction of iron acetylacetonate ($\text{Fe}(\text{acac})_3$) with 1,2-hexadecanediol in the presence of oleic acid and oleylamine. Resulting nanoparticles were monodisperse and size could be tuned from 4 nm to 20 nm [59].

The decomposition of iron pentacarbonyl in the presence of oleic acid at 300 °C allows the production of magnetite nanoparticles with a very good crystallinity, the size of the particles obtained range from 4 to 16 nm [58]. The size and shape of the particles are determined by the reaction time, temperature, precursors, concentration, ratios of the reactants, and solvent. The oleic acid on the surfaces of the particles is used to stabilize the colloid solution.

The thermal decomposition of iron carbonyl in the presence of octyl ether and oleic acid and using consecutive aeration can produce hydrophobic magnetite nanoparticles with narrow size distribution [60].

The thermal decomposition of iron oleate or iron pentacarbonyl in organic solvent at different temperatures can produce particles with size between 4 and 11 nm [61].

This method produced nanoparticles dispersible in organic solvents but not in aqueous medium. The decomposition of $\text{Fe}(\text{acac})_3$ or FeCl_3 in refluxing 2-pyrrolidone can produce water-dispersible particles in acidic or basic media [62, 63]. The decomposition of $\text{Fe}(\text{acac})_3$ in high boiling organic solvent allowed to obtain particles with size of 4, 6, 9 or 12 nm and very narrow size distribution. They are coated by 2,3-dimercaptosuccinic acid and are dispersible in water [64].

The decomposition of $\text{Fe}_3(\text{CO})_{12}$ in the presence of oleic acid and diethylene glycol diethyl ether has also been described [65]. The particles are then annealed at 300, 700, and 900 °C. The annealing temperature helps to control the size, size distribution, composition, and magnetic properties of the particles.

To control the oxides crystal growth and to get uniform size and shape of nanoparticles, the decomposition is performed in low polar solvents and high boiling point. The solvents in thermolysis sometimes can be used as the stabilizing agent, for their high dielectric constant or high donor number [66, 67]. $\text{Fe}(\text{acac})_3$ is mostly used as the Fe-source for forming Fe_3O_4 by thermolysis. To achieve the control over the nucleation and growth processes, assistant agents interacting with metal atoms and solvents are required, which mostly employ polymers with nucleophilic groups, capping ligands and organoaluminium agents. Superparamagnetic Fe_3O_4 nanoparticles with different sizes were synthesized from high-temperature reaction of iron acetylacetonate and 1,2-hexadecanediol in high-boiling phenyl ether solvent in the presence of oleic acid and oleylamine [59]. CoFe_2O_4 and MnFe_2O_4 nanoparticles were synthesized by the same method but with addition of $\text{Co}(\text{acac})_2$ and $\text{Mn}(\text{acac})_2$, respectively. It was found that particle diameter can be tuned from 3 to 20 nm by varying reaction conditions such as refluxing temperature or by seed-mediated growth. The key to obtain monodisperse nanoparticles was the pretreatment of mixture solution at 200 °C for some time before refluxing. Gao and his coworkers reported the synthesis of Fe_3O_4 nanoparticles via the thermal decomposition of $\text{Fe}(\text{acac})_3$ dissolved in 2-pyrrolidone, which was used as the polar solvent and also the ligand agents for controlling the crystal growth and preventing the agglomeration [68]. For the application as MRI CAs, Fe_3O_4 nanoparticles obtained by thermolysis were transferred from hydrophobic to hydrophilic, using hydrophilic polymers like monocarboxyl-terminated poly(ethylene glycol) (MPEG-COOH) [69, 70] and by amphiphilic molecular like oleyl alcohol [71].

Size of the nanoparticles synthesized using by thermal decomposition method depends strongly of the reaction temperature, the iron to surfactant ratio and the reaction time. Nanoparticles obtained are soluble in organic solvents, they are stabilized and protected from aggregation by a surfactant surface coating as lauric or oleic acid: the polar head group of the surfactant is oriented on the nanoparticle surface and the hydrophobic tail is extended away from the nanoparticles as shown in Fig. 5.9 [72, 73].

5.2.4 Sol-Gel Methods

The sol-gel process is based on the hydrolysis and condensation of precursors in a colloidal solution (sol). A metal oxide network (gel) can be obtained by removing solvent or chemical reaction: a colloidal gel is produced by basic catalysis and a polymeric form of the gel is produced by acidic catalysis [74].

The rates of hydrolysis and condensation influence the properties of particles formed. A lower and more controlled hydrolysis rate allows to produce smaller particles. The size of the particles can also be correlated to the solvent, concentration, pH, and temperature [75].

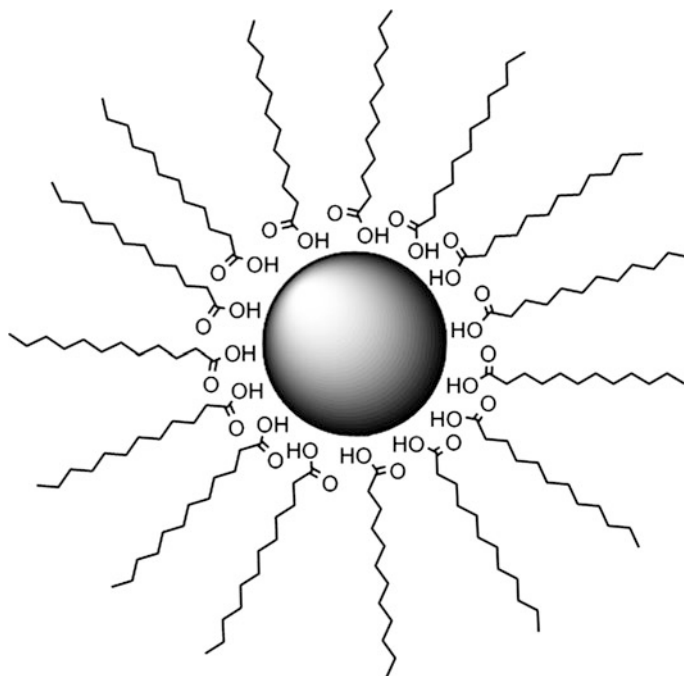


Fig. 5.9 Representation of SPIONs synthesized by thermal decomposition method. SPION surface is coated with lauric acid during the synthesis

After a treatment at 400 °C, Fe_2O_3 particles with a size between 6 and 15 nm are formed [76]. With this method, Fe_2O_3 nanoparticles can be coated in an inert and inorganic silica matrix [77, 78].

5.2.5 Microemulsions

A microemulsion is a thermodynamically stable dispersion of two immiscible liquids. In water-in-oil microemulsion systems, the microdroplets of the aqueous phase surrounded by surfactant molecules are dispersed in a continuous oil phase [38]. The role of surfactant molecules is the limitation of the nucleation, the growth, and thus the agglomeration of the particles [79, 80]. The growth of the particles is due to an interdroplet exchange and nuclei aggregation [81, 82].

Water-in-oil (w/o) microemulsions are formed by well-defined nanodroplets of the aqueous phase, dispersed by the assembly of surfactant molecules in a continuous oil phase. Vidal-Vidal et al. [83] have reported the synthesis of

monodisperse maghemite nanoparticles by the one-pot microemulsion method. Due to the nanometer size of the aqueous core, the particles formed usually have a size less than 15 nm and a very narrow size distribution. The biggest advantage of this method is the control of the particle size.

5.2.6 Polyol Methods

The polyols, like polyethyleneglycol, have some good properties: (i) a high dielectric constant, (ii) an ability to dissolve inorganic compounds, and (iii) a high boiling temperature. They offer a wide range of operating temperature for the production of inorganic compounds [84]. Nanoparticles can be produced by the reduction of dissolved iron salts and precipitation in the presence of polyol [85, 86].

The polyols are used as reduction agents and also as stabilizers, they can control the growth of particles and prevent aggregation. In this method, the precursor is suspended in the liquid polyol. The suspension is stirred and heated to reach the boiling point of polyol. The precursor becomes soluble in the diol during the process. The size and shape of the nanoparticles can be regulated by controlling the kinetic of the process.

The yield of iron oxide nanoparticles depends on the kind of polyols and ferrous salts, but also on the concentration and temperature. The size and yield of the particles are related to the reduction potential of the polyols [87].

Non-aggregated magnetite nanoparticles have been produced by a modified polyol method [88]. Different polyols, including ethylene glycol, diethyleneglycol, triethylene glycol, and tetraethylene glycol, have been used to react with $\text{Fe}(\text{acac})_3$ at high temperature. The non-aggregated magnetite nanoparticles with narrow size distribution and uniformed shape are only produced when using triethylene glycol.

5.2.7 Electrochemical Methods

Nanoparticles with a size between 3 and 8 nm have been synthesized by electrochemical method [89]. In this method, the particles are prepared from an iron electrode in an aqueous solution of dimethylformamide and cationic surfactant; the nanoparticle size is controlled by the current density.

5.2.8 Aerosol/Vapor Method

Aerosol methods, including spray and laser pyrolysis, are continuous chemical processes allowing high rate production.

Spray pyrolysis consists in a process where a solution of ferric salts and a reducing agent in organic solvents is sprayed into a series of reactors, then the solvent is evaporated and the solute condenses. The size of the original droplets controls the particle diameter. Using different iron precursors in alcoholic solution, maghemite particles with size ranging from 5 to 60 nm and with different shapes can be produced.

Laser pyrolysis is a method to reduce the reaction volume. In this method, laser is used to heat a flowing gaseous mixture of iron precursor, and non-aggregated particles with small size and narrow size distribution can be obtained. By controlling the experimental conditions, maghemite particles can have a size between 2 and 7 nm and a very narrow size distribution [90, 91]. For example, maghemite nanoparticles with a size of 5 nm have been synthesized by continuous laser pyrolysis of $\text{Fe}(\text{CO})_5$ vapors [92].

5.2.9 Sonolysis/Thermolysis

The decomposition of organometallic precursors by sonolysis can also produce iron oxide nanoparticles. Polymers, organic capping agents, or structural hosts allow to limit the particle growth [93, 94]. The rapid collapse of cavities generated by sonochemistry gives very high temperature hot spots. These spots allow the conversion of ferrous salts into magnetic nanoparticles. For example, magnetite nanoparticles have been produced by the sonolysis of $\text{Fe}(\text{CO})_5$ aqueous solution in the presence of sodium dodecyl sulfate [95].

In another example, well-defined supermagnetic iron oxide nanoparticles have been produced by sonolysis [96]. Stable particles coated with oleic acid can be dispersed in chitosan solution and have a size of 65 nm.

For biomedical applications, the control of particle size, size distribution, particle shape, and magnetic properties is very important. Different physico-chemical characteristics can be obtained by different fabrication methods (Table 5.3).

According to the comparison of the different synthetic way to form iron oxide nanoparticles, thermal decomposition of metal iron precursors appears as the most profitable and the most promising synthetic method to produce monodispersed crystalline magnetic nanomaterials. This process forms well-controlled iron oxide particles without the use of a size selection step at the end of the synthesis.

5.3 Stabilization of Nanoparticle Suspensions

Colloidal suspensions are stable dispersions of small solid particles ranging in size from 1 nm to 1 μm . Stable nanoparticle dispersions are colloidal suspensions. The behavior of such solutions is dictated by the rules of interface and colloid science [97].

Table 5.3 Summary of the synthetic method of iron oxide nanoparticles

Synthetic methods	Synthesis comments	Reaction temperature (°C)	Reaction period	Solvent	Size distribution	Shape control
Co-precipitation	Very simple, ambient conditions	20–90	Minutes	Water	Relatively narrow	Poor
Thermal decomposition	Complicated, inert atmosphere	100–320	Minutes hours	Organic compound	Very narrow	Very good
Microemulsion	Complicated, ambient conditions	20–50	Hours	Waterorganic compound	Relatively narrow	Good
Hydrothermal synthesis	Simple, high pressure	130–250	Hours–days	Water or/and organic compound	Very narrow	Very good
Polyol	Simple, high temperature and inert conditions	120–280	Minutes days	Polyol compound	Relatively broad	Good
Sol-gel	Complicated, high temperature	200–400	Minutes days	Water or/and organic ompound	Relatively broad	Good

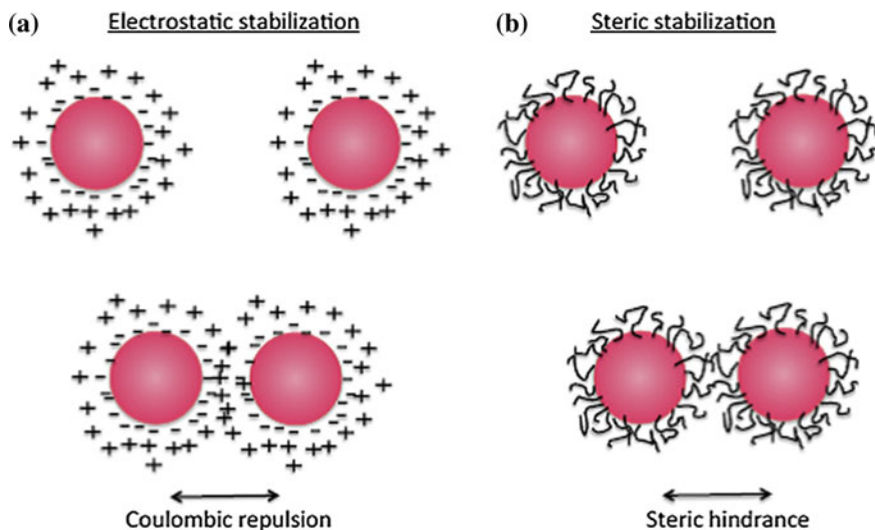


Fig. 5.10 Representation of the two possible ways for particle stabilization. **a** Electrostatic stabilization, **b** steric stabilization

Due to their high surface-to-volume ratio, nanoparticles tend to aggregate in order to lower their interfacial energy. The destabilization is caused by interparticle attractions. The dispersion state of nanoparticle solutions is an important factor while synthesizing nanoparticles for biomedical applications because it tends to modify their physico-chemical properties. The DLVO theory (named after DeJaguin, Landau, Verwey and Overbeek) [98–104] describes the stability of colloid suspensions and will be presented later.

Production of stable nanoparticle suspensions is challenging and primordial for biomedical applications. Stability of nanoparticles in a colloidal system is related to their tendency to aggregate under the action of gravity. Destabilization results from attractive van der Waals interactions.

One way to stabilize nanoparticles is to introduce interparticle repulsions that avoid van der Waals attraction. This can be achieved (A) by charging particles so that the surface charge leads to interparticle repulsion forces or (B) by covering the nanoparticles with big molecules to create interparticle steric repulsions.

The first strategy is known as electrostatic stabilization and the second is the steric stabilization (Fig. 5.10).

5.3.1 Stability of Charged Nanoparticles

This section highlights the factors that control stability. Nanoparticles can acquire a charge by four different mechanisms [97]:

- ionization of surface groups where the degree and nature of which are controlled by the pH of the solution,
- ion adsorption as for instance the adsorption of a cationic surfactant,
- isomorphous substitution,
- dissolution in ionic solids.

To understand the stabilization mechanism, it is primordial to study what happens when a charged particle is in solution. Let's take the case of a positively charged nanoparticle. When placed in a solution containing inert ions, anions will be attracted by the nanoparticle surface and cations repelled from the interface. Electroneutrality is reached when the magnitude of the negative charge of the layer near the interface is equal to the magnitude of the nanoparticle's surface charge.

This charged surrounding is known as the electrochemical double layer. It is characterized by a potential drop across the solid/liquid interface which is described by the Stern-Gouy-Chapman (SGC) model. According to this latter, the surface charge is counterbalanced by two distinctive regions, the Helmholtz layer (or inner layer) and the Gouy-Chapman layer (or diffuse layer) (Fig. 5.11). They both contribute to the total interfacial charge density.

The innerlayer includes the inner Helmholtz plane (IHP) and the outer Helmholtz plane (OHP). Ions directly adsorbed to the surface are located in the IHP and OHP is defined as the plane of closest approach of fully solvated ions. The charge in OHP is counterbalanced by a dynamic ionic atmosphere forming the diffuse layer.

The surface potential at the diffuse layer is called zeta potential (ζ) and is an experimentally measurable value.

The SGC model considers that the solvent is a dielectric continuum and that ionic species are non-interacting point charges. It assumed that the total interfacial charge density depends on ionic species concentration in solution and on the permittivity of the solvent.

Fig. 5.11 Representation of the electrochemical double layer in accordance with the SGC model. Reproduced from Ref. [105]

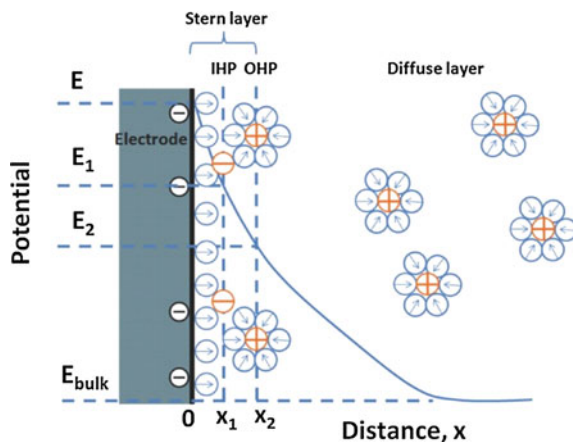
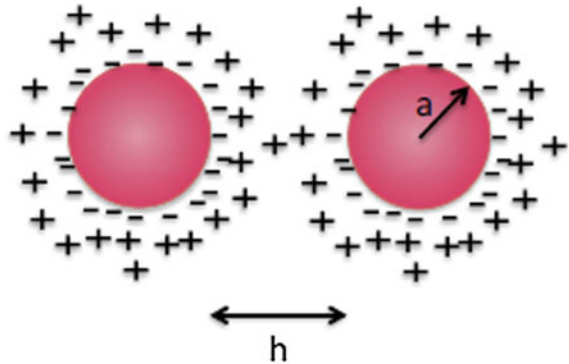


Fig. 5.12 Representation of two colloidal particles coming close together



Stability of a whole colloidal system can be predicted by studying what happens when two nanoparticles come close together because of the Brownian motion. The colloidal pair potential governs the stability of colloidal dispersions, it is defined as the potential energy of interaction between two colloidal particles. The most relevant components for interaction between charged particles are the van der Waals and the repulsive forces (Fig. 5.12).

The potential energy resulting from attractive forces (U_A) is proportional to the particle radius (a), a material constant named the Hamaker constant (A) and the inverse of the distance of separation (h) [106]. It is given by equation.

$$U_A = -\frac{A}{12} \left[\frac{1}{x(x+2)} + \frac{1}{(x+1)^2} + 2 \ln \frac{x(x+2)}{(x+1)^2} \right]$$

where $\chi = h/2a$

If the particles are very close ($h \ll 2a$), then the last equation can be simplified and the potential energy becomes,

$$U_A = -\frac{Aa}{12h}$$

The Hamaker constant depends on the electronic polarisability and the density of the material. Table 5.4 provides the value of the Hamaker constant for some media and materials.

$$A = \left(\sqrt{A_{particle}} - \sqrt{A_{medium}} \right)^2$$

The electrostatic repulsion is an important stabilization mechanism of charged nanoparticles dispersed in aqueous solutions. When two of them come close together, two cases exist: their surface charge doesn't change and the surface potential compensates or the opposite.

Table 5.4 Value of the Hamaker constant of some components [99]

Media	Hamaker constant (J/10 ⁻²⁰)
Water	3.7
Ethanol	4.2
Cyclohexane	5.2
Particles	Hamaker constant (J/10 ⁻²⁰)
Silica	6.5
Titanium dioxide	19.5
Metals (Au, Ag, ...)	40

Hogg, Healy and Fuersteneau have developed equations describing the electrostatic interaction between two non-identical particles under constant charge (U_R^σ) and constant potential (U_R^ψ) [107]

$$U_R^\psi = \frac{\varepsilon a_1 a_2 (\psi_{01}^2 + \psi_{02}^2)}{4(a_1 + a_2)} \left[\frac{2\psi_{01}\psi_{02}}{\psi_{01}^2 + \psi_{02}^2} \ln\left(\frac{1 + e^{-\kappa h}}{1 - e^{-\kappa h}}\right) + \ln(1 + e^{-2\kappa h}) \right]$$

$$U_R^\sigma = \frac{\varepsilon a_1 a_2 (\psi_{01}^2 + \psi_{02}^2)}{4(a_1 + a_2)} \left[\frac{2\psi_{01}\psi_{02}}{\psi_{01}^2 + \psi_{02}^2} \ln\left(\frac{1 + e^{-\kappa h}}{1 - e^{-\kappa h}}\right) + \ln(1 - e^{-2\kappa h}) \right]$$

where κ^{-1} is the Debye length defined as the thickness of the electrical double layer, ε is the dielectric constant of the suspending medium, ψ_{01} , ψ_{02} are the total double layer potentials of the respective particles, h is the distance between the particles and a_1 and a_2 are the particle radii.

For two identical particles, $a_1 = a_2 = a$, $\psi_{01} = \psi_{02} = \psi_0$ and these two last equations become,

$$U_R^\psi = \frac{\varepsilon a \psi_0^2}{2} \ln(1 + e^{-\kappa h})$$

$$U_R^\sigma = \frac{\varepsilon a \psi_0^2}{2} \ln(1 - e^{-\kappa h})$$

and if $\kappa a < 3$, then the general expression is given by equation

$$U_R = 2\pi\varepsilon a \psi_0^2 e^{-\kappa h}$$

According to the DLVO theory, the colloidal stability of charged particles relies on the total potential resulting from the interaction of colloidal particles (Fig. 5.13). It is equal to the linear sum of the attractive and the repulsive potentials. The attractive potential results from attractive van der Waals forces and the repulsive one from repulsive electrical double layer forces. The result is a typical curve for charge-stabilized colloid particles.

Fig. 5.13 Representation of the DLVO theory showing the interparticle interaction as the result of the van der Waals attraction and the electrostatic repulsion

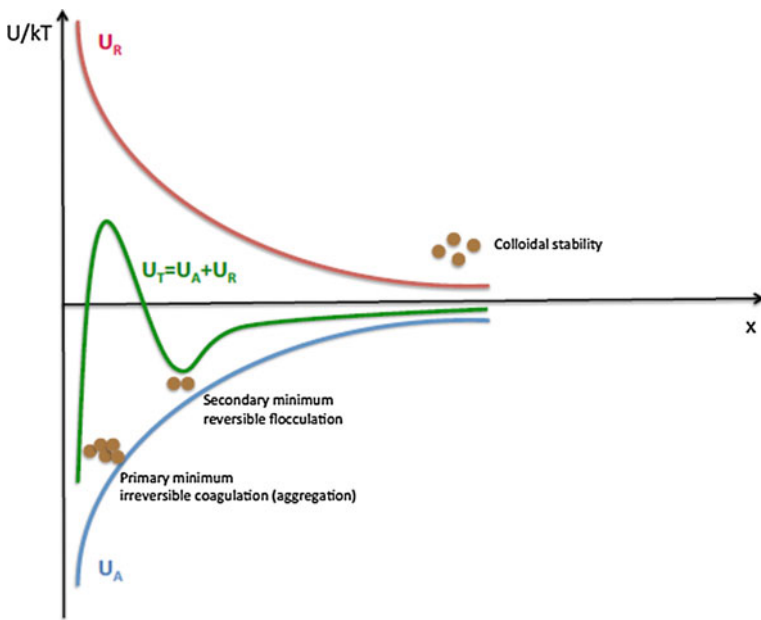
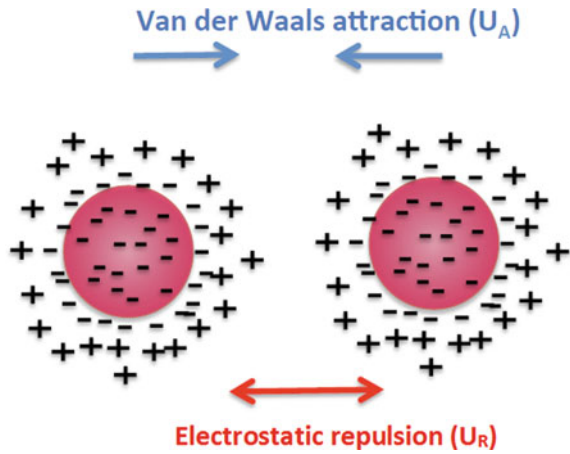


Fig. 5.14 Shape of the total potential energy (U_T) as a function of the distance (x)

$$U_T = U_A + U_R$$

From low to high interparticle distance, the shape of the curve presents, first, a primary minimum leading to irreversible coagulation (aggregation), then, a potential energy barrier and finally, a secondary minimum resulting in reversible flocculation (Fig. 5.14).

Nevertheless, while the DLVO theory works reasonably well to predict the stability of colloidal solutions at low salt concentrations ($< 5 \times 10^{-2}$ M), it has been shown to fail at higher ionic salt concentrations. The magnitude of forces varies greatly by changing counter-ion and is not in good agreement with the experiment anymore. This is explained by the fact that the theory ignores ion effects and dispersion forces acting on the ions.

At low salt concentration, electrostatic forces dominate so that the DLVO theory that describes interparticle interaction as the result of attractive VDW forces and repulsive electrical double layer forces is sufficient. But at higher salt concentration, buffer specificity is influential, dispersion forces are expected to dominate electrostatic forces and must be treated at the same level [108]. This is a major problem particularly when samples are devoted to biomedical applications because biological salt concentrations are more or less equal to 0.1 M. It can be solved by properly including dispersion forces acting on ions.

5.3.2 Steric Stabilization

The colloidal stability resulting from steric stabilization, for example the adsorption of polymer chains on the particle surface, is based on steric hindrance. The total potential function is the linear sum of the attractive potential (U_A) due to van der Waals interaction and a steric potential (U_S) and is given by the following equation.

$$U_S = 4\pi akT(0.5 - \chi)\Gamma^2 e^{(1-\frac{h}{\sigma})}$$

where h is the interparticle distance, Γ is the amount of adsorbed or grafted polymer, σ is the layer thickness and χ is the Flory polymer/solvent interaction. In the ideal case of stability, $\chi < 0.5$ and U_S is repulsive; if $\chi > 0.5$, U_S is attractive and the solution is not stable.

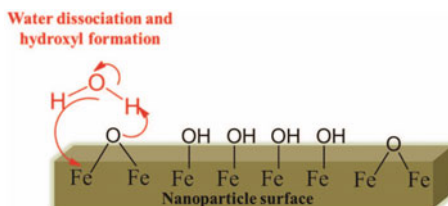
$$U_T = U_A + U_S$$

5.3.3 The Surface of Iron Oxide Nanoparticles

In order to describe the different stabilization methods, it is essential to define the surface nature of iron oxide nanoparticles. During the nanoparticle formation, surface is covered by epoxy functions which naturally react with water molecules to form hydroxyl groups on surface (Fig. 5.15).

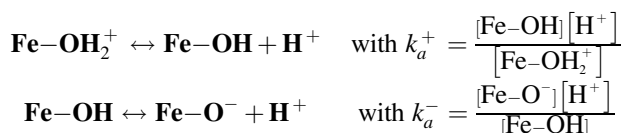
The hydroxyl groups on nanoparticle surface exhibit amphoteric properties and thus their charge can be modified with pH variation. These charge modification can

Fig. 5.15 Schematic representation of hydroxyl formation on iron oxide surface



modulate the surface with positive or negative charges as illustrated by following relations:

As a function of pH, acido-basic equilibriums are:



The superficial electrical charge (q_0) is given by:

$$q_0 = \left(\frac{F}{S}\right) \{ [\text{Fe-OH}_2^+] - [\text{Fe-O}^-] \}$$

where S is nanoparticle area by volume unit ($\text{m}^2 \text{L}^{-1}$), F is Faraday constant ($96,500 \text{ C mol}^{-1}$) and the second factor correspond to the difference between the concentrations of both charged species (mol L^{-1}).

The charge of nanoparticle surface depends on the pH and on the ionic force of the solution: the superficial electrical charge can be positive, negative and neutral as a function of the medium condition. Zero Point Charge (ZPC) corresponds to the pH value which the electrical charges cancel out [109]. ZPC depends on material nature and is represented as an average between the dissociation constants of species present on surface:

$$\text{ZPC} = \frac{pk_a^+ + pk_a^-}{2}$$

In the case of magnetite (Fig. 5.16), ZPC is around to 6.5 [110–112]. Below the ZPC, hydroxyl sites absorb the protons and become positive by the formation of FeOH_2^+ species. Above the ZPC, the protons are desorbed and the deprotonation leads to negative species Fe-O^- .

When iron oxide nanoparticles are put in contact with a solution, a biphasic system is created with solid/liquid interface. At this interface, some phenomena can appear and influence the colloidal stability, especially, the creation of a double electrical layer.

In the presence of water molecules, the surface can be hydrated by the surrounding water molecules. A water layer will thus be absorbed on nanoparticle

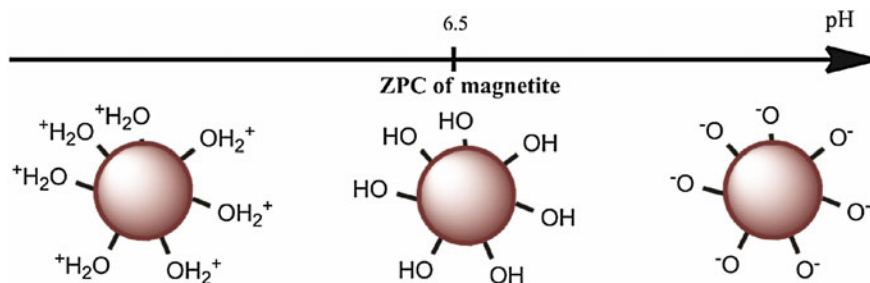


Fig. 5.16 Schematic representation of the charges present on nanoparticle surface as a function of pH

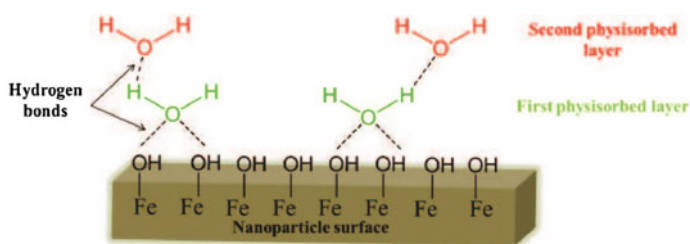


Fig. 5.17 Schematic representation of solvated surface of iron oxide nanoparticles

surface and the structure is stabilized by hydrogen bonds as shown in Fig. 5.17 [113]. Depending on the oxide nature, the thickness of solvated layer can vary from 1 to 10 Å.

The surface depends on two parameters: the charge and the hydration states. However, it is also important to distinguish the difference between the ZPC and the isoelectric point (IEP). ZPC is the pH value at which the surface is neutral by the absence of positive or negative charges. On the contrary, the isoelectric point is the pH value when the surface charge is exactly offset by the counter-ions.

The structure of the double electrical layer is described by different models [114–119]. In our work, Stern model was used to explain the composition of the interface between the nanoparticles and the solution (Fig. 5.18). It is based on the models of Helmholtz and Gouy-Chapman. The double electrical layer is composed of two distinct layers: a **compact layer** (Stern layer) and a **diffuse layer** (Fig. 5.18b).

The compact film of the double electrical layer corresponds to the Stern layer in which the ions (H^+ or OH^-) can protonate or deprotonate the hydroxyl groups bonded on iron oxide surface. The Stern layer can be separated into two sets:

- (A) The first part of Stern layer is composed of ions presenting a high affinity with surface. Their main role is to neutralize charges of the surface. These ions are away from the surface until a distance called Interne Helmholtz Plan (IHP).

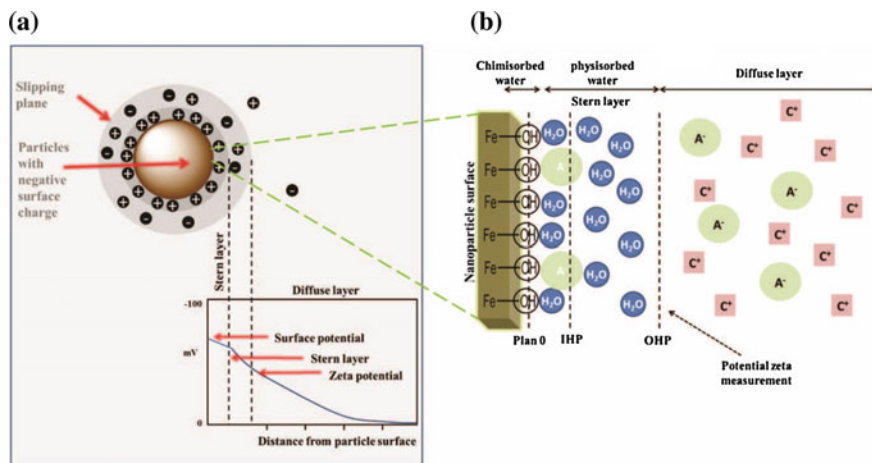


Fig. 5.18 **a** Schematic representation of the electrical double layer and the evolution of electrostatic potential as a function of the distance with negative charges on particle surface. **b** Schematic representation of the water absorption on the surface of iron oxide nanoparticles where A^- and C^+ are the anions and the cations present in solution respectively. IHP is the inner Helmholtz plan and OHP is the Outer Helmholtz plan. Wih pemission of Ref. [120]

(B) The second part of Stern layer can be considered as a frontier between the IHP layer and the diffuse layer. This layer is included in the Stern layer until the Outer Helmholtz Plan (OHP).

Beyond the Outer Helmholtz Plan, the second layer of the double electrical layer starts. This one is called the diffuse layer and is mainly composed of the counter ions. The global electric charge of the diffuse layer leads to the charge compensation, inducing the electric neutrality of the nanoparticles. These different layers explain why the hydrodynamic diameter of nanoparticles is always larger than the diameter measured by the transmission electron microscopy.

The electrostatic potential decreases as a function of the distance with the nanoparticle surface. However, this decrease is more affected by the Outer Helmholtz plan also called slipping plan as illustrated in Fig. 5.18a. At this plan, the potential observed is named the zeta potential (ξ). This value is the unique measurement of an observable and measurable potential. It is used as a reference to evaluate the stability of colloidal suspension.

The magnitude of zeta potential gives an indication of the stability of a nanoparticle suspension. In the case of a high value of zeta potential (negative or positive), the colloidal suspensions are stable and the presence of agglomerates is not observed. On the contrary, when low values of zeta potential are observed, flocculation generally occurs in solution. This parameter is strongly influenced by pH value. Zeta potential value varies and allows thus to estimate the pH region where the suspension is stable or not. At a given pH value, the zeta potential is close to zero when all charges are compensated by the counter-charged molecules versus

to the surface; this point is the isoelectric point (previously described). Although the pH is the most important factor influencing the zeta potential properties, the ionic strength of the media also affects the value of zeta potential, and especially, the nature of the ions interacting with the surface.

The thickness of the double layer depends on the ion concentrations in solution and can be correlated to the ionic strength of the medium. According to the ionic force, the double layer can be collapsed (at high ionic force) or extended (at low ionic force).

Two kinds of ions can interact with a charged surface:

- The **nonspecific ions** exhibit a low electrostatic interaction with the surface. When their concentrations increase in solution, the thickness of the electrical double layer is reduced without any effect on the value of the isoelectric point;
- The **specific ions** are attracted towards the surface with the electrostatic force inducing their adsorption on the surface and the isoelectric point is thus modified. In some cases, the adsorption of specific ions can affect the nature on charge surface.

The knowledge and the study of the surface state give indications on the stability of colloidal suspensions. Moreover, the measurements of zeta potential are generally used to confirm the surface modification process.

5.3.4 *Stabilization Strategies*

The surface of nanoparticle is the first area interacting with the body fluids when iron oxide suspensions are injected. This is the major reason why the nanoparticle surface is extensively studied and characterized. The problematic of uncoated nanoparticles is their trend to agglomerate. This explains why the development of stabilization methods are required to promote the repulsive forces between the particles that are opposed to attractive Van der Waals forces. The strategy generally used is to insert capping agents on nanoparticle surface.

Two complementary approaches can be envisaged: **electrostatic stabilization** and **steric stabilization** (Fig. 5.19) [121–124].

The goal of the surface protection is to decrease the interfacial energy in order to obtain a strong barrier that limits the distance of closest approach between nanoparticles. In the literature, the main coating agents are polymers (synthetic or naturals), small ionisable organic molecules, silica coating and biological molecules. Some examples are illustrated in Fig. 5.20.

The following part briefly describes some examples for each stabilizing agent.

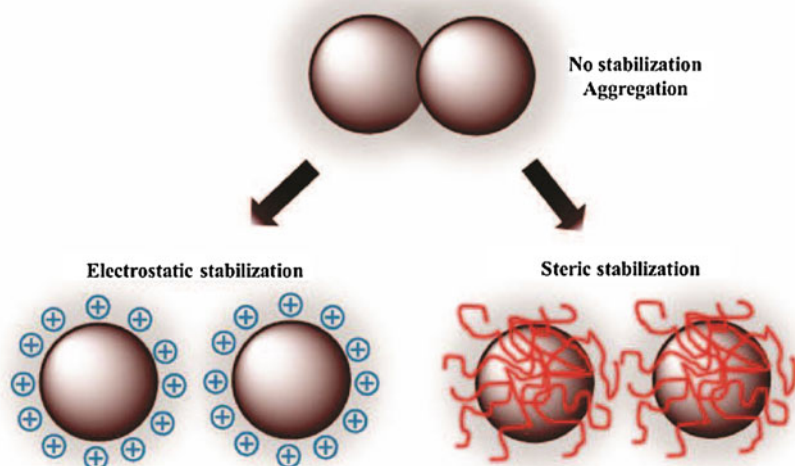
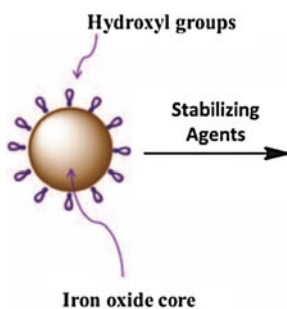


Fig. 5.19 Schematic representation of stabilization strategies: electrostatic and steric protections

Fig. 5.20 Summary of the main coating agents used for the stabilization of iron oxide nanoparticles



- | | |
|--------------------------------|----------|
| Natural polymers | |
| Dextran | Gelatin |
| Chitosan | Pullulan |
| Synthetic polymers | |
| Polyethylene Glycol | PEG |
| Polyvinyl Alcohol | PVA |
| Polystyrene | PS |
| Poly(vinylpyrrolidone) | PVP |
| Poly(acrylic acid) | PAA |
| Silica, silane | |
| TEOS | |
| Alkoxysilane | |
| Small organic molecules | |
| Carboxylate | |
| Phosphate | |
| Sulphate | |
| phosphonate | |
| Biological molecules | |
| Avidin | |
| Human serum albumin HSA | |

5.3.4.1 Silica

The most common strategy used for the surface modification is the formation of a silica shell. Protocols including trialkoxysilane molecules or tetraethyl orthosilicate (TEOS) are often reported [125]. The advantage of these molecules is that the silane groups can be covalently grafted onto nanoparticle surface through the reaction between the hydroxyl groups present on iron oxide surface and the alkoxy silane functions ($-\text{Si}-\text{O}-\text{R}$ where R is methyl or ethyl groups) [126]. The cross-linking reactions induce the formation of a silica layer surrounding the particles [127]. A large choice of terminal functional groups (alcohol, amine, epoxy, thiol or carboxylate) [128–130] can be used to protect and stabilize iron oxide nanoparticles (Fig. 5.21).

Coating agents with precious metal (such as gold) can also provide an effective protection to avoid the surface oxidation and to reduce nanoparticle agglomeration in aqueous solution [132, 133].

5.3.4.2 Polymers

Another important and widely employed stabilizing agent is polymer (synthetic or natural). This type of coating allows a biocompatible and biodegradable surface but also improves the blood circulation times depending on the polymer nature [134, 135]. The polymers can be natural [136] or synthetic [134, 137].

One of the most used natural polymers is dextran [138]. This biocompatible and biodegradable polysaccharide can be strongly absorbed on nanoparticle surface, due to a strong interaction with hydrogen bonds formed between the hydroxyl functions on the polymer chains and the surface of iron oxide cores [139, 140]. Several MRI contrast agents have been elaborated with a dextran coating or its derivatives (carboxydextran and carboxymethyl dextran) [141–144]. Some dextran coatings are based on the cross-linked polymer after the nanoparticle attachment using epichlorohydrin [145, 146]. The system has demonstrated a high circulation half-life in blood with no acute toxicity.

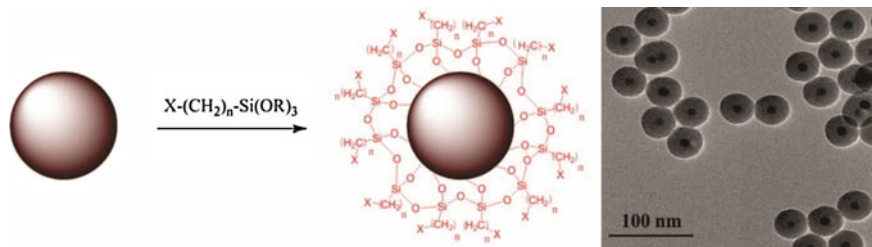


Fig. 5.21 General silica coating method and TEM images of silica coated iron oxide nanoparticles [131]

Duguet et al. [147–149] described the modification of the dextran structure with silane molecules. This strategy allows a covalent grafting on the surface. Other polymers can be used such as chitosan [150–152], gelatin [153], alginate [154] and pullulan [155] as stabilizing agents.

Another natural and biodegradable polymer, polylactic acid, has been used for the preparation of stable colloid suspension with a typical range of hydrodynamic diameter included between 10 and 180 nm [156, 157].

Among the synthetic polymers, the polyethylene glycol (PEG) is widely used due its properties such as the increase of the blood circulation time, the hydrophilicity and the biocompatibility. PEG can also be used for the coupling with other polymer to increase the hydrophilic properties. Two approaches are currently used to coat nanoparticles and consist in the addition of the surfactants during the synthetic process or post-synthesis. Other polymers and copolymers which have been used to coat magnetic nanoparticles are polyvinyl alcohol (PVA) [158, 159], polystyrene (PS) [160, 161], poly(vinylpyrrolidone) (PVP) [162, 163], poly(acrylic acid) (PAA) [164], poly(ethylenimine) (PEI) [165, 166], PAA-chitosan [167], ...

5.3.4.3 Small Organic Molecules

Small organic molecules are frequently used to obtain stable colloidal suspension. The chemical functions used generally are carboxylates, phosphates and sulfates due to their high affinity for iron oxide surface. These strong interactions result from an ionic interaction between the acidic functions of the coating agents and the surface of nanoparticles. The most used carboxylic acids are citric and dimercaptosuccinic acids (Fig. 5.22) [168–172]. These polyacids form a stable colloidal suspension resulting from the high coordination on metal surface. However, the ionic bonds between the carboxylic functions and the iron oxide surface are labile and can be easily broken by the elevation of temperature or by carboxylic compounds presenting a much higher affinity with the surface.

Phosphate and phosphonate derivatives also are promising stabilizing molecules. Their absorption on metal surface is very stable and they are able to form a strong interaction in aqueous solution [173–176]. Bisphosphonate compounds (Fig. 5.22) are preferred in order to anchor double functions on the metal surface, involving the strengthening of the nanoparticle stability [177–179].

5.3.4.4 Biological Molecules

The stabilization performed by biological molecules is not a common method. Some examples describe the surface covering with proteins such as avidin [180] or human serum albumin (HSA) [181, 182]. This process allows the formation of stable and biocompatible magnetic fluids.

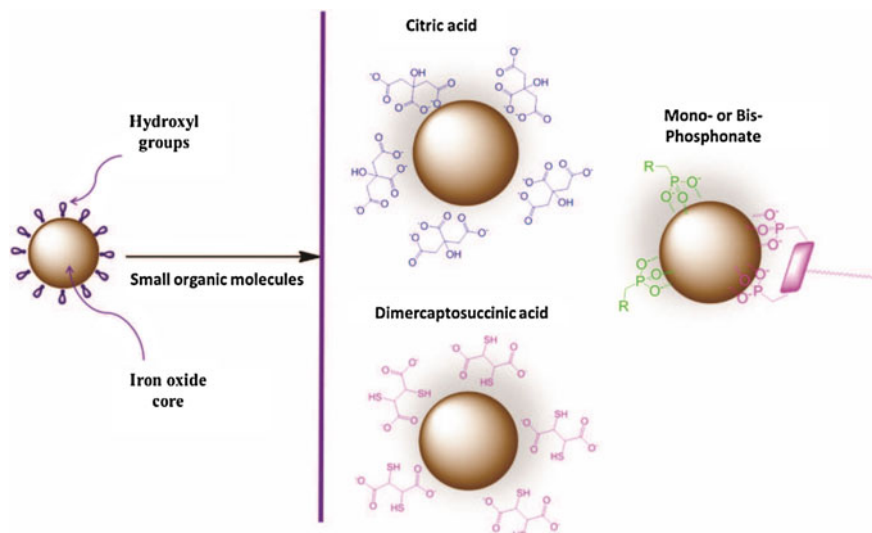


Fig. 5.22 Some examples of the nanoparticle stabilization with small organic molecules as citric acid, dimercaptosuccinic acid and mono- or bis-phosphonate molecules

5.3.5 Recent Advances on the Stabilization of Hydrophobic Iron Oxide Nanoparticles

The biocompatibility and the colloidal stability in water are primordial characteristics in order to use nanoparticles for clinical applications. Iron oxide nanoparticles synthesized in organic medium present the advantages to have controlled magnetic properties depending on the size and on the size distribution.

However, it is essential to modify the surface in hydrophilic one by the use of appropriate coating.

Two main ways are: **ligand addition** or **ligand exchange** (Fig. 5.23).

5.3.5.1 Stabilization by Ligand Addition

The ligand addition approach consists in the addition of amphiphilic molecules interacting with the initial stabilizing agent. The hydrophobic segments interact with the post-synthesis hydrophobic capping groups and the hydrophilic segments contribute to a better dispersion of nanoparticles in water. As a result, this interaction forms a double layer on the nanoparticle surface (Fig. 5.23). In this way, the stabilizing molecules are considered as phase transfer agents that allow the transition from an organic phase to an aqueous solution. The reported main surfactants

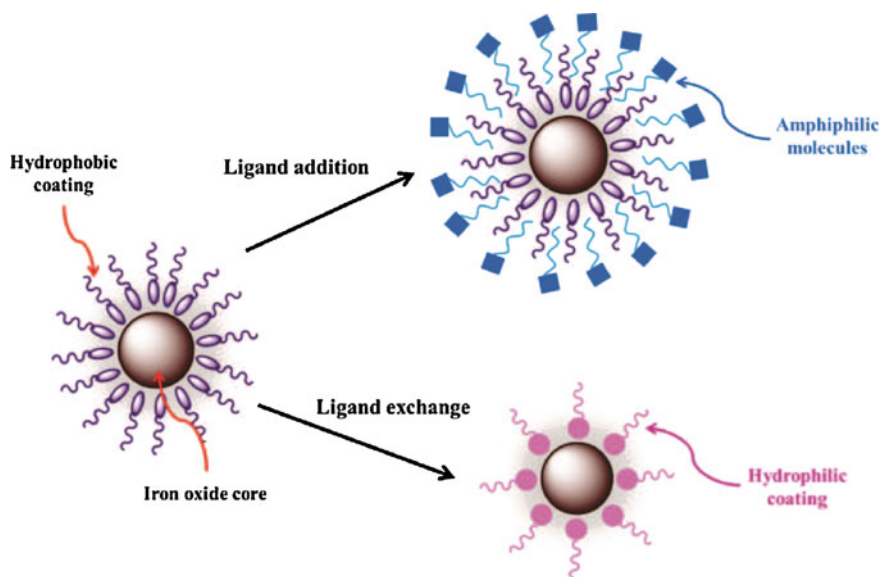


Fig. 5.23 Transfer of hydrophobic iron oxide nanoparticles in water media thanks to the ligand addition or ligand exchange

are polymers, alkylammonium salts and lipids; this part briefly describes some main examples.

Polymers

Many strategies have been developed using polymers as a surfactant. An example widely used is pluronic copolymers composed of a hydrophobic poly(propylene oxide) (PPO) chain flanked on each side with hydrophilic poly(ethylene oxide) (PEO) chains [183–187]. This is a triblock polymer (PEO-PPO-PEO) where hydrophobic PPO segments are located between the oleic acid chains and the PEG segments (Fig. 5.24).

Several similar processes have been described in the literature for diblock or triblock copolymers [188–192].

Alkylammonium Salts

Another common class of phase transfer agents is alkylammonium compounds. For example, cetyltrimethylammonium bromide (CTAB) is usually used [193, 194] as shown in Fig. 5.25.

A better water dispersion is observed when CTAB interacts with initial oleic acid coating. The nanoparticle size is inevitably increased.

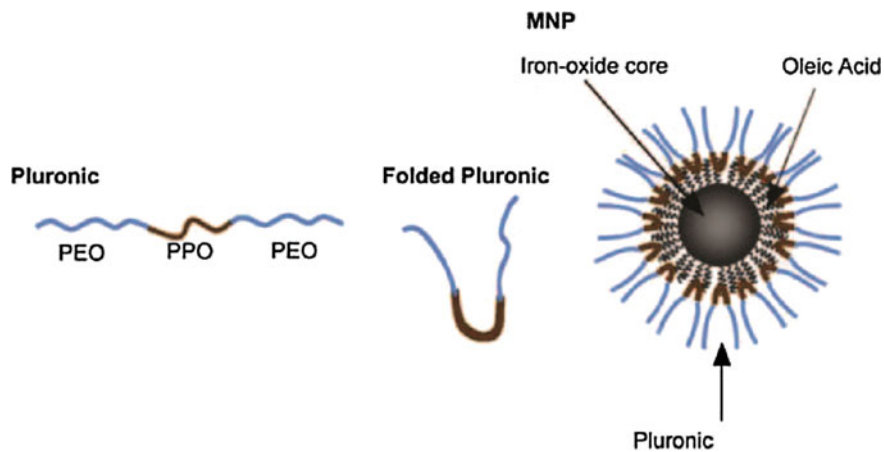


Fig. 5.24 Water stabilization of iron oxide nanoparticles by pluronic polymers (with permission of reference [184])

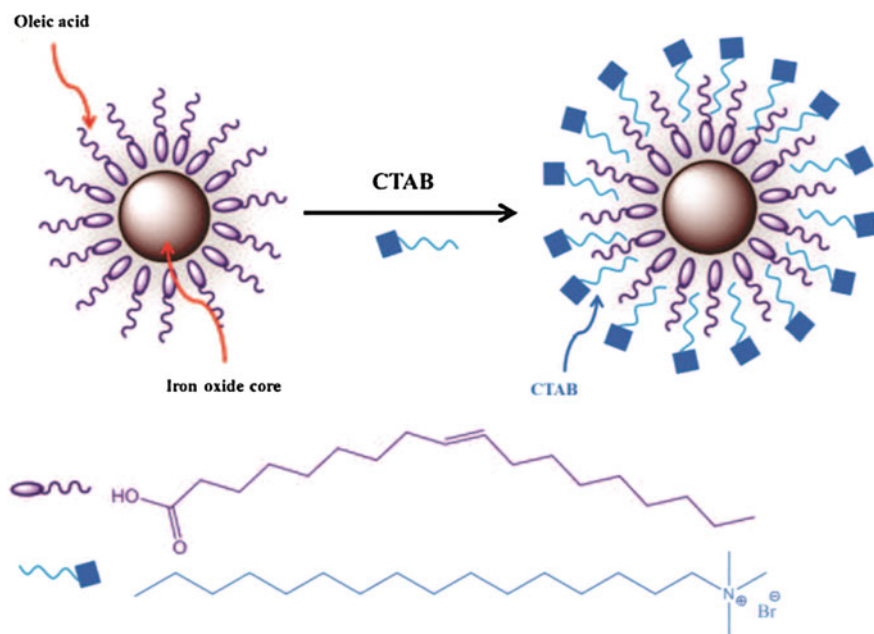


Fig. 5.25 Ligand addition of CTAB on oleic acid coated iron oxide nanoparticles

Lipids

A similar approach is exploited by the addition of lipids (Fig. 5.26) [195, 196] on nanoparticle surface. Phospholipids and fatty acids are the most widely used as amphiphilic ligands in order to transfer hydrophobic particles in water.

Deng et al. [197] reported a strategy to functionalize the hydrophobic surface of iron oxide nanoparticles with phospholipids such as 1,2-dioleoyl-snglycero-3-phosphoethanolamin-*N*(succinyl) (carboxylated phospholipid). This coating was selected for several reasons. This phospholipid is amphiphilic, biocompatible and able to disperse nanoparticles in water when it interacts with long carbon chains initially present on nanoparticle surface. Other phospholipids as phosphatidylcholine and phosphatidylethanolamine were also used [198].

Fatty acids (with 10–18 carbons) are also used to form a hydrophobic-hydrophilic bilayer for the transfer of hydrophobic nanoparticles in water [199, 200].

In summary, ligand addition transfers hydrophobic particles in water by the use of amphiphilic compounds (polymers, alkylammoniums, lipids, ...). The strong hydrophobic interaction of amphiphilic molecules with nonpolar organic surface of nanoparticles leads to a complete encapsulation of the magnetic core and preserves the original coating. As a result, the agglomeration phenomena are generally minimized due to the conservation of the initial coating and a strong steric

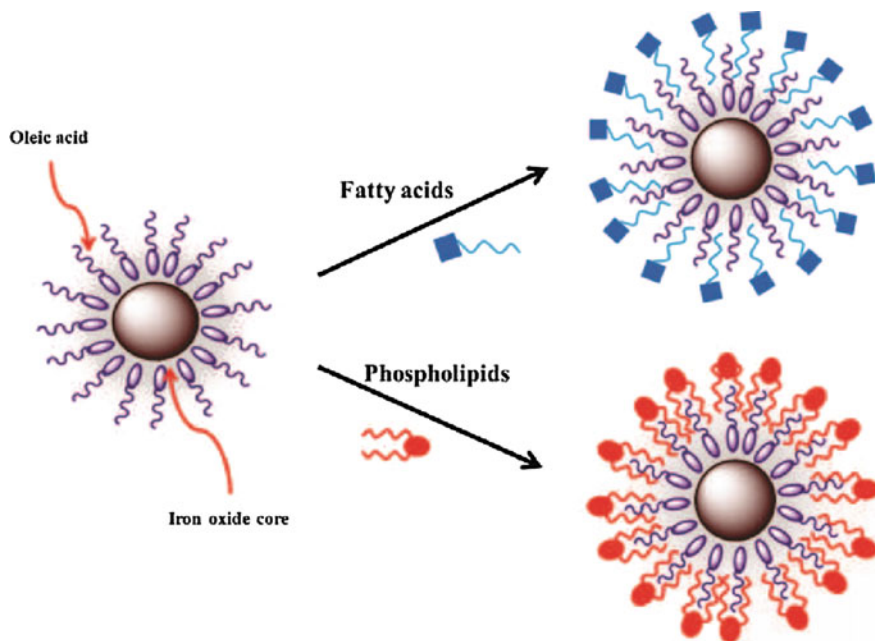


Fig. 5.26 Stabilization of hydrophobic nanoparticles by the use of fatty acids or phospholipids

stabilization. However, some drawbacks are observed such as the increase of nanoparticle size due to the addition of new coating and the destabilization of double layer by the elevation of temperature [159].

5.3.5.2 Stabilization by Ligand Exchange

Ligand exchange is defined as a replacement of initial capping agent with other coating, which is put onto the nanoparticle surface through exchange reactions. This approach is considered as a most versatile process to provide a better colloidal stability. According to the ligand nature, the link between the coating molecules and the nanoparticle surface can be covalent or ionic. In the following part, some major examples are described such as the ligand exchange by small charged molecules, by polymers or by silica.

Small Charged Molecules

The main small charged molecules are carboxylic acid derivatives. As previously described, the interaction between the nanoparticle surface and the carboxylic acid of oleic acid can be easily broken by temperature increase or by exchange with another carboxylic acid compound. An easy way is to select carboxylate molecules containing many carboxylic functions so that it could be anchored by two or more sites on the particle. Citric acid [159, 201] and dimercaptosuccinic acid (DMSA) [202–205] are the most widely used polyacids in which a multiple coordination of carboxylate functions on magnetic core can be observed. These ligands give a better stability in water by a strong ionic interaction.

Tetramethylammonium hydroxide (TMAOH) is a second common transfer agent used in order to replace oleic acid. This small molecule electrostatically stabilizes nanoparticles in water. The phase transfer is composed of a mixture between nanoparticles dispersed in non-polar solvent and an aqueous TMAOH solution. A double phase is created and the solution can be mixed either by magnetic agitation or by the use of ultrasounds [206–208].

These strategies are generally easy to perform. However, their stabilization is often not sufficient in order to conserve nanoparticle suspensions during long time storage.

Polymers

Polymers have been also widely used for the surface modification by ligand exchange. Firstly, the polymer chains can be attached on nanoparticle surface thanks to available chemical functions to create a strong interaction between the nanoparticles and the polymer.

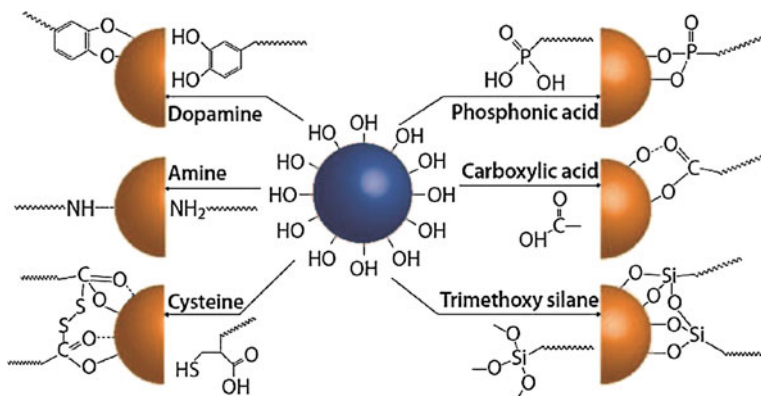


Fig. 5.27 Main chemical functions for the attachment of polymers on nanoparticle surface. With permission of Ref. [134]

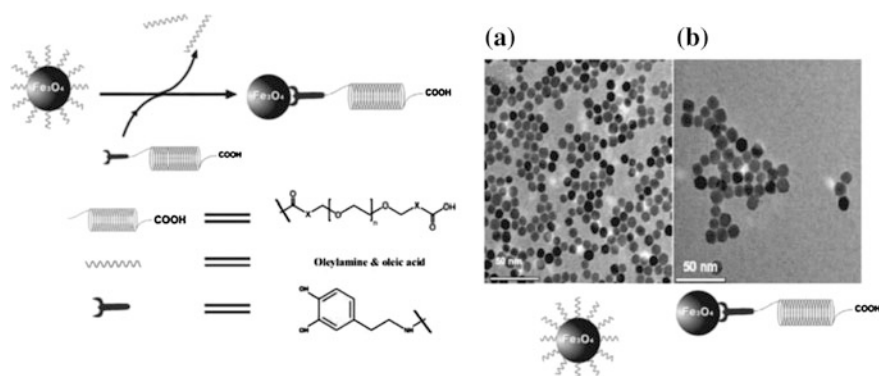


Fig. 5.28 Surface modification of oleic acid coated nanoparticles with dopamine modified PEG diacid and TEM images before and after their surface modification [212]

Several chemical functions [209] can be used to graft the polymer on the surface. Amongst others, we can quote dopamine [210–212], cysteine [213], phosphonic acid, carboxylic acid [214] and alkoxy silane [128, 129, 215] (Fig. 5.27).

As an example, the PEGylation of oleic acid coated nanoparticles can be performed via the reaction with dopamine modified PEG diacid (DPA-PEG-COOH) (Fig. 5.28). The first step is the modification of PEG diacid derivative with dopamine. Then, the resulting DPA-PEG-COOH is used to replace oleate compound and the particle surface is converted from hydrophobic to hydrophilic [212, 216].

Another method to form water-soluble iron oxide nanoparticles from the preparation of high thermal decomposition is to combine two ligand exchange strategies (Fig. 5.29) (small charged molecules and polymers) [159].

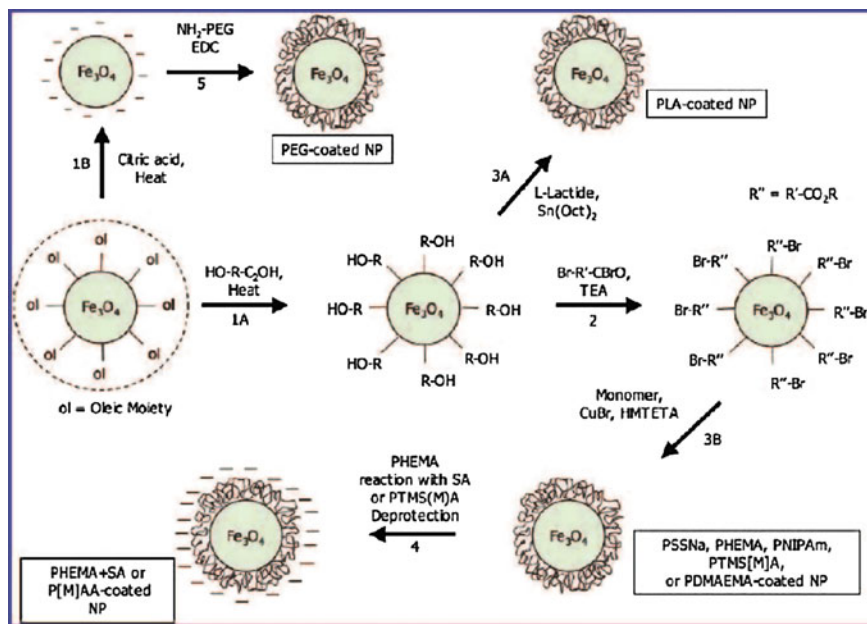


Fig. 5.29 General scheme of stabilization strategies described by Lattuada et al. [217]. Steps 1A and 1B: ligand exchange reactions with polyelectrolytes. Step 2: acylation of hydroxyl groups to prepare the surface with polymerization initiators. Step 3A: surface-initiated the polymerization with L-lactide. Step 3B: polymerization start up via the addition of monomers. Step 4: deprotection or additional reaction after polymerization. Step 5: grafting of end functionalized PEG chains onto nanoparticle surface using amidation chemistry. With permission of Ref. [217]

In the first steps, authors proposed the use of polyelectrolyte compounds such as ricinoleic acid or citric acid to remove oleic acid on nanoparticle surface, ensuring the nanoparticle dispersion in water (Fig. 5.29). The second step promotes the formation of a polymer shell. Two general pathways are described: (i) the first one links the polymer chains by the reaction of carboxylate functions available on nanocrystal surface or (ii) the polymerization process can be directly initiated on nanoparticle surface by addition of initiator.

Another alternative is to combine the effect of small charged molecules and polymers in using a unique compound. Short hydrophilic polyelectrolyte molecules such as poly(acrylic acid), poly(allylamine) or poly(sodium styrene sulfonate) exhibit several functional groups presenting a strong coordination on nanocrystal surface and are thus able to remove initial hydrophobic coating.

For example, Ge et al. [216, 218] proposes a simple process using polyacrylic acid. This method is performed at high temperature in glycol solvent (Fig. 5.30). Due to a high boiling point (245 °C), a high dissolving capability for polyelectrolytes and a high miscibility with water or with organic solvents, diethylene glycol is widely used.

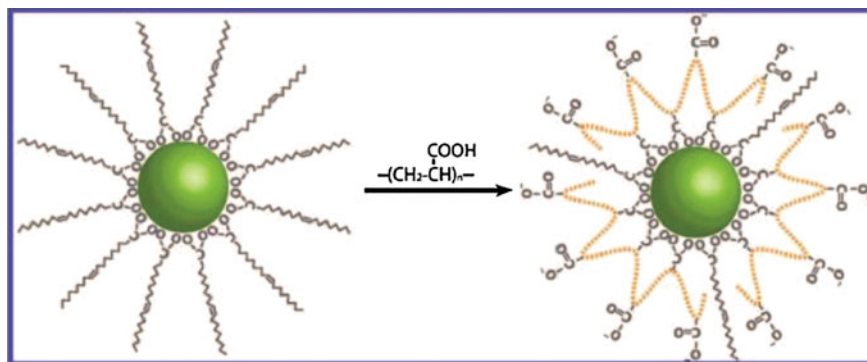


Fig. 5.30 Schematic representation of ligand exchange of oleic acid with polyacrylic acid in diethylene glycol [216, 218]. Reproduced with Ref. [216]

This approach presents several advantages such as:

- the high reaction temperature favors the desorption of oleic acid;
- the polyelectrolyte bonds are strong with multiple anchoring points on nanoparticle surface;
- due to the presence of the large quantities of the polar end functionalities, the final particles are water-soluble.

Silica

Silica coating is extensively used for nanoparticle stabilization in water. Indeed, silica shell is chemically inert and biocompatible [219]. Due to the ability to interact covalently with the surface, they are excellent candidates.

Major methods described in the literature for the silanization of hydrophobic nanoparticles can be separated into three general approaches:

- Direct silanization in non-polar solvents or a mixture of polar and non-polar solvents;
- Silanization by reverse microemulsion;
- Two-steps silanization: phase transfer followed by silanisation.

For the direct silanisation, several research groups proposed to replace oleic acid initially present on nanoparticle surface by organosilanes.

De Palma et al. [127] described a versatile method to functionalize the nanoparticles with different silane coupling agents. This group developed a method consisting in ligand exchange with oleic acid and silanes bearing different chemical end (Fig. 5.31). Briefly, the reaction occurs by the mixing of particles dispersed in n-hexane and selected trialkoxysilane in presence of acetic acid. The resulting solution is then mixed during three days at room temperature. Numerous silanes

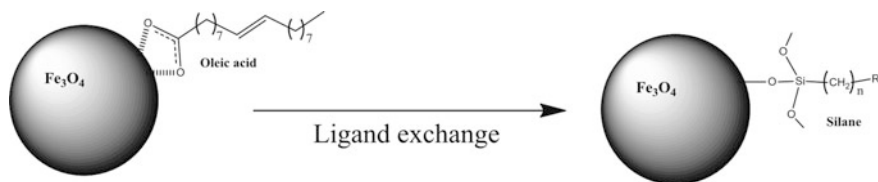


Fig. 5.31 Ligand exchange method to make hydrophilic iron oxide nanoparticles with silane agents. Reproduced with permission of Ref. [127]

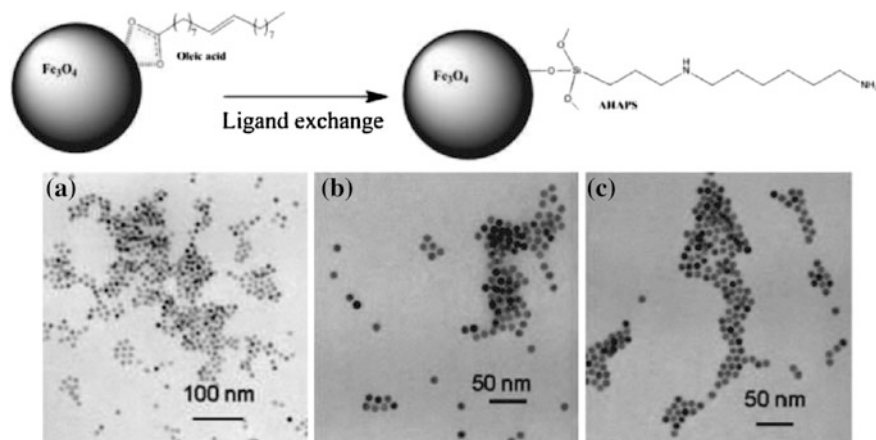


Fig. 5.32 TEM images of iron oxide nanoparticles stabilized with AHAPS from **a** THF, **b** ethanol or **c** water at pH = 3. With permission of Ref. [220]

with different chemical functions were used such as amine, aldehyde, thiol, carboxylic acid and cyano groups. The authors reported the efficient phase transfer of nanoparticles in water.

Another similar approach was proposed by Huang et al. [220]. The reaction is performed in tetrahydrofuran (THF) at 50 °C during a time varying from 100 to 210 min. Two types of silane were tested: *N*-(6-aminohexyl)-aminopropyl-trimethoxysilane (AHAPS) and 3-(triethoxysilyl)propylsuccinic anhydride (TEPSA). Interestingly, this strategy keeps the initial narrow size distribution (Fig. 5.32).

The ligand exchange with silane is time consuming. Therefore, Bloemen and collaborators [221] recently proposed an alternative method to reduce the reaction time. Thanks to the sonication during 5 h, the nanoparticles are dispersed in toluene and are mixed with trimethylamine and selected silane. After the reaction, a silane coating was formed.

Although the direct silanization is an efficient method to stabilize the nanoparticles, the control of the silica layer thickness is a poorly controlled parameter.

Consequently, the reverse microemulsion system was investigated in order to control this parameter. The reverse micelle system can be compared to nanoreactors allowing the control of the size, the shape and the reactant concentration.

Widely inspired by the works on hydrophobic semiconductor nanoparticles [222–225], Narita et al. [226] reported an easy method to control the silica layer thickness by tuning the reaction time and by limiting the reaction rate rather than by changing the solution components. Influenced by the reaction time and the pH, the thickness of silica layer can be tuned from 2 to 14 nm. However, the formation of silica coated particles with multiple magnetic cores has been observed.

Recently, Ding et al. [227] described a similar approach. The preparation of a single magnetic core encapsulated with different silica shell thicknesses for different size of iron oxide nanoparticles was reported. The reverse microemulsion system used is composed of five compounds: ammonia, Igepal-CO520, TEOS, cyclohexane and the hydrophobic iron oxide nanoparticles. IgepalCO520 is an amphiphilic copolymer made of hydrophilic polyethyleneglycol chains and a short hydrophobic alkylphenyl derivative. It is used as a surfactant to form reverse micelle in cyclohexane.

TEOS can be used in order to initiate the formation of silica shell after its hydrolysis by ammonia in solution. The general reaction is illustrated in Fig. 5.33.

Thanks to the control of experimental parameters, authors demonstrated the ability to produce silane coated iron oxide nanoparticles with thickness control. Additionally, the silane coverage of magnetic particles presenting different sizes was also proven (Fig. 5.34).

Reverse microemulsion system is an efficient and innovative method to produce nanoparticles covered with a controlled silica shell. Although the silane thickness can be modulated, the preparation of a large quantity needs the use of large surfactant and solvent amounts. Moreover, nanoparticles initially prepared with TEOS

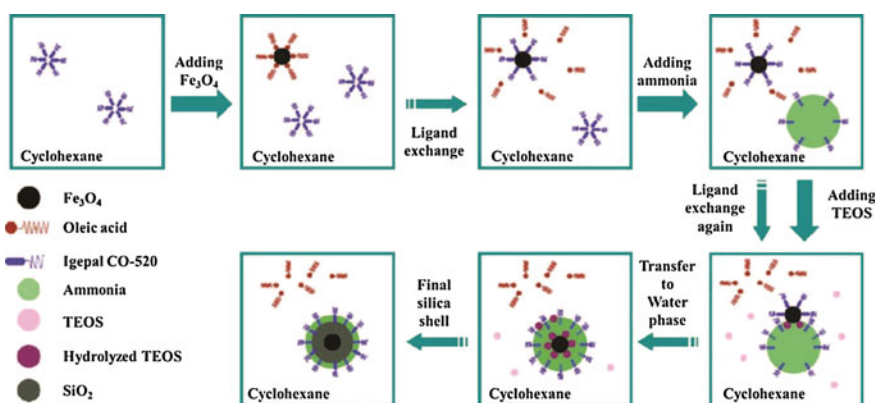


Fig. 5.33 Schematic representation of the silica coating mechanism on the surface of iron oxide nanoparticles via the reverse microemulsion method published by Ding et al., with permission of Ref. [227]

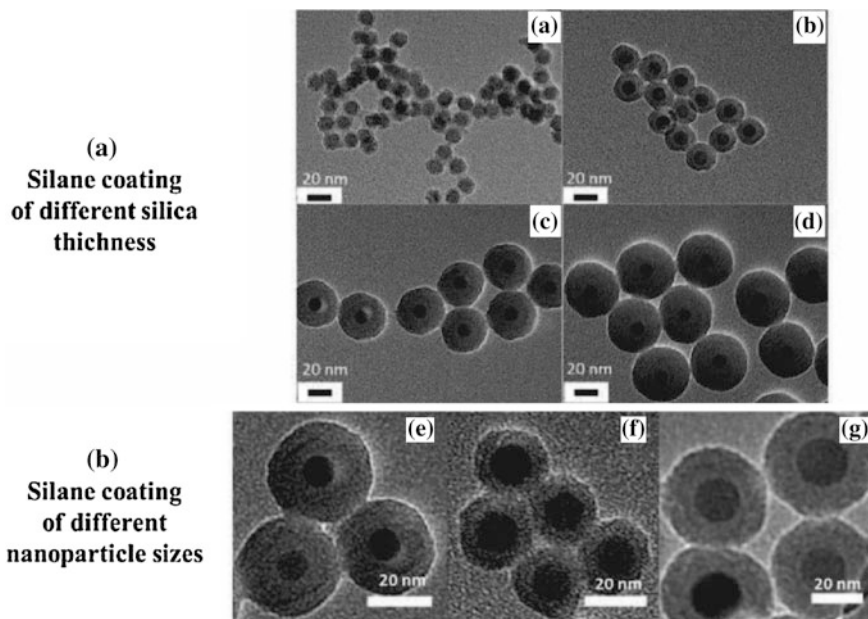


Fig. 5.34 TEM images of silica coated iron oxide nanoparticles: **a** with different silica shell thicknesses of 2 nm (a), 6.3 nm (b), 14.1 nm (c) and 19.8 nm (d) and **b** with magnetic core sizes of 10.1 nm (e), 13.5 nm (f) and 19.1 nm (g). With permission of Ref. [131]

often require an additional step in order to functionalize the surface for future targeting [226].

The last method uses two steps. Typically, the first step is a ligand exchange or a ligand addition in order to transfer hydrophobic particles in water. The second step involves the formation of silica shell.

For example, Kim et al. [193, 228] developed a two-steps method to modify hydrophobic nanoparticles with cetyltrimethylammonium bromide (CTAB) in order to disperse nanomaterials in water (Fig. 5.35). The carbon chains of CTAB interact

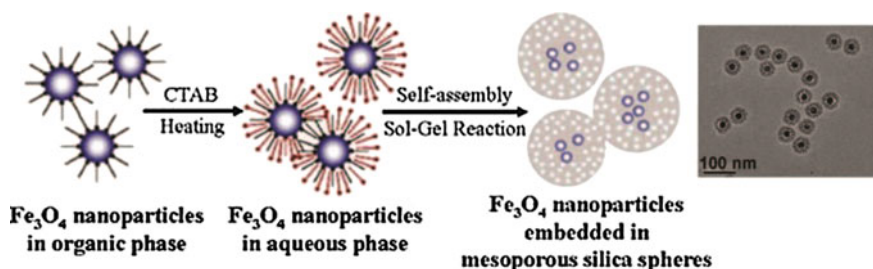


Fig. 5.35 Schematic representation of two-steps silanization and TEM image of the resulting materials. Reproduced with permission of Ref. [228]

with oleic acid chains through hydrophobic interactions and the polar group of CTAB (ammonium function) is oriented towards aqueous solution. This first step is a phase transfer via a ligand addition. The subsequent reaction is a ligand exchange in order to form silica coating by addition of TEOS performed in water medium.

Some authors used this method of phase transfer with polymers as a primary step before the silanization. As an example, Shen et al. [229] proposed the use of silane modified poly(ethylene glycol). Firstly, methoxy-poly(ethylene glycol) silane (MPEG-sil) replaces oleic acid. The nanoparticles are thus coated with polymer and can be dispersed in a more hydrophilic media. The silane function present on the polymer allows the formation of silica coating by the addition of TEOS.

In a similar way, it has been reported that the primary ligand exchange step can be also realized by poly(acrylic acid) (PAA) [230]. After the first ligand exchange, nanoparticles can be dispersed in an aqueous ethanol solution and then, the particles can be easily encapsulated with a uniform silica coating using TEOS as a silica source.

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Chapter 6

MRI Applications: Classification According to Their Biodistribution

Abstract MRI contrast agents are known for about 30 years. Most of clinical compounds are extracellular agents without tissue specificity. They are excreted by the kidneys (Gd-DTPA, Gd-DOTA, Gd-DTPA-BMA or Gd-HP-DO3A for example). More recently, intracellular agents (Gd-EOB-DTPA, Gd-BOPTA, ... undergoing hepatocyte uptake), and blood pool contrast agent (MS-325, binding albumin) have been developed. These Gd complexes have an extended clearance time and allow longer contrast-enhanced imaging sessions. These systems are called “ T_1 or positive” agents because they increase the water signal of the tissue areas where they are concentrated. A second kind of contrast agents is made of superparamagnetic iron oxide nanoparticles (USPIO or SPIO). These nanosystems have a high efficiency and are generally “ T_2 or negative” agents, inducing a darkening of the region where they accumulate. Such nanoparticles with a diameter of approximately 20–200 nm can be used as contrast agents for liver, lymphatic system (because of macrophage uptake, mainly as negative agent), or blood vessels (because of low extravasation, mainly as positive agent). The different kinds of mechanisms through which they affect the nuclear magnetic relaxation time of the water are briefly described. Nowadays, researches are focused on the development of molecular imaging contrast agents, to target receptors which are overexpressed in pathologies.

The classification of contrast agents according to their distribution in the body may also be considered. [1].

6.1 Non-specific Agents (Interstitial Diffusion of Gadolinium Complexes)

6.1.1 Toxicity of Free Gadolinium and Gd-Complexes

Gadolinium ions have extremely high toxicity. Precipitation of gadolinium chloride at physiological pH results in blockage of the reticuloendothelial system (liver toxicity). On the other hand, the Gd^{3+} ions compete with the calcium at the level of myocardial contractility, the coagulation, the Ca^{2+} dependent enzymes, mitochondrial respiration and neurotransmission. [2].

In order to be injected, the toxicity of gadolinium must be greatly reduced by complexing the Gd^{3+} ion with an organic ligand. Chelates (or ligands) have little effect on the paramagnetic properties of gadolinium as they leave free a coordination site for water molecules.

Complexation with chelate does not involve covalent bonds, a release of the gadolinium ion in vivo is still possible. Six linear or macrocyclic commercial chelates, ionic or nonionic (Fig. 6.1) have r_1 and r_2 relaxivities of the same order of magnitude (Table 6.1) but some differences can be observed in terms of stability, dissociation constants, decomplexation rate or osmolarity.

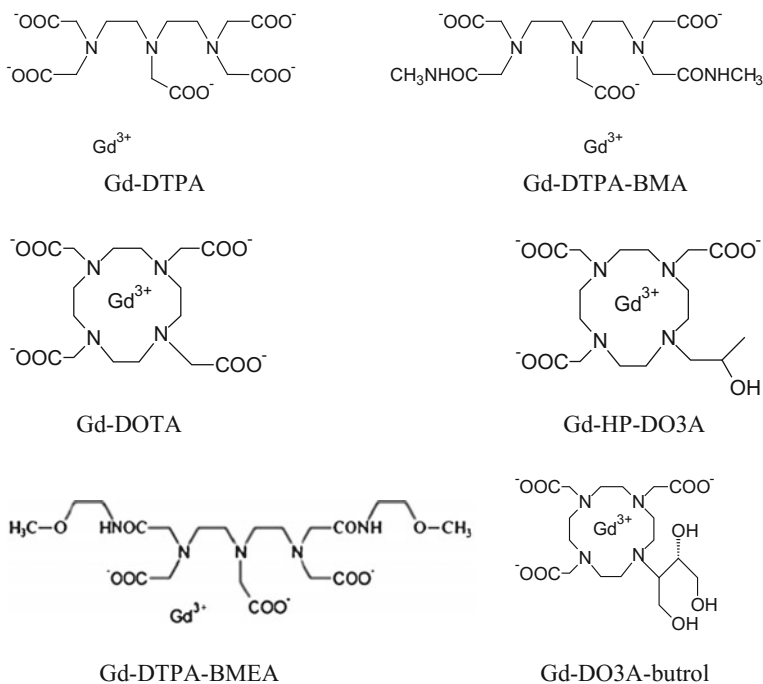


Fig. 6.1 Structure of the non specific clinically-accepted Gd-complexes

Table 6.1 Extracellular contrast agents in clinical use

Contrast agents	Relaxivity ($s^{-1} \text{ mM}^{-1}$) at 1.5 T and 37 °C
Gd-DTPA, gadopentetate dimeglumine Magnevist [®] , Schering AG, Germany	$r_1 = 3.3$ $r_2 = 3.9$
Gd-DTPA-BMA, gadodiamide Omniscan [®] , GE-Healthcare, USA	$r_1 = 3.3$ $r_2 = 3.6$
Gd-DTPA-BMEA, gadoversetamide Optimark [®] , Mallinckrodt, USA	$r_1 = 3.8$ $r_2 = 4.2$
Gd-DOTA, gadoterate meglumine Dotarem [®] , Guerbet, France	$r_1 = 2.9$ $r_2 = 3.2$
Gd-HP-DO3A, gadoteridol ProHance [®] , Bracco, Italy	$r_1 = 2.9$ $r_2 = 3.2$
Gd-BT-DO3A, gadobutrol Gadovist [®] , Schering AG, Germany	$r_1 = 3.3$ $r_2 = 3.9$

Gd gadolinium; *DTPA* diethylenetriaminepentaacetic acid

The release of free Gd ion depends on the affinity of the metal for the ligand. It is measured by the selectivity constant and by the thermodynamic constant.

The kinetics of decomplexation is much slower for the macrocyclic complexes (Gd-DOTA, Gd-HP-DO3A, Gd-DO3A-BT) than for the linear compounds (Gd-DTPA, Gd-DTPA-BMA, Gd-DTPA-BMEA). If the residence time in the body is much less than the decomplexation half-life, the compound is completely eliminated even before it could release free Gd ions and the affinity constant becomes less important. In fact, decomplexation kinetics and affinity constant are related. Macrocyclic chelates exhibit both a high affinity constant and very long decomplexation kinetics.

In theory, if a chelate dissociation occurs *in vivo*, free Gd ions could express their toxicity and free chelate could fix other ions that have a constant in the same order than that of gadolinium affinity. In particular, there may be a chronic chelation of zinc, or acute toxicity by calcium chelation.

The affinity constant for the different calcium chelates is weak in comparison with that of gadolinium, the risk of transmetallation is low. With zinc, the risk is more important [3]. *In vitro* transmetallation test has also been developed recently [4].

After intravenous injection, Gd-DTPA and Gd-DOTA diffuse rapidly in the extracellular space and are then excreted by the kidney exclusively by glomerular filtration. They do not bind to albumin and do not cross the healthy blood-brain barrier. Their plasma half-life is about 90 min. Pharmacokinetics is very close to that of iodinated contrast media used in radiology. They result in an increase of signal (T_1 effect). The T_2 effect (signal decrease) is observable only in the excretory bladder or renal cavities wherein the compound is highly concentrated.

Figure 6.2 shows another example of MR images obtained after injection of Gd-HP-DO3A. A slice at the level of the heart shows the contrast medium in blood vessels. The clinical safety of these agents is excellent, with less than 1 % minor

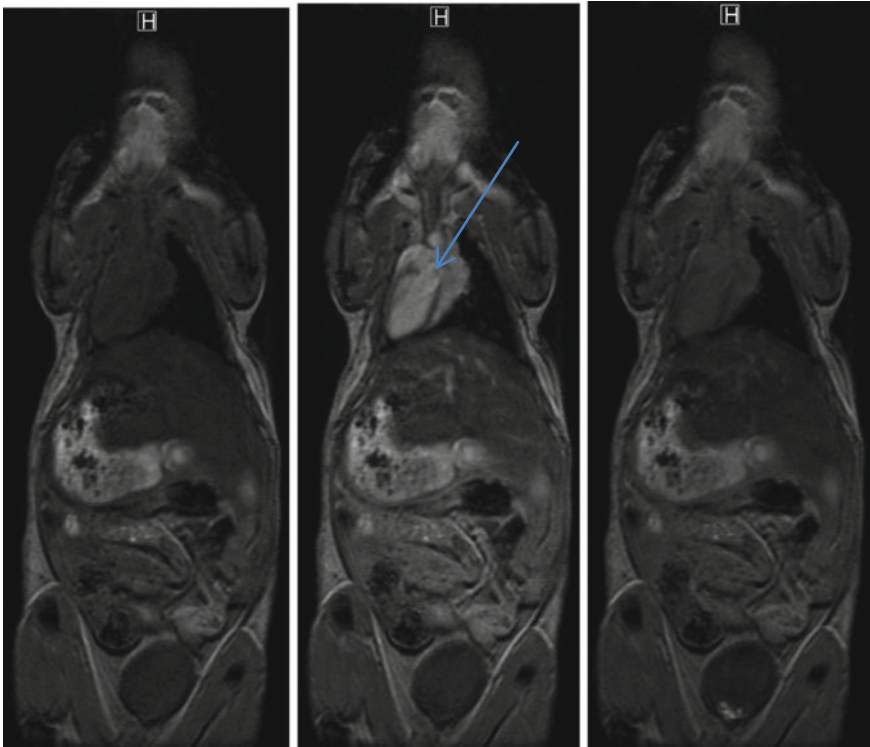


Fig. 6.2 MR images; pre-contrast, one minute, and 5 min after iv injection of Gd-HP-DO3A, gadoteridol (ProHance[®]) at a dose of 45 micromoles/kg. The presented slice shows the heart (arrow) enhanced by the presence of contrast medium in blood vessels of a mouse (with permission of Ref. [6])

effects and exceptional anaphylactoid reactions that do not justify the systematic premedication in allergic patients [5].

6.1.2 NSF Disease [7–14]

Many cases of nephrogenic systemic fibrosis (NSF) (or nephrogenic fibrosing dermopathy, NFD) were observed in patients with renal insufficiency who underwent MRI with intravenous Gd chelates. Nephrogenic systemic fibrosis results in fibrosis of certain tissues such as skin, skeletal muscle, pleura, pericardium and myocardium. This is particularly important in fibrosis of the skin where it can limit movement and causing disability. Renal failure is now considered by many as contraindication for injection of Gd complexes. Several cases of NSF have been reported in association with three contrast agents: Omniscan[®], Magnevist[®] and

Optimark[®]. A majority of reported cases have been associated with exposure to Omniscan[®]. No case has been reported in association with macrocyclic Gd complex. The pathophysiology of this disease remains unclear and therefore the link between cause and effect cannot be determined with certainty. However, this link appears possible because researchers have demonstrated the presence of Gd in skin biopsies of patients with NSF disease. The differences in stability between the reported Gd chelates could explain the disease in patients with severe renal impairment and in which the persistence of contrast agent is the longest. The stability of the Gd chelates is linked to their macrocyclic structure.

6.2 Hepatic Agents

The interest of liver contrast agents is to improve liver tissue characterization for better detectability of lesions. There are several types of contrast agents for the liver [15, 16].

6.2.1 Gadolinium Complexes with Biliary Excretion: Gd-EOB-DTPA and Gd-BOPTA

By adding a side chain containing a benzene ring on the Gd-DTPA complex, a more lipophilic compound (Gd-BOPTA and Gd-EOB-DTPA) (Fig. 6.3), which allows specific capture by the liver is obtained [17, 18]. After passage through the hepatocytes, they are excreted by the bile. These paramagnetic agents induce a significant enhancement of the liver by increasing the signal of more than 200 %. Thus an increase in contrast is obtained between a tumor and the healthy liver. Indeed, a tumor lacking the organic anion transporter system which allows for contrast agent uptake will remain hypointense relative to the liver [19, 20]. With

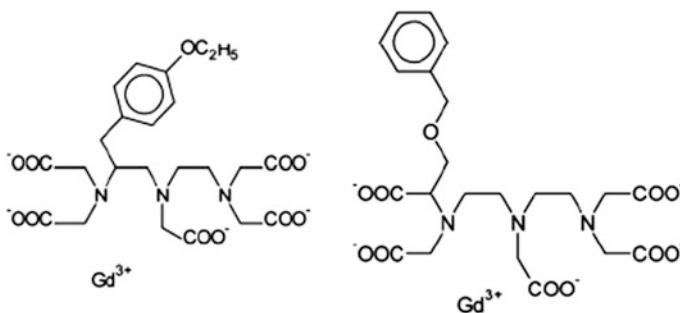


Fig. 6.3 Structure of the Gd-EOB-DTPA and Gd-BOPTA

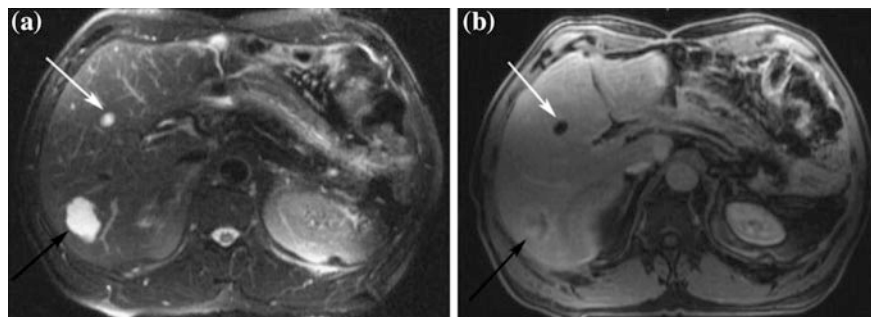


Fig. 6.4 Distinction between metastasis and focal nodular hyperplasia, thanks to a liver contrast agent (Gd-EOB-DTPA) by MRI (T_1 -weighted FLASH sequence, fast low angle shot) 3D. During the arterial phase of the contrast agent, two hypervascular lesions are visible (*white arrow*) in a patient with a clinical history of breast cancer (a). During the hepatospecific phase of Gd-EOB-DTPA (20 min after injection), MRI showed a “wash-out” of the contrast agent in the area indicated by the *white arrow*, corresponding to metastasis. During this same phase, the region indicated by the *black arrow* has captured the contrast agent homogeneously with a better definition of a central scar characteristic of a focal nodular hyperplasia (b). The nature of these lesions was confirmed after surgical resection of metastasis and biopsy of the focal nodular hyperplasia. Figure reproduced with permission of Ref. [27]

Gd-BOPTA, liver enhancement is more prolonged and somewhat lower. Once the compound have been excreted into bile, a very strong enhancement of the biliary tree is obtained which allows to consider application of these contrast agents in MRI cholangiography. Gd-BOPTA and Gd-EOB-DTPA are approved for clinical use. In the imaging of biliary tract, a dose of $10 \mu\text{mol/kg}$ is sufficient. Beyond appears the T_2 effect induced by the high concentration of the contrast agent.

Gd-BOPTA and Gd-EOB-DTPA allow for the detection of primary or secondary liver tumors [21–24]. With hepatic enhancement extended over two hours, it is possible to collect images from several sequences after injection. Furthermore, these two complexes bind to albumin, which causes an increase in relaxivity and provides an interesting property for their use as contrast agent for angiography [25, 26].

A better characterization of hepatocellular lesions such as hepatocellular adenoma or nodular and focal hyperplasia is also permitted. Enhanced lesions in the arterial phase of Gd-EOB-DTPA are identifiable during its hepatospecific phase: in a patient, a focal and nodular hyperplasia appears clearer than metastasis in T_1 -weighted MRI [27] (Fig. 6.4).

6.2.2 Superparamagnetic Iron Oxide Nanoparticles as Specific Agents of Kupffer Cells

The main agents based on iron oxide nanoparticles known are AMI-25 (Feridex® or Endorem®) [28], SHU555A (Resovist® or Cliavist®) [29] and AMI-227

(Sinerem®) [30]. These particles are made of crystalline iron oxide core, coated with a dextran derivative. The overall size of the particle is determined by the thickness of the dextran layer. After intravenous injection, particles are captured by cells of the reticuloendothelial (mononuclear phagocytic) system (Kupffer cells in the liver, spleen, lymph nodes, bone marrow, lung macrophages) [31, 32].

SPIO have a relatively short plasma half-life (10–15 min in rats, 20–40 min in humans) with an extremely rapid hepatic uptake. The agent persists in Kupffer cells for about seven days, leaving considerable time for imaging acquisition after administration. Gradually, the product is degraded in lysosomes of Kupffer cells and is integrated into the pool of iron in the body.

USPIO particles are smaller with a longer blood half-life (24 h in humans), which allows them to have contrast properties related to their vascular compartmentalization, thus for the study of blood vessels.

The AMI-25 and AMI-227 have high relaxivities r_1 and r_2 (Table 6.2), which allow them to have two possible enhancement effects: increasing signal (T_1 effect) and lowering signal (T_2 effect).

The T_2 effect is predominant with nanoparticles and direct clinical application is the detection of liver tumors [33, 34]. The particles act on the T_2 relaxation by spin-spin interaction. Moreover, the aggregation of particles within the Kupffer cells will create very intense local heterogeneities of magnetic field inducing a dephasing of the protons and a signal loss. It is a phenomenon called magnetic susceptibility (T_2^* effect).

In the zones where normal liver Kupffer cells are present, a signal decrease is observed after injection, while a tumor lacking phagocytic activity does not take up the nanoparticles and its signal remains unchanged (Fig. 6.5). The tumor therefore appears bright in a black liver (negative contrast effect) using a T_2 -weighted MRI sequence. Because of their relatively high r_1 relaxivity, the SPIO and USPIO are also able to enhance the MRI signal, especially at low concentration and with T_1 -weighted sequences. This effect is easily observable on aqueous dilutions of the particles, but in vivo, other physiological phenomena are superimposed, making it more difficult to demonstrate a positive enhancement. [35].

A study showed a positive signal enhancement of the liver was possible in the first five minutes after AMI-227 injection in rats [36]. In humans, such an observation has also been reported at the end of the AMI-227 slow infusion [37] and the enhancement of hepatic hemangiomas after the Endorem® infusion T_1 -weighted [38].

Table 6.2 Relaxivity of iron oxide nanoparticles (37 °C, 1.5 T)

Nanoparticles	Hydrodynamic diameter	Relaxivity ($s^{-1}mM^{-1}$)
AMI-227, Sinerem®, Combidex® (dextran)	17–30 nm	$r_1 = 19.5$ $r_2 = 87.6$
AMI-25, Endorem®, Feridex®, Ferumoxides (dextran)	80–150 nm	$r_1 = 9.95$ $r_2 = 158$

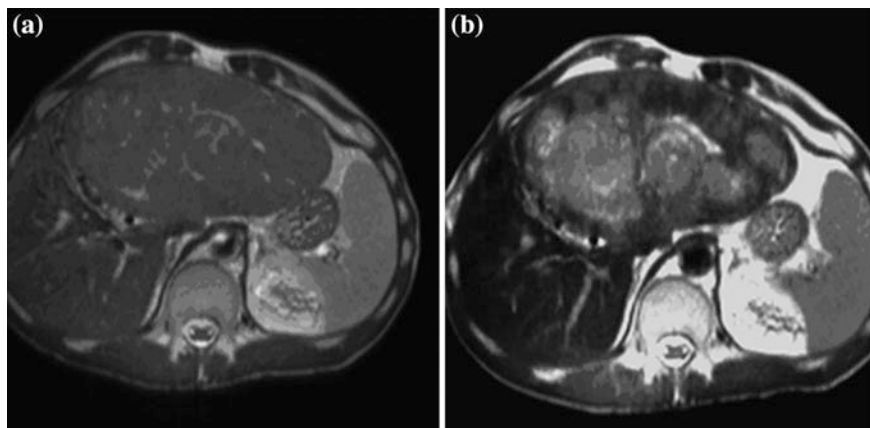


Fig. 6.5 Well differentiated hepatocellular carcinoma in a patient's liver. Axial slice acquired with T_2 -weighted turbo spin echo sequence before (a) and after (b) injection of Endorem[®]. We can observe in the tumor (b) darkened peripheral regions (due to the accumulation of iron oxide nanoparticles in the cells), while the central region remains clear (with permission of Ref. [6]).

After Endorem[®] injection, the liver signal decreases. On a T_1 -weighted sequence, the signal of liver becomes close to that of the tumor (most tumors are hypointense on T_1). The contrast is thus decreased. On proton density and T_2 -weighted sequences however, the contrast is increased as the lesion appears white in a black liver. The overall image quality is improved and the lesions are more easily discernible. The best sequence after injection seems to be proton density-weighted which is sensitive to the presence of particles and has a sufficiently short TE to obtain a good signal to noise [39] ratio. The injection of Endorem[®] gives higher sensitivity in the detection of small lesions under 1 cm [40].

6.2.3 Contrast Agents for Lymphatic System

The clinical interest is important: ganglion curettages are made systematically to distinguish inflammatory lymph nodes from metastatic nodes.

Superparamagnetic nanoparticles are captured by different cells of the mononuclear phagocytic system, particularly in lymph nodes. Percentage of nanoparticle captured into the lymph is quite low after intravenous injection, and varies depending on the type of particle. For small particles (AMI-227, Sinerem[®]) [41], the lymphatic uptake is greater than for AMI-25 (Endorem[®]) which undergo predominant hepatic uptake.

Several pre-clinical experiments have shown that particles cluster in macrophages of lymph node sinusoids, both in normal or inflammatory lymph nodes with

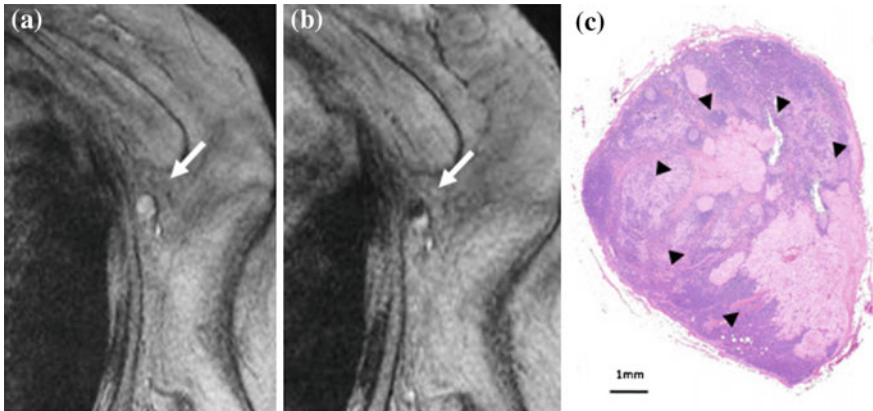


Fig. 6.6 Sentinel lymph node (breast cancer) having a high signal intensity on a portion of more than 50 % after injection of SPIO (T_2^* weighted MRI to a magnetic field of 1.5T, gradient echo sequence: TR = 613, TE = 30 ms, 4 mm slice thickness, flip angle 30° , resolution of 1×0.65 mm). **a** The axillary lymph node shown by the *arrow* has a high signal intensity before injection of SPIO. **b** Eighteen to twenty-four hours after intradermal injection of SPIO (Resovist®), the node pointed by the arrow is dimmed, but has a hyper-signal zone, diagnosed as malignant. **c** Histology shows the presence of macro-metastasis in the lymph node (*arrowheads*) (Reproduced according to Ref. [45])

T_2 effect as a consequence. In metastatic lymph nodes, tumor is devoid of phagocytic cells and does not capture particles.

The signal may change in tumor-containing node after injection; T_1 effect was found at 1.5T probably due to unclustered particle leakage in the tumor interstitium [42]. Clinical trials in healthy humans (cervical lymph nodes) were also partially completed. The development of such a technique requires: a particle having a high percentage of node capture and imaging technique with high spatial resolution to analyze small nodes [43, 44] (Fig. 6.6).

6.3 Vascular Contrast Agents

Vascular contrast agents have a prolonged time of intravascular persistence allowing imaging of organ perfusion after the first minutes of injection (initial rise of the signal), vascular imaging performed on the plateau phase after initial increase (MRA application), imaging of the capillary permeability of tissues based on the study of contrast agent leakage in the interstitium.

6.3.1 *Superparamagnetic Nanoparticles*

USPIO nanoparticles (USPIO, AMI-227) have a long plasma half-life (two hours in rats 24 h in humans), a large size and a very high r_1 relaxivity. So they have the properties to be used as contrast agents of the vascular area [44, 46–48]. Using their T_1 or T_2 effect, several experimental studies have shown their application in the imaging of myocardial ischemia [49], renal vasculature [50] and tumor capillary permeability.

6.3.2 *Macromolecular Contrast Agents* *(Albumin-Dextran, Polylysine-Gd-DTPA)*

They are composed of a carrier macromolecule (albumin, dextran, polylysine) to which several Gd chelates are covalently attached [51, 52].

6.3.3 *Applications*

Tumor angiogenesis induced proliferation of hyperpermeable vessels. Indeed, tumors are developing new vessels from existing vasculature. This process of angiogenesis is essential for tumor growth. Tumors can be characterized by measuring perfusion parameters with MRI (reflecting vessel permeability, volume of extra and intravascular compartments). Perfusion imaging is usually performed using a dynamic approach of T_1 -weighted imaging after bolus injection of contrast agent (dynamic contrast enhanced MRI). The size of these compounds influences their volume distribution: macromolecular agents do not pass the capillary barrier, have a prolonged blood half-life, and can be used as vascular contrast agents. Contrast agents of average molecular weight (1–60 kDa) and higher molecular weight (>60 kDa) were called blood pool agents due to their prolonged intravascular retention and allow to evaluate the modification tumor vessel permeability. Albumin-(Gd-DTPA)₃₅ is a prototype of contrast agents for the blood compartment (molecular weight 92 kDa). Its use in humans, however, is limited by its immunogenicity. MS-325 (Vasovist®), a Gd chelate reversibly binding to endogenous albumin is currently marketed [53–55]. Particulate contrast agents are another class of blood pool agents. Among them, the USPIO particles (Sinerem®) which have a diameter of 10–50 nm and a molecular weight higher than 100 kDa were particularly studied but never marketed. However, the contrast agents of high molecular weight have the major disadvantage of a very slow kinetic enhancement due to very low rates of extravasation.

Contrast agents of intermediate molecular weight are used to obtain images with good signal to noise ratio in a relatively short time (5–7 min). These agents, such as

Gadomer-17 (in vivo Contrast) with a molecular weight of 17 kDa and P792 (Vistarem®) with a molecular weight of 6.47 kDa, represent a good compromise between low and high molecular weight agents but cannot be used in human patients [56, 57].

6.4 Conclusions

Nowadays, only Gd chelates are approved and available as MRI contrast agent for clinical use. Approved iron oxide nanoparticles were taken off the market because of lack of sales [58]. However, several types of iron oxide nanoparticles are available for pre-clinical MRI. In contrast media research, compound size as well as their contrast and surface properties can be adapted for optimizing their distribution, circulation time, elimination way and MRI detectability, according to a desired in vivo fate suitable for vascular, tissue, cellular or molecular MRI. Indeed, current research focuses on the development of molecular imaging contrast agents, capable of targeting overexpressed molecular receptors to diagnose or characterize disease and treatment efficiency at an early stage. The main problem is the detection threshold: contrast agents are detectable in the range of millimolar concentrations, which is much higher than the intracellular concentrations of protein or nucleic acid. It is therefore necessary to develop contrast agents with lower detectability threshold. A second difficulty is to find agents that have a good affinity and high specificity for the target. Mimetic peptide sequences (RGD) or polysaccharides (Sialyl Lewis X) have been developed and grafted on Gd complexes or iron oxide nanoparticles. The identification of sequences having a high affinity for molecular targets of diagnostic interest can be performed by the systemic evolution of ligands by exponential enrichment (SELEX) method. The aim of this technique is to identify sequences in a library of random oligonucleotides. This method comprises several steps including the incubation of ribonucleic (RNA) or deoxyribonucleic acid (DNA) with the target of interest, the selection of sequences that have bound to the target, the amplification of these sequences by RT-PCR (reverse transcription-polymerase chain reaction) and their transcription in vitro. These selection steps and amplification must be repeated (about 8–15 times) until the RNA/DNA candidates having the strongest affinity for the target are isolated; these candidates called aptamers. The SELEX method was used to generate aptamers directed against target molecules of widely varying structure and size such as nucleic acids, small organic molecules (e.g., ATP) and more complex targets such as membrane receptors or whole cells.

Many laboratories have chosen the phage display technology to select the right vector. This method utilizes a library of phages bearing combinations of all peptides of a given length (generally between 6 and 15 amino acids). Incubation of this library with the target allows for the selection of candidates showing high affinity for the target. The primary structure of the peptide is determined by sequencing the DNA of selected phages. This technique has already led to the selection of specific

peptides, targeting angiogenesis, apoptosis, or atherosclerotic plaques These peptides are then linked to contrast agents, forming “molecular imaging” contrast agents. The first tests look very promising [59–63].

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